Context-Dependent Hormone-Refractory Progression Revealed through Characterization of a Novel Murine Prostate Cancer Cell Line

Philip A. Watson,[1] Katharine Ellwood-Yen,[1] Jennifer C. King,[1] John Wongvipat,[1,2] Michelle M. LeBeau,[3] and Charles L. Sawyers[1,2]

[1]Division of Hematology/Oncology, Department of Medicine, and [2]Howard Hughes Medical Institute, University of California at Los Angeles, Los Angeles, California; and [3]Section of Hematology/Oncology, University of Chicago, Chicago, Illinois

Abstract

Insights into the molecular basis of hormone-refractory prostate cancer have principally relied on human prostate cancer cell lines, all of which were derived from patients who had already failed hormonal therapy. Recent progress in developing genetically engineered mouse prostate cancer models provides an opportunity to isolate novel cell lines from animals never exposed to hormone ablation, avoiding any potential bias conferred by the selective pressure of the castrate environment. Here we report the isolation of such a cell line (Myc-CaP) from a *c-myc* transgenic mouse with prostate cancer. Myc-CaP cells have an amplified androgen receptor gene despite no prior exposure to androgen withdrawal and they retain androgen-dependent transgene expression as well as androgen-dependent growth in soft agar and in mice. Reexpression of c-Myc from a hormone-independent promoter rescues growth in androgen-depleted agar but not in castrated mice, showing a clear distinction between the molecular requirements for hormone-refractory growth *in vitro* versus *in vivo*. Myc-CaP cells represent a unique reagent for dissecting discreet steps in hormone-refractory prostate cancer progression and show the general utility of using genetically engineered mouse models for establishing new prostate cancer cell lines. (Cancer Res 2005; 65(24): 11565-71)

Introduction

Patients receiving androgen deprivation therapy for locally recurring or metastatic prostate cancer almost inevitably experience treatment failure, with their cancers progressing to a hormone-refractory state (1). Unfortunately, current treatment modalities for hormone-refractory prostate cancer are mostly palliative and the prognosis of these patients remains especially dire, with a median survival of only 2.5 years (1). The molecular mechanisms responsible for the emergence of hormone-refractory prostate cancer are poorly understood but several hypotheses have been proposed. These include amplification, overexpression, or mutation of the androgen receptor (AR) as well as cross talk of AR and kinase signaling pathways (2).

The prostate cancer research community has relied heavily on the use of human cancer cell lines to explore the underlying causes of prostate cancer progression from androgen dependence to a hormone-refractory or androgen-independent stage. The three most commonly used lines, LNCaP (3), DU 145 (4), and PC-3 (5), were all isolated from metastatic lesions in patients that had progressed after receiving hormonal therapy. Both DU 145 and PC-3 are androgen independent but they do not express AR (6, 7). Consequently, the relevance of these cell lines in studying human hormone-refractory prostate cancer may be limited as most such tumors continue to express AR (8). In contrast, LNCaP is AR positive and androgen dependent in culture and in mice despite the fact that the line was derived from a patient with hormone-refractory disease. Whether this prior treatment history affects the conclusions made from studying LNCaP cells is unknown but it would be desirable to derive prostate cancer lines that have never been exposed to androgen ablation *in vivo*.

Historically it has been difficult, if not impossible, to derive stable cell lines from hormone-naive men with prostate cancer. However, establishment of cell lines from genetically defined mouse models of prostate cancer may offer an opportunity to gain a molecular insight into all stages of prostate carcinogenesis, including progression to hormone independence, due to the ability to control experimental conditions. Among the available mouse models of prostate cancer, cell lines have been described only from the TRAMP mouse which develops cancer due to prostate-specific expression of the SV40 large T antigen (9, 10). Paradoxically, the three TRAMP cell lines no longer express the SV40 T antigen (11).

Our laboratory recently described the Hi-Myc and Lo-Myc mice with a prostate-specific *c-myc* transgene, which drives carcinogenesis in a stepwise fashion from prostatic intraepithelial neoplasia to invasive cancer (12). Here, we report the characterization of a novel androgen-dependent prostate cancer cell line (Myc-CaP) from a *c-myc* transgenic mouse with prostate cancer and no prior hormonal therapy. Interestingly, Myc-CaP underwent amplification of AR despite not being subjected to androgen withdrawal. Furthermore, we discovered that variables that define hormone-refractory growth differ between the *in vitro* and *in vivo* setting.

Materials and Methods

Generation of Myc-CaP. A large prostate carcinoma dissected from a 16-month-old Hi-Myc transgenic mouse was minced, digested with 0.5% collagenase type I, and tissue fragments were filtered through a 40-μm mesh. Fragments trapped by the mesh were plated in tissue culture dishes coated with type I collagen (BD Biosciences, Mountain View, CA). Epithelial cells growing out of the tissue fragments were collected and single cells plated into a 96-well plate. The Myc-CaP cell line was established as a spontaneously immortalized line from one well. The line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 25 μg/mL bovine pituitary extract, 5 μg/mL bovine insulin, and 6 ng/mL recombinant human epidermal growth factor (all from Sigma-Aldrich, St. Louis, MO). The cells can also be passaged in DMEM or Iscove's

Requests for reprints: Charles L. Sawyers, University of California at Los Angeles, 9-240 Factor, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail: csawyers@mednet.ucla.edu.

doi:10.1158/0008-5472.CAN-05-3441

media with only 10% fetal bovine serum but we have not extensively characterized Myc-CaP grown in the absence of the additional growth factor supplements.

Quantitative reverse transcription-PCR. Total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, CA) and then treated with TURBO DNase (Ambion, Austin, TX). cDNA synthesis was carried out using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the protocol of the manufacturer. Quantitative PCR was done in the My iQ thermal cycler (Bio-Rad, Hercules, CA) using the 2× iQ SYBR Green Supermix (Bio-Rad). Quantitative PCR for each sample was run in triplicate and each reaction contained 1 μL of cDNA in a total volume of 20 μL. ΔC_t for each gene was determined after normalization to β-actin and $\Delta\Delta C_t$ was calculated relative to the designated reference sample. Gene expression values were then set equal to $2^{-\Delta\Delta Ct}$ as described (Applied Biosystems). All PCR primers were synthesized by Operon Biotechnologies (Huntsville, AL) and designed for the mouse sequence unless otherwise specified. The following primer pairs (written 5′ to 3′) were used at the indicated final concentration: *β-actin* (TGTTACCAACTGGGACGACA and GGGGTGTTGAAGGTCTCAAA at 600 nmol/L), human *c-myc* (AGCGACTCTGAGGAGGAACA and CTCTGACCTTTTGCCAGGAG at 300 nmol/L), mouse *c-myc* (CAACGTCTTGGAACGTCAGA and TCGTCTGCTTGAATGGACAG at 300 nmol/L), *AR* (GGACCATGTTTTACCCATCG and TCGTTTCTGCTGGCACATAG at 600 nmol/L), *Nkx3.1* (TCCGTCTTTTGGCTCTGAGT and GTGAAAGTGCACGCTGAAAA at 600 nmol/L), *Psca* (GCTCACTGCAACCATGAAGA and GCTAAGTAGGTGGCCAGCAG at 300 nmol/L), *chromogranin A* (GGGAGCTGGAACATAAGCAG and TGTCCTCCCATTCTCTGGAC at 300 nmol/L), *synaptophysin* (TGATCGTGTGTTGCCATTTT and AACAATACCGAAGGGCACAG at 600 nmol/L), *CK5* (ACCTTCGAAACACCAAGCAC and TTGGCACACTGCTTCTTGAC at 300 nmol/L), *CK14* (GACTTCCGGACCAAGTTTGA and CCTTGAGGCTCTCAATCTGC at 300 nmol/L), *CK8* (ATCGAGATCACCACCTACCG and TGAAGCCAGGGCTAGTGAGT at 300 nmol/L), *CK18* (ACTCCGCAAGGTGGTAGATG and GCCTCGATTTCTGTCTCCAG at 300 nmol/L), *TAp63* (AGACAAGCGAGTTCCTCAGC and CTGAGTCTTGCATGCGGATA at 300 nmol/L), *ΔNp63* (GGAAAACAATGCCCAGACTC and GAGAGAGGGCATCAAAGGTG at 300 nmol/L), and *Sca-1* (TTTGTTGTGGATTGCTGCTC and CTTCACTGTGCTGGCTGTGT at 600 nmol/L).

Immunofluorescence. Myc-CaP plated onto poly-L-lysine coated coverslips were fixed with ice-cold methanol, permeabilized with 0.1% Triton X-100, and nonspecific binding blocked with 10% goat serum (Vector Laboratories, Burlingame, CA). AR staining was accomplished using rabbit polyclonal anti-AR (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG; Invitrogen). Coverslips were then mounted and fluorescent images digitally captured using a Nikon Eclipse E800 microscope. Normal rabbit IgG was substituted for AR antibody as a negative control.

Western blotting. Whole-cell lysates were prepared in 2× SDS sample buffer with protease inhibitor cocktails (EMD Biosciences, San Diego, CA). Equal amounts of protein were run on a 10% SDS-PAGE gel. Immunoblotting was done with anti-AR (Santa Cruz Biotechnology) or anti-human c-Myc (Santa Cruz Biotechnology or Chemicon, Temecula, CA). An anti-β-actin antibody was used as a loading control (Sigma-Aldrich). Antimouse and rabbit horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Immunoblots were developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Retroviral transduction. A human *c-myc* cDNA was cloned into the *Bam*HI and *Eco*RI sites of the retroviral vector pQCXIN (BD Biosciences). pQCXIN/Myc was transfected into GP2-293 pantropic retroviral packaging cells (BD Biosciences) and the collected retrovirus used to infect Myc-CaP. Infected cells were selected with 800 to 1,000 μg/mL G418 (Invitrogen) and the surviving pool of cells was designated Myc-CaP/+Myc.

Soft agar colony formation assay. Soft agar assays were essentially done using the procedure of Lugo and Witte (13). The cell suspension was made with 0.3% noble agar and 20% fetal bovine serum or charcoal-stripped fetal bovine serum in Iscove's media. In some cases, R1881 was added to each of the layers. Cells were plated at a density of 1×10^4 per 6-cm dish or 3×10^4 per 10-cm dish and were evaluated for colonies after 14 to 21 days. Plates were photographed and the number of colonies was determined from images using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Mammary fat pad transplantation. 2×10^5 Myc-CaP were resuspended 1:1 in media/Matrigel (BD Biosciences) in a final volume of 30 μL. The cells were injected into the cleared abdominal mammary fat pads (two grafts per mouse) of adult sygeneic FVB male mice (Taconic, Germantown, NY) according to the procedure of DeOme et al. (14). In some experiments, the mice were castrated 3 weeks before grafting of the cells. When included, a 12.5-mg, 90-day-release testosterone pellet (Innovative Research of America, Sarasota, FL) was implanted 18 days after castration and 4 days before grafting of cells. For determining the effect of castration on established tumors, 2×10^4 Myc-CaP were grafted into mice and they were subsequently castrated when developing a tumor measuring 7 to 10 mm in one dimension. Tumor size was then periodically measured. The Mann-Whitney rank-sum test was used to statistically compare tumor burden among the treatment groups. Statistical analysis was carried out using SigmaStat (Systat Software, Point Richmond, CA).

Results

A novel prostate cancer cell line from the Hi-Myc transgenic mouse has phenotypic properties shared by the prostate transit amplifying and stem cells with genomic amplification of the wild-type androgen receptor. The generation of established prostate cancer cell lines from genetically defined mouse models of prostate cancer could facilitate a better understanding of prostate tumorigenesis. We therefore set out to create a cell line from our transgenic c-Myc driven model of prostate cancer. Primary prostate cells were isolated from a large carcinoma occupying all prostatic lobes in a 16-month-old Hi-Myc transgenic mouse. The Myc-CaP cell line subsequently emerged from a single-cell derived clone after spontaneous immortalization.

As the prostate cell lines derived from the TRAMP mouse did not maintain expression of the SV40 T antigen (11), our first objective was to assess whether Myc-CaP continued to express the human *c-myc* transgene. Using an antibody specific for human c-Myc, we did Western blotting on lysates from Myc-CaP and a prostate carcinoma from a Hi-Myc transgenic mouse. The TRAMP-C1 cell line served as a negative control for human c-Myc. Myc-CaP retained expression of c-Myc that was equivalent to the level seen in transgenic prostate carcinomas (Fig. 1*A*).

Given the importance of AR in prostate carcinogenesis, we also evaluated the AR status of Myc-CaP. Compared with both Hi-Myc carcinomas and TRAMP-C1, Myc-CaP unexpectedly displayed greatly elevated AR overexpression (Fig. 1*A*). Immunofluorescence staining confirmed that all Myc-CaP cells were uniformly positive for AR (Fig. 1*B*). Fluorescence *in situ* hybridization (FISH) with a bacterial artificial chromosome clone containing the mouse *AR* gene revealed that Myc-CaP had acquired extensive genomic amplification of *AR*. The amplified segments occurred as homogeneously staining regions on multiple chromosomes (Fig. 1*C*). We were unable to detect any mutations in *AR* after complete sequencing of all exons (data not shown). This *AR* amplification is remarkable in the sense that it occurred without subjecting Myc-CaP or the donor animal to hormonal ablation.

It is well established that the prostates of both humans and rodents consist of basal and luminal epithelium that can be distinguished on the basis of differential expression of phenotypic markers. However, there is a rare population of cells in the adult prostate that displays expression of both basal and luminal markers, a property that is shared by the epithelium of the embryonic urogenital sinus (15), which is the fetal precursor of the

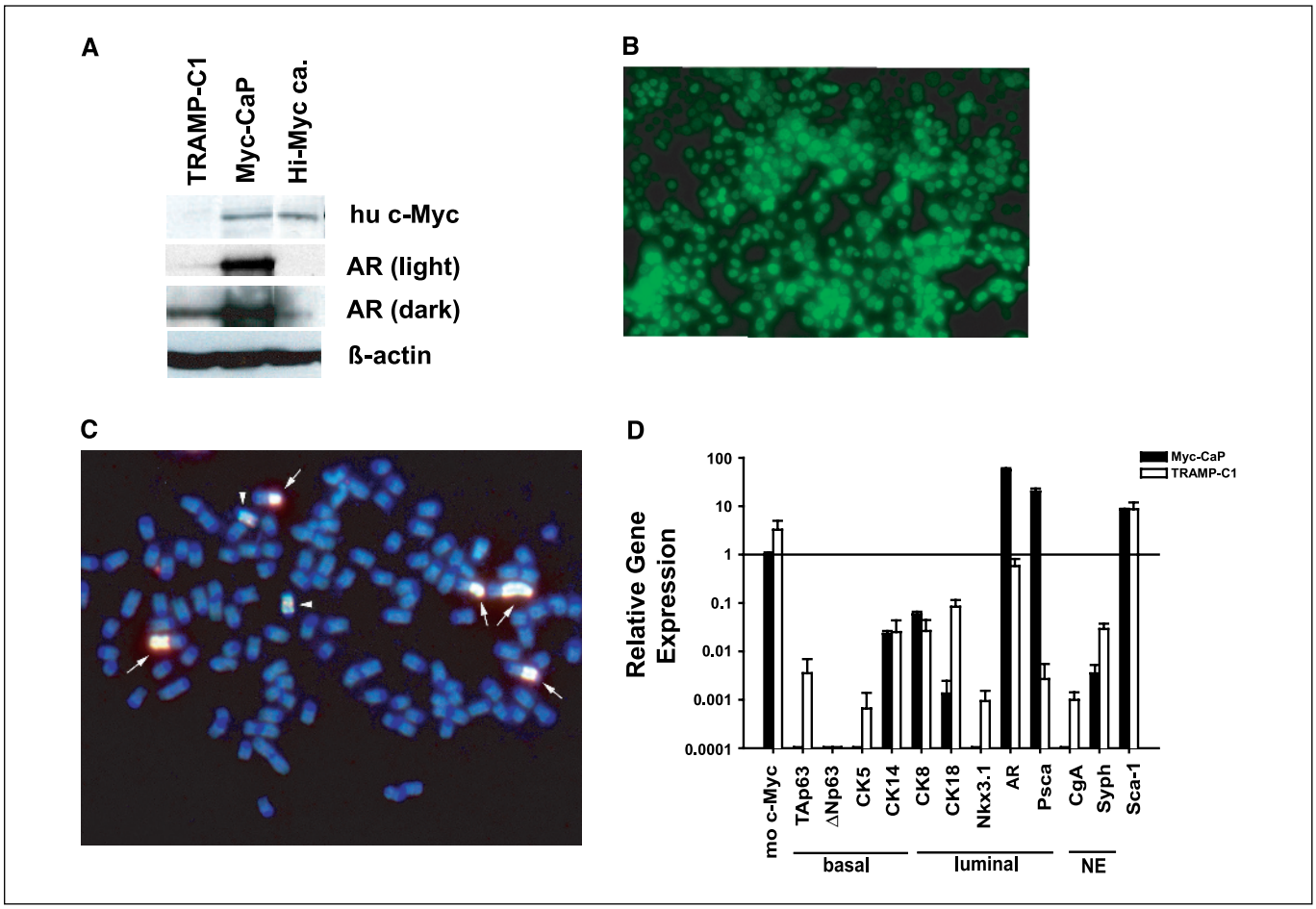

Figure 1. The Myc-CaP prostate carcinoma cell line overexpresses the wild-type AR and displays a phenotypic marker profile similar to prostate stem or transit amplifying cells. *A,* Western blots for human c-Myc and AR in TRAMP-C1, Myc-CaP, and a prostate carcinoma (*ca.*) from a Hi-Myc transgenic mouse. AR blots are shown with both light and dark exposures to highlight the overexpression in Myc-CaP. *B,* AR in Myc-CaP visualized by immunofluorescence after overnight treatment with 1 nmol/L R1881 (×200). *C, AR* amplification in Myc-CaP shown using dual-color FISH. A murine BAC clone containing the *AR* gene (RP23 191K6) labeled with digoxigenin and detected with rhodamine-conjugated antidigoxigenin antibodies revealed amplification of the *Ar* locus in homogeneously staining regions on multiple rearranged chromosomes (*arrows*). A control BAC clone (RP23 227O4) from the X chromosome (band XA2) labeled with biotin and detected with fluorescein identified two normal X chromosomes (*arrowheads*). *D,* quantitative RT-PCR quantification of prostate markers in Myc-CaP and TRAMP-C1. Values are expressed relative to mouse c-Myc of Myc-CaP. These markers are associated with the neuroendocrine (*NE*) cells or the basal or luminal cells of the prostate. *CK,* cytokeratin; *CgA,* chromogranin A; *Syph,* synaptophysin.

prostate. It has been proposed that this population of epithelial cells represents the progenitor/stem cell pool of the adult prostate (15). The specific type of epithelial cell where prostate cancer naturally originates is unknown but it has recently been shown in a prostate reconstitution system that a Sca-1$^+$ murine prostate progenitor population can be experimentally transformed by constitutive Akt overexpression (16).

We used quantitative reverse transcription-PCR (RT-PCR) to determine the pattern of phenotypic markers expressed by Myc-CaP in the interest of deducing the possible cell type of origin. A parallel analysis was done on TRAMP-C1 to reveal any similarities or differences between these two murine prostate cancer cell lines (Fig. 1*D*). After normalization to β-actin, the expression value for each gene was quantified relative to that of endogenous mouse *c-myc* in Myc-CaP. Mouse *c-myc* was arbitrarily designated as the reference gene due to a similarly intermediate abundance in both cell lines. Both Myc-CaP and TRAMP-C1 were concomitantly positive for basal (CK14) and luminal (CK8) associated cytokeratins. Western blots of Myc-CaP for each of the cytokeratins were consistent with the quantitative RT-PCR results (data not shown). Furthermore, both cell lines expressed Psca, which has been ascribed to a population of prostate cells with a differentiation profile intermediate between that of basal and luminal (17). However, the level of Psca expression was considerably higher in Myc-CaP. Recent reports have identified Sca-1 as marking a subpopulation of epithelial cells within the mouse prostate with stem cell properties (16, 18). Interestingly, Myc-CaP and TRAMP-C1 were also positive for Sca-1 by quantitative RT-PCR. Immunofluorescence and flow cytometry analysis confirmed that >99% of Myc-CaP expressed Sca-1 (data not shown). Collectively, this profile of expressed phenotypic markers suggests that prostate cancer cell lines originating from both *c-myc* and TRAMP transgenic mice may be aberrant descendants of a normal prostatic transit amplifying or stem cell.

The *in vitro* and *in vivo* growth of Myc-CaP is androgen dependent. *AR* is amplified in ~30% of hormone-refractory human prostate cancers but this rarely, if at all, occurs in prostate tumors before androgen deprivation therapy (19, 20). Consequently, it is believed that *AR* amplification provides a mechanistic means of progressing to androgen independence. Therefore, we evaluated

whether Myc-CaP had acquired the ability to grow independently of androgens in part through overexpression of AR. Initial experiments using cells growing in liquid media revealed that the androgen dependency of Myc-CaP was variable, depending on culture conditions. In an effort to develop a more robust *in vitro* assay for hormone dependence, we assessed the growth of Myc-CaP in soft agar in the presence or absence of androgen. Cells were plated into agar containing 10% FBS or 10% charcoal-stripped serum (CSS) plus the synthetic androgen R1881 in concentrations ranging from 0 to 1.0 nmol/L. After 17 days, the plates were photographed and scored for colony formation. Representative images are shown in Fig. 2*A* with the number of colonies graphically represented in Fig. 2*B*. There was minimal growth with CSS but Myc-CaP efficiently formed colonies with FBS (6.2-fold higher compared with CSS). R1881 at 0.1 to 1.0 nmol/L rescued the ability of Myc-CaP to grow in soft agar with CSS.

Because colony formation in soft agar often correlates with *in vivo* tumorigenesis, we next tested whether Myc-CaP would form tumors when grafted into the gland-cleared mammary fat pad of syngeneic male mice. Using 2×10^5 cells per graft, 100% of the sites developed tumors by 4 to 5 weeks. Histologic analysis revealed that Myc-CaP carcinomas were uniformly composed of sheets of undifferentiated cells (data not shown). We next determined the response of established Myc-CaP tumors to androgen withdrawal. 2×10^4 Myc-CaP were grafted bilaterally into intact mice (n = 12 graft sites) and each mouse was subsequently castrated 37 days later when at least one of its graft sites had developed a large tumor ($\sim$500 mm^3). Because of the heterogeneous growth rate of individual tumors, there was a wide range of tumor volumes at the time of castration (tumor volume range, 81-1,458 mm^3; median, 466 mm^3). Tumor volumes were then measured at multiple time points after castration (designated as day 0) until day 13 when all of the mice were sacrificed. To simplify presentation of the data, individual tumors are plotted together based on volume at day 0 (<500 or >500 mm^3; Fig. 2*C* and *D*, respectively).

Eleven of twelve tumors partially regressed by 3 days after castration. The mean percent reduction in tumor volume was larger for those tumors <500 mm^3 at day 0 compared

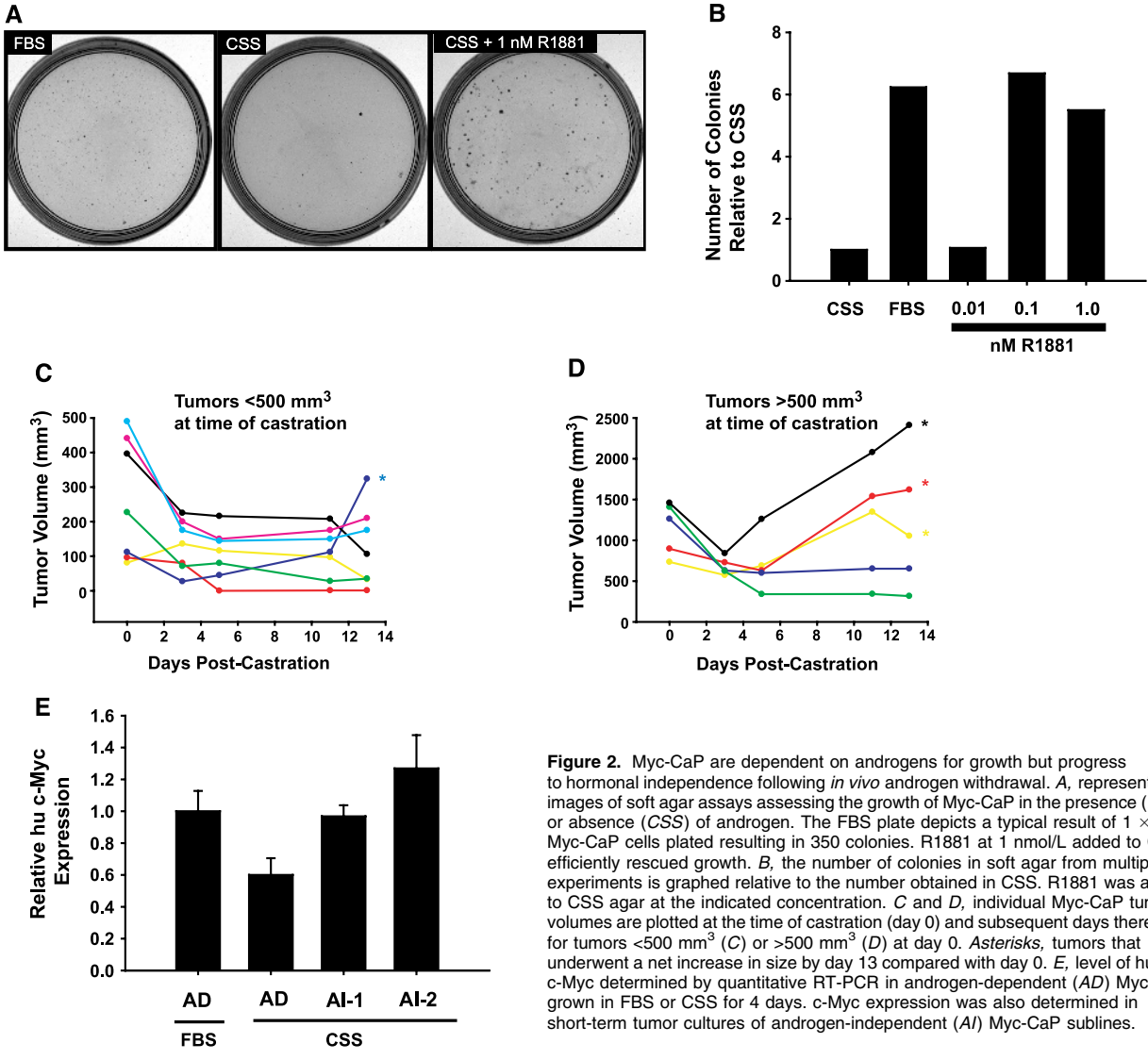

Figure 2. Myc-CaP are dependent on androgens for growth but progress to hormonal independence following *in vivo* androgen withdrawal. *A,* representative images of soft agar assays assessing the growth of Myc-CaP in the presence (*FBS*) or absence (*CSS*) of androgen. The FBS plate depicts a typical result of 1×10^4 Myc-CaP cells plated resulting in 350 colonies. R1881 at 1 nmol/L added to CSS efficiently rescued growth. *B,* the number of colonies in soft agar from multiple experiments is graphed relative to the number obtained in CSS. R1881 was added to CSS agar at the indicated concentration. *C* and *D,* individual Myc-CaP tumor volumes are plotted at the time of castration (day 0) and subsequent days thereafter for tumors <500 mm^3 (*C*) or >500 mm^3 (*D*) at day 0. *Asterisks,* tumors that underwent a net increase in size by day 13 compared with day 0. *E,* level of human c-Myc determined by quantitative RT-PCR in androgen-dependent (*AD*) Myc-CaP grown in FBS or CSS for 4 days. c-Myc expression was also determined in short-term tumor cultures of androgen-independent (*AI*) Myc-CaP sublines.

with those >500 mm^3 at day 0 (60% versus 38%, respectively). At 13 days after androgen withdrawal, seven tumors were present at a smaller size than before castration and one tumor had completely regressed. In contrast, four tumors had undergone a net increase in size compared with day 0 (range, 43-189% tumor volume increase) after the initial period of regression. We extended the observation period postcastration in an additional two mice bearing small tumors (n = 3 tumors; range, 36-72 mm^3) at the time of castration. Complete regression of all three tumors, as determined by palpation, occurred after 8 days. Nonetheless, at the time of necropsy at 35 days postcastration, all three graft sites had large tumors. These results revealed that although the Myc-CaP tumors are androgen dependent, hormone-refractory sublines can be reproducibly generated once exposed to androgen deprivation.

The *c-myc* transgene in normal and neoplastic prostate tissue of the transgenic mice is dependent on androgens for maximal induction (12) due to the nature of the promoter that directs prostate-specific expression (21). To determine if Myc-CaP displayed the same androgen-dependent transgene expression, we quantified human c-Myc levels by quantitative RT-PCR in Myc-CaP cells maintained in FBS or 4 days after androgen withdrawal (CSS). Not surprisingly, removal of androgens decreased c-Myc expression by 40% (Fig. 2*E*). The fact that Myc-CaP tumors can acquire the capacity to grow in castrated animals raised the issue of whether this was occurring in the absence of transgene expression. Two of the tumors that had increased in size after castration [AI-1 (yellow plot) and AI-2 (black plot); Fig. 2*D* and *E*] were harvested on day 13 after castration and placed into short-term culture without androgen (CSS) where they continued to proliferate. Expression of the *c-myc* transgene was determined by quantitative RT-PCR. Remarkably, both AI tumor samples displayed a level of c-Myc mRNA equal to or greater than the parental androgen-dependent Myc-CaP cells grown in FBS (Fig. 2*E*). This finding suggests that there is renewed AR function in the hormone-refractory Myc-CaP tumors despite a castrate level of androgens. This is analogous to reexpression of prostate-specific antigen in hormone-refractory human prostate cancers.

Continuous maintenance of c-Myc relieves the dependency on androgens for growth *in vitro* but not *in vivo*. We wanted to explore whether the continual maintenance of elevated c-Myc would be adequate to convert the androgen-dependent Myc-CaP cells to androgen independence. To uncouple c-Myc from its androgen dependency, we created a subline of Myc-CaP containing an additional human c-Myc expression construct under the regulatory control of the constitutive cytomegalovirus (CMV) promoter. We then assessed the levels of human c-Myc by Western blotting in Myc-CaP and Myc-CaP/+Myc in FBS and CSS (Fig. 3*A*). CMV-driven c-Myc could be distinguished from ARR_2PB (transgene) c-Myc by molecular weight due to additional sequences in the transgenic c-Myc construct. In agreement with the quantitative RT-PCR results, there was a substantial reduction in ARR_2PB regulated c-Myc protein levels in CSS relative to FBS for both Myc-CaP (*lane 1* versus *lane 3*) and Myc-CaP/+Myc (*lane 2* versus *lane 4*). In contrast, the CMV-driven c-Myc of Myc-CaP/+Myc was not decreased in CSS (*lane 2* versus *lane 4*).

We used the CSS soft agar assay to address whether c-Myc add-back was sufficient to overcome the androgen growth dependency of Myc-CaP cells. As expected, parental Myc-CaP grew poorly in CSS agar; however, Myc-CaP/+Myc cells formed colonies either in CSS or FBS agar (Fig. 3*B*). Importantly, there was an equivalent number of colonies for Myc-CaP/+Myc grown in CSS compared with parental cells with FBS, showing that ectopic c-Myc expression was sufficient to confer hormone-refractory growth to Myc-CaP cells *in vitro*.

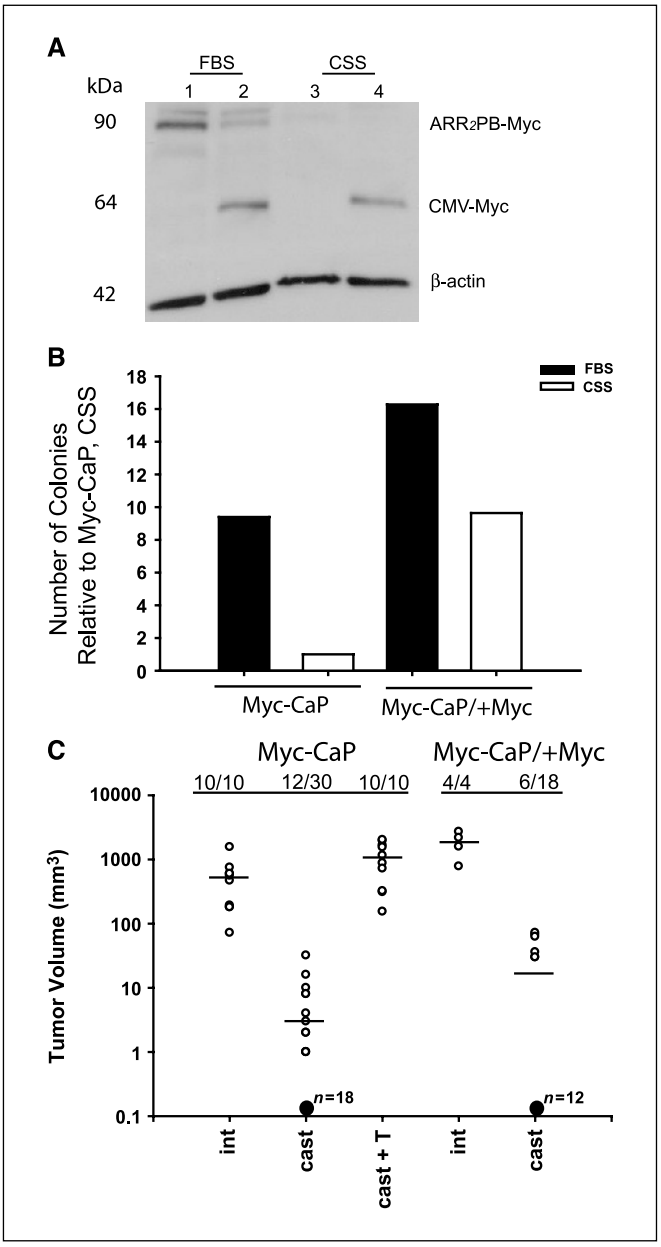

Figure 3. Constitutive ectopic expression of c-Myc can rescue the *in vitro*, but not the *in vivo*, growth of Myc-CaP in the absence of androgen. *A,* immunoblotting for c-Myc in Myc-CaP (*lanes 1* and *3*) or Myc-CaP/+Myc (*lanes 2* and *4*) grown in FBS or CSS. Myc-CaP/+Myc cells have c-Myc bands from both the transgene (ARR_2PB) and the CMV promoters. *B,* Myc-CaP and Myc-CaP/+Myc cells were grown in soft agar with (*FBS*) or without (*CSS*) androgen. The average from two independent experiments is shown as the number of colonies relative to that obtained with Myc-CaP in CSS. *C,* individual tumor volumes at necropsy are plotted for Myc-CaP or Myc-CaP/+Myc grafted into either intact (*int*) or castrated (*cast*) mice. Where indicated, testosterone pellets were implanted into castrated mice before grafting of the cells. Because of overlapping values, not all data points are visible. *Horizontal line,* mean tumor volume within each group. *Black circles,* grafts with no tumor, with the number of such cases noted. The ratios at the top indicate the number of graft sites positive for tumor at necropsy relative to the total number of grafts for each group.

Next, we expanded the analysis to evaluate the tumorigenic potential of Myc-CaP/+Myc cells grafted into the mammary fat pads of mice castrated 3 weeks earlier (Fig. 3*C*). Intact mice also received grafts and served as positive controls for tumor formation. All mice were sacrificed 28 to 30 days after grafting due to the size of tumors in the intact groups. Sixty percent of the sites grafted with Myc-CaP in castrates were tumor-free. The remaining 40% of Myc-CaP grafts in castrated mice contained tumors of negligible size (mean tumor volume, 2.9 mm^3). This contrasts with the mean tumor volume in intact hosts of 516 mm^3 ($P < 0.001$ compared with castrates). As expected, administration of testosterone to castrated mice completely rescued tumor formation. However, in contrast to the *in vitro* soft agar assays, c-Myc add-back did not rescue tumor growth in castrated hosts. Similar to parental cells, 67% of Myc-CaP/+Myc graft sites in castrated mice were tumor-free. Thirty-three percent of the fat pads contained tumors with a mean volume of 16.6 mm^3. Compared with castrated mice receiving parental Myc-CaP, the increase in tumor volume was not statistically significant ($P = 0.741$). Hence, the maintenance of elevated c-Myc levels was not sufficient for androgen-independent tumor formation by Myc-CaP.

Discussion

The Myc-CaP cell line described here has a number of properties that make it a useful reagent to study the progression of prostate cancer to a hormone-refractory stage. Myc-CaP cells are easy to maintain in culture and are readily transfectable and infectable. Furthermore, Myc-CaP cells express wild-type AR and display a clear androgen dependency both in soft agar and in mice. In addition, hormone-refractory Myc-CaP tumors can be reproducibly derived after exposure to androgen deprivation. Of note, the primary prostate tumor that gave rise to Myc-CaP had never been exposed to hormonal ablation. This property differs from hormone-dependent human prostate cancer cell lines, such as LNCaP and LAPC4 (22), which were obtained from metastatic lesions in patients previously exposed to hormonal therapy. Therefore, Myc-CaP may offer certain advantages for studying the progression of prostate cancer.

Amplification of *AR* occurs in ~30% of hormone-refractory human prostate cancers (19, 20). It is believed that overexpression of AR provides a selective advantage in the castrate setting due to increased sensitization to the residual level of androgens present in men treated with hormone therapy. Our results clearly show, however, that *AR* amplification can occur without prior androgen ablation. This phenomenon is not simply a unique feature of mouse prostate cancer cell lines as the TRAMP-C1 line does not have AR overexpression. In the absence of selective pressure, it is interesting to speculate why Myc-CaP underwent amplification and overexpression of AR. One possibility is that increased AR simply maintains elevated levels of c-Myc, thereby giving AR amplified cells a growth advantage. Alternatively, AR may itself promote tumorigenesis, as recently suggested in studies of immortalized human prostate epithelial cells (23). The fact that Myc-CaP cells remain androgen dependent despite high AR levels is of interest because we have previously shown that overexpression of AR was sufficient to promote hormone-refractory growth of two human prostate cancer cell lines (LNCaP and LAPC4; ref. 24). These distinct phenotypes of AR overexpression in Myc-CaP versus LNCaP and LAPC4 might be explained by the timing of AR dysregulation relative to additional oncogenic events required for prostate cell transformation. In the Myc-CaP system, c-Myc and AR amplification may be relatively early events that cooperate with additional unknown lesions to promote hormone-refractory growth. It should be possible to identify these lesions through the generation and characterization of hormone-refractory Myc-CaP sublines. In contrast, LNCaP and LAPC4 human prostate cancer cell lines may have already acquired these additional oncogenic hits and simply need increased AR levels to promote hormone-refractory progression. Genetically engineered mouse models offer an opportunity to order the timing of distinct molecular events, enabling these issues to be directly addressed.

c-myc is often amplified in late-stage human prostate cancers (25, 26). However, it is unclear if this simply marks a more aggressive form of the disease or whether c-Myc plays a specific role in mediating hormonal independence. One recent study reported that c-Myc could rescue the *in vitro* growth of LNCaP in androgen-depleted media (27), analogous to our finding that c-Myc rescues Myc-CaP growth in androgen-depleted soft agar. However, because Myc-CaP cells evolved with expression of the c-Myc transgene, the c-Myc-associated rescue observed in this system may not specifically address its role in hormone dependence. Nevertheless, our *in vivo* results indicate that constitutive expression of elevated c-Myc is insufficient for disease progression and point to the importance of additional events for hormone-independent growth.

It is of interest to consider potential mechanisms for the differential rescue of hormone-refractory growth by c-Myc *in vitro* versus *in vivo*. One possibility is that there is a requirement that specific genes, normally regulated by androgen, must be expressed by the cancer cell to become hormone-refractory *in vivo*, but these genes are not necessary for *in vitro* hormone-refractory growth. Alternatively, there could be androgen-regulated paracrine factors secreted by the stroma that modulate the progression to hormonal independence. There is clear precedent that mesenchymal tissue can influence the growth of normal or neoplastic prostate epithelium. For example, cancer-associated fibroblasts can stimulate the tumorigenic progression of immortalized nontumorigenic human prostate epithelial cells (28, 29). Conditional inactivation of the transforming growth factor-β type II receptor in mouse stroma resulted in prostatic intraepithelial neoplasia (30). Furthermore, androgen-regulated soluble factors have been implicated in the inductive effects of urogenital mesenchyme on the differentiation of the urogenital sinus epithelium (31, 32). Conversely, the growth of a human prostate cancer line was not affected by loss of AR expression in the stromal cells (33). Using the Myc-CaP cell line described here, it should be possible to address the relative role of cell intrinsic versus stromal factors in regulating the progression of prostate cancer to hormone independence.

Acknowledgments

Received 9/26/2005; accepted 10/7/2005.

Grant support: U.S. Army Medical Research and Material Command grants DAMD17-03-1-0086 (P.A. Watson) and W81XWH-04-1-0822 (J.C. King), the Giannini Family Foundation Award no. 04012566 (K. Ellwood-Yen) and the National Cancer Institute grants U01 CA084221 (M.M. LeBeau) and U01 CA084128 (C.L. Sawyers).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Sternberg CN. Highlights of contemporary issues in the medical management of prostate cancer. Crit Rev Oncol Hematol 2002;43:105–21.
2. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001; 1:34–45.
3. Horoszewicz JS, Leong SS, Chu TM, et al. The LNCaP cell line—a new model for studies on human prostatic carcinoma. Prog Clin Biol Res 1980;37:115–32.
4. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF. Isolation of a human prostate carcinoma cell line (DU 145). Int J Cancer 1978;21:274–81.
5. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 1979;17: 16–23.
6. van Bokhoven A, Varella-Garcia M, Korch C, et al. Molecular characterization of human prostate carcinoma cell lines. Prostate 2003;57:205–25.
7. Tilley WD, Wilson CM, Marcelli M, McPhaul MJ. Androgen receptor gene expression in human prostate carcinoma cell lines. Cancer Res 1990;50:5382–6.
8. Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res 1995; 55:3068–72.
9. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A 1995;92:3439–43.
10. Gingrich JR, Barrios RJ, Morton RA, et al. Metastatic prostate cancer in a transgenic mouse. Cancer Res 1996;56:4096–102.
11. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res 1997; 57:3325–30.
12. Ellwood-Yen K, Graeber TG, Wongvipat J, et al. Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer Cell 2003;4:223–38.
13. Lugo TG, Witte ON. The BCR-ABL oncogene transforms Rat-1 cells and cooperates with v-myc. Mol Cell Biol 1989;9:1263–70.
14. DeOme KB, Faulkin LJ, Jr., Bern HA, Blair PB. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 1959;19: 515–25.
15. Wang Y, Hayward S, Cao M, Thayer K, Cunha G. Cell differentiation lineage in the prostate. Differentiation 2001;68:270–9.
16. Xin L, Lawson DA, Witte ON. The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proc Natl Acad Sci U S A 2005;102:6942–7.
17. Tran CP, Lin C, Yamashiro J, Reiter RE. Prostate stem cell antigen is a marker of late intermediate prostate epithelial cells. Mol Cancer Res 2002;1:113–21.
18. Burger PE, Xiong X, Coetzee S, et al. Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue. Proc Natl Acad Sci U S A 2005;102:7180–5.
19. Visakorpi T, Hyytinen E, Koivisto P, et al. *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995; 9:401–6.
20. Koivisto P, Kononen J, Palmberg C, et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 1997;57:314–9.
21. Zhang J, Thomas TZ, Kasper S, Matusik RJ. A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids *in vitro* and *in vivo*. Endocrinology 2000;141:4698–710.
22. Klein KA, Reiter RE, Redula J, et al. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nat Med 1997;3:402–8.
23. Berger R, Febbo PG, Majumder PK, et al. Androgen-induced differentiation and tumorigenicity of human prostate epithelial cells. Cancer Res 2004;64:8867–75.
24. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9.
25. Jenkins RB, Qian J, Lieber MM, Bostwick DG. Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence *in situ* hybridization. Cancer Res 1997; 57:524–31.
26. Nupponen NN, Kakkola L, Koivisto P, Visakorpi T. Genetic alterations in hormone-refractory recurrent prostate carcinomas. Am J Pathol 1998;153:141–8.
27. Bernard D, Pourtier-Manzanedo A, Gil J, Beach DH. Myc confers androgen-independent prostate cancer cell growth. J Clin Invest 2003;112:1724–31.
28. Hayward SW, Wang Y, Cao M, et al. Malignant transformation in a nontumorigenic human prostatic epithelial cell line. Cancer Res 2001;61:8135–42.
29. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999;59:5002–11.
30. Bhowmick NA, Chytil A, Plieth D, et al. TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 2004;303:848–51.
31. Shima H, Tsuji M, Elfman F, Cunha GR. Development of male urogenital epithelia elicited by soluble mesenchymal factors. J Androl 1995;16:233–41.
32. Cunha GR, Lung B. The possible influence of temporal factors in androgenic responsiveness of urogenital tissue recombinants from wild-type and androgen-insensitive (Tfm) mice. J Exp Zool 1978;205: 181–93.
33. Gao J, Isaacs JT. Development of an androgen receptor-null model for identifying the initiation site for androgen stimulation of proliferation and suppression of programmed (apoptotic) death of PC-82 human prostate cancer cells. Cancer Res 1998;58: 3299–306.