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Androgen-Induced Differentiation and Tumorigenicity of Human Prostate Epithelial Cells

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ABSTRACT

Androgen ablation is the primary treatment modality for patients with metastatic prostate cancer; however, the role of androgen receptor signaling in prostate cancer development remains enigmatic. Using a series of genetically defined immortalized and tumorigenic human prostate epithelial cells, we found that introduction of the androgen receptor induced differentiation of transformed prostate epithelial cells to a luminal phenotype reminiscent of organ-confined prostate cancer when placed in the prostate microenvironment. Moreover, androgen receptor expression converted previously androgen-independent, tumorigenic prostate epithelial cells into cells dependent on testosterone for tumor formation. These observations indicate that androgen receptor expression is oncogenic and addictive for the human prostate epithelium.

INTRODUCTION

Prostate cancer is the most frequently diagnosed, nondermatologic cancer in men. Androgen receptor (AR) signaling plays a critical role in the normal development, proliferation, and differentiation of the prostate (1–3). Moreover, androgen ablation therapy remains one of the only treatments that prolong life for men with metastatic prostate cancer (4). However, AR expression in normal human prostate epithelial cells (PrECs) is associated with differentiation, suggesting that alterations in the genetics or environment of the PrECs occur to convert the response of such cells to AR signaling from differentiation to transformation. At present, we lack a comprehensive understanding of the genetic events sufficient for prostate epithelial transformation and the genetic alterations that modulate the cellular response to AR.

To study the molecular alterations associated with prostate cancer, several groups have developed human and murine experimental systems (5, 6). However, prostate cancer cell lines have proven difficult to isolate and often fail to recapitulate early stage disease (7). As a result, much of our knowledge of prostate cancer biology is based on a few prostate cancer cell lines derived from patients with metastatic disease (8–10) and represent a small subset of this disease. Furthermore, the available cell lines harbor an unknown collection of genetic alterations, making the identification and characterization of the roles of specific molecular pathways difficult. In particular, the majority of these cell lines fail to express AR.

Although infection with oncogenic DNA tumor viruses (11–13) also has been used to transform PrECs, these strategies select for rare cells that survive extended passage in culture. Several groups more recently have produced transgenic murine models that develop prostate hyperplasia or prostate cancer following prostate-specific overexpression of oncogenes or knockout of tumor suppressor genes previously implicated in prostate cancer (14). Although these models will certainly provide critical insights into the cell autonomous and non–cell autonomous interactions that cooperate to program prostate cancer, the cost and time required to develop and characterize these models are significant. Moreover, because the murine and human prostate clearly differ with respect to developmental biology and anatomy, it will be important to compare these observations in murine models with human prostate cancer specimens.

We and others have shown that a number of primary human cells are immortalized by the introduction of the telomerase catalytic subunit human telomerase reverse transcriptase (hTERT) and manipulation of the retinoblastoma (pRB) and p53 pathways (15–20). Such immortal cells are converted into transformed cells capable of tumorigenic growth by the further introduction of an oncogenic allele of H-*ras* and the SV40 early region oncoprotein small t antigen (ST; ref. 15). Here, we apply this system of human cell transformation to human PrECs to understand the role of AR in PrEC differentiation and transformation. Immortalized and tumorigenic PrECs generated in this manner recapitulate many features of the normal and malignant human prostate. Using these experimental models, we find that AR expression is oncogenic and addictive for the human prostate epithelium.

MATERIALS AND METHODS

Development of Prostate Epithelial Cells. Human PrECs were obtained from BioWhittaker (Rockland, ME) and propagated in defined medium (PrEGM) as recommended. PrECs were infected with combinations of amphotropic retroviruses encoding the SV40 large T antigen (LT), ST, *hTERT*, H-*ras*, phosphatidylinositol 3′-kinase (PI3K), and *MYC* as described previously (16, 21). Wild-type AR was introduced using an AR cDNA cloned into the pWZL retrovirus with a blasticidin selection cassette (22). PrEC LH cells express SV40 LT and *hTERT*. PrEC LHS cells express LT, *hTERT*, and ST. PrEC LHSR cells express SV40 LT, *hTERT*, ST, and H-*ras*. PrEC LHMK cells express SV40 LT, *hTERT*, *MYC*, and PI3K. PrEC LHS-AR and PrEC LHSR-AR indicate the cell lines that express wild-type AR.

Androgen Stimulation. PrEC LHSR-AR and LHS-AR cells were propagated in defined media for 2 days, stimulated with 1 nmol/L of R1881, and collected at times ranging from 0 to 5 days. During androgen stimulation, cells were counted at each passage to assess cumulative population doublings.

Immunoblot Analysis and Immunofluorescence. PrECs were starved overnight in PrEBM without supplements and then lysed in 1.25% SDS, 0.0125 $NaPO_4$ (pH 7.2), 50 mmol/L NaF, 2 mmol/L EDTA, 1.25% NP40, 1 mmol/L sodium vanadate, and a pellet of complete protease inhibitor mixture (Roche, Indianapolis, IN). Lysates were sonicated, centrifuged at 12,000 × *g*

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for 10 minutes at 4°C to remove insoluble material, boiled (100 μg for each sample) for 5 minutes, separated by 10% SDS-PAGE, transferred to nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Piscataway, NJ), and immunoblotted with the indicated antibodies.

Monolayers were fixed in 2% formalin (Sigma, St. Louis, MO) at room temperature for 25 minutes, washed three times in PBS/glycine (130 mmol/L NaCl, 7 mmol/L Na_2HPO_4, 3.5 mmol/L NaH_2PO_4, and 100 mmol/L glycine) for 15 minutes and blocked in immunofluorescence buffer (130 mmol/L NaCl, 7 mmol/L Na_2HPO_4, 3.5 mmol/L NaH_2PO_4, 7.7 mmol/L NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, and 0.05% Tween 20) plus 10% goat serum and 20 μg/mL goat antimouse $F(ab')_2$ for 1 to 2 hours. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. After washing three times, antimouse secondary antibodies coupled to Alexa Fluor dyes (Molecular Probes, Eugene, OR) were diluted in immunofluorescence buffer containing 10% goat serum and incubated for 45 to 60 minutes. The structures were incubated for 15 minutes with PBS containing 0.5 ng/mL 4′,6-diamidino-2-phenylindole (Roche, Indianapolis, IN) before mounted with the antifade agent Prolong (Molecular Probes). Confocal analyses were performed with Zeiss LSM410 confocal microscopy systems (Oberkochen, Germany).

Antibodies were obtained from the following sources: anti–phospho-Akt (Ser473) and anti-Akt from Cell Signaling Technologies (Beverly, MA); anti–H-*ras* (C-20), anti– c-*myc* (9E10), and anti–SV40-TAg from Santa Cruz Biotechnology (Santa Cruz, CA); anti-AR from Upstate (Lake Placid, NY); anti-p63 from (Pharmingen, San Diego, CA); anti-FLAG M2, antitubulin, and anti–β-actin from Sigma-Aldrich Chemical (St. Louis, MO); anti-HMCK and anti-CK8 from ABcam (Cambridge, MA); and anti–prostate-specific antigen (PSA) from DakoCytomation (Glostrup, Denmark).

Expression Analysis of Prostate Epithelial Cells. Expression analyses were performed on RNA derived from triplicate cultures of asynchronously dividing PrECs. RNA was isolated after direct solubilization on plastic plates with TRIzol; 15 μg of total RNA were processed for hybridization to U133A microarrays (Affymetrix, Santa Clara, CA) as described previously (23); and expression information was obtained using MAS5 (Affymetrix). Thirty-five of the 36 microarrays were of sufficient quality for analysis, and standard parameters were used for all of the experiments that applied thresholds and/or filters to expression data (Supplemental Methods and Supplemental Table 3).

Genes with significant differential regulation ($P < 0.001$) caused by *RAS* expression [LHS ± AR ($n = 12$) versus LHSR ± AR ($n = 11$)] were identified using a signal-to-noise metric and permutation testing as described previously (23). All of the genes matching the correlation between each genotype with a significance of $P = 0.001$ based on permutation testing were identified. Hierarchical clustering analysis was performed using dCHIP (24, 25) on scaled and filtered array data from PrEC ($n = 3$), LH ($n = 3$), LHSR ($n = 3$), and LHMK ($n = 3$).

The genes with significant differential expression associated with H-*ras* expression (identified previously) were mapped from the U133A Affymetrix microarrays to genes on the U95 arrays using the "best match" table provided by Affymetrix. All of the genes with matched probes on U95Av2 were used to perform a hierarchical cluster of 50 benign and 52 malignant primary tissues (26) using Gene Cluster and visualized with TreeView (http://rana.lbl.gov/EisenSoftware.htm). To determine the statistical significance of such organization, the same number of genes was randomly chosen from the data set of malignant and benign tissues before performing the identical clustering protocol with Gene Cluster. After 10^4 iterations, the number of times the randomly chosen gene lists exceeded the separation between benign and malignant samples seen with the experimental data was used as a measure of significance (see Supplemental Methods).

Gene Set Enrichment Analysis. A variation of gene set enrichment analysis (27) was used to test whether genes differentially expressed between immortalized PrECs and the tumorigenic LHSR and LHMK PrECs are overexpressed as a group in prostate tumors (benign and metastatic) compared with normal prostate tissue (see Supplemental Methods for details). The 200 genes with increased expression in LHSR or LHMK when compared with naïve PrECs were identified and mapped from the U133A microarray to the U95Av2 microarray using "best match." The LHSR and LHMK gene sets were tested for increased expression in primary ($n = 52$) and metastatic ($n = 13$) tumors compared with local benign prostate samples ($n = 50$). The running score was compared with 1000 random permutations to determine whether significant enrichment was present.

Orthotopic Implantation of Tumor Cells. Immunodeficient mice (Taconic, Germantown, NY) were anesthetized with Avertin (Sigma); a lower-midline incision was made; and PrECs (5×10^5/10 μL) in PrEGM were implanted into the anterior, dorsolateral, and ventral prostate lobes using a 30-gauge needle with a 0.1-mL syringe. For every implantation, one cell line was placed in each mouse. The prostate was returned to the abdominal cavity, and the abdominal wall was sutured closed. Testosterone pellets (Innovative Research of America, Sarasota, FL) were implanted under the skin on the day of surgery in the indicated groups of animals. Mice were sacrificed at 4, 6, and 12 weeks after the intraprostatic implantation of tumor cells. Blood samples were obtained before sacrificing mice, and plasma testosterone levels were measured using Testosterone ELISA kit (American Laboratory Products Company, Windham, NH).

Surgical Castration. An incision was made in the scrotum of anesthetized mice and in the tunica. The testis, vas deferens, and attached testicular fat pad were pulled out of the incision, and the blood vessels supplying the testis were cauterized. The testis, vas deferens, and fatty tissue were severed just below the site of the cauterization, and this procedure was repeated on the contralateral side before suturing the scrotum. All of the animal work was performed in accordance to our institutional animal care committee guidelines.

Immunohistochemistry. Xenografts were fixed in formalin and embedded in paraffin. Paraffin sections (5 μm) were deparaffinized, rehydrated, and heated in 10 mmol/L citrate buffer (pH 6.0; BioGenex, San Ramon, CA) in a 750-W microwave oven for 15 minutes. Slides were cooled at room temperature for 30 minutes. For cells implanted orthotopically, the entire prostate was removed and completely sectioned. Hematoxylin and eosin staining was performed every 10th section to look for the presence of microscopic tumors. Sections were blocked in 10% goat serum (30 minutes), incubated with primary antibodies in 1% bovine serum albumin (12 hours at 4°C), washed with PBS, incubated with secondary antibody (1:200; 30 minutes), and detected with the ABC kit (Vector Labs, Burlingame, CA). Peroxidase activity was localized with 3,3-diaminobenzidine or 3,3-diaminobenzidine/nickel chloride. Standardized development time periods were used to allow accurate comparison between all of the samples. The sections were counterstained with hematoxylin, rehydrated, and mounted for microscopic examination.

RESULTS

Immortalization and Transformation of Prostate Epithelial Cells with SV40ER, *hTERT*, and H-*ras*. Most normal and malignant human prostate cell lines lack AR and fail to recapitulate many of the characteristics associated with the secretory prostate epithelium. To study the role of AR in prostate cancer, we used primary PrECs to establish immortalized and tumorigenic human cell lines. Like many human epithelial cells, normal PrECs exhibit a limited lifespan in culture and enter senescence after only 10 population doublings. To determine whether telomere shortening is the primary factor limiting the proliferative potential of human PrECs, we introduced the telomerase catalytic subunit *hTERT* into early passage human PrECs using retroviral-mediated gene transfer. PrECs expressing *hTERT* exhibit readily detectable telomerase activity (Fig. 1*A*), and telomere shortening is arrested (data not shown). However, similar to other types of human epithelial cells (17, 28, 29), these cells enter senescence at the same time as PrECs infected with a control vector (Fig. 1*B*).

Previous studies have linked mutations in *pRB* and *p53* tumor suppressor genes to the development of prostate cancer, and loss of heterozygosity at the *pRB* locus has been observed in up to 60% of clinical cases (30–33). Because the immortalization of other epithelial cells requires the additional ablation of the pRB and p53 tumor suppressor pathways, we also introduced the SV40 LT into parallel PrEC cultures (Fig. 1*A*). Expression of LT alone fails to immortalize these PrECs, whereas coexpression of LT and *hTERT* (PrEC LH) suffices to immortalize PrECs (Fig. 1*B*).

These immortal PrECs lack the ability to grow in an anchorage-

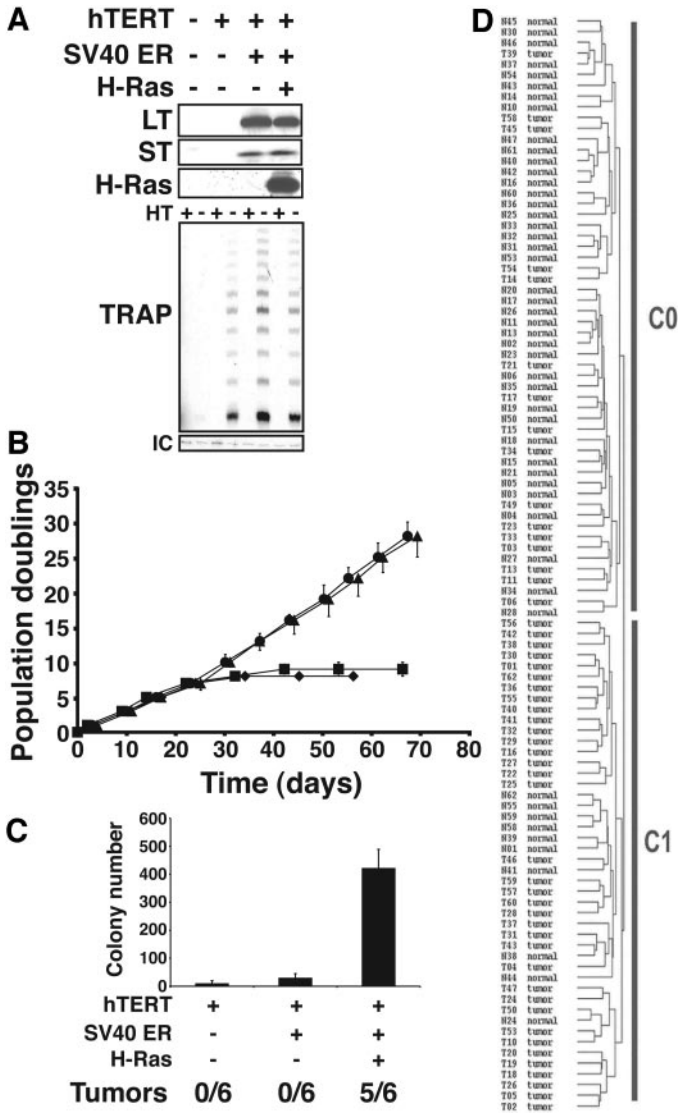

Fig. 1. Genetic manipulation of PrECs. *A.* The expression of SV40 early region (SV40ER) oncoproteins, LT and ST, and H-*ras* was confirmed by immunoblotting whole cell lysates (100 μg). Telomerase activity was assessed by conventional telomere repeat amplification protocol (TRAP) assay (100 ng). HT refers to heat-treated samples, and IC denotes internal PCR control for the TRAP assay. *B,* population doublings (mean ± SD; $n = 3$) for PrECs expressing a control retrovirus (♦), expressing *hTERT* (■), expressing *hTERT* and the SV40 ER (●), or expressing *hTERT,* SV40 ER, and *H-RAS* (▲). *C,* the number of colonies formed in soft agar (mean ± SD; $n = 3$) for PrECs expressing *hTERT* alone, *hTERT* and SV40ER, or *hTERT*, SV40 ER, and H-*ras*. Tumor formation in immunodeficient mice is reported as number of tumors identified/number of injection sites. *D.* The expression of genes significantly associated with tumorigenicity ($n = 716$ on U95Av2) organized prostate samples (50 normal "N," 52 tumor "T") into two major clusters (C0 and C1). A hazard matrix of sample membership for these two major clusters and sample identity [normal (*N*) or tumor (*T*)] is a nonrandom distribution (χ^2, $P \leq 0.001$). The observed nonrandom distribution of the *RAS* gene set was achieved at a frequency of <1 in 1,000 when 10,000 random sets of 716 genes were selected from prostate sample expression data (see Supplemental Fig. 1*C*).

independent manner and fail to form tumors in animal hosts (Fig. 1*C*). However, the additional introduction of an oncogenic allele of H-*ras* and the SV40 ST (PrEC LHSR) confers the ability to grow in an anchorage-independent fashion and to form poorly differentiated tumors when placed subcutaneously in immunodeficient mice (Fig. 1*C*). Coexpression of H-*ras* (PrEC LHR) or ST (LHS) with LT and *hTERT* fails to render these cells tumorigenic (data not shown). Collectively, these observations indicate that, like other human epithelial cells, PrECs are immortalized by the expression of *hTERT* and LT and are rendered tumorigenic by the additional expression of H-*ras* and ST.

Relevance to Human Prostate Tumors. Although these manipulations allowed us to develop immortalized and tumorigenic PrECs, we wished to understand whether such experimental models reflect the changes observed in human prostate cancers. We first identified the global gene expression consequences of transformation in PrECs by performing a supervised analysis of oligonucleotide microarray data and by determining how the expression of genes altered during transformation reflects the differences seen between normal and malignant prostate samples. Despite similar proliferation rates (Fig. 1*B*), we observed profound, statistically significant gene expression differences between the immortalized (PrEC LHS) and tumorigenic (PrEC LHSR) cells (1207 genes at $P < 0.001$; Supplemental Table 1 and Supplemental Fig. 1*A*). When we used the expression of these genes to organize a previously described set of benign ($n = 50$) and malignant ($n = 52$) human prostate samples (ref. 26; hereafter referred to as tumor-normal clustering), we noted a significant separation of the normal prostate samples from prostate tumors ($P = 0.001$; Fig. 1*D* and Supplemental Fig. 1*B* and *C*), supporting the notion that these PrECs recapitulate some of the transcriptional hallmarks of spontaneously originating human prostate cancers.

However, because activating mutations of the *RAS* family occur infrequently in human prostate cancers (34), we tested whether oncogenes more commonly implicated in prostate cancer also transform PrECs. In previous work, we found that substitution of ST and H-*ras* with an activated version of PI3K and c-*myc* also allows human mammary epithelial cells to grow in an anchorage-independent manner (21). Because c-*myc* is amplified in ~25% of advanced prostate cancers (35) and because activation of the PI3K pathway through disruption of the *PTEN* tumor suppressor gene occurs in many prostate cancers (36), we introduced *c-myc* and a myristoylated version of the p110α subunit of PI3K (Myr-FLAG-p110α PI3K) alone and in combination into PrECs expressing *hTERT* and LT (Fig. 2*A* and *B*). We confirmed that this Myr-FLAG-p110α PI3K was active by analyzing the phosphorylation of AKT at Ser473 (Fig. 2*A*). Coexpression of c-*myc* and Myr-FLAG-p110α PI3K in PrECs expressing LT and *hTERT* (LHMK) conferred the ability to grow in an anchorage-independent manner (Fig. 2*C*). Unlike human mammary epithelial cells (21), this combination of introduced genes also sufficed to permit the formation of small (0.1 to 0.2 cm in diameter) tumors when these cells were injected orthotopically into the murine prostate gland (four tumors of six surgical implantations). Because these PrECs (LHMK) failed to form tumors when implanted subcutaneously (zero tumors in four implantations), these observations suggest that specific interactions between these PrECs and the prostate microenvironment cooperate with alterations in c-*myc* and PI3K signaling to transform PrECs.

We then applied global expression analysis to determine whether we could identify specific transcriptional phenotypes associated with these various immortalized and tumorigenic PrECs (LH, LHSR, and LHMK). When we organized normal PrECs, immortalized PrECs (LH), tumorigenic PrECs expressing H-*ras* and ST (LHSR), and tumorigenic PrECs expressing c-*myc* and PI3K (LHMK) based on the expression of all of the genes passing a minimal filter ($n = 6586$), we found that the phenotypic behavior of the PrECs (mortal, immortalized, or tumorigenic) was the primary organizing factor (Fig. 3*A*). Specifically, we found a clear distinction between immortal cells and tumorigenic cells. Although some genes were uniquely expressed in either the two types of tumorigenic PrECs (LHSR and LHMK), the overall transcriptional signature of these tumorigenic PrECs was more similar to each other than mortal or immortal PrECs (Fig. 3*A*).

We used two methods to determine whether the gene expression changes between the mortal PrECs and the tumorigenic cell lines (LHSR and LHMK) reflect differences between benign and malignant prostate samples. First, we again applied tumor-normal clustering (as

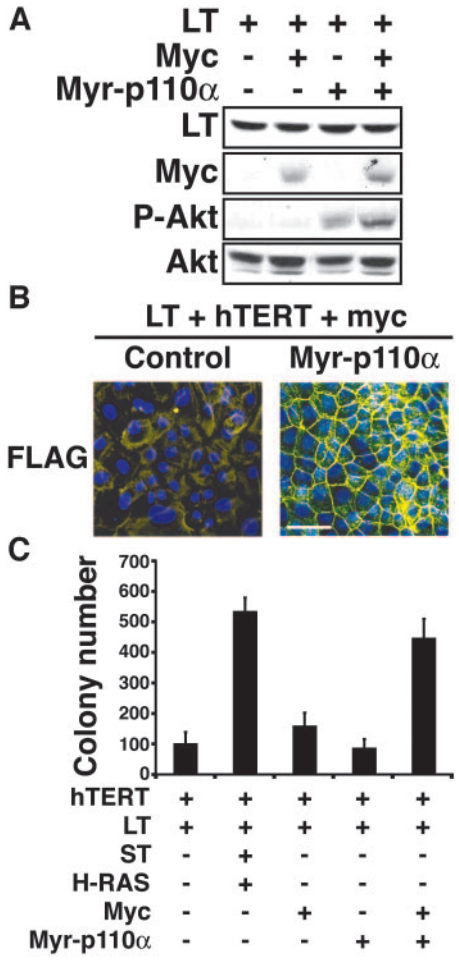

Fig. 2. Substitution of *MYC* and PI3K for *RAS* and ST. *A.* In place of SV40 ST and H-*ras*, PrECs expressing SV40 LT (LT) and *hTERT* were infected with retroviruses containing the *MYC* (Myc) and separately a FLAG-tagged, myristoylated PI3K (Myr-p110α). The phosphorylation status of Akt at position S473 (P-Akt) was assessed to determine the activity of Myr-FLAG-p110α PI3K. *B.* Indirect immunofluorescence confirms the membranous expression of Myr-FLAG-p110α PI3K. Staining was performed using a FLAG epitope-specific monoclonal antibody; *bar,* 50 μm. *C.* Anchorage-independent growth of PrECs. The mean ± SD for three experiments is shown.

described previously) and found that both sets of the top 200 genes up-regulated in LHSR and LHMK compared with mortal PrECs successfully separated benign and malignant prostate specimens (χ^2, $P < 0.001$; Fig. 3*B*, Supplemental Table 2, and Supplemental Fig. 1*D* and *E*). Second, we applied the more specific analytic method of gene set enrichment analysis (27) to determine whether the same sets of genes up-regulated in the tumorigenic PrECs were overexpressed in prostate tumors when compared with benign samples. Although the results for the LHSR and LHMK gene sets were similar, we found that the LHMK gene set was significantly enriched in local ($n = 53$; $P = 0.02$) and metastatic tumors ($n = 13$; $P = 0.02$) when compared with benign prostate tissue, whereas the LHSR gene set enrichment did not reach statistical significance ($P = 0.08$ for local; $P = 0.14$ for metastatic; Fig. 3*C*). Collectively, these observations indicate that these PrECs identify a set of genes that delineate a tumorigenic phenotype and recapitulate some of the transcriptional alterations found in human prostate cancers.

Investigating the Effects of Androgen Receptor Expression Using Immortalized and Tumorigenic Prostate Epithelial Cells. Normal human PrECs exhibit a basal epithelial cell phenotype (37). When propagated in culture and as tumor xenografts, the immortalized and tumorigenic PrECs described previously retain this basal phenotype as gauged by the expression of high molecular weight cytokeratins (data not shown) and the basal cell epithelial marker p63 (Fig. 4*A*). Consistent with previous descriptions of the basal epithelial cell phenotype (37, 38), these immortalized and transformed PrECs fail to express the AR.

Because virtually all of the prostate cancers display a secretory phenotype characterized in part by the expression of AR and absence of p63 expression (39), we introduced AR in these immortalized (LHS-AR) and tumorigenic (LHSR-AR) PrECs (Fig. 4*A*) to investigate the role of androgen signaling in prostate epithelial differentiation and tumorigenicity. The expression of AR in immortalized and tumorigenic PrECs confers a marked decrease in the rate of cell proliferation when we added the synthetic androgen R1881 (Fig. 4*B*). Coincident with androgen stimulation and the decrease in proliferation, we observed a rapid down-regulation of p63 expression (Fig. 4*D*) and the secretion of small amounts of PSA into the culture medium (Fig. 4*C*), suggesting that AR signaling induces several elements of luminal differentiation in these PrECs (37). These effects were specific for AR because control PrECs lacking AR expression (PrEC LHS and LHSR) failed to down-regulate p63 or secrete PSA after androgen stimulation, and bicalutamide (a competitive inhibitor of androgen) blocked the down-regulation of p63 expression during treatment with R1881 (Fig. 4*D*). However, in neither the immortalized nor the transformed cell lines was AR expression in the presence of R1881 sufficient to induce all of the molecular markers associated with luminal cell differentiation because these cells continue to express high molecular weight cytokeratins when propagated on plastic culture dishes (data not shown). Thus, expression of AR in these PrECs induces some but not all of the features of prostatic luminal differentiation when such cells are propagated on plastic culture dishes.

Several lines of evidence indicate that prostate cancer development is influenced by interactions with other nontumorigenic cells in the prostate (40). To test whether the prostatic microenvironment influenced PrEC differentiation, we injected LHS, LHS-AR, LHSR, LHSR-AR, LHMK, and LHMK-AR PrECs orthotopically in the ventral, anterior, and dorsolateral prostate of immunodeficient mice. In our initial studies, we noted that immortalized PrECs (LH, LHS, or LHR) failed to form tumors when placed subcutaneously, whereas tumorigenic PrECs (LHSR) formed poorly differentiated carcinomas (data not shown); the expression of AR failed to alter the kinetics, morphology, or behavior of these PrECs when placed subcutaneously (data not shown). However, when we implanted these immortalized PrECs expressing AR orthotopically, we found that the expression of AR permitted immortalized PrECs (LHS-AR) to form tumors (four tumors in four orthotopic injections; Fig. 5*A*). These tumors, 6 weeks after implantation, were smaller (0.2 to 0.5 cm in diameter) than the orthotopic tumors derived from LHSR (2 to 3 cm in diameter, 20 tumors in 20 implantations) or LHSR-AR (2 to 3 cm in diameter, 45 tumors in 45 implantations). Furthermore, the introduction of AR into LHMK cells, which we previously found displayed limited capability of forming tumors, rendered these cells highly tumorigenic (20 tumors in 20 implantations) with tumor sizes averaging 2 to 3 cm in diameter. In the time frame of these experiments (12 weeks), we have yet to detect distant metastases following orthotopic implantation of each of these cell lines.

Histologically, tumors derived from LHS-AR PrECs invaded the host parenchyma and displayed acinar and cribriform patterns, reminiscent of human prostate Gleason III and IV tumors. Compared with orthotopic PrEC tumors without AR (LHSR), we also found evidence of androgen-induced luminal differentiation with down-regulation of p63, increased expression of cytokeratin 8, increased expression of FKBP51 (another androgen-induced gene in the prostate), and expres-

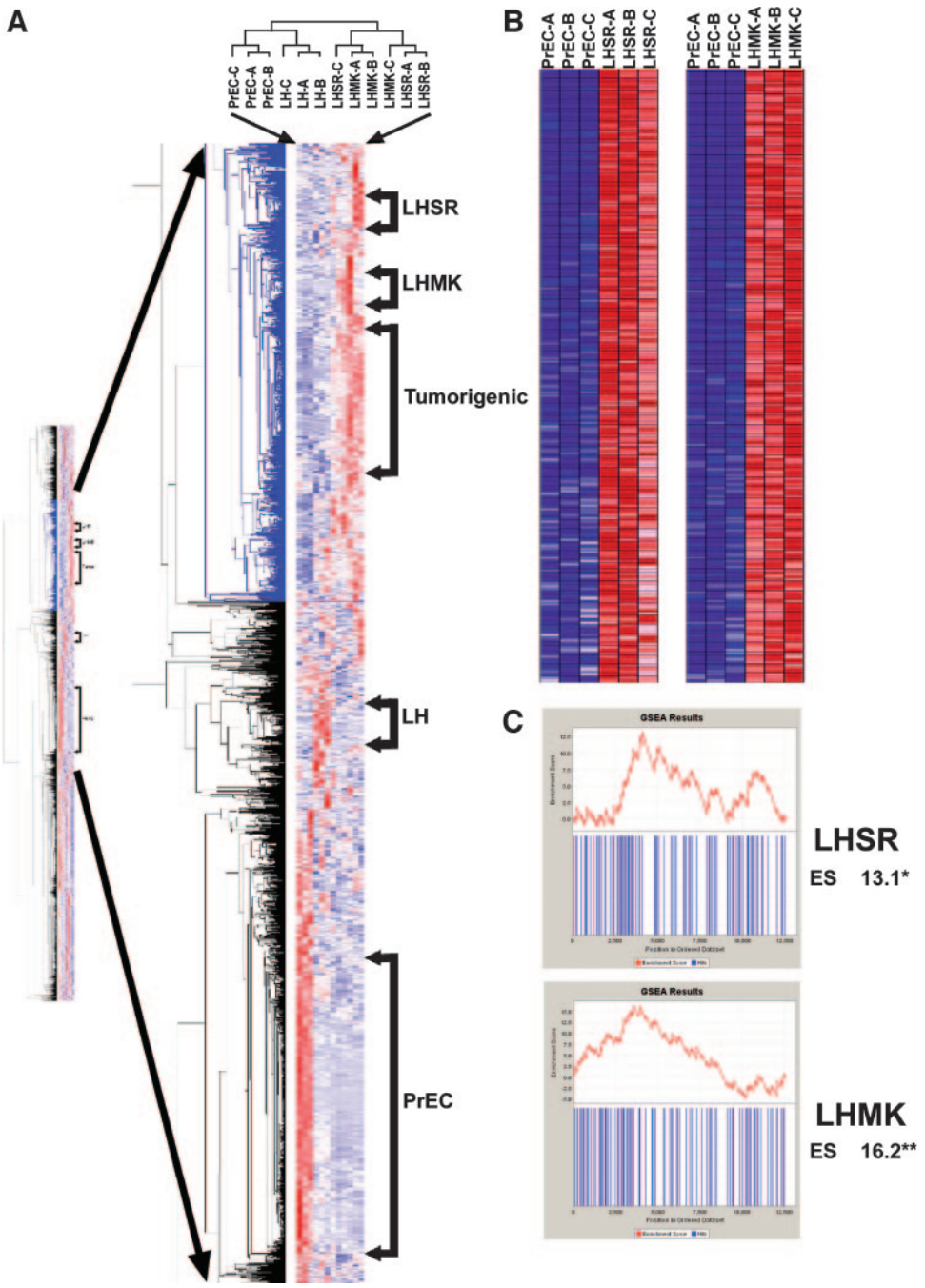

Fig. 3. Expression analysis of PrECs. *A*. A hierarchical clustering of genes and samples ($n = 3$ of each cell line) separated samples into three major groups: the parental cell line (PREC), the immortalized cell line (LHS), and the tumorigenic cell lines (LHSR and LHMK). More genes whose expression was increased were shared between LHSR and LHMK (*Tumor*) than were uniquely expressed in either LHSR or LHMK alone. *B,* normalized expression representing the top 200 genes found to have increased expression in either LHR or LHMK tumorigenic PrECs compared with parental PRECs ranked according to the signal-to-noise metric. *C.*These two gene sets were tested for expression enrichment in localized prostate cancer samples ($n = 52$) compared with benign samples ($n = 50$). The LHMK gene set showed a significant enrichment score (*ES*; $**P = 0.02$), whereas the LHR gene set had a borderline ES ($*P = 0.08$). The red line indicates the running ES and the position of the genes within each gene set (LHSR or LHMK) within the ordered data set of genes ranked using the signal-to-noise metric to measure their increased expression in local prostate cancer compared with benign prostate samples (blue lines in ordered data set).

sion of PSA (Fig. 5*A*). The expression of AR in tumorigenic PrECs (LHSR) also induced a luminal phenotype when such cells were placed orthotopically (three tumors in four orthotopic injections). These effects on prostate epithelial tumor formation and differentiation were specific for AR signaling because immortalized PrECs lacking AR failed to form tumors (no tumors in six orthotopic injections) and tumorigenic PrECs lacking AR formed anaplastic tumors with a basal epithelial phenotype (Fig. 5*B*). Collectively, these observations identify AR signaling as a key regulator of prostate epithelial cell differentiation and transformation and show that PrECs with a basal phenotype differentiate into luminal-appearing PrECs under the influence of AR signaling and the prostate microenvironment.

Manipulation of Androgen Levels. To investigate the role of AR in these phenotypes, we next studied the kinetics, morphology, and tumorigenicity of these PrECs expressing AR in mice in which we altered systemic androgen levels. LHSR, LHSR-AR, and LHMK-AR PrECs were implanted subcutaneously and orthotopically in groups of 20 mice for each cell line. Each group of 20 mice was divided as follows: Five mice were subjected to surgical castration on the day of PrEC implantation (castration on day 0). Testosterone pellets were placed subcutaneously on the day that PrECs were injected in 10 mice. Five mice were castrated 3 weeks after the cells and testosterone pellets were implanted, and the testosterone pellets also were removed (castration on day 21). The remaining five mice were injected with the various PrECs with no additional treatment. As expected, testosterone serum levels correlated with these manipulations (Fig. 6*A*). Supplemental testosterone induced rapid tumor growth in AR-expressing LHSR PrECs that was reversed with castration on day 21 (Fig. 6*B* and *D*), showing that these AR-expressing PrECs are dependent on circulating androgen for tumor growth. This treatment failed to influence

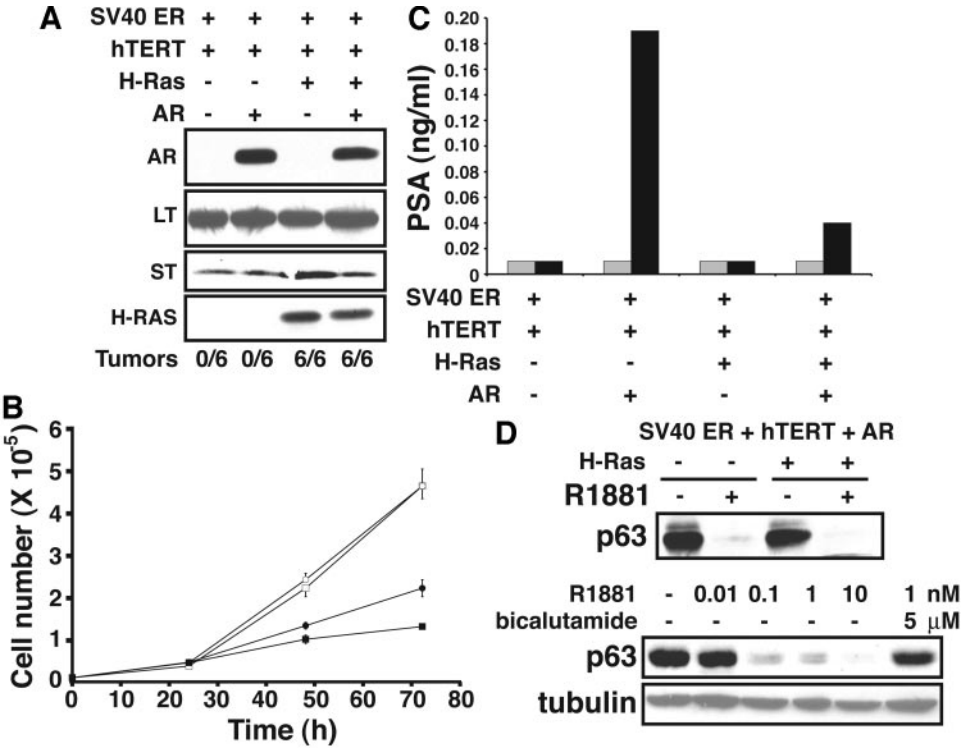

Fig. 4. Introduction of AR in PrECs. *A,* expression of AR in immortalized and transformed PrECs as shown by immunoblot analysis. Subcutaneous tumor formation was unaffected by AR expression and is reported as number of tumors identified/number of injection sites. *B.* Treatment with R1881 inhibits PrEC proliferation. Immortalized (LHS, *squares*) or tumorigenic (LHSR, *circles*) PrECs lacking (*open symbols*) or expressing (*closed symbols*) AR were treated with 0.1 nmol/L R1881 and cells counted at the indicated times. *C.* PSA secretion by PrECs. PrECs expressing the indicated genes were treated with 0.1 nmol/L R1881 for 7 days, and PSA was measured by a clinical grade immunoassay. *D.* R1881 suppression of p63 expression is shown by immunoblot analysis of whole cell lysates (100 μg) of immortalized and tumorigenic PrECs after treatment with R1881. Dose response of R1881-induced suppression of p63 expression is shown in bottom panel.

the growth of LHSR cells lacking AR (compare Fig. 6*B* and *C*). Surprisingly, we found that AR-expressing LHSR PrECs exhibited a markedly increased latency in castrated mice compared with LHSR PrECs lacking AR (Fig. 6*C*). We observed similar effects when we tested the LHMK-AR PrECs (Fig. 6*D*). These observations indicate that the introduction of AR into PrECs renders such previously androgen-independent cells dependent on AR for tumor growth.

When we sacrificed these mice and analyzed the orthotopic tumors, we found that the size and weight of these orthotopic tumors derived from LHSR-AR and LHMK-AR PrECs correlated directly with blood androgen levels (Fig. 6*A* and *D*). Histologically, we noted primarily nuclear AR staining in tumors derived from mice harboring implanted testosterone pellets, whereas tumors derived from castrated mice showed mixed AR staining in the nucleus and cytoplasm (Fig. 6*D*), as is the case in human prostate cancers following adjuvant androgen ablative therapy.[10] Thus, these engineered PrEC tumors recapitulate the natural history of the human disease, including the histologic appearance, AR localization, and response to castration. Collectively, these observations implicate AR signaling as a key contributor to the malignant phenotype in the prostate epithelium.

DISCUSSION

Because none of the available prostate cancer cell lines fully recapitulate the phenotype and behavior of the human prostate cancer, we created a series of genetically defined immortalized and tumorigenic human PrECs to investigate the role of AR in the malignant transformation of the prostate. Although these PrECs initially exhibited a basal epithelial phenotype, the introduction of AR together with orthotopic implantation sufficed to convert immortalized PrECs into differentiated secretory PrEC tumors that recapitulate many of the cell and molecular phenotypes associated with human prostate cancer. Surprisingly, AR not only provides an oncogenic signal for immortalized PrECs but also makes previously androgen-independent, transformed cells dependent on androgen for tumorigenic growth. These findings define specific roles for AR in the malignant transformation of the prostate epithelium.

The development of these immortalized and tumorigenic PrECs represents an important new tool for the further investigation of prostate cancer biology. The majority of prostate cancer cell lines in use are derived from metastatic lesions, and we still lack a comprehensive understanding of the genetic alterations harbored by such cells (7–10, 41). Most previously reported transformed primary PrECs have involved the introduction of oncogenes followed by a long period of selection, during which time further, uncontrolled, and largely unknown genetic events accumulate to result in transformation (13, 42–45). These experimental PrEC models will facilitate the functional dissection and elucidation of prostate cancer–associated genetic changes in the pathogenesis of prostate cancer.

Interestingly, these manipulations corroborate recent observations in genetically altered mice harboring activated AKT, loss of PTEN, or increased levels of c-*myc* in the prostate (46–49). When the global expression changes resulting from the *in vitro* transformation of PrECs were compared with the expression differences between normal and malignant human prostate samples, the changes occurring in the cell lines containing PI3K and c-*myc* were more reflective of spontaneous prostate cancer than the changes in the ST- and H-*ras*–containing cells. Because genetic changes occurring in prostate cancer continue to be discovered, these cells now provide a useful model for assessing oncogenic potential within genetically defined prostate epithelial cells. Genetic substitutions also can be assessed for their relevance to human disease by comparing the global expression changes induced during transformation with those observed in human prostate samples.

Although *RAS* mutations are found in prostate cancer from Japanese men (50, 51), such mutations are uncommon among most patients with prostate cancer (34). Despite the use of H-*ras* to create tumorigenic PrECs, transcriptional profiles derived from *RAS*-

[10] Unpublished observations.

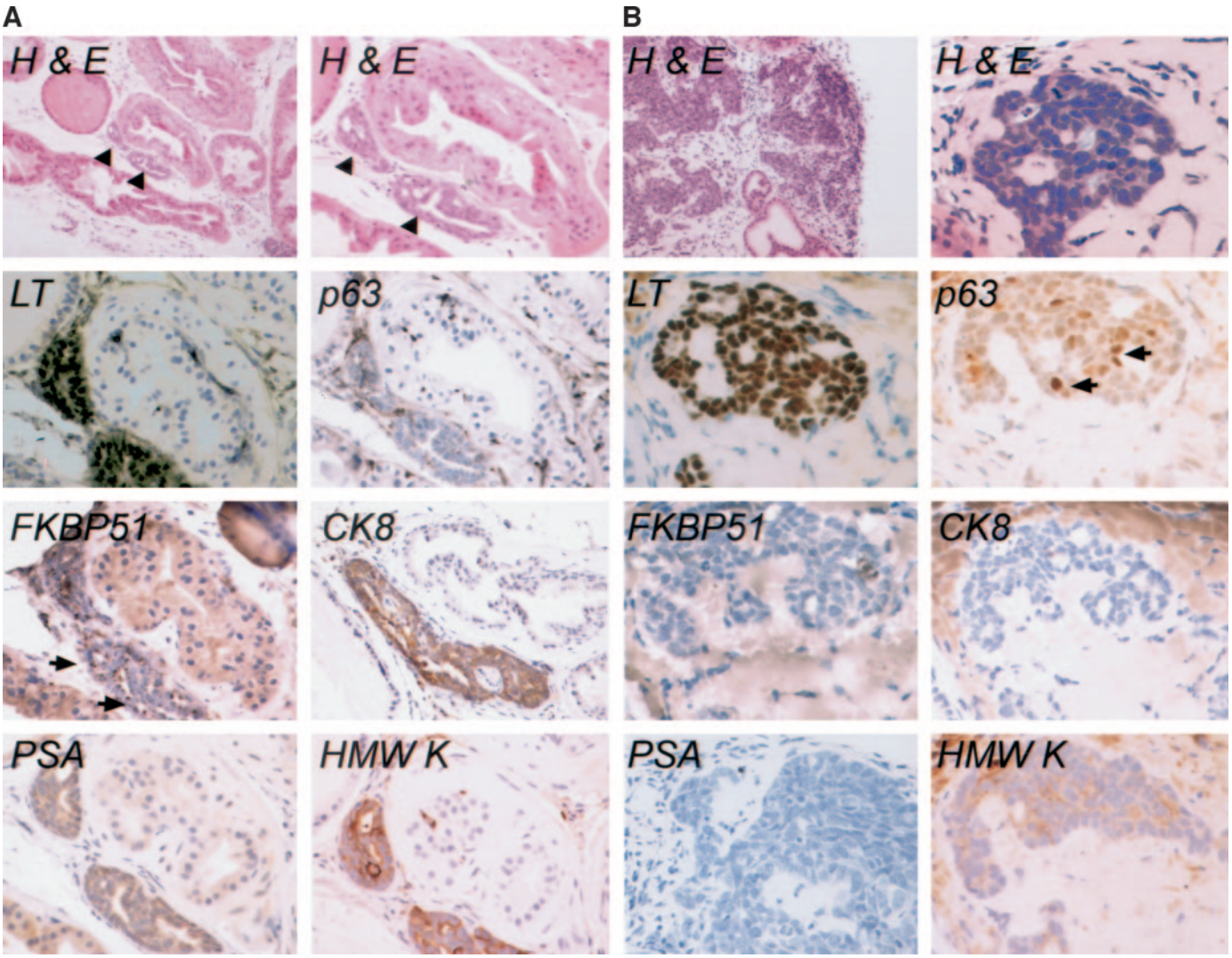

Fig. 5. Effects of AR signaling on orthotopically implanted PrECs. Immortalized and H-*ras*--expressing PrECs were implanted in the murine prostate. Prostate glands were isolated 28 days after implantation. Immortalized PrECs lacking AR failed to form tumors. Immortalized PrECs expressing AR (*A*) formed discrete tumor nodules interspersed throughout the murine prostate that stained for LT, FKBP51, cytokeratin 8 (*CK8*), and PSA and failed to stain for p63. Tumorigenic PrECs lacking AR formed anaplastic tumors that expressed LT, p63, and high molecular weight keratins (*HMW K*). Panels were stained with H&E or anti-LT, anti-p63, anti-FKBP51, anti–cytokeratin 8, anti-PSA, and anti–high molecular weight keratin antibodies. The p63 staining pattern in *B* is typical of basoluminal cells. Magnification of the left H&E panels is 200×; all of the other panels are shown at 400×. Triangles indicate well-differentiated xenograft tumors. Arrows in the FKNP51 panel indicate Golgi staining, and arrows in the p63 panels indicate cells expressing p63.

expressing PrECs permitted us to reliably organize expression profiles taken from benign and malignant prostate samples (26). Thus, although *RAS* mutations may occur infrequently in most prostate cancers, the downstream effectors induced by expression of an activated allele of H-*ras* may be a common manifestation in prostate cancer tumors. The transcriptional profiles of PrECs expressing activated PI3K and c-*myc* more closely resemble transcriptional profiles derived from human prostate cancer specimens. Moreover, by combining genetically defined models with orthotopic implantation, these PrECs expressing PI3K and c-*myc* represent the first human cell lines that do not require *RAS* overexpression to achieve tumorigenicity.

Consistent with previous studies (37, 52), these PrECs fail to express AR and exhibit a molecular phenotype most closely related to basal PrECs. AR expression renders these cells responsive to androgen and induces some luminal differentiation. However, these observations confirm that non–cell autonomous factors are necessary for AR-mediated differentiation because more complete luminal differentiation required AR expression and orthotopic implantation. Such factors likely involve cell-cell interactions between epithelial cells, stroma, and possibly inflammatory cells (53). Thus, AR signaling within the prostatic microenvironment results in differentiation of PrECs from a basal phenotype to a luminal phenotype, and although we cannot exclude the possibility that luminal PrECs are a final product of more than one differentiation program, these observations support the notion that basal and luminal PrECs share a common lineage.

AR expression in immortalized PrECs growing orthotopically also sufficed to permit tumor formation. Although it has been clear that AR expression plays a central role in the development and growth of prostate cancer, it has been difficult to characterize the AR as an oncogene with specific transforming capability in prostate cells. In these experiments, although AR expression alone was insufficient for cellular transformation as defined by soft agar colony formation or subcutaneous tumor formation, AR expression sufficed to permit tumor formation when immortalized cells were placed orthotopically. Thus, although the AR is not an autonomous oncogene in these PrECs, the environment of the prostate potentiates the effects of AR during the transformation of PrECs. These observations reinforce previous studies that showed the importance of orthotopic implantation for tumor formation (54) and the metastatic phenotype (55).

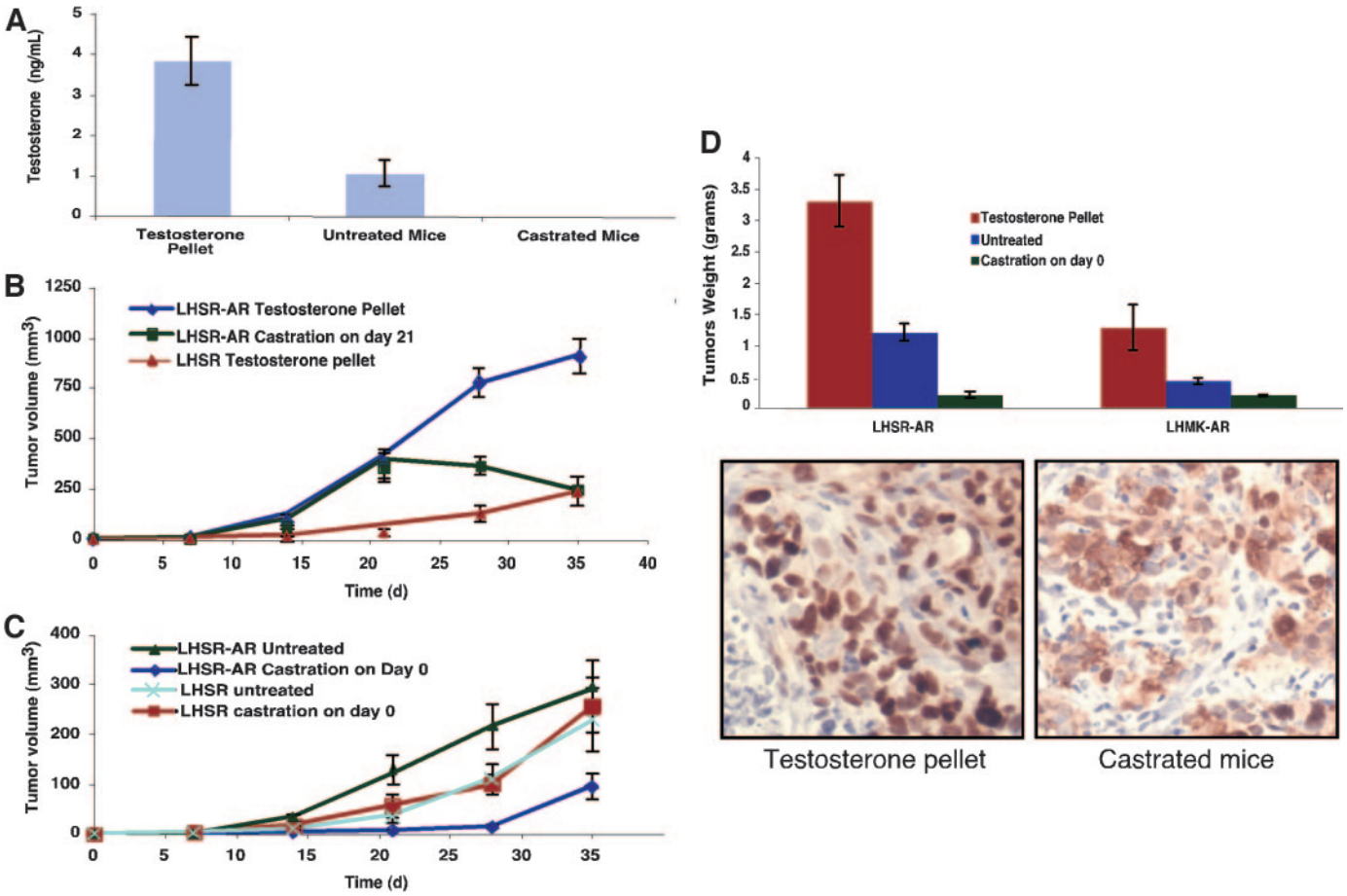

Fig. 6. Effects of androgen level manipulation on the behavior of PrEC-derived tumors. LHSR-AR and LHMK-AR PrECs were implanted subcutaneously and orthotopically in the prostate. *A.* Tumors were permitted to grow in the presence of supplemental androgen (testosterone pellet), unmanipulated androgen levels (untreated mice), and minimal androgen levels (castrated mice). Testosterone was undetectable in the serum of castrated mice. *B.* LHSR and LHSR-AR PrECs were implanted in mice harboring testosterone pellets. Five of the 10 mice were castrated after 21 days, and the pellets were removed (castration on day 21). Subcutaneous tumor growth is presented as tumor volume ± SE. *C,* subcutaneous tumor growth for PrECs implanted in mice castrated on day 0 and untreated mice. Tumor growth is presented as tumor volume ± SE. *D, top,* effects of androgen level manipulation on orthotopic tumor growth. Orthotopic tumors were harvested and weighed 35 days after implantation. *Bottom,* AR staining in tumors derived in the presence of supplemental androgen (testosterone pellet) or from mice castrated on day 21 (castrated mice). Tumors from mice harboring testosterone pellets showed primarily a nuclear AR staining pattern, whereas nuclear and cytoplasmic AR was seen in castrated mice.

These findings also complement previous work that showed that AR expression in the stroma cooperates to allow a spontaneously immortalized prostate cell line (BPH-1) to form tumors (40, 56) and that transgenic mice expressing AR in the prostate develop high-grade prostatic intraepithelial neoplasia as they age (57). Although additional work is necessary to delineate molecular interactions among immortalized PrEC prostate stromal cells and other components of the prostate microenvironment that lead to tumor growth, these systems provide a platform to investigate these important interactions.

When we manipulated systemic androgen levels in mice harboring these genetically altered PrECs, we found that tumorigenic cells expressing AR recapitulated the effects of androgen stimulation and ablation long observed in patients. Surprisingly, the expression of AR in tumorigenic PrECs renders such cells dependent on androgen for tumor formation. This observation suggests that beyond its effects in promoting cell proliferation and differentiation, AR plays a crucial role in the transformation process. Consistent with these observations, a recent study showed that increases in AR mRNA and protein levels was necessary and sufficient to convert prostate cancer cells from a hormone-sensitive to a hormone-refractory state and that these effects required a functional ligand-binding domain (3). Moreover, this addictive phenotype (58) is reminiscent of other oncogenes such as *RAS* (59), *MYC* (60, 61), and *Bcr-Abl* (62), whose continued expression are required for tumor maintenance and help explain the salutatory effects of androgen ablation in patients with metastatic prostate cancer. These observations provide a rationale for developing other AR-specific agents to manage hormone-naïve and hormone-refractory prostate cancer. Moreover, these experimental models should prove useful for the identification and validation of novel antineoplastic agents specific for prostate cancer.

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