

Androgen-Regulated and Highly Tumorigenic Human Prostate Cancer Cell Line Established from a Transplantable Primary CWR22 Tumor

Ayush Dagvadorj,¹ Shyh-Han Tan,¹ Zhiyong Liao,¹ Luciane R. Cavalli,² Bassem R. Haddad,² and Marja T. Nevalainen¹

Abstract **Purpose:** One of the major obstacles in understanding the molecular mechanisms underlying the transition of prostate cancer growth from androgen dependency to a hormone-refractory state is the lack of androgen-regulated and tumorigenic human prostate cancer cell lines. **Experimental Design:** We have established and characterized a new human prostate cancer cell line, CWR22Pc, derived from the primary CWR22 human prostate xenograft tumors. **Results:** The growth of CWR22Pc cells is induced markedly by dihydrotestosterone, and CWR22Pc cells express high levels of androgen receptor (AR) and prostate-specific antigen (PSA). Importantly, PSA expression in CWR22Pc cells is regulated by androgens. Stat5a/b, Stat3, Akt, and mitogen-activated protein kinase were constitutively active or cytokine inducible in CWR22Pc cells. The AR in CWR22Pc cells contains the H874Y mutation, but not the exon 3 duplication or other mutations. When inoculated subcutaneously into dihydrotestosterone-supplemented castrated nude mice, large tumors formed rapidly in 20 of 20 mice, whereas no tumors developed in mice without circulating dihydrotestosterone. Moreover, the serum PSA levels correlated with the tumor volumes. When androgens were withdrawn from the CWR22Pc tumors grown in nude mice, the tumors initially shrank but regrew back as androgen-independent tumors. **Conclusions:** This androgen-regulated and tumorigenic human prostate cancer cell line provides a valuable tool for studies on androgen regulation of prostate cancer cells and on the molecular mechanisms taking place in growth promotion of prostate cancer when androgens are withdrawn from the growth environment. CWR22Pc cells also provide a model system for studies on the regulation of transcriptional activity of mutated H874YAR in a prostate cancer cell context.

The median duration of response to androgen deprivation therapy of primary prostate cancer is less than 3 years (1, 2). The molecular mechanisms underlying development of androgen-independent growth of prostate cancer are largely

unknown and no effective therapies for hormone-refractory prostate cancer exist at present. One of the key problems in conducting studies to identify growth factors and signaling pathways that can replace androgens in the growth control of prostate cancer cells is the lack of androgen receptor (AR)-positive human prostate cancer cell lines that are regulated by androgens and would be tumorigenic in nude mice. LNCaP cells respond by accelerated growth rate to androgens (3), but are able to grow relatively well in the absence of androgens (4). Moreover, tumor incidence of LNCaP cells after s.c. inoculation into nude mice is low and the tumors grow slowly (3).

We have characterized a new human prostate cancer cell line established from primary transplantable human CWR22 prostate tumors. CWR22 tumors were originally derived from a Gleason score 9 primary prostate cancer with bone metastases (5, 6). The primary CWR22 prostate tumors are highly responsive to androgen deprivation with marked tumor regression after castration, mimicking the course of the human disease. After androgen deprivation-induced regression of the original tumor, CWR22 prostate cancer recurs within 7 to 9 months and the recurrent CWR22 tumors (CWR22R) are not dependent on androgens for growth (7). CWR22Rv1 is an androgen-independent human prostate cancer cell line that has been established from one of the hormone-refractory recurrent CWR22R tumors (8). However, no cell lines displaying the

Authors' Affiliations: ¹Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania and ²Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, District of Columbia

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Requests for reprints: Marja T. Nevalainen, Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, BLSB 309, Philadelphia, PA 19107. Phone: 215-503-9250; Fax: 215-503-9245; E-mail: marja.nevalainen@jefferson.edu.

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Translational Relevance

Currently, one of the major obstacles in understanding the molecular mechanisms underlying the transition of prostate cancer growth from androgen dependency to hormone-refractory state is the lack of well-characterized androgen-regulated and tumorigenic human prostate cancer cell lines. We have established and characterized a new human prostate cancer cell line, CWR22Pc, derived from the primary CWR22 human prostate xenograft tumors. We show that the CWR22Pc cell line genetically originates from the primary CWR22 tumors. The growth of CWR22Pc cells is strictly regulated by androgens, and CWR22Pc cells express high levels of androgen receptor and prostate-specific antigen. Importantly, when androgens were withdrawn from the established CWR22Pc tumors grown in nude mice, the tumors initially shrank but regrew back as androgen-independent tumors. The androgen-regulated CWR22Pc cell line provides a new much-needed experimental model system for studies on androgen-regulated growth of prostate cancer cells and for research on genetic changes and kinase signaling pathways involved in growth promotion and dedifferentiation of androgen-deprived prostate cancer cells. Furthermore, CWR22Pc cells provide a model system for studies on the regulation of transcriptional activity of mutated H874YAR in a prostate cancer cell context. Because CWR22Pc cells produce prostate-specific antigen in an androgen-regulated manner, and the serum prostate-specific antigen levels correlate with the CWR22Pc xenograft tumor volumes in mice, CWR22Pc cells/nude mouse tumor system will provide a valuable model for development of new diagnostics and therapeutics for prostate cancer.

growth characteristics of the original androgen-regulated primary CWR22 tumors have existed until now.

In this work, we have established an androgen-regulated human prostate cancer cell line named CWR22Pc from the primary CWR22 tumors. We show by comparative genomic hybridization and by DNA fingerprinting that the CWR22Pc cell line genetically originates from the primary CWR22 tumors. We further show that the growth of CWR22Pc cells is regulated by androgens in cell culture. Importantly, CWR22Pc cells form androgen-regulated, rapidly growing tumors at high incidence in nude mice. Moreover, CWR22Pc cells produce prostate-specific antigen (PSA) in an androgen-regulated manner in culture, and the serum PSA levels correlate with the CWR22Pc xenograft tumor volumes in mice. Similar to the parental primary CWR22 tumors, CWR22Pc cells express AR containing the H874Y mutation without the tandem duplication of exon 3. We show that several key growth factor-related signaling pathways are cytokine inducible or constitutively active in CWR22Pc cells. Finally, androgen deprivation of the CWR22Pc tumors in nude mice induced tumor regression, which was followed by androgen-independent recurrence of the CWR22Pc tumors. The androgen-regulated CWR22Pc cell line provides a new experimental model system for studies on androgen-regulated growth of prostate cancer cells and for research on

genetic changes and kinase signaling pathways involved in growth promotion and dedifferentiation of androgen-deprived prostate cancer cells.

Materials and Methods

Cell culture. The suspension of CWR22Pc cells was prepared from androgen-dependent human primary prostate tumor xenograft, CWR22P (a gift from Dr. Thomas Pretlow, Case Western Reserve University, Cleveland, OH), using a method described previously for the dissociation of human prostate cancer tissue (9). Briefly, under sterile conditions, six tumors were dissected from the mice and minced. The minced tumor was washed thrice in RPMI 1640 (Life Technologies) supplemented with 20% fetal bovine serum (FBS; Life Technologies) and the tumor tissue was digested serially with 0.1% Pronase E (EMD Pharmaceuticals) in Joklik-modified MEM (Sigma). The Pronase E digest fractions were filtered through a single layer of a NITEX 250- μ m-porosity membrane (Safer America) and centrifuged at $97 \times g$ for 7.5 min at 4°C. The pellets were resuspended in RPMI 1640 supplemented with 20% FBS. The cells were counted and the cell viability was confirmed by trypan blue exclusion. The cells were cultured in RPMI 1640 containing 10% FBS, 2.5 mmol/L L-glutamine, and penicillin-streptomycin (100 IU/mL and 100 μ g/mL, respectively) in the presence of 0.8 nmol/L dihydrotestosterone (5 α -androstane-17-ol-3-one, Sigma) at 37°C with 5% CO₂. CWR22Rv1, LNCaP, and DU145 cells (American Type Culture Collection) were cultured in RPMI 1640 (Biofluids) containing 10% FBS, 2.5 mmol/L L-glutamine, and penicillin-streptomycin (100 IU/mL and 100 μ g/mL, respectively) at 37°C with 5% CO₂. LNCaP cells were cultured in the presence of 0.8 nmol/L dihydrotestosterone. For testing of inducibility of Stat5a/b and Stat3 by cytokines, the cell lines were serum starved 16 h after which the cells were stimulated with 10 nmol/L human prolactin or 4 nmol/L interleukin-6 (IL-6; Upstate) before harvesting the cells for immunoprecipitations.

Growth of CWR22Pc cells as xenograft tumors in athymic nude mice. Castrated male athymic mice were purchased from Taconic and cared for according to the institutional guidelines. Briefly, 20×10^6 CWR22Pc cells were mixed with one half of the total injection volume of 0.2 mL with Matrigel (BD Bioscience). One week before the tumor cell inoculation (2 sites per mouse), sustained-release dihydrotestosterone pellets (12.5 mg/pellet, 1 pellet/mouse; Innovative Research of America) were implanted s.c. in half of the mice. The tumor sizes were measured twice a week, and the tumor volumes were calculated using the formula $3.14 \times \text{length} \times \text{width} \times \text{height}/6$ (ref. 10). When the tumors reached 15 to 20 mm in diameter, mice were sacrificed, the tumor tissues were harvested, and the blood samples were collected. In the second set of experiments, CWR22Pc cells were inoculated s.c. to the flanks of castrated athymic nude mice ($n = 34$) supplied with dihydrotestosterone pellets as described above (1 site per mouse). When the tumors reached 10 mm in diameter, the dihydrotestosterone pellets were removed and the tumor sizes were measured twice a week.

Serum PSA determinations. Serum PSA levels were determined using the commercial kit DSL-10-9700 ACTIVE PSA (Diagnostic Systems Laboratories, Inc.) according to the manufacturer's instructions. Briefly, the standards, the controls, and the serum samples were incubated in microtiter wells coated with an anti-PSA monoclonal antibody. The antigen binding was quantified by a polyclonal anti-PSA antibody labeled with horseradish peroxidase enzyme and subsequent addition of the horseradish peroxidase substrate tetramethylbenzidine. The degree of enzymatic turnover of the substrate was determined by dual-wavelength absorbance measurement at 450 and 620 nm.

Protein solubilization and immunoblotting. Pellets of CWR22Pc cells were solubilized in lysis buffer [10 mmol/L Tris-HCl (pH 7.6), 5 mmol/L EDTA, 50 mmol/L sodium chloride, 30 mmol/L sodium

PPi, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, 1 µg/mL pepstatin A, and 2 µg/mL leupeptin], rotated end-over-end at 4°C for 60 min, and insoluble material was pelleted at 12,000 × g for 30 min at 4°C. The protein concentrations of clarified cell lysates were determined by simplified Bradford method (Bio-Rad Laboratories). For immunoblotting of PSA and AR, we used anti-PSA polyclonal antibody (pAb; 1:1,000, DakoCytomation) and anti-AR monoclonal antibody (mAb; 1:1,000; BioGenex). For Akt and mitogen-activated protein kinase (MAPK) immunoblottings, primary antibodies were used at the following concentrations: anti-phospho-Akt (Thr³⁰⁸) pAb (1:500), anti-phospho-Akt (Ser⁴⁷³) pAb (1:500), anti-Akt pAb (1:500), anti-phospho-42/44 MAPK (T202/Y204) mAb (1:500; Cell Signaling), anti-pan-ERK mAb (1:1,000; Transduction Laboratories, Inc.), and anti-actin pAb (1:4,000; Sigma). In addition, whole-cell lysates were immunoprecipitated for 1 h at 4°C with polyclonal rabbit antisera against either Stat5a or Stat5b (4 µL/mL; Advantex Bioreagents) or Stat3 (1 µg/mL, K-15; Santa Cruz Biotechnologies). Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Pharmacia Biotech). Samples were run on a 4% to 12% SDS-PAGE under reducing conditions. For Western blotting of the immunoprecipitations, the primary antibodies were used at the following concentrations—anti-phosphotyrosine-Stat5a/b (Y694/Y699) mAb (1 µg/mL, Advantex BioReagents), anti-Stat5ab mAb (1:250; Transduction Laboratories, Inc.), anti-phosphotyrosine-Stat3 (Y705) pAb (1:1,000; Cell Signaling), and anti-Stat3 pAb (1:1,000; K-15; Santa Cruz Biotechnologies)—and detected by horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence.

Cell viability assay. The cell viability was determined by counting attached cells by hemacytometer and trypan blue exclusion. For comparison of other prostate cancer cell lines, CWR22Pc, LNCaP, and CWR22Rv1 cells were grown in the presence or absence of 0.8 dihydrotestosterone in 3% CS-FBS containing medium for 9 d. The medium was changed every other day and cells were counted manually every 3rd day. Representative photographs were taken 9 d after cell plating.

DNA fragmentation ELISA assay. Fragmentation of DNA was determined by photometric enzyme immunoassay according to the manufacturer's instructions (cell death detection ELISA^{PLUS}; Roche Molecular Biochemicals). Briefly, cells were centrifuged at 200 × g, and cytoplasmic fractions containing fragmented DNA were transferred to streptavidin-coated microtiter plates that had been incubated with biotinylated monoclonal anti-histone antibody. The amount of fragmented DNA bound to anti-histone antibody was evaluated by peroxidase-conjugated monoclonal anti-DNA antibody using ABTS as a substrate at 405 nm.

Conventional cytogenetic analysis. Chromosome preparation and G-banding were done using standard protocols (11). Chromosomes were identified and classified according to standard cytogenetic nomenclature proposed by the International System for Human Cytogenetic Nomenclature (12).

Comparative genomic hybridization. Total genomic DNA was extracted from primary CWR22 tumor tissues, CWR22Pc cells from two different passages (test DNA), and from the lymphocytes of a karyotypically normal female control (control DNA), using Wizard Genomic DNA Purification Kit (Promega). Comparative genomic hybridization analysis was done using standard protocols that we have previously published (13). Quantitative evaluation of the hybridization was completed using a commercially available software package (Applied Imaging). Average ratio profiles were computed as the mean value of 8 ratio images and were used to identify changes in chromosome copy number.

Cell cycle and ploidy analysis. Cells were stained with propidium iodide (50 µg/mL) with RNase A (50 µg/mL). Duplicate samples were run with and without human peripheral blood lymphocytes added as a standard. Cells were run on a Becton Dickinson FACSort. Twenty

thousand cells were collected. Data were modeled with ModFit Software (Verity Softwarehouse).

DNA fingerprinting. Total genomic DNA was extracted from primary CWR22 tumor tissues and CWR22Pc cells from two different passages using Wizard Genomic DNA Purification Kit (Promega). DNA fingerprinting was done using the commercially available kit, PowerPlex 1.2 System (Promega). This system allows the coamplification and two-color detection of nine loci (eight short tandem repeat loci and the Y-specific Amelogenin). This approach provides a powerful level of discrimination in excess of 1 in 10⁹. The following markers were tested: D5S818, D13S317, D7S820, D16S539, vWA, TH01, Amelogenin, TPOX, and CSF1PO. The PCR amplification was done according to the manufacturer's recommended protocol. Allele size was determined by electrophoresis of the PCR products in 6% denaturing polyacrylamide gels and compared with ROX 500 size standards (Applied Biosystems), using the automated sequencer, ABI 377 (Applied Biosystems). The fluorescent signals from the different size alleles were recorded and analyzed using GENESCAN version 3.1 and GENOTYPER version 2.1 software, respectively (Applied Biosystems). Allele size evaluation and data analysis were done by two independent observers. The experiments were repeated twice.

The AR sequence analysis. Total RNA from primary CWR22 xenograft tumors, CWR22Rv1, CWR22Pc, and LNCaP cells was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with SuperScriptII reverse transcriptase (Invitrogen) using oligodeoxy-TMP primers. The conditions for PCR for all reactions were 94°C for 2 min, followed by 30-s denaturation at 94°C, 30-s annealing at 60°C, 30-s extension at 72°C, and final extension period of 10 min. The PCR products were size-separated on a 2% Tris-borate EDTA-agarose gel. To analyze the AR cDNA, primer pairs were designed to amplify four overlapping segments (I, II, III, and IV) encompassing the entire AR coding region plus 5' and 3' untranslated sequences. Primers for PCR amplification and sequencing for segments were designated based on the human AR mRNA reference sequence (NM_000044) deposited in the Genbank database: segment I (forward 5'-GCCAAGCTCAAG-GATGGA-3' and reverse 5'-ATCTCAGTGCTTGCCTG-3'), segment II (forward 5'-CATTGGCCGAATGCAAAGGT-3' and reverse 5'-CGGCTCTTTGAAGAAGACC-3'), segment III (forward 5'-GAA-GACCTGCCTGATCTGTG-3' and reverse 5'-ACATCCGGGACTTGTG-CATG-3'), and segment IV (forward 5'-CCTTACCAATGTCAACTCC-3' and reverse 5'-AGTGCAGAGTTATAACAGGC-3'). Sequencing was done on automatic sequencer ABI 377 (Applied Biosystems) and sequences were analyzed using ClustalW multiple sequence alignment tool.³

Results and Discussion

CWR22Pc cell line is derived from the androgen-dependent re-transplantable primary CWR22 tumors. CWR22 prostate tumor system was originally established from a Gleason score 9 human prostate cancer obtained from prostatectomy. The prostate cancer tissue pieces were grown in male nude mice as subcutaneous tumors and the tumor system is maintained by serial regrafting. The tumors were named primary CWR22 tumors, and they are dependent on androgens for growth. In response to long-term androgen deprivation, the tumors regress but recur back within 7 to 9 months as androgen-independent tumors. The androgen-independent secondary tumors were named recurrent CWR22 tumors and a cell line established from the recurrent CWR22 tumors was named CWR22Rv1.

In this work, we established a new prostate cancer cell line from the primary CWR22 tumors. To confirm the genetic

³ <http://www.ebi.ac.uk/Tools/clustalw/>

lineage identity of CWR22Pc cell line as a derivative of the primary CWR22 tumors, we compared the DNA fingerprinting pattern of two passages of CWR22Pc line with that of the CWR22 primary tumors at nine different genetic markers (eight short tandem repeat markers and the Amelogenin locus).

CWR22Pc cell line showed an identical DNA fingerprinting pattern to the primary CWR22 tumors (number of alleles and allele size) at all eight short tandem repeat markers. The Y-specific Amelogenin locus showed two alleles (size 213 and 219 bp) in both CWR22Pc cells and in the primary CWR22

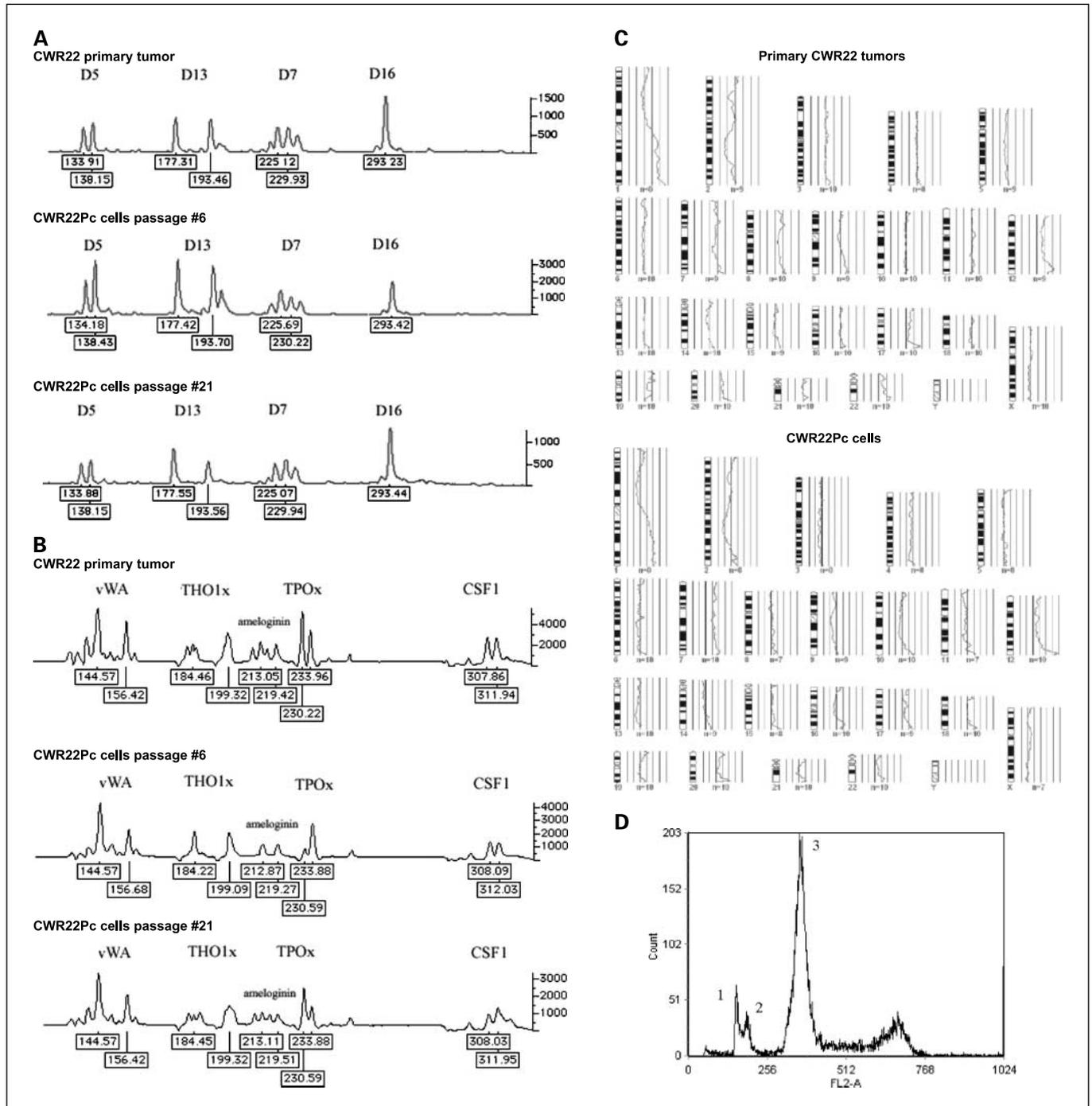


Fig. 1. The genetic lineage identity of CWR22Pc cells is similar to that of CWR22 primary prostate tumors. DNA fingerprinting analysis of the CWR22 primary tumor and the cell line CWR22Pc (passages 6 and 21) show a similar pattern for all nine markers analyzed. *A*, patterns of alleles at markers D5S818 (*D5*), D13S317 (*D13*), D7S820 (*D7*), and D16S539 (*D16*). *B*, the results at markers vWA (*vWA*), THO1 (*THO1x*), Amelogenin, TPOX (*TPOx*), and CSF1PO (*CSF1*). The allele sizes are indicated under each allele. *C*, the comparative genomic hybridization analysis shows gains at 1q, 7, 8p, and 12, and losses of chromosomes 2 and X in both primary CWR22 tumors and in CWR22Pc cells. *D*, flow cytometric analysis of CWR22Pc cells shows that the cell line consists of a mixed population of cells with DNA indices of 1.11 and 2.23. 1, the G₁ peak of the human peripheral blood lymphocytes (added as a control). 2, the G₁ peak of the cells with a lower ploidy (DNA index = 1.1). 3, the G₁ peak of the cells with a higher ploidy (DNA index = 2.23).

tumors, indicating the presence of an X and a Y chromosome. Figure 1A and B shows a comparison between the results of DNA fingerprinting analysis of the primary CWR22 tumor and the two passages of CWR22Pc cells (nos. 6 and 21) at the 9 loci

studied. Moreover, we compared the chromosomal alterations in the CWR22Pc line with the ones in the primary CWR22 tumor. Specifically, we evaluated DNA obtained from two passages of CWR22Pc cells and DNA from the primary CWR22

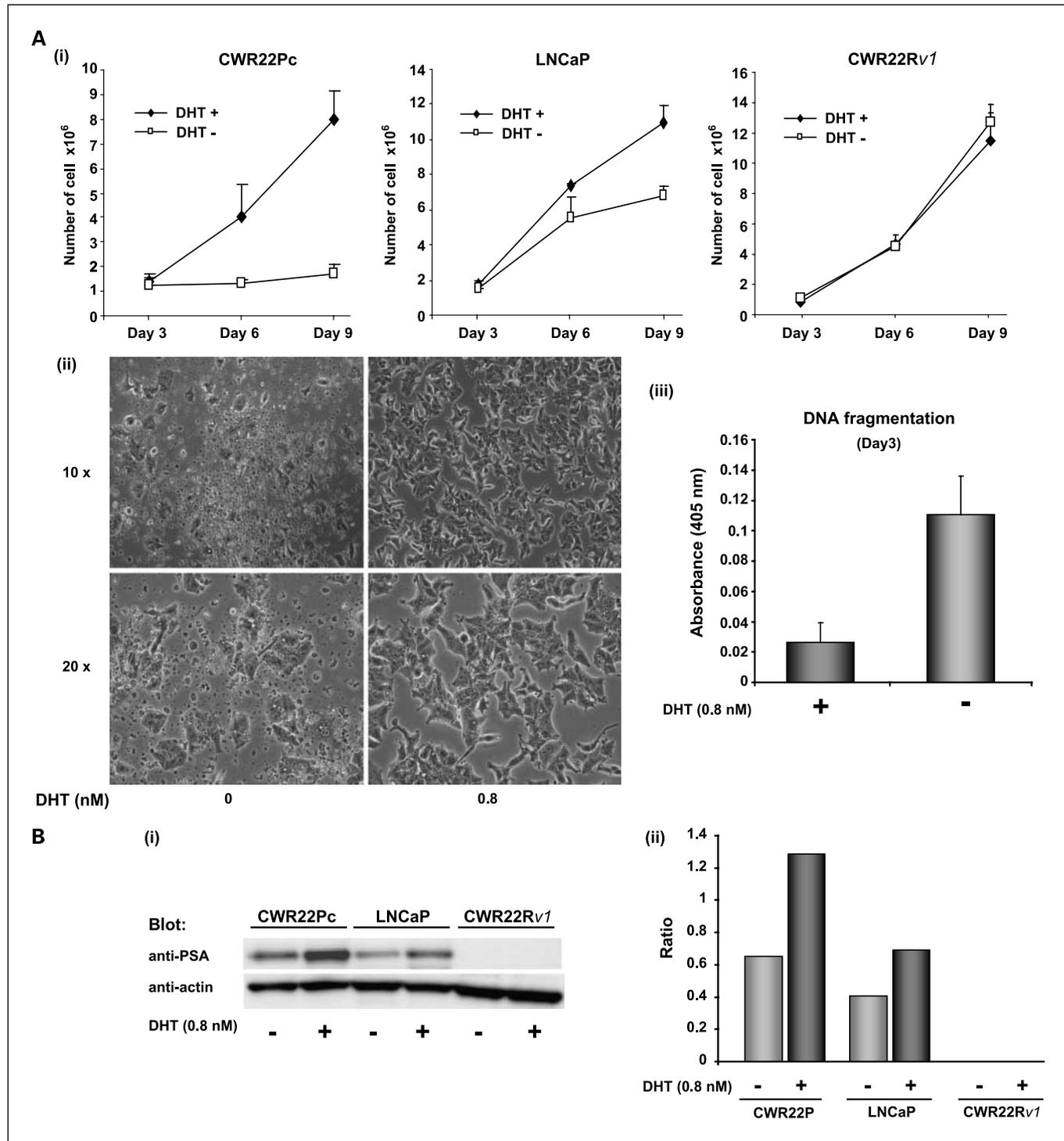
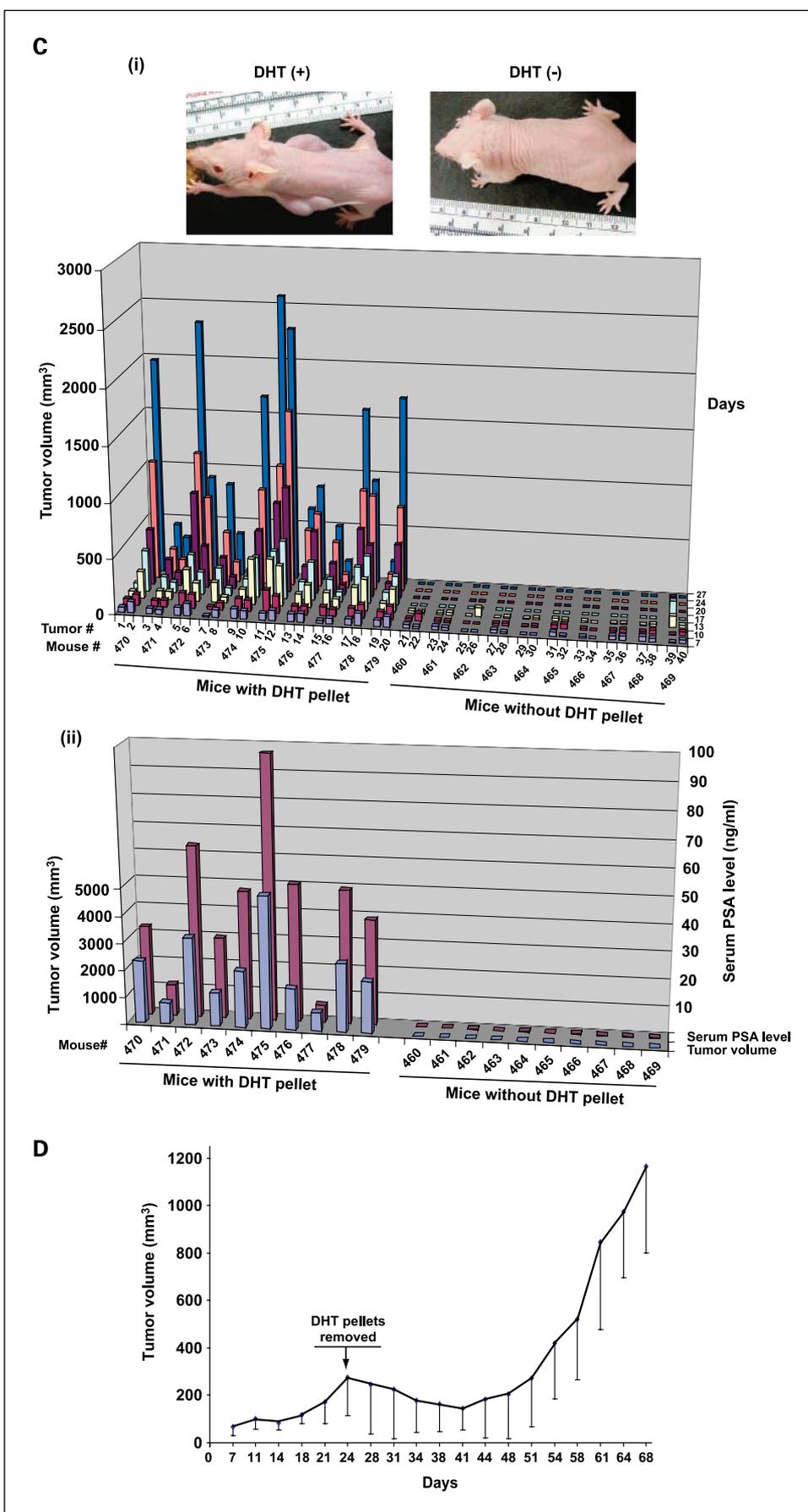


Fig. 2. The growth of CWR22Pc cells is increased by androgens. *A*, CWR22Pc, LNCaP, and CWR22Rv1 cells were grown in the medium containing 3% CS-FBS in the presence or absence of 0.8 nmol/L dihydrotestosterone (*DHT*) for 9 d. Cells were counted every 3rd day. The means of three independent experiments are presented with SDs (*i*). The absence of dihydrotestosterone had marked effects on the cell morphology of CWR22Pc cells. In the absence of dihydrotestosterone, stereomicroscope photographs show that the majority of the cells were dead and floating (*ii*) with increased DNA fragmentation (*iii*). *B*, PSA expression is regulated by androgens in CWR22Pc and LNCaP cells. CWR22Pc, LNCaP, and CWR22Rv1 cell lines were cultured in RPMI with or without 0.8 nmol/L dihydrotestosterone for 12 d. Cells were harvested, lysed, and immunoblotted with anti-PSA pAb. Stripped filters were reblotted with anti-actin pAb to show equal loading (*i*). Densitometric normalization and comparison of the PSA levels (*ii*).

Fig. 2 Continued. C, the growth of CWR22Pc cells as subcutaneous xenograft tumors in athymic nude mice is regulated by androgens. CWR22Pc cells were inoculated s.c. into flanks of castrated nude mice supplied with sustained-release 5 α -dihydrotestosterone pellets ($n = 10$ /group, 2 tumors per mouse, 20×10^6 CWR22Pc cells per site). The tumor incidence and growth were measured twice a week for 36 d. *i,* tumor volumes were calculated using the formula $3.14 \times \text{length} \times \text{width} \times \text{depth}/6$. *ii,* serum PSA levels in mice carrying CWR22Pc tumors correlated with the volumes of the tumors. **D,** CWR22Pc tumors recur after androgen deprivation – induced regression of the tumors. CWR22Pc cells were inoculated s.c. into flanks of castrated athymic nude mice supplied with sustained-release 5 α -dihydrotestosterone-pellets ($n = 4$ mice, 1 tumor per mouse, 20×10^6 CWR22Pc cells per site). Once the tumors reached 10 mm in diameter, the dihydrotestosterone pellets were removed and the tumor growth was measured twice a week. Tumor volumes were calculated as described above.



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tumor using comparative genomic hybridization. Comparative genomic hybridization analysis is an ideal method to detect overall chromosomal gains and losses in the genome. The comparative genomic hybridization analysis showed that the pattern of chromosomal alteration of the CWR22Pc cell line was very similar to that of the primary CWR22 tumor. Chromosomal gains included gains at 1q, 7, 8p, and 12, and losses of chromosomes 2 and X (Fig. 1C). The findings of the DNA fingerprinting analysis and comparative genomic hybridization confirmed that the CWR22Pc cell line was indeed derived from the primary CWR22 tumors. To evaluate the ploidy status of the CWR22Pc cells, we did a G-banding analysis of over 300 metaphase spreads and a ploidy analysis by flow cytometry. Metaphase analysis showed that ~80% of the cells were near tetraploid (chromosomal count ~100) and 20% of the cells showed a chromosomal count of ~50 chromosomes. These findings were consistent with the cell cycle and ploidy analysis that showed a mixed population of cells with DNA content of 1.11 and 2.23 (Fig. 1D).

In summary, our results of the G-banding and the ploidy analysis of the CWR22Pc cells are in line with the mosaic karyotype reported previously for the primary CWR22P tumors (6, 14), the recurrent CWR22R xenografts (14), and the CWR22Rv1 cell line established from the recurrent CWR22R tumors (8). In detail, primary CWR22 xenograft tumors were reported to have a mixture of two karyotypes where part of the cells displayed an unidentified marker chromosome (6). In addition, the relapsed strains of CWR22R tumors after androgen deprivation showed each a different karyotype (14) and the CWR22Rv1 cell line consists of a mixed population of hyperdiploid (90%) and near-tetraploid (10%) cells (15). The results of our study reported here indicated a mosaic karyotype of CWR22Pc cells. It will be both important and interesting for the future studies to determine whether the ploidy status of CWR22Pc cells will change during a long-term androgen deprivation *in vitro* and when CWR22Pc cells are grown as xenograft tumors in nude mice *in vivo*.

Growth and PSA protein expression of CWR22Pc cells is regulated by androgens. Given that the CWR22Pc cell line was established from primary CWR22 tumors, which are regulated by androgens, we first aimed to determine the effects of androgens on the growth of CWR22Pc cells in culture. CWR22Pc cells, LNCaP cells, and androgen-independent CWR22Rv1 cells were cultured in the presence or absence of 0.8 nmol/L dihydrotestosterone for 9 days, and the number of attached viable cells was determined every 3rd day. On day 6 of the experiment, the number of CWR22Pc cells was increased by 3-fold, whereas the number of LNCaP cells was increased only by 40% (Fig. 2A, i). Moreover, on day 9 of the experiment, the number of CWR22Pc cells was increased by 6-fold by androgens, whereas the number of LNCaP cells was increased only by 60% (Fig. 2A, i). At the same time, the growth rate of CWR22Rv1 cells was not significantly affected by dihydrotestosterone during a 9-day period. Deprivation of CWR22Pc cells from androgens induced apoptotic death of the cells as shown by cell morphology on the day 9 of the experiment (Fig. 2A, ii). Specifically, androgen deprivation induced extensive detachment of the cells, cell fragmentation, shrinkage, and blebbing, all of which are morphologic changes consistent with apoptotic cell death (Fig. 2A, ii). Moreover, DNA fragmentation was increased by 5-fold on the day 3 of

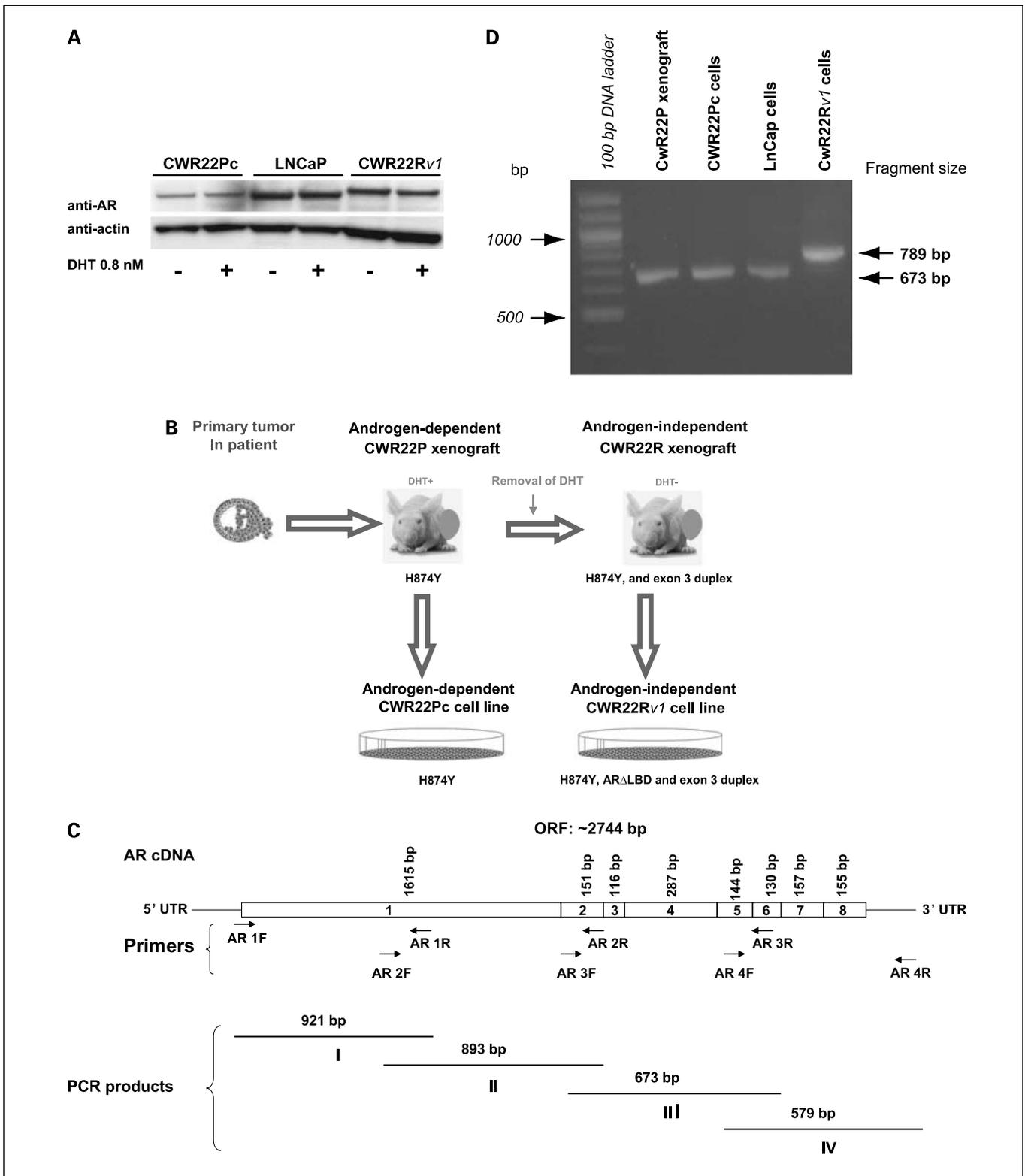
the experiment in the cells cultured in the absence of dihydrotestosterone (Fig. 2A, iii).

To further examine androgen regulation of CWR22Pc cells in culture, we compared the effect of dihydrotestosterone on PSA protein expression in CWR22Pc versus LNCaP or CWR22Rv1 cells. The promoter region of PSA is known to contain an androgen response element. The cells were cultured in the presence or absence of 0.8 nmol/L dihydrotestosterone, and the levels of PSA protein in whole-cell lysates were determined by Western blotting. The PSA protein levels were increased by 3-fold in CWR22Pc cells when the cells were cultured in the presence of dihydrotestosterone (Fig. 2B). Dihydrotestosterone increased PSA protein expression in LNCaP cells by ~2-fold. The levels of PSA protein in CWR22Rv1 cells were undetectable when compared with both CWR22Pc and LNCaP cells (Fig. 2B).

CWR22Pc cells are highly tumorigenic in nude mice and the tumor growth is regulated by androgens. Once we had established that CWR22Pc cells were genetically similar to the primary CWR22 tumors and the growth of CWR22Pc cells in culture is regulated by androgens, we next focused on evaluating whether CWR22Pc cells are tumorigenic in athymic nude mice and whether *in vivo* growth of CWR22Pc cells is regulated by androgens. To investigate whether CWR22Pc cells would grow as xenograft tumors in nude mice, we injected CWR22Pc cells (20×10^6 per site) s.c. to the flanks of athymic nude mice ($n = 20$; two tumors per mouse). The nude mice were castrated and half of the mice ($n = 10$) were implanted with sustained-release 5α -dihydrotestosterone pellets to normalize the circulating androgen levels. In the mice supplied with dihydrotestosterone pellets, tumors started to form on day 7 with a 100% incidence (Fig. 2C, i). Importantly, the tumors in mice supplied with the dihydrotestosterone pellets grew rapidly, whereas both the tumor incidence and the growth rate were low in mice without dihydrotestosterone pellets (Fig. 2C, i). These results suggested that CWR22Pc human prostate cancer cells are highly tumorigenic in athymic nude mice and the tumor growth is regulated by androgens.

Because CWR22Pc cells in culture produced high levels of PSA, and the PSA protein expression was regulated by androgens, our next aim was to investigate whether serum PSA levels would correspond with the volumes of the CWR22Pc xenograft tumors in mice. Serum PSA levels were determined from the blood samples collected on day 27 of the experiment (Fig. 2C, ii) from the athymic nude mice carrying CWR22Pc tumors presented in Fig. 2C, i. The results of the serum PSA assay showed that PSA protein levels in serum of the mice closely correlated with the volumes of the CWR22Pc subcutaneous xenograft tumors (Fig. 2C, ii). Specifically, serum PSA levels were almost undetectable in mice without dihydrotestosterone pellets carrying small tumors at low incidence. In contrast, serum PSA levels closely followed tumor volumes in dihydrotestosterone-supplied mice, which developed large CWR22Pc xenograft tumors with a high incidence (Fig. 2C, ii; the tumor volume is the mean of the two tumors in each mouse). Collectively, the results presented here indicate that the cells in subcutaneous CWR22Pc xenograft tumors secrete high levels of PSA to the circulation of the mice. Moreover, the serum PSA levels in these mice correlate with the volumes of the xenograft tumors.

CWR22Pc tumors recur after androgen withdrawal. In the next set of experiments, we first grew CWR22Pc cells as



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Fig. 3. Androgen receptor expression in CWR22Pc cells. *A*, AR expression in CWR22Pc, LNCaP, and CWR22Rv1 cells. Cells were cultured in the presence or absence of 0.8 nmol/L dihydrotestosterone, lysed, and immunoblotted with an anti-AR mAb. Filters were re-blotted with anti-actin pAb to show the protein loading. AR protein expressed in CWR22Rv1 is of higher molecular weight than in CWR22Pc and LNCaP cells. *B*, schematic presentation of AR mutations in primary CWR22 prostate tumors (CWR22P), CWR22Pc cells, recurrent CWR22 tumors (CWR22R), and CWR22Rv1 cells. *C*, AR gene sequencing primers. PCR primers were designed to amplify and sequence four overlapping segments (I, II, III, IV) encompassing the entire AR coding region plus 5' and 3' untranslated sequences. Segment I covers a part of the 5' untranslated region and the 5' end part of exon 1; segment II covers the 3' end of exons 1 and 2; segment III covers exons 2, 3, 5 and a part of exon 6. Segment IV covers a part of exons 5, 6, 7, 8 and a part of 3' untranslated region. *D*, RT-PCR products of the AR segment III in different human prostate cancer xenograft tumors and prostate cancer cell lines. The RT-PCR product for segment III yielded the expected amplicon size of 673 bp in CWR22Pc cells, in primary CWR22 xenograft tumors, and in LNCaP cells, whereas the CWR22Rv1 RT-PCR reaction yielded an ~100-bp larger RT-PCR product.

subcutaneous athymic nude mice supplied with dihydrotestosterone pellets in two consecutive experiments ($n = 34$ mice, 1 tumor per mouse and $n = 30$, 2 tumors per mouse). When the tumors reached 10 mm in diameter, the dihydrotestosterone pellets were removed and the tumor growth was followed by consecutive tumor volume measurements. The removal of the dihydrotestosterone pellets resulted in both experiments in a regression that reached the maximum in 15 days (Fig. 2D). Importantly, the tumors regrew back in the androgen-deprived nude mice with the following 20 to 30 days (Fig. 2D).

The human prostate cancer cell line CWR22Pc established from the primary CWR22 tumors provides several valuable advantages as a model system for androgen-regulated growth and development of androgen independence of prostate cancer cells. First, androgen-promoted tumor growth of CWR22Pc cells inoculated as subcutaneous tumors in nude mice occurs fast (in 3 weeks) with 100% tumor incidence. Second, development of hormone-refractory tumors is highly reproducible and the tumor regrowth takes place rapidly. Third, the CWR22Pc tumor system allows easy genetic *in vitro* genetic manipulation of the cells that form the tumors.

CWR22PC cells express ARs. To analyze the AR protein expression level in CWR22Pc cells, we immunoblotted CWR22Pc, LNCaP, and CWR22Rv1 cell lysates for AR using an anti-human AR mAb (Fig. 3A). The filters were stripped and reblotted with anti-actin pAb to show the loading of total proteins. The results show that AR protein is expressed at a high level in all three cell lines. Moreover, the results show that the size of AR in CWR22Rv1 cells is slightly larger compared with the AR in LNCaP and CWR22Pc cells. This was the expected result because the relapsed CWR22 tumors (CWR22R) and the CWR22Rv1 cell lines established from the relapsed tumors are known to express an AR gene that contains an in-frame tandem duplication of exon 3. This region encodes the second zinc finger of the AR DNA-binding domain and the AR protein product has an ~5-kDa increase in protein size relative to the LNCaP AR (16). Moreover, the AR gene in the relapsed CWR22 tumors and in CWR22Rv1 cells has the H874Y mutation in the ligand binding domain of AR, which enables the receptor to bind adrenal androgen DHEA in addition to estradiol, progesterone, and hydroxyflutamide (ref. 17; Fig. 3B). The AR gene also in the primary CWR22 tumors contains the H874Y (histidine to tyrosine) mutation. However, the AR gene in the primary CWR22 tumors does not have the tandem duplication in exon 3 present in CWR22Rv1 (Fig. 3B).

As the next step, we wanted to characterize the AR transcript in CWR22Pc cells by reverse transcription-PCR mapping and by sequencing to determine whether the AR gene in CWR22Pc cells contains the H874Y mutation found in the AR in the primary CWR22 tumors. Moreover, we wanted to determine whether the AR gene in CWR22Pc cells contains any additional mutations. To that end, we did RT-PCR mapping using primer pairs designated to amplify overlapping AR mRNA segments of ~600 to 900 bp and spanning the entire length of the AR coding sequence. The exons and the 5'- and 3'-untranslated sequences amplified by each of the primer sets are described in Fig. 3C. RT-PCR reactions done using RNA isolated from primary CWR22Pc cells, primary CWR22 xenograft tumors (CWR22P), LNCaP cells, and CWR22Rv1 cell line yielded

expected sizes for the amplification products of the segments I, II, and IV of the AR gene. The RT-PCR product for segment III, which spans the exon 3 of the AR gene, yielded the expected amplicon size of 673 bp in CWR22Pc cells, in primary CWR22 xenograft tumors, and in LNCaP cells, whereas the CWR22Rv1 RT-PCR reaction yielded an ~100-bp larger RT-PCR product due to the exon 3 tandem duplication as reported previously (ref. 16; Fig. 3D). We then purified the segments I-IV amplification products obtained from CWR22Pc cells and analyzed them by automated DNA sequencing followed by ClustalW-driven pairwise alignment with the reference AR cDNA sequence in Genbank (National Center for Biotechnology Information). The sequence comparison showed the H874Y mutation in the AR gene in CWR22Pc cells, whereas no additional mutations in the AR gene were found in CWR22Pc cells. In summary, these results showed that the sequence of the AR gene in the CWR22Pc cells has the H874Y mutation but not the exon 3 duplication. Moreover, the data indicate that the AR gene is identical to that in the primary CWR22 xenograft tumors, which indirectly supports the notion that CWR22Pc cell line is derived from the primary CWR22 tumors and is genetically different from the androgen-independent CWR22Rv1 cell line.

Currently, only few androgen-dependent human prostate cancer cell lines exist, such as LNCaP (3), LAPC-4 (18), and MDA PCa 2b (13, 19). Androgen-independent cell lines PC-3 (20) and DU145 (21) were derived from metastatic lesions to the bone and brain, respectively. Moreover, CWR22Rv1 human prostate cancer cell line (8) is independent of androgens for growth and was established from a xenograft tumor derived from an untreated primary prostate cancer (5). It is important to note that AR expression persists in clinical prostate cancer despite progression to hormone-refractory state (22). AR in prostate cancer typically undergoes genetic alterations, including AR gene amplification during hormone therapy. Moreover, 10% to 30% of prostate cancers acquire a point mutation in the AR gene (23). Both types of known AR gene alterations lead to increased sensitivity of the receptor to low levels of circulating androgens and also to the receptor's ability to recognize a broadened spectrum of ligands as potent agonists of AR action. DU145 and PC-3 cells are both AR-negative prostate cancer cell lines, whereas LNCaP, MDA pCa 2b, LAPC-4, and CWR22Rv1 cells express the AR protein. In LNCaP cells, the AR has a point mutation T877A, which makes the AR more sensitive to flutamide, estradiol, and progesterone (24). LAPC-4 cells express wild-type AR, whereas AR in MDA PCa 2b cells contains two mutations, T877A and L701H, which reduces the AR affinity to androgens but enhances binding of adrenal corticosteroids to the AR (25, 26). CWR22Rv1 cells are AR positive, but the AR contains a mutation H874Y in addition to a tandem duplication in exon 3 (16). The AR(H874Y) in CWR22Rv1 cells has been reported to be more sensitive to adrenal androgen DHEA and antiandrogen hydroxyflutamide. Our work presented here characterizes a new androgen-dependent human prostate cancer cell line that expresses AR having the H874Y mutation without the tandem duplication in exon 3. This is the first time such a human prostate cancer cell line is reported. CWR22Pc cells will provide a valuable experimental tool to investigate the importance of H874Y mutation in the AR for androgen regulation of prostate cancer cells as well as for development of androgen independence.

Cytokine signaling pathways are active in CWR22Pc cells. One of the key molecular mechanisms that promote prostate cancer cell growth involves protein kinase growth factor signaling pathways. These cell signaling pathways regulate prostate cancer cell survival, proliferation, and/or differentiation independently of AR or by affecting the transcriptional activity of AR. To investigate the activation status of the key known signaling pathways influencing growth and AR activity in prostate cancer cells, phosphorylation and total protein levels of Stat5a/b, Akt, MAPK, and Stat3 (27–36) were examined in exponentially growing CWR22Pc, CWR22Rv1, LNCaP, and DU145 cells. Immunoprecipitation and immunoblotting of Stat5a/b shows that Stat5a/b is active in the CWR22Pc cell line, whereas Stat3 is constitutively active only in DU145 cells but not in CWR22Pc cells (Fig. 4A, top). The filters were stripped and reblotted for total Stat5a/b and Stat3 protein levels. In addition, parallel samples of the cell lysates were immunoblotted with anti-actin to show the total protein level in each lane. Immunoblotting of whole-cell lysates with anti-phospho-MAPK-p44/42 antibody showed that MAPK is activated at a high level in exponentially growing CWR22Pc cells, whereas it is phosphorylated at a lower level in LNCaP, CWR22Rv1, and DU145 cells (Fig. 4B). Furthermore, Akt is also phosphorylated in CWR22Pc cells (Fig. 4B). Parallel

samples were immunoblotted to show total ERK and AKT levels as well as actin expression to show equal protein loading.

In the next set of experiments, we wanted to investigate the inducibility of Stat5a/b and Stat3 activation by cytokines prolactin and IL-6 in CWR22Pc cells compared with LNCaP, CWR22Rv1, and DU145 cells. The cells were first grown in a regular growth medium containing 10% FBS, serum-starved overnight, and stimulated with human prolactin (10 nmol/L) or IL-6 (4 nmol/L) for 15 min. Stat5a, Stat5b (Fig. 4C), or Stat3 (Fig. 4D) were immunoprecipitated and blotted with antibodies recognizing phosphorylated Stat5a/b or Stat3, respectively. The filters were stripped and reblotted with anti-Stat5ab or anti-Stat3 mAbs to show equal loading. Prolactin stimulation of CWR22Pc cells induced predominant activation of Stat5b, whereas the level of Stat5a expression in CWR22Pc cells was generally low. In CWR22Rv1 cell line, Stat5a and Stat5b were expressed at equal levels and prolactin induced phosphorylation of both Stat5a and Stat5b in CWR22Rv1 cells, whereas prolactin did not activate Stat5a/b in DU145 or LNCaP cells (Fig. 4C). IL-6 stimulated phosphorylation of Stat3 in CWR22Pc cells. In addition, IL-6 induced activation of Stat3 in LNCaP and DU145 cells, but not in CWR22Rv1 cells. In conclusion, Stat5a/b and Stat3 in CWR22Pc cells are activated by prolactin and IL-6, respectively.

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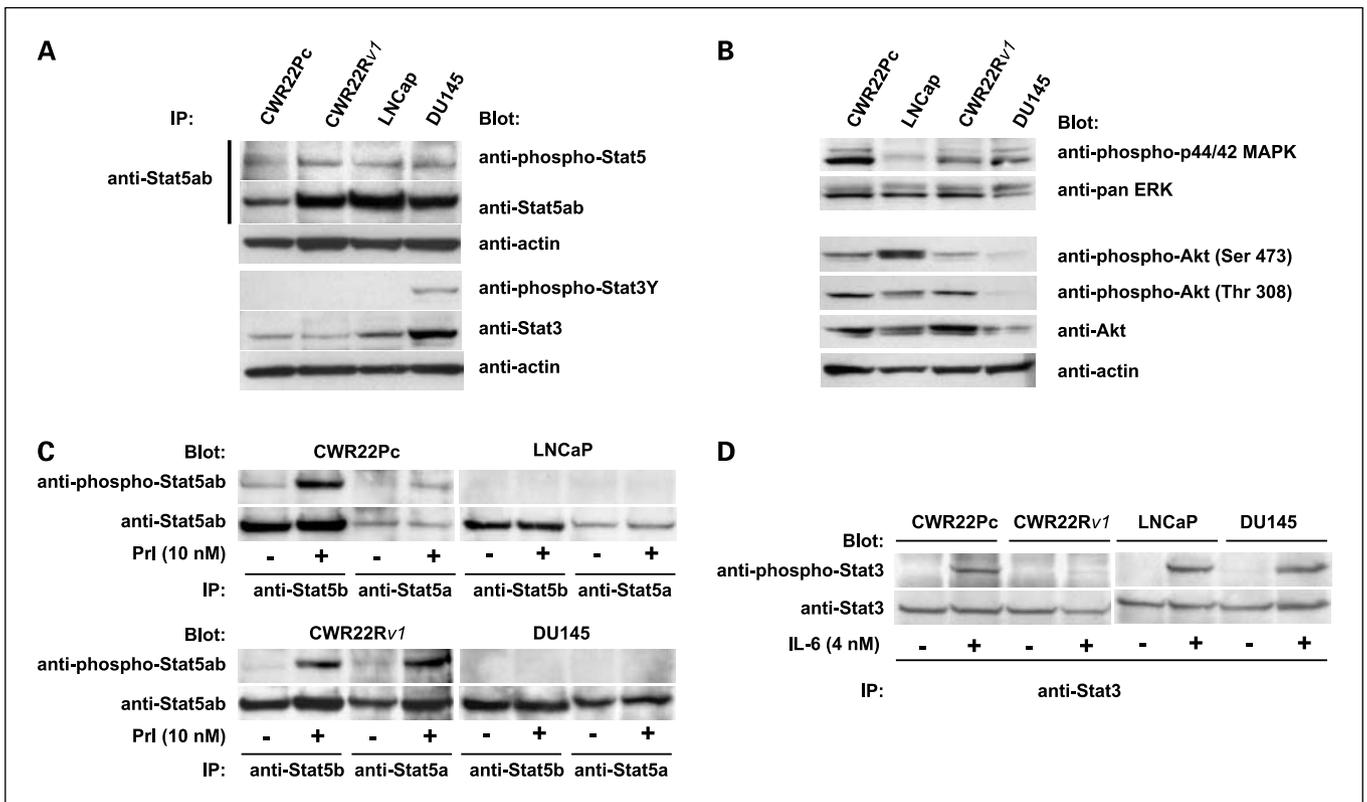


Fig. 4. Protein kinase signaling pathways are active in CWR22Pc cells. Transcription factor Stat5a/b, MAPK(p44/42), and AKT are constitutively active in CWR22Pc cells. **A**, Stat5ab or Stat3 were immunoprecipitated (IP) from exponentially growing CWR22Pc, CWR22Rv1, LNCaP, and DU145 cells using anti-Stat5ab or anti-Stat3 pAb, respectively, and blotted with anti-phospho-Stat5a/b or anti-phospho-Stat3 antibody as indicated. Filters were stripped and reblotted with anti-Stat5ab mAb or anti-Stat3 mAb and whole-cell lysates before immunoprecipitates were immunoblotted with anti-actin pAb. **B**, whole-cell lysates of exponentially growing CWR22Pc, LNCaP, CWR22Rv1, and DU145 cells were immunoblotted with antibodies to phosphorylated MAPK (anti-phospho-p44/42 MAPK), ERK (anti-pan-ERK), serine-phosphorylated AKT (anti-phospho-AKT Ser473), threonine-phosphorylated AKT (anti-phospho-AKT Thr³⁰⁸), AKT (anti-AKT), or actin (anti-actin). To investigate cytokine activation of Stat5a/b and Stat3 in CWR22Pc cells, all four cell lines (CWR22Pc, LNCaP, CWR22Rv1, and DU145) were serum-starved for 16 h and stimulated for 15 min with 10 nmol/L human prolactin (Prl; **C**) or 4 nmol/L IL-6 (**D**) and harvested. Stat5a, Stat5b, and Stat3 were immunoprecipitated and blotted with anti-phospho-Stat5ab or anti-phospho-Stat3 antibodies as indicated. The filters were stripped and reblotted with anti-Stat5ab mAb or anti-Stat3 mAb to show equal loading.

The CWR22Pc cell line provides a useful research tool to address a number of key questions pertinent to the basic biology of prostate cancer as well as clinical management of the disease. First, because growth of CWR22Pc cells in culture is strictly regulated by androgens, CWR22Pc cells will be able to provide critical information about androgen-regulated growth mechanisms in a prostate-specific cell context. Second, the CWR22Pc cell line provides a model system where significance of different protein kinase signaling pathway activation can be tested for their ability to replace androgens for growth promotion and maintenance of prostate cancer cell viability *in vitro* and *in vivo*. Third, CWR22Pc cells have the H874Y mutation in the AR gene without additional genetic changes and will therefore enable studies of AR (H874Y) transcriptional activity in a biological setting where prostate-specific coactivators and corepressors are expressed and present. Importantly, the regrowth of CWR22Pc tumors

in vivo in nude mice after androgen deprivation mimics the course of development of hormone-refractory prostate cancer in humans. Future studies need to characterize genetically and epigenetically the cell clones that recur when CWR22Pc cells are grown as xenograft tumors under the biological pressure of androgen deprivation. Finally, it will be important to establish whether CWR22Pc cells metastasize when inoculated orthotopically in nude mice.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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