

# A novel neoplastic primary tumor-derived human prostate epithelial cell line

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**Abstract.** Research into molecular and genetic mechanisms underlying prostate carcinogenesis would be greatly advanced by *in vitro* models of prostate tumors representing primary tumors. The generation of immortalized primary prostate cancer cells that will accurately reflect the *in situ* characteristics of malignant epithelium is greatly needed. We have successfully established a neoplastic immortalized human prostate epithelial (HPE) cell culture derived from a primary tumor. The RC-9 cells transduced through infection with a retrovirus vector expressing the E6 and E7 genes (E6E7) of human papilloma virus-16 (HPV-16) are currently growing well at passage 40, whereas RC-9 cells senesced at passage 7. RC-9/E6E7 cells exhibit epithelial morphology and high level of telomerase activity. More importantly, these immortalized cells produced tumors (SCID5038D) when inoculated into SCID mice. RC-9/E6E7 cells and SCID-5038D cells exhibit a high level of telomerase activity and androgen-responsiveness when treated with R1881. Expression of prostate specific antigen (PSA), androgen receptor (AR), prostate stem cell antigen (PSCA), an androgen-regulated prostate specific gene (NKX3.1), p16, cytokeratins 8, 15 and HPV-16 E6 gene was detected in both of these cells. RC-9/E6E7 and SCID5038D cells also showed growth inhibition when exposed to retinoic acid and transforming growth factor (TGF)- $\beta$ 1, potent inhibitors of prostate epithelial cell growth. A number of chromosome alterations were observed including the loss of chromosomes 2p, 3p, 8p, 13, 14, 16, 17, 18, 21 and the gain of 7 and 20 in the tumor cell line (SCID5038D). These results demonstrate that this primary tumor-derived HPE cell line retained its neoplastic phenotypes

and its prostate-specific markers and should allow studies to elucidate molecular and genetic alterations involved in prostate cancer. This is the first documented case of a malignant AR and PSA positive established human prostate cancer cell line from a primary tumor of a prostate cancer patient.

## Introduction

Prostate cancer is the most common male cancer in the United States, as well as in the Western world, and the second leading cause of male cancer death in the United States (1). The recent progress made in identifying cancer genes and understanding cancer genetics is impressive. However, our understanding of the molecular genetic mechanisms underlying prostate carcinogenesis remains limited, particularly when compared with other cancers i.e., colon, renal, and breast (2).

*In vitro* human cell culture models are critical for defining the mechanism of prostate cancer progression and for testing preventive and therapeutic regimens. The generation of immortalized HPE cell culture that will accurately reflect the *in situ* characteristics of malignant prostate epithelium is imperative. *In vitro* cell culture models of human prostate carcinogenesis have not been widely available or well characterized until recently (3). To date, only three readily available and well-studied long-term human prostate carcinoma cell lines (DU145, PC-3, and LNCaP) exist. All three were isolated from metastatic lesions, thus leaving a void in reagents representing long-term human cell lines derived from primary localized adenocarcinoma of the prostate. To study early genetic and molecular lesions of prostate cancer, cell lines derived from primary tumors are urgently needed.

Androgen regulation of prostate growth as well as the widely-used androgen deprivation therapy for prostate cancer treatment necessitates a better understanding of the role of androgen in prostate cancer biology. Well-characterized prostate cancer cell line (LNCaP) is known to express AR but it is a mutant receptor. Therefore, generation of primary prostate tumor-derived cell lines expressing AR will have significant impact in evaluating the role of androgen signaling pathway. However, such cell lines are presently not available.

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The establishment and maintenance of long-term HPE cell lines which retain their original malignant phenotypes of primary tumors have been unsuccessful in the absence of *in vitro* immortalization. Successful generation of immortal HPE cell lines from primary prostate cancer specimens by HPV has been described (4,5). However, these immortalized cells are usually not tumorigenic in nude mice and do not express AR as well as PSA. To our knowledge, no successful establishment of AR and PSA-positive primary human prostate cancer cell lines with neoplastic phenotypes has been reported.

## Materials and methods

**Cell culture and medium.** The tumor tissue (RC-9) used for generating cell lines was obtained from the radical prostatectomy specimen of a 61-year old patient. The presence of prostatic adenocarcinoma with moderate - poor differentiation (Gleason 3 + 4) was confirmed under light microscopy. A fresh prostatectomy specimen was obtained under sterile conditions by an experienced pathologist. On gross inspection tumor tissue was dissected separately for the purpose of generating cell culture. The tumor tissue was chopped into small fragments, 1-2 mm in size, with a sterile blade. The small cell clumps were placed into several type 1 collagen-coated dishes (Becton-Dickinson, Boston, MA) containing growth medium and allowed to attach for a week to the bottom surface of the culture dishes. The cells were incubated at 37°C in a humidified air of 5% CO<sub>2</sub> until reaching semiconfluency. Aliquots of the primary cultures were then frozen down and stored in liquid nitrogen until the cells were re-established in secondary culture for further serial passages. For serial passages, routine trypsinization was used once a week in the collagen-treated culture dishes, and the split ratio of the cells was 1-2. Keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract and recombinant epidermal growth factor (Life Technologies, Inc., Grand Island, NY) was used for the growing and maintaining the cells (6,7). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 5 µg/ml of insulin (Invitrogen, Carlsbad, CA) (DMEM+F+I) were also used.

**Generation of RC-9/E6E7 cell line.** At passage 1, the actively proliferating RC-9 cells grown in K-SFM were infected with a recombinant retroviral construct, LXSN-E6E7 (generously provided by Dr D.A. Galloway, Seattle, WA) containing the E6 and E7 genes of HPV-16 and a neomycin resistance gene (8). Briefly, cells were transduced through infection using polybrene at the concentration of 10 µg/ml and incubated at 37°C in 5% CO<sub>2</sub> overnight. The infected cells were washed with PBS, then incubated and subcultured weekly for further serial passages. No G418 selection was necessary because the uninfected RC-9 cells senesced at passage 7. RC-9/E6E7 cells (passage 13) were later adapted to grow in DMEM+F+I medium by stepwise fashion. The RC-9/E6E7 were grown for 2 weeks with mixtures of K-SFM and DMEM+F+I medium in ratios of 1:1, 1:3 and 1:7 for 2 passages each. The adaption process took six weeks for 6 passages.

**Growth curves.** RC-9/E6E7 cells (passage 24) were thoroughly trypsinized and plated in triplicate into 6-well tissue culture

plates to obtain 0.5, 1.0, 2.0, and 4.0x10<sup>4</sup> cells/cm<sup>2</sup>. Plates were incubated at 37°C in 5% CO<sub>2</sub> by changing media twice a week. At 3, 6, 9, 12, and 15 days, cells were counted using a Coulter counter (Beckman Coulter, Miami, FL). The data represent each average value through independent double experiments.

**Tumorigenicity in SCID mice.** To determine tumorigenicity, 1x10<sup>7</sup> cells in 0.2 ml of phosphate-buffered saline (PBS) were injected subcutaneously into the mid-dorsal intrascapular region of 7-day-old male suckling SCID mice. The mice were observed for 6 months for tumor development.

**Telomerase assay.** Cellular extracts were assayed for telomerase activity with telomeric repeat amplification protocol (TRAP) assay (9). This is a PCR-based assay utilizing a telomerase extension (TS) primer (5'-AATCCGTCGAGCAGAGTT-3') and a downstream (CX) primer (5'-CCCTTACCCTTACCCTTACCCTAA-3'). This assay amplifies telomeric repeats; repeat length is longest in cells exhibiting telomerase activity and shortest in cells exhibiting little or no telomerase activity. LNCaP cell line was used as a positive control because it is known to exhibit a high telomerase activity (10).

**Reverse transcriptase polymerase chain reaction (RT-PCR).** RT-PCR assay was done as previously described (11). Briefly, total RNAs from culture cells were extracted with RNazol B (TEL-TEST Inc., Friendswood, TX) according to manufacturer's protocol and quantified with Nucleic Acid Quantitation Kit (NBI, Plymouth, MN). Total RNA (1 µg) was reverse transcribed into cDNA with RNA PCR-kit (Perkin Elmer, Foster, CA) and 1/10 of the reverse-transcribed product from each sample was used for PCR to amplify AR, PSA, NKX3.1, PSCA, CK 8 and 15, p16, and HPV-16 E6 genes respectively. The expression of CK 8 was used as an internal control for input RNA as well as the marker for epithelial cells, because NKX3.1 expression was restricted to epithelial cells. To verify the validity of CK 8 as the internal control, we compared CK 8 and the house-keeping gene, GAPDH in the same cDNA samples. The condition of PCR for the individual gene was optimized to analyze the amplified product in the linear range of amplification by adjusting amplification cycles for each set of primers. The primer sequences and the expected size of PCR products were the same as described (11).

**Androgen sensitivity assays.** One hundred thousand cells were plated in duplicate into sterile 6-well plates with designated media. After 24 h, when cells were settled down completely, each well was changed with fresh medium supplemented with R1881 (methyltrienolone) (New England Nuclear Life Sciences, Boston, MA) at various concentrations ranging from 0 to 10 nM. After a 6-day treatment, changing media every 48 h, cells were counted using a Coulter counter (Beckman Coulter). The data represent each average value through independent triple experiments.

**Growth factor response.** Both human recombinant transforming factor-β1 (TGF-β1, Boehringer Mannheim, Germany)

Table I. Properties of HPV-16 E6E7-transduced RC-9/E6E7 cells and its derivative tumor cell line, SCID5038D.

|                             | RC-9/E6E7        | SCID5038D |
|-----------------------------|------------------|-----------|
| Life span                   | >40              | >20       |
| Telomerase activity         | +                | +         |
| Gene expression by RT-PCR   |                  |           |
| E6                          | +                | +         |
| AR                          | +                | +         |
| PSA                         | +                | +         |
| NKX3.1                      | +                | +         |
| PSCA                        | +                | +         |
| CK 8                        | +                | +         |
| CK 15                       | +                | +         |
| p16                         | +                | +         |
| GAPDH                       | +                | +         |
| Tumorigenicity in SCID mice | 4/4 <sup>a</sup> | ND        |

<sup>a</sup>Tumors were re-established in tissue culture and confirmed as human cells; their resemblance to the cell of origin was determined by karyological analysis. RC-9/E6E7 cells (passage 19) was tested. ND, not done.

and RA (Sigma Chemicals, St. Louis, MO) were tested for their inhibitory effect on the RC-9/E6E7 and SCID5038D cells. One hundred thousand cells were plated in duplicate into sterile 6-well plates with designated media. After 24 h, when cells were settled down completely, each well was changed with fresh medium supplemented with TGF- $\beta$ 1 or RA at various concentrations ranging from 0 to 10 ng/ml. After a 6-day treatment, changing media every 48 h, cells were counted using a Coulter counter. The data represent the average value through double independent experiments.

**Cytogenetic analysis.** Chromosome counts, ploid distribution, and Giemsa (G)-banded karyotypes were prepared by standard protocol as described previously (12,13).

## Results

**Immortalization of the RC-9 cells with HPV-16 E6E7 genes.** To determine whether human prostate cancer cells will be immortalized by the expression of HPV-16 E6E7 genes, we introduced a retrovirus construct expressing HPV-16 E6E7 genes into early-passage (passage 1) RC-9 cells through overnight infection in the presence of polybrene (10  $\mu$ g/ml). Non-infected cells could not be propagated serially beyond 7 subcultures. In contrast, infected RC-9 cells have an apparently unlimited lifespan and have been successfully subcultivated for more than 40 passages over the course of 1 year with no evidence of decreased proliferation capacity (Table I). The RC-9/E6E7 cells had the typical transformed morphology (Fig. 1A). The cells grew as adherent cells and were more piled-up on each other in some areas. RC-9/E6E7 cells were grown in a dose-dependent manner, but they did

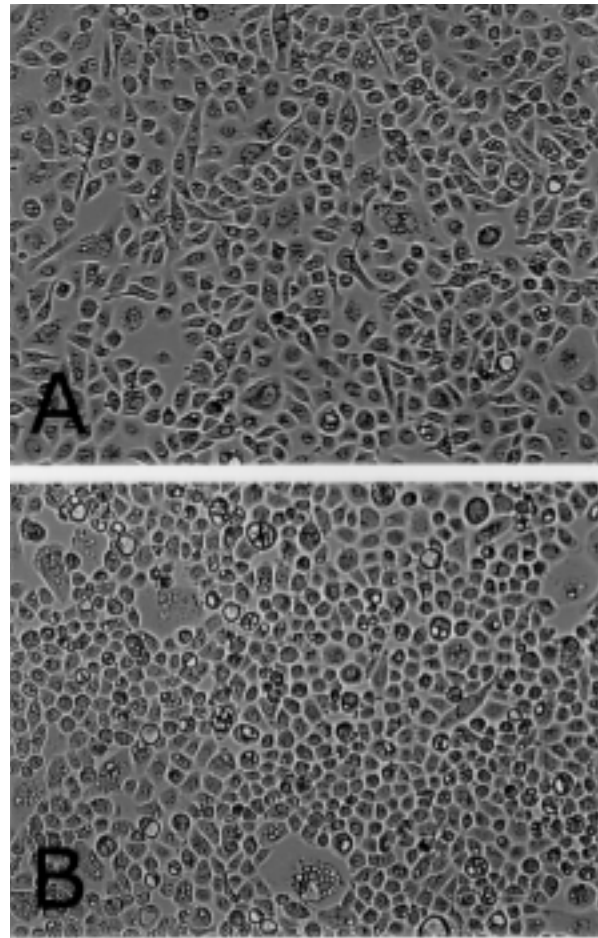


Figure 1. Morphological features of RC-9/E6E7 (A) and DMEM-adapted RC-9/E6E7 (B) cell cultures.

not require much density for cell proliferation (Fig. 2A). The optimal density for cell growth ranged from 0.5 to 1.0x10<sup>4</sup> cells/cm<sup>2</sup>, respectively. A high level of telomerase activity was detected in the RC-9/E6E7 cells (Fig. 3).

**Induction of tumors in SCID mice by the RC-9/E6E7 cells.** To determine tumorigenicity *in vivo* for the RC-9/E6E7 cell line (passage 19), 1.0x10<sup>7</sup> cells were injected subcutaneously into SCID mice. All the animals developed tumors within 2 1/2 months at the sites of inoculation (Table I). Microscopic examination of sections of the tumors revealed carcinoma. Cultures established from the tumors (SCID5038D) resembled the RC-9/E6E7 cells (Fig. 1B).

**Characteristics of RC-9/E6E7 and SCID5038D cell lines.** To confirm that the RC-9/E6E7 and SCID5038D cells contain the transduced HPV-16 E6E7 genes, RT-PCR was performed. RC-9/E6E7 and SCID5038D cells expressed the HPV-16E6 gene (Fig. 4). RNA samples from RC-9/E6E7, SCID5038D and 267B1 cells were further analyzed to determine the expression of specific markers by RT-PCR. The 267B1 cells were used as a positive control. RC-9/E6E7 and SCID5038D cells expressed AR, PSA, NKX3.1, and CK 8 and CK 15. PSCA and p16 was also expressed in these cell lines (Fig. 4).

Telomerase activity is found in prostate cancer and immortalized HPE cell lines (10,14). It was thought to be

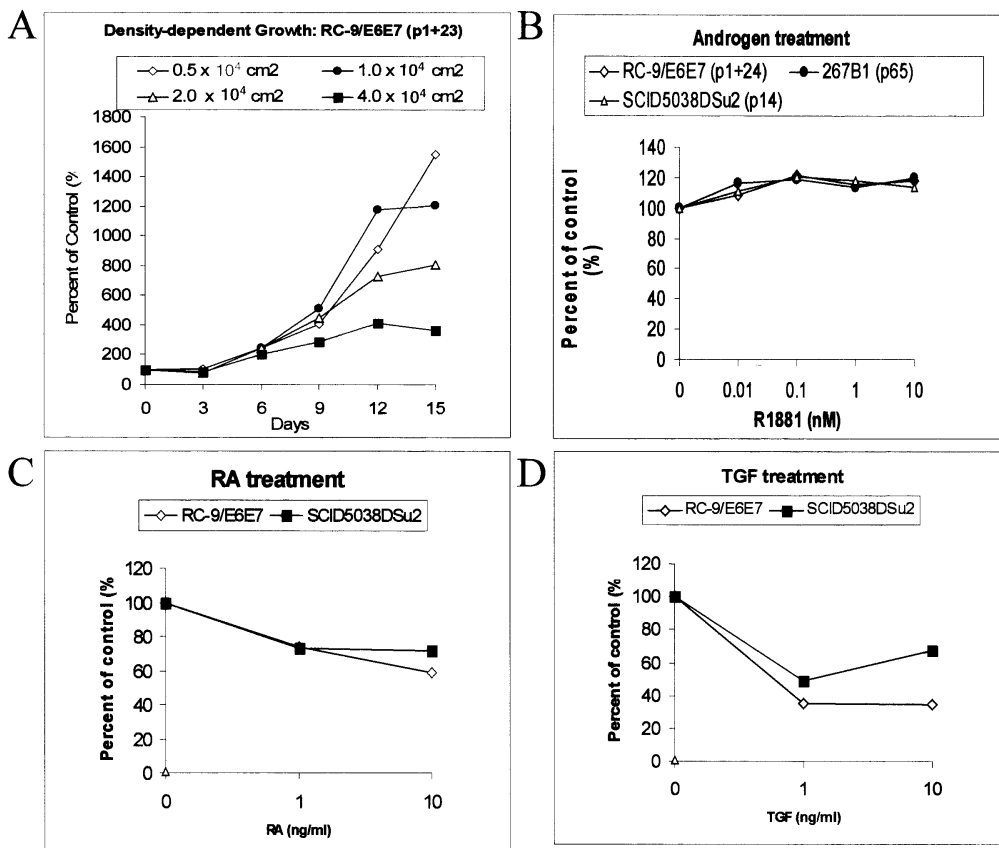


Figure 2. A, density-dependent growth of RC-9/E6E7 cells at the density of 0.5 (◇), 1.0 (●), 2.0 (△), 4.0 (■) x10<sup>4</sup> cells/cm<sup>2</sup>. B, cell growth by treatment with 0.01, 0.1, 1, and 10 nM of R1881 in RC-9/E6E7 (◇), SCID5038D (△), and 267B1 (●) cells. Inhibition of cell growth by treatment with 1 and 10 ng/ml of RA (C) or TGF-β1 (D) in RC-9/E6E7 (◇) and SCID5038D (■) cells.

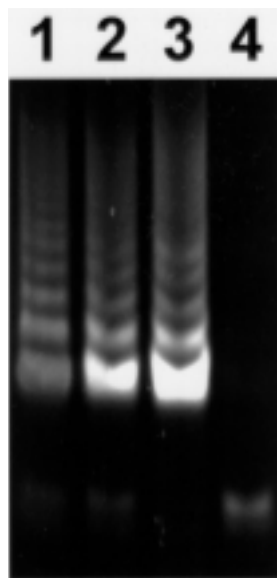


Figure 3. Telomerase activities in extracts from RC-9/E6E7 (1), SCID5038D (2), and LNCaP (3) cells. 1X CHAPS buffer was used as a negative control.

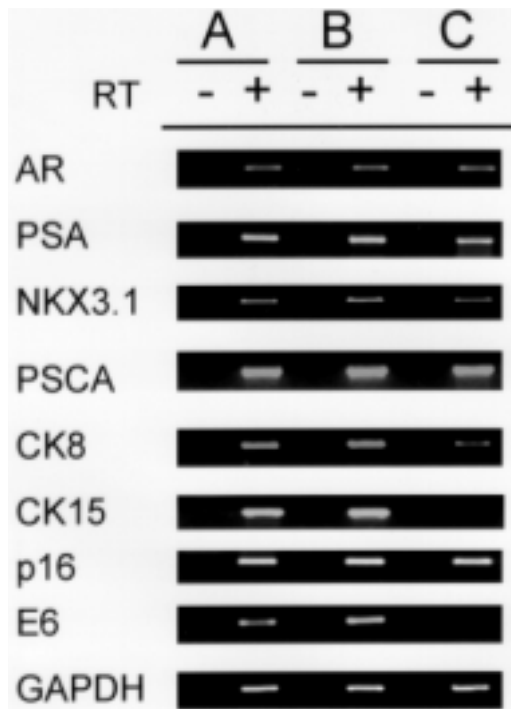


Figure 4. Analysis of RT-PCR products generated from RC-9/E6E7 (A), SCID5038D (B), and 267B1 (C) cells. 267B1 are an immortalized human prostate epithelial cell line that is already known to express a variety of genes, and is therefore used as a positive control. Each cell line was also evaluated without reverse transcriptase activation which resulted in an internal control. This figure shows the representative data through triple experiments.

necessary to maintain telomeric length in order for cells to continue to proliferate. Using the TRAP assay, high levels of telomerase activity were detected in both cell lines (Fig. 3).

The RC-9/E6E7 and SCID5038D cell lines were further analyzed for their abilities to respond to androgen treatment



Figure 5. Karyotypes of the RC-9/E6E7 (upper) and SCID5038D (lower) cell lines.

in order to confirm that they are AR-positive cell lines. Both cell lines responded to androgen treatment with maximum increase of 20% in comparison to that of the control, which was quite similar to that of 267B1 cells known as an androgen-responsive cell line (Fig. 2B). Both cell lines were also analyzed for their abilities to respond to growth-inhibitory factors known to regulate prostate cell growth (15). Both RC-9/E6E7 and SCID5038D cells showed growth inhibition when exposed to RA (Fig. 2C) in a dose-dependent manner. TGF- $\beta$ 1 also inhibited growth of both cell lines, but the effect was maximal at the concentration of 1 ng/ml (Fig. 2D).

Cytogenetic characteristics of the RC-9/E6E7 and SCID5038D cell lines were examined (Fig. 5; Table II). The

RC-9/E6E7 cell line was hyperdiploid while SCID5038D cell line was hypotetraploid. There was observed trisomy 9 and addition of extra material of unknown origin on one copy of chromosome 15 in the RC-9/E6E7 cell line. The additional copy of chromosome 9 observed in the RC-9/E6E7 cell line was lost in the SCID5038D tumor cell line. The random loss of chromosomes 2p, 2q, 3p, 6q, 7p, 7q, 8p, 10p and addition of material with 11q observed in the RC-9/E6E7 cell line were established as markers in the SCID5038D cell line (Fig. 4; Table II). The most significant markers in the tumor SCID5038D cell line were M2, M3, M4, M5 and M10. These are observed in majority of karyotypes. Looking at the karyotypes it is evident that there is loss of chromosomes 2p, 3p,

Table II. Karyological characterization of RC-9/E6E7 and SCID5038D cell lines.

| Cell line            | Ploidy         | Count | Model no. | Karyotype description  |
|----------------------|----------------|-------|-----------|--|
| RC-9/E6E7 P(1+19)    | Hyperdiploid   | 45-48 | 47        | 47, XY +9 -15+(M1=15p+)  |
| SCID5038D/SU-2 p(12) | Hypotetraploid | 72-80 | 78        | 70-80, XXYY-(2, 2, 3, 3, 4, 5, 8, 10, 10, 11, 11, 12, 13, 13, 14, 14, 14, 14, 15, 16, 17, 18, 19, 19, 19, 21.) +(7, 20, 20)+M1-M10 |

Marker description: M1, 15p+; M2, der(11)t(3?/8?;11)(q21/q22?;q22); M3, der(14?); M4, der(19?); M5, t(10q;14q); M6, 21p+; M7, t(3q?:19?); M8, del(3)(q10); M8A, I(3q); M9, 16q+; M10, del(2)(p16p24).

8p (8q may be present in markers M2, M3 and M4, which can be tested by FISH), 13, 14, 16, 17, 18, 21 and the gain of chromosomes 7 and 20 in the SCID5038D cell line.

## Discussion

The present study appears to represent the first documented case of the neoplastic HPE cell line established from a primary tumor of a prostate cancer patient. The RC-9/E6E7 cells grown originally in K-SFM were able to grow in the DMEM+F+I medium and produced tumors in inoculated SCID mice. They expressed AR and PSA, featuring typical markers of prostate origin, as well as CK 8, NKX3.1, PSCA, and p16. They showed androgen-responsiveness when treated with synthetic androgen (R1881), and also growth inhibition when exposed to RA and TGF- $\beta$ 1. The cell line was hyperdiploid and showed typical marker chromosomes M1(15p+) and M2(11q+). The M2(11q+) marker chromosome has been observed as common chromosome directly due to the immortalization with HPV-16 E6E7 (16).

Our group and others have previously developed reliable methods for generating and characterizing prostate cancer derived from primary tumors using HPV genes (4,5). These models are well-established, promising a powerful *in vitro* tool for the study of early carcinogenic mechanisms underlying prostate cancer. However, these models do not mimic primary tumors of prostate cancer because these immortalized cells are not tumorigenic. In addition, these immortalized cells do not express AR and PSA. A recent study showed that CA-HPV-10 cells immortalized by HPV-18 from a primary tumor specimen induced tumors after a few passages in SCID mice. Stepwise genetic change was associated with progression of this non-tumorigenic CA-HPV-10 cell line to a malignant phenotype (17).

It is interesting to note that the high passage RC-9/E6E7 cells adapted to grow in the DMEM+F+I medium were tumorigenic in SCID mice. Some genetic alterations might be required during stepwise changes of the medium like those already reported in *in vivo* situation (17). The addition of insulin might be another important factor to achieve tumorigenicity. Although there has been controversy about the effects of insulin, insulin-like factor (IGF) or IGF-binding protein (IGF-BP) on prostatic epithelial cells (18). IGFs and IGF-BPs have been implicated as strong mitogens and to have potential to accelerate the progression in prostate cancer models (19).

Recently, an epidemiological study revealed the strong association between prostate cancer and serum levels of insulin (20). Studies focused on clarifying the potential role of insulin or IGFs in underlying the tumorigenicity of the RC-9/E6E7 cells are currently in progress.

Prostate cancer is a complex, multifactorial disease with genetic and environmental factors involved in its etiology. The search for genetic determinants involved in the disease has proven to be challenging, in part because such complex diseases are often not amenable to characterization by linkage analysis and positional cloning as is the case for diseases with simple Mendelian genetic inheritance. The molecular mechanisms underlying the development and progression of prostate cancer are poorly understood. Epidemiological studies have suggested that 9% of all prostate cancers are familial, and numerous chromosomal loci have been associated with prostate cancer in multicenter linkage studies (21-24). However, no putative susceptibility genes harbored in these chromosomal regions have thus far been identified. Several recurrent chromosomal alterations in prostate cancer have been detected in comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analysis (21). The target genes for many of these aberrations are still unknown. Looking at our data some of the same recurrent chromosomal alterations already reported in prostate cancer are evident. There is loss of chromosomes 2p, 3p, 8p (as 8q may be present in markers M2, M3 and M4, which can be tested by FISH), 13, 14, 16, 17, 18, and 21 and gain of 7 and 20 tumor SCID5038D cell line.

The results obtained here have demonstrated that this immortalized primary tumor-derived HPE cell line retained its original malignant phenotypes of a primary tumor and expresses its prostate specific markers. The androgen-responsive properties of this cell line should help answer questions related to androgen regulation of prostate cells. This novel *in vitro* model will become a powerful tool for elucidation of prostatic carcinogenesis and also provide the means for testing new modalities for both prevention and progression of prostate cancer, as well as provide the methods for testing both chemopreventive and chemotherapeutic agents.

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