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Phenotypic Characterization of Immortalized Normal and Primary Tumor-Derived Human Prostate Epithelial Cell Cultures

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BACKGROUND. Cell lines can provide powerful model systems for the study of human tumorigenesis. However, the human prostate cancer cell lines studied most intensively by investigators (PC3, DU145, and LNCaP) were established from metastatic lesions, and it is unlikely that they accurately recapitulate the genetic composition or biological behavior of primary prostate tumors. Cell lines more appropriate for the study of human prostate primary tumors would be those derived from spontaneously immortalized cells; unfortunately, explanted prostate cells survive only short-term in culture, and rarely immortalize spontaneously. Therefore, we examined whether cell lines developed through viral gene-mediated immortalization of human normal or primary tumor prostate epithelium express aspects of the normal or malignant phenotypes, and could serve as appropriate models for normal or transformed human prostatic epithelium.
METHODS. To accomplish these goals, we assessed the phenotypic expression of cell cultures established through the immortalization of normal (1532N, 1535N, 1542N, and PrEC-T) or malignant (1532T, 1535T, and 1542T) human prostate epithelium with the E6 and E7 genes of HPV-16, or the large T antigen gene of SV40.
RESULTS. Examination of these cell lines for their proliferative rates and their abilities to grow with or without serum or androgen stimulation, to form colonies in soft agar, or to form tumors in vivo, suggests that they may serve as valid, useful tools for the elucidation of prostate tumorigenesis. Moreover, the observation of structural alterations involving chromosome 8, including gain of 8q in 3 of the 4 cell lines expressing aspects of the malignant phenotype, implies that these cell lines accurately recapitulate the genetic composition of primary prostate tumors.

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CONCLUSIONS. Taken together, these data suggest that cell lines generated from immortalized normal or primary tumor epithelium may be useful for the elucidation of early transforming events in the prostate. *Prostate 44:164–171, 2000.* © 2000 Wiley-Liss, Inc.

KEY WORDS: **tumorigenesis; transformation; cell lines; primary tumors; prostate**

INTRODUCTION

Prostate cancer is a major health concern worldwide. There will be approximately 180,400 new cases of prostate cancer diagnosed in 2000, making it the most commonly diagnosed cancer in the United States [1]. Unfortunately, very little is known about the process of prostate cancer initiation and progression to the malignant phenotype. The uncertain time course and heterogeneity of the disease hinder elucidation of prostate tumorigenesis, and make the procurement of clinical specimens for study from appropriate time points during prostate tumor progression largely impossible.

Therefore, we and others have developed prostate cancer cell lines as model systems to examine the process of malignant transformation. In particular, the establishment of long-term cell cultures through the immortalization of normal and primary tumor-derived epithelium from radical prostatectomy specimens with the E6 and E7 genes of the human papilloma virus 16 (HPV-16), or the large T gene of the simian virus 40 (SV40), have provided valuable tools toward the analysis of tumor development in the prostate [2].

In this study, we report on the expression of the malignant phenotype in primary tumor-derived human prostate cell cultures. Our observations suggest that prostate epithelium immortalized through transfection with the HPV-16 E6 and E7 or SV40 large T genes may serve as valid, useful tools for the elucidation of malignant transformation in the prostate.

MATERIALS AND METHODS

Cell Culture

The HT-29 human colon cell line was grown in minimum essential medium (MEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD) and 1% penicillin/streptomycin/fungizone antibiotic mixture (BioWhittaker). All other cell lines were grown in defined keratinocyte-SFM (Gibco BRL), 5% FBS, and 1% penicillin/streptomycin/fungizone antibiotic mixture (BioWhittaker). All cell lines were grown at 37°C in a humidified incubator with 5% CO_2.

Immortalization

The 1532N, 1535N, 1542N, 1532T, 1535T, and 1542T cell lines were produced through immortalization of primary human prostate epithelial cells obtained from radical prostatectomy by transduction with a recombinant retrovirus encoding the E6 and E7 genes of HPV-16, as previously described [2]. The PrEC-T cells were produced through immortalization of normal prostate epithelial cells (Clonetics, Inc., Walkersville, MD) by transfection with the pMT10D plasmid (Japanese Cancer Research Resources Bank, Tokyo, Japan) containing sequences encoding the simian virus 40 (SV40) large T antigen.

Growth Curves

Cells were detached using 0.05% trypsin and plated in triplicate at a density of 10,000 cells/well in sterile six-well tissue culture plates (Fisher Scientific, Hanover Park, IL). PrEC-T cells were plated at a density of 5,000 cells/well. Each cell line was grown in media either with or without serum supplemented with 0.1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) for a period of 2 weeks, with media changes every 2–3 days. Plates were incubated at 37°C in 5% CO_2. Twenty-four hours later (time point 0 hr), media were aspirated and wells were washed with 5 ml of sterile 1 × phosphate-buffered saline (PBS). PBS was aspirated, and 2 ml of hypotonic solution (0.01 M HEPES and 0.0015 M $MgCl_2$) were added to each well. Plates were incubated for 10–15 min at room temperature on a rocking platform. Two hundred microliters of lysis buffer (0.13 M c-ethylhexadecyldimethylammonium bromide and 3% acetic acid) were added to each well. Plates were incubated for 10 min at room temperature on a rocking platform. Ten milliliters of isoton II diluent solution (Coulter Corp., Miami, FL) were added to each well and gently mixed. Nuclei were counted using a Coulter Counter (Coulter Corp.). Averages and standard deviations of cell number were calculated for each time point.

Colony Formation Assays

Five hundred microliters of media and 500 μl of 1% agar at 50°C were mixed in sterile 15-ml conical tubes (Fisher Scientific) and quickly poured into each well of six-well sterile tissue culture plates. The plates were allowed to solidify for several hours. Cells were added to 500 μl of warm media at densities of 10^5, 10^4, or 10^3, followed by the addition of 200 μl of sterile warm

TABLE I. Genotypic and Phenotypic Characteristics of Immortalized Prostate Cell Lines

Cell line	Tissue derivation	Doubling time	Number of colonies (SD)	Growth in low serum	Androgen sensitivity	Tumor formation *In vivo*	8p status by allelotyping/cytogenetics[a]
1532N	Normal prostate	32 hr	None	Yes	No	ND	2 copies of intact 8p/nd/
1535N	Normal prostate	41 hr	None	Yes	No	ND	2 copies of intact 8p/nd/
1542N	Normal prostate	48 hr	None	Yes	No	ND	2 copies of intact 8p/nd/
PREC-T	Normal prostate	36 hr	76 (6)	Yes	ND	ND	nd/gain of 8q
1532T	Malignant prostate	24 hr	308 (17)	Yes	No	No	One copy of 8p deleted/[iso(8q)]
1535T	Malignant prostate	27 hr	168 (11)	Yes	No	No	2 copies of intact 8p/nonclonal deletion of one copy of 8p
1542T	Malignant prostate	31 hr	132 (17)	Yes	No	No	One copy of 8p deleted/ structural alterations of chromosome 8
HT-29[c]	Malignant colon	25 hr	2,240 (339)	ND	ND	Yes[b]	1 copy of 8p deleted/[iso(8q)])

[a]As reported here and in Bright et al. [2] and Macoska et al. [7].
[b]As reported in Gustafson et al. [31].
[c]Included as a control; not derived from prostate tissue.
ND = not determined.

water and 300 µl sterile 1% agar (50°C). Tubes were quickly mixed and poured onto the solidified layer of the six-well plates and allowed to solidify for several hours. Each plate was fed with 1 ml of the appropriate media and placed at 37°C in a humidified incubator containing 5% CO_2. Each well was fed with 1 ml of media every 3 days. After 3 weeks, cells were stained by overnight incubation with 10 µg/ml tetrazolium violet (Sigma Chemical Co.) in the media at 37°C with CO_2. Each well was viewed and colonies were counted. HT-29 cells were used as positive controls for colony formation.

Tumor Formation in Athymic Mice

To determine tumorigenicity in vivo for the 1532T, 1535T, and 1542T cell lines, 1×10^7 cells in 0.2 ml of phosphate-buffered saline (PBS) were injected subcutaneously into the mid-dorsal interscapular region of adult athymic mice. The mice were observed for 5 months for tumor development.

Androgen Sensitivity Assays

Ten thousand cells of the 1535N, 1535T, 1532T, or 1542T lines were plated into sterile six-well plates. Media containing no serum was supplemented with 0.1% BSA with no R1881 (methyltrienolone) (New England Nuclear Life Sciences, Boston, MA) as controls or concentrations of 10^{-7}, 10^{-8}, or 10^{-9} MR1881. Plates were placed into 37°C humidified incubators with 5% CO_2 for 1 week with changes of media every 2 days. Cells were lysed and nuclei were counted as described above.

Immunohistochemistry

Cells were detached using trypsin and plated into sterile chamber slides (Fisher Scientific). Media were aspirated and the wells were washed with 1 × PBS. Cells were fixed using a 50% methanol/50% acetic acid solution for 15 min at 4°C. Wells were washed with 1 × PBS, and cells were stained using a pan cytokeratin antibody mixture containing clones C-11, PRK-26, CY-90, KS-1A3, M20, and A53-B/A2 or an isotype control antibody (Sigma Chemical Co.), using the UniTect™ immunohistochemistry detection system (Oncogene Science, Inc., Cambridge, MA), following the manufacturer's protocol. Slides were viewed using light microscopy.

RESULTS

Expression of the malignant phenotype was assessed for all cell lines by evaluating their proliferative rates (doubling times), their ability to grow in low serum and form colonies in soft agar and in vivo in athymic mice, and their sensitivity to androgen.

Growth Curves

Differences in cell doubling times for cells grown in the presence or absence of fetal bovine serum (FBS) were examined. The 1532T, 1535T, and the 1542T human malignant prostate cell lines had faster doubling times than the 1532N, 1535N, and 1542N human normal prostate cell lines. Specifically, the doubling times for the malignant cell lines were in the range of 24–31 hr, whereas the range of the normal cell lines was from

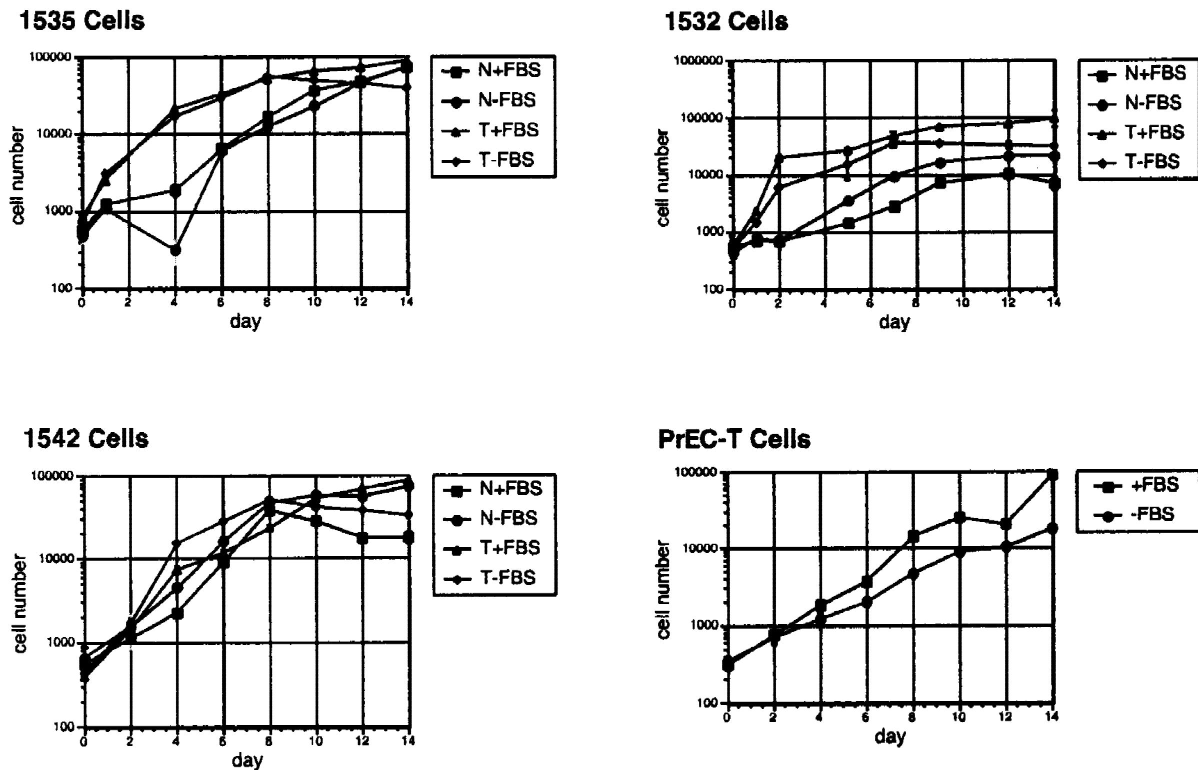

Fig. 1. Effect of fetal bovine serum (FBS) on cell growth. Cell number (y-axis, logarithmic scale) at consecutive time points over a 2-week period (x-axis, linear scale) is shown for normal-derived cells grown in the presence (solid squares) or absence (solid circles) of FBS, and for tumor-derived cells grown in the presence (solid triangles) or absence (solid diamonds) of FBS. **Upper left,** 1535 cells; **upper right,** 1532 cells; **lower left,** 1542 cells; **lower right,** PrEC-T cells.

32–48 hr (Table I). The PrEC-T cell line, which was established from normal prostatic epithelium after stable transfection with the SV40 large T antigen gene, had a doubling time of 36 hr, which was intermediate to those of the normal and tumor-derived prostate cell lines. No significant differences in growth rates were observed for any of the cell lines when grown in the presence or absence of FBS (Fig. 1). Hence, the tumor lines, as well as the normal lines, were able to grow in low serum.

Colony Formation

The ability of cells to form colonies in soft agar denotes that they have lost contact inhibition, a trait of the malignant or transformed phenotype. In order to determine whether cells demonstrated this trait, they were embedded in 0.5% agar and allowed to grow for 3 weeks, using HT-29 cells as a positive control for colony formation. The only cell lines that were able to form colonies in this assay were the HT-29, 1532T, 1535T, 1542T, and PrEC-T cell lines. The ability of prostate cell lines to form colonies in soft agar was 1532T > 1535T > 1542T > PrEC-T. The 1532N, 1535N, and 1542N cell lines were unable to produce any colonies in this assay (Table I).

Tumor Formation in Athymic Mice

Subcutaneous inoculation of 1×10^7 cultured cells into athymic mice failed to produce measurable tumors from the 1532T, 1535T, or 1542T cell lines after 5 months of observation.

Androgen Sensitivity Assays

In order to see if the cell lines were sensitive to androgen, the 1535N, 1532T, 1535T, and 1542T cells were grown in serum-free media at 0, 10^{-7}, 10^{-8}, and 10^{-7} M R1881. Less than a twofold difference in growth was observed for cells exposed to androgen compared to the controls in any of the cell lines tested (Fig. 2).

Immunohistochemistry

In order to see if the cell lines were entirely epithelial in origin and without fibroblastic or neuroendo-

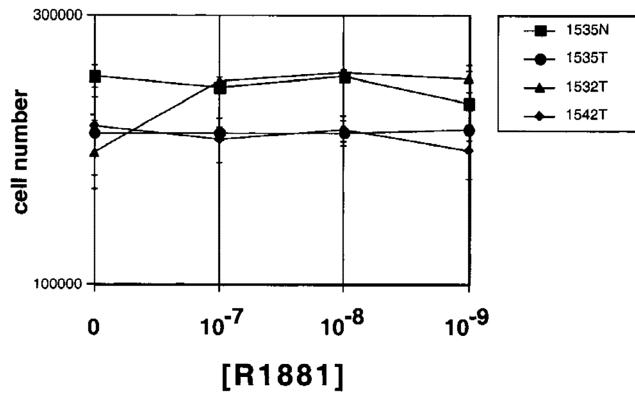

Fig. 2. Androgen sensitivity assay. Sensitivity to androgen was assessed for 1535N, 1532T, 1535T, and 1542T cells after growth in serum-free media at 0, 10^{-7}, 10^{-7}, and 10^{-7}M R1881. Less than a twofold difference in growth was observed for cells exposed to a synthetic androgen, methyltrienolone (R1881), compared to the controls in any of the cell lines tested.

crine contamination, cells were stained using a cytokeratin antibody mixture containing anti-cytokeratin antibodies and compared to cells stained with an isotype control (Table I). All of the cells examined stained uniformly and completely for cytokeratins, demonstrating that the cell lines were entirely epithelial and without fibroblast contamination (Fig. 3).

DISCUSSION

Cell lines can provide powerful model systems for studying the acquisition and expression of the malignant phenotype for the tissues from which they were derived. This is especially true when correlations between gross genotypic changes and expression of the malignant phenotype are attempted, as such studies using mouse models are often confounded by problems associated with mouse-human synteny. Even so, the human prostate cancer cell lines studied most intensively by prostate cancer investigators (PC3, DU145, LNCaP, and TSU-Pr1) were all established from metastatic lesions [3–6]. As such, it is unlikely that these cell lines accurately recapitulate the genetic composition or biological behavior of primary prostate tumors. Cell lines more appropriate for the study of human prostate primary tumors would be those derived from spontaneously immortalized cells; unfortunately, explanted prostate cells survive only short-term in culture, and rarely immortalize spontaneously. The use of viral transforming proteins to immortalize normal and primary tumor-derived human prostatic epithelium has allowed the continual propagation of these cells in vitro [2]. The cell lines examined in the present study were created through the

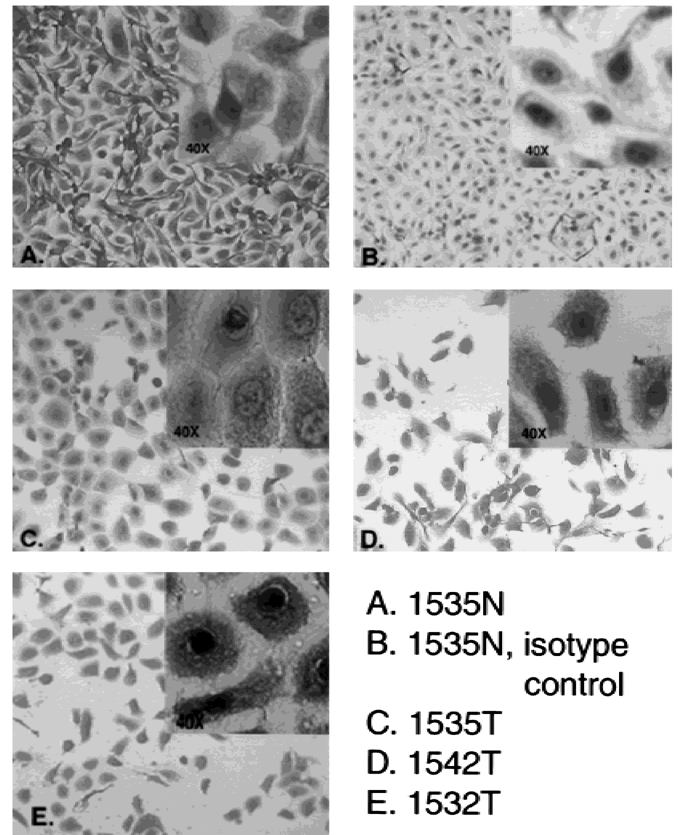

Fig. 3. Cytokeratin expression. Cells were stained using a panel of anti-cytokeratin antibodies. Photomicrographs of the stained slides taken at 10× and 40× (insets) magnification are shown for cells stained with anti-cytokeratins in (**A**) 1535N cells, (**C**) 1535T cells, (**D**) 1542T cells, and (**E**) 1532T cells, or (**B**) the isotype control in 1535N cells.

transduction, and subsequent immortalization, of normal and malignant prostatic tissues with the E6 and E7 transforming genes of HPV-16 or with the large T antigen gene of SV40 [2,7].

The experiments reported here were designed to assess the extent to which cell lines developed through immortalization of normal or primary tumor-derived prostatic epithelium with viral genes would express aspects of the normal or malignant phenotypes. This information would indicate whether such cell lines successfully recapitulated the biological behavior of the normal or malignant prostatic epithelium from which they were derived, and hence, whether they could serve as useful model systems for prostate biology and malignant transformation.

We first examined the growth of HPV-16 E6/E7 immortalized cell lines in the presence or absence of growth factors. All prostate cell lines examined (1532N, 1535N, 1542N, 1532T, 1535T, and 1542T) were able to grow in the absence of serum, with or without androgen supplementation, at rates comparable to

those achieved with serum added to the media. We then examined the doubling times of the HPV-16 E6/E7 immortalized cells under normal growing conditions (with serum). Interestingly, doubling times for the malignant cell lines were 24–31 hr, considerably shorter than those of the normal cell lines at 32–48 hr. Thus, the first observed phenotypic difference between HPV-16 E6/E7 immortalized cells was shorter doubling times for primary tumor- compared to normal-derived prostatic epithelial cell lines. The second phenotypic difference observed for the HPV-16 E6/E7 immortalized cells was that all three primary tumor-derived cell lines, but none of the corresponding normal-derived cell lines, were able to form colonies in soft agar. Thus, the primary tumor-derived cell lines were clearly distinguishable from the normal-derived cell lines by both a rapid doubling rate and the demonstration of anchorage independence. Not surprisingly, an inverse relationship was observed between doubling time and ability to form colonies in soft agar. Of the primary tumor-derived cell lines, the one with the shortest doubling time, 1532T, was also the one with the greatest ability to form colonies in soft agar (308 ± 17). In contrast, the primary tumor-derived cell line with the longest doubling time, 1542T, was least able to form colonies in soft agar (168 ± 11). Thus, if doubling time and anchorage independence can be used as criteria to measure expression of the malignant phenotype, then these cell lines may be ranked by their abilities to do so as 1532T > 1535T > 1542T.

The PrEC-T cell lines demonstrated a phenotype with aspects common to both the HPV-16 E6/E7 immortalized normal- and primary tumor-derived cells. Like the HPV-16 E6/E7-immortalized cells, PrEC-T cells were able to grow in low serum and did not respond to androgen stimulation. However, the doubling time for PrEC-T cells was 36 hr, which was intermediate to those of the normal- and primary tumor-derived prostate cell lines. Also, PrEC-T cells were the only normal-derived cells to demonstrate any ability to form colonies in soft agar, though this ability was limited (76 ± 6). Thus, it appears that transfection with large T antigen resulted in both the immortalization and early transformation of PrEC-T cells.

These observations are consistent with other studies suggesting that the mechanism by which expression of the HPV-16 E6 and E7 genes results in cellular immortalization is through inactivation of the p53 protein by viral E6, destabilization of the Rb protein by viral E7, and activation of telomerase [8–11]. The combined effect of these activities is to bypass cell cycle checkpoints, reestablish telomere lengthening, and overcome replicative senescence, i.e., become immortalized. None of these events, however, are sufficient for cellular transformation or expression of the malignant phenotype [12]. In contrast, the SV40 large T antigen, which also abrogates the p53 and Rb pathways, can mediate both cellular immortalization and transformation [13–15]. Cells transformed by SV40 typically express some aspects of the malignant phenotype, including reduced dependency on growth factor (e.g., serum) requirements and anchorage independence [16]. It has been proposed that the ability of SV40 large T antigen to induce transformation may be due to mutations in other cellular genes not directly effected by T antigen [17]. Therefore, the ability of the SV40 large T-transfected, but not HPV-16 E6/E7-transfected, normal prostate cells to express aspects of the malignant phenotype consistent with initial transformation may be due to differences in the genetic background of these cells. These differences are likely due to genetic alterations acquired due to, or consequent to, expression of viral proteins and the immortalization process. For example, many cytogenetic changes have been described for cells immortalized through transfection with HPV or SV40 [11,18–21].

None of the HPV-16 immortalized human prostate primary tumor-derived cell lines examined here were able to form detectable tumors in vivo in athymic mice. These findings are similar to those reported for CA-HP-10 cells, an HPV-18 immortalized human prostate adenocarcinoma-derived cell line, which were able to form only small nodules of nonproliferating cells in athymic mice [22]. Bright et al. also reported that subcutaneous injection of HPV-16 E6/E7 immortalized primary tumor-derived prostate cancer cell lines 1510-CPTx, 1512-CPTx, or 1519-CPTx failed to produce tumors in vivo in athymic mice [2]. Taken together, these studies suggest that cell lines developed from human prostate primary tumors are unable to express the full spectrum of the malignant phenotype. Rather, these cells express phenotypic traits perhaps more consonant with the primary, localized tumors from which they were derived (faster proliferation rate, reduced dependence on growth factors, and anchorage independence) and are unable to express phenotypic traits associated with more aggressive, metastatic tumors (e.g., the ability to form tumors in vivo). It is perhaps not surprising that human prostate cell lines capable of forming tumors in vivo were all derived from metastatic lesions and possess very aberrant genotypes [3–6,23]. It is possible, then, that genetic alterations in addition to those demonstrated by the cell lines examined here (see below) may be required for full expression of the malignant phenotype.

Of the genetic changes observed in the cell lines utilized in this study, it is interesting to note that 4 of the 5 cells lines expressing aspects of the malignant phenotype (1532T, 1542T, PreC-T, and HT-29) demon-

strated structural alterations of chromosome 8 [2,7,18]. The 1532T and HT-29 cell lines are characterized by i(8q) chromosome formation; the 1542T karyotype includes i(8q), der(8;20)(q10;p10), and der(8;21)(p10;q10) chromosomes; and PrEC-T demonstrates gain of the 8q chromosomal region [7]. Therefore, gain of all or part of 8q was observed in three prostate-derived cell lines (1532T, 1542T, and PrEC-T), and was accompanied by the loss of 8p sequences in 2 of the 3 cell lines (1532T and 1542T). Deletion of one or both copies of 8p is a very frequent and likely early event in prostate tumorigenesis [24–26]. Moreover, other studies have reported 8q gain in advanced prostatic cancer [27–30]. Therefore, it is certainly possible that loss of 8p sequences and/or gain of 8q sequences contributed to the ability of these cells (especially PrEC-T) to express aspects of the malignant phenotype. If so, these cell lines, and others like them, generated from normal or primary tumor epithelium, may offer unique models for prostate tumorigenesis, and may help elucidate early transforming events in the prostate.

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