hTERT-Immortalized Prostate Epithelial and Stromal-Derived Cells: an Authentic *In vitro* Model for Differentiation and Carcinogenesis

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Abstract

Prostate cancer is the most commonly diagnosed type of cancer in men, and there is no available cure for patients with advanced disease. *In vitro* model systems are urgently required to permit the study of human prostate cell differentiation and malignant transformation. Unfortunately, human prostate cells are particularly difficult to convert into continuously growing cultures. We report here the successful immortalization without viral oncogenes of prostate epithelial cells and, for the first time, prostate stromal cells. These cells exhibit a significant pattern of authentic prostate-specific features. In particular, the epithelial cell culture is able to differentiate into glandular buds that closely resemble the structures formed by primary prostate epithelial cells. The stromal cells have typical characteristics of prostate smooth muscle cells. These immortalized cultures may serve as a unique experimental platform to permit several research directions, including the study of cell-cell interactions in an authentic prostate microenvironment, prostate cell differentiation, and most significantly, the complex multistep process leading to prostate cell transformation. (Cancer Res 2006; 66(7): 3531-40)

Introduction

Prostate cancer is the most commonly diagnosed type of cancer in men.[5] Localized tumors are conventionally treated by radical prostatectomy (1). In cases of advanced disease, initially effective hormone therapy eventually fails, leading to the development of hormone-refractory, drug-resistant malignancy (2). Despite unremitting efforts put into prostate cancer research, there is still no cure available for patients with advanced disease (3). The difficulty in developing treatments is due, in part, to the fact that the existing model systems are not able to address important questions concerning this malignancy. In general, cancer research relies mainly on animal and tissue culture systems as experimental models. To that end, large numbers of cell lines have been established from different tissue origins. However, human prostate cells are known to be one of the most difficult cell types to develop into continuously growing culture (4). Furthermore, several mouse models for prostate cancer as well as for other types of cancer have been established using transgenic animals. These models offer a very important tool for cancer research. However, fundamental differences between mouse and human mechanisms of immortalization and transformation make it imprudent to rely on mouse models alone (5). Thus, *in vitro* models using human cells represent an indispensable tool for cancer research in general and for prostate cancer research in particular.

Nonmalignant human primary cells undergo terminal growth arrest following several divisions *in vitro* (6). This growth arrest is termed “replicative senescence” and it is thought to also occur *in vivo*, representing one of the mechanisms that block transformation (6). Since the discovery of DNA tumor viruses, it became possible to create immortal cultures from normal human tissues and from primary tumors. Immortalized lines from various tissues, including prostate, were successfully established by overexpression of viral oncogenic products (7–10). An alternative and more recent tool to immortalize human normal cells is the overexpression of the human telomerase catalytic subunit (hTERT; ref. 11). Immortalization of cells with hTERT has an advantage over viral proteins. Although the viral immortalization mediators display oncogenic properties, such as inactivation of the major tumor suppressor proteins (12), hTERT solely elongates chromosomal ends, which prevents genomic catastrophe or crisis associated with cell death (13). Although hTERT introduction readily induces immortalization of human fibroblasts, immortalization of other human cell types requires additional modifications (6), the most common of which is the inactivation of the pRb/p16^{INK4a} pathway (14). It is well accepted that early-passage hTERT-immortalized cells faithfully represent the physiologic properties of normal cells *in vivo* (13). Thus, we chose this strategy as a starting point for our model aimed at deciphering the molecular mechanism that underlies malignant transformation of the prostate.

The prostate consists of several cell types that may be involved in the emergence of cancer. Epithelial cells serve as the primary targets for malignant transformation, and stromal cells also contribute to this process (15, 16). We have successfully immortalized normal human prostate epithelial cells and stromal smooth muscle cells by hTERT introduction, generating continuously growing cultures. Although some modifications in gene expression patterns were noticed, both immortalized epithelial and stromal cells exhibited authentic prostate-specific features, including the ability of

[5] This information was obtained from http://www.cancer.org/docroot/STT/stt_0.asp.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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epithelial cells to differentiate *in vitro* into glandular buds. We believe that these human prostate epithelial and stromal cell cultures immortalized by hTERT comprise an improved model for prostate tumorigenesis, as they do not express viral-derived oncogenes (17, 18).

It is well accepted that immortalization represents an important step in the development of human cancer, because indefinitely dividing cells are susceptible to multiple genetic alterations necessary for malignant transformation (19). Therefore, we believe that our established immortalized cultures are a more faithful representation of the authentic prostate microenvironment and may serve as a suitable experimental platform to study transformation of prostate cells and the stem cell origins of prostate cancer. Furthermore, they should facilitate the investigation of prostate differentiation and cell-cell interactions.

Materials and Methods

Establishment of primary cell cultures. Primary epithelial and stromal cell cultures were established from normal prostate tissue obtained from patients undergoing radical prostatectomy for prostate cancer as previously described (20, 21). EP156 cells were derived from a radical prostatectomy specimen of a 66-year-old patient. Pathologic tumor stage was pT3a, Gleason score 7 (4 + 3). The specimen had a positive margin, meaning that the tumor extended the prostate capsule. Seminal vesicles were tumor-free. PM151 cells derived from a radical prostatectomy specimen of a 62-year-old patient. Pathologic tumor stage was also pT3a, Gleason score 5 (3 + 2). The specimen had a positive margin, meaning that the tumor extended the prostate capsule. Seminal vesicles were tumor-free. Tissue samples used for the establishment of the cultures were removed by the pathologist from a transition zone site that was free of tumor. Detailed procedure and growth media components are available in supplementary information.

To calculate cell growth curves, cells were split close to confluence by incubation with trypsin and replated at a cell density of 2,500 per cm^2. Population doublings at each passage were calculated using the formula: population doublings = log (cell output / cell input) / log 2.

Tumor cell lines. PC3 and LNCaP cells were grown in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% FCS (PAA Laboratories, Coelbe, Germany) and antibiotics. Amphotropic and ecotropic Phoenix retrovirus-producing cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% FCS and antibiotics.

Retroviral construct. pBabe-hTERT-puro was kindly provided by Dr. J. Shay (University of Texas Southwestern Medieval Center).

Retroviral infection. For hTERT infection, amphotropic Phoenix packaging cells were transfected with 10 μg DNA of the pBabe-hTERT-puro construct by a standard calcium phosphate coprecipitation procedure. The precipitate was removed, and fresh MCDB-131$^+$ or MCDB-153$^+$ media were added to the transfected Phoenix cells 24 hours after transfection. After 24 hours, culture supernatants were collected and filtered. Primary prostate stromal and epithelial cells were infected with the filtered viral supernatants in the presence of 4 μg/mL polybrene (Sigma, St. Louis, MO) for 12 hours, after which the medium was changed. Fresh viral suspensions were added after a 24-hour interval for an additional 12 hours. Infected cells were selected with 1 μg/mL Puromycin (5 days).

Telomere length measurement by the telomeric repeat binding fragment assay. Genomic DNA was extracted by GenElute Mammalian Genomic DNA kit (Sigma) according to the manufacturer's recommendations. Next, 2 μg of genomic DNA was reacted using the Telo TAGGG Telomere Length Assay kit (Roche Applied Science, Mannheim, Germany). Washed membranes were exposed to phosphorimaging plates for 5 to 60 minutes. Telomere lengths were compared according to the position of the bands.

Western blotting analysis. For Western blotting, total cell extracts were fractionated by gel electrophoresis, and proteins were transferred to nitrocellulose membranes. The following primary antibodies were used: mouse monoclonal anti-p53 (DO-1; kindly provided by Dr. David Lane, Ninewells Hospital and Medical School, Dundee, Scotland); rabbit polyclonal anti-p53 (produced in our laboratory); anti-p21^{waf1} (C-19, Santa Cruz Biotechnology, Santa Cruz, CA); anti-p16^{INK4a} (C-20, Santa Cruz Biotechnology); anti-β-tubulin (SAP.4G5, Sigma); anti-p63 (4A4, Santa Cruz Biotechnology); anti-androgen receptor (anti-AR; BioGenex, San Ramon, CA); anti-cytokeratin 14 (BD PharMingen, San Diego, CA); anti-cytokeratin 18 (BD PharMingen), anti-cytokeratin 5, anti-cytokeratin 6, and anti-cytokeratin 8 (Abcam, Cambridge, MA); anti-cytokeratin 7 and anti-cytokeratin 8 (CAM5.2, BD PharMingen); anti-β-actin (Chemicon, Temecula, CA); anti-estrogen receptor (Upstate, Lake Placid, NY); anti-smooth muscle α-actin 1A4 (Sigma); anti-myosin heavy chain (Biocytex, Marseilles, France); anti-calponin (hCP, Sigma); anti-progesterone receptor (AB-52, Santa Cruz Biotechnology). The protein-antibody complexes were detected by horseradish peroxidase–conjugated secondary antibodies followed by the enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights).

Total RNA isolation. Total RNA for the quantitative real-time PCR (QRT-PCR) was isolated using RNAeasy kit (Qiagen, Chatsworth, CA), according to the manufacturer's protocol.

QRT-PCR. A 2-μg aliquot of the total RNA was reverse transcribed using Moloney murine leukemia virus RT (Promega, Madison, WI) and random hexamer primers. QRT-PCR was done using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7000 instrument (Applied Biosystems). The primers for QRT-PCR were designed using Primer Express software. The values for the specific genes were normalized to the GAPDH housekeeping control. Primer sequences for SYBR Green PCR will be provided upon request.

Endothelin A treatment. Cells were seeded in Matrigel-coated six-well plates. After 24 hours, they were stimulated to contract with 2 nmol/L Endothelin A under a microscope equipped with a heating chamber to maintain the temperature at 37°C and a time lapse video camera. Cells were recorded for 2 hours at 1 frame per second (22).

Transforming growth factor-β treatment. Subconfluent PM151 cells and their hTERT derivatives were brought to quiescence in serum-free medium for 24 hours and exposed to control medium (serum-free) or medium containing 1 ng/mL transforming growth factor-β1 (TGF-β1; R&D Systems, Abingdon, United Kingdom) for 24 hours. For longer treatment, cells were seeded in six-well plates, and after 24 hours, they were treated with 1 ng/mL of TGF-β1 for 6 or 9 days.

Cisplatin treatment. Subconfluent cell cultures were treated with 1 or 2 μg/mL of cisplatin (Abic, Netanya, Israel) for 24 hours. Stock solution was prepared as 1 mg/mL in water.

Three-dimensional cell culture. Primary prostate epithelial cell cultures were established as described above and selected with keratinocyte serum-free medium (KSFM; Life Technologies, Paisley, United Kingdom) supplemented with KSFM supplement (containing insulin, epidermal growth factor, and fibroblast growth factor), bovine serum albumin (2.5 mg/mL), transferrin (2 μg/mL), and minimal essential amino acids (1%; ref. 23). Cells were detached using trypsin/EDTA and used for three-dimensional cell cultures based on a method described for mammary epithelial cells (24). EP156T cells or primary epithelial cells were seeded on eight-well chamber slides (Lab Tek; 5,000 per well) coated with Matrigel (BD Biosciences, San Jose, CA) and cultured for 14 days in MCDB-153$^+$ medium or KSFM, respectively, with 2% Matrigel and in the presence of the synthetic androgen R1881 (1 nmol/L). Medium was replaced every 4 days. For immunohistochemistry, spheroids were removed together with the Matrigel from the chamber slide using a pipette and mixed with an equal amount of Tissue-Tek (ornithine carbamyl transferase compound). The collected spheroids were snap frozen, and serial 7-μm sections were cut and mounted on Super Frost microscope slides. For immunohistochemistry, slides were acetone fixed for 10 minutes at −20°C and air-dried before staining. After blocking in 10% normal goat serum, sections were incubated with antibodies against: cytokeratin 5 (RCK 103, Dr. F. Raemakers, University of Maastricht, the Netherlands); cytokeratin 18 (DC10), p63 (Clone 4A4), and prostate-specific antigen (PSA; all from DAKO, Glostrup, Denmark); and AR (N-20, Santa Cruz Biotechnology). As secondary

antibody, either an Alexa Fluor 594–labeled goat anti-mouse or goat anti-rabbit was used (Molecular Probes, Eugene, OR). Sections were mounted with fluorescent mounting medium (DAKO) and examined with a fluorescence microscope.

Results

Immortalization of Primary Human Prostate Epithelial and Stromal Cells by hTERT Overexpression: Characterization of Cell Growth

Prostate epithelial cells. Primary cells were established from benign tissue from four different radical prostatectomies (see Materials and Methods). In our hands, the four individual primary human prostate epithelial (hPEC) cultures (designated EP152, EP153, EP156, and EP157) accumulated 13 to 16 population doublings before they ceased growth *in vitro* (Fig. 1). This is in agreement with previous studies, which reported that hPECs undergo 10 to 40 population doublings in culture before they enter senescence (4, 18, 25). To obtain immortalized populations, the four individual hPEC cultures (at ~12 population doublings) were infected with a recombinant retrovirus encoding hTERT, giving rise to the EP152T, EP153T, EP156T, and EP157T cultures, respectively. After recovery from antibiotic selection, the cells were serially passaged in drug-free medium. At each passage, the cells were harvested and counted before they attained confluence. The number of population doublings was determined by counting the cells at each passage (see Materials and Methods). As can be seen in Fig. 1*A*, the EP156T culture was successfully immortalized by hTERT. Two separate cultures, derived from the EP156T hTERT-infected pool, continued to proliferate beyond the replicative senescence checkpoint. The cells initiated from the initially infected culture (designated EP156T) underwent up to 200 population doublings, and the cells from the second culture (designated EP156T3) attained 80 population doublings (Fig. 1*A*). The noninfected EP156 cells gradually ceased proliferating after ~17 population doublings. In our present study, we focused mainly on the EP156T culture, unless otherwise indicated. One of the other hTERT cultures, EP153T, is also being successfully passaged *in vitro*. However, because this culture is at an earlier passage, its growth curve is not presented here. The other two infected cultures, EP152T and EP157T, ceased growing after 22 and 20 population doublings, respectively. In contrast, their noninfected counterparts divided only 14 and 13 times, respectively (Fig. 1*B* and *C*). Although EP152T and EP157T cultures did not become immortalized, their proliferative life span was extended following infection with hTERT.

As loss of telomeric sequences with each division cycle has been proposed to limit proliferation of normal human somatic cells (6), we assumed that the establishment of the immortalized line resulted from successful expression of hTERT. We therefore first wished to evaluate the activity of hTERT in our cultures. To this end, we measured telomere length, which reflects the ectopic expression of telomerase (26). Telomeric repeat binding fragment assays were done at different time points of three hTERT-infected prostate epithelial cultures (Fig. 1*D*). As expected, telomere elongation was evident in the immortalized EP156T culture. Interestingly, the EP152T culture, which was not immortalized and eventually died, also displayed longer telomeres at the later passage, indicating successful expression of the introduced hTERT. It, therefore, seems that telomere elongation alone was not sufficient to immortalize this culture. Taken together, these results suggest that in addition to the telomeric elongation mediated by hTERT expression, other factors must have contributed to the successful immortalization of the EP156T cells.

To further characterize the immortalized cultures, we compared their morphology to that of primary benign prostate epithelial cultures. The primary prostate epithelial cells at passage 5 were large, vacuolated, and heterogeneous (Fig. 1*E, a* and *b*). The

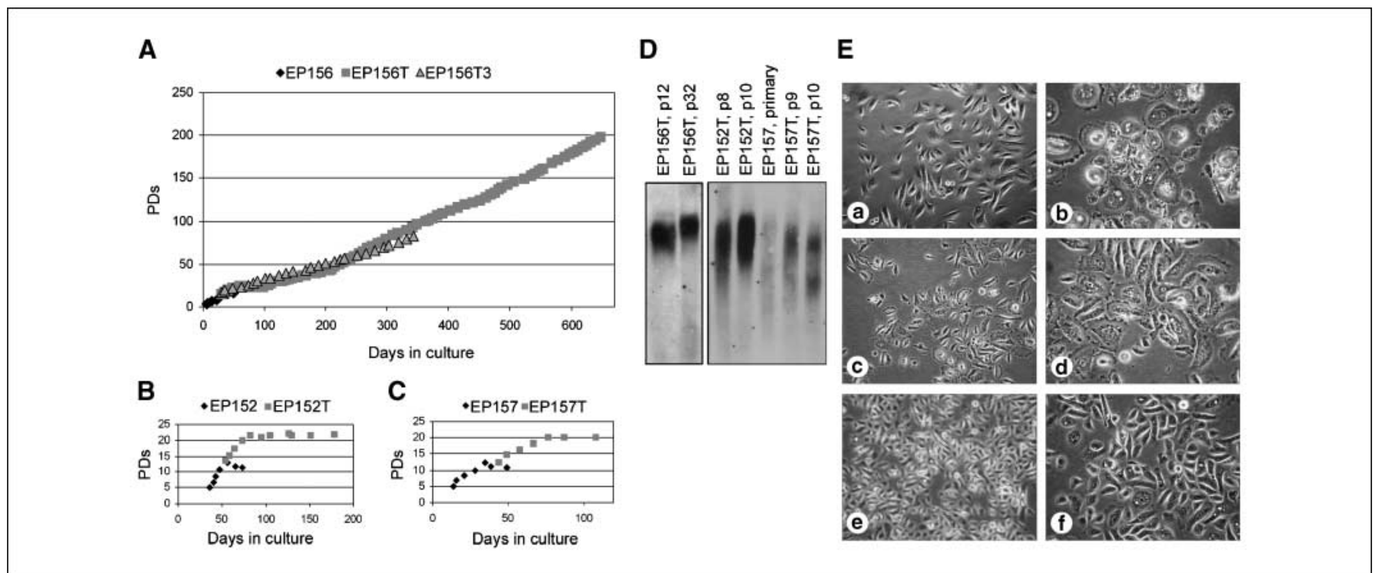

Figure 1. hTERT introduction induces immortalization of primary prostate epithelial cells. *A-C,* proliferation curves of prostate epithelial cells infected with hTERT (EP156T/EP156T3, EP152T, and EP157T) and their noninfected counterparts (EP156, EP152, EP157). To calculate the exact number of population doublings (*PD*), cells were harvested and counted before they attained confluence. *D,* telomeric length was evaluated by telomere repeat binding fragment analysis. Genomic DNA was isolated from noninfected (primary) and hTERT-infected prostate epithelial cells at various passages (p8-p32), and telomere repeat binding fragment assays were done as described in Materials and Methods. *E,* morphology of benign prostate epithelial primary and immortalized cultures: *a* and *b,* primary cells at passage 5; *c* and *d,* hTERT-immortalized cells (EP156T) at passage 31; *e* and *f,* hTERT-immortalized cells (EP156T) at passage 101 (*b, d*, and *f* are higher magnifications of *a, c*, and *e*, respectively).

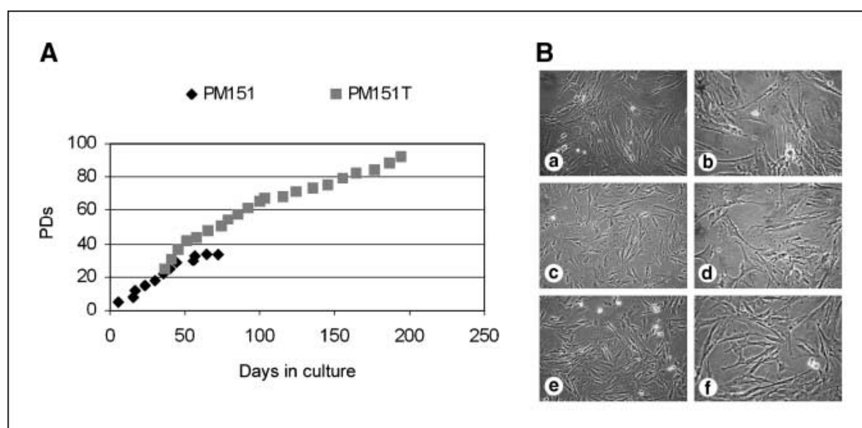

Figure 2. hTERT introduction induces immortalization of prostate stromal cultures. *A,* proliferation curves of prostate stromal cells infected with hTERT (PM151T) and their noninfected counterparts (PM151). *B,* morphology of prostate stromal primary and immortalized cultures: *a* and *b,* primary cells at passage 5; *c* and *d,* hTERT-immortalized cells (PM151T) at passage 37; *e* and *f,* hTERT-immortalized cells (PM151T) at passage 103 (*b, d,* and *f* are higher magnifications of *a, c,* and *e*, respectively).

immortalized EP156T cells at passage 31 were large and flat, suggestive of a senescent phenotype (Fig. 1*E, c* and *d*), whereas at passage 101, they were smaller and more uniform in their morphology (Fig. 1*E, e* and *f*). In general, the immortalized cells, similarly to their nonimmortalized counterparts, seemed to exhibit epithelial morphologic features.

Prostate stroma cells. Stroma-derived cells were isolated from prostate tissue as described in Materials and Methods. Cells were infected with the hTERT-containing retroviral vector, and their proliferation was characterized in the same manner as that of the epithelial cells. The primary noninfected stromal culture, PM151, accumulated 34 population doublings in 72 days *in vitro* and then stopped proliferating (Fig. 2*A*). Following hTERT introduction, the cells, designated PM151T, were able to proliferate for >190 days in culture, which is equivalent to ~90 population doublings (Fig. 2*A*). These cells have now been passaged >100 times (data not shown). Morphologically, the immortalized PM151T cells were similar to the primary stromal cell culture (Fig. 2*B*) and are clearly different from the epithelial cultures. Thus, ectopic expression of hTERT-induced immortalization of prostate stromal cells.

Expression Pattern of Endogenous Cell Cycle–Related Proteins in the Immortalized Prostate-Derived Cultures

Expression of $p16^{INK4a}$. The *INK4a* locus encodes two tumor suppressor genes, $p16^{INK4a}$ and $p14^{Arf}$, which play a central role in terminal growth arrest of cultured cells (27). $p16^{INK4a}$ functions as a positive upstream regulator of pRb by inhibiting cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) activity (27). $p16^{INK4a}$ is one of the most common genes silenced during immortalization of several types of human cells (14). Transcriptional inactivation of $p16^{INK4a}$ by deletion or promoter hypermethylation is found in >50% of advanced prostate cancers (17). It was therefore of interest to examine possible spontaneous changes in $p16^{INK4a}$ expression in the hTERT-immortalized cultures. By QRT-PCR analysis of the EP156T culture, we found that $p16^{INK4a}$ expression gradually declined and completely disappeared after passage 62 (Fig. 3*A, left*). Likewise, Western blotting for $p16^{INK4a}$ showed that its protein levels were also gradually down-regulated until the loss of expression at passage 58 (Fig. 3*A, right*). To further investigate this phenomenon, we treated the cells with 5-aza-2′-deoxycytidine, a known inhibitor of DNA methylation that often reactivates promoters silenced by methylation (28). This treatment resulted in a dose-dependent up-regulation of $p16^{INK4a}$ expression, whereas $p21^{waf1}$ levels were not affected (Fig. 3*B*). As seen in Fig. 3*B*, both $p16^{INK4a}$ mRNA and protein were increased under these conditions. In primary prostate epithelial cultures tested, $p16^{INK4a}$ mRNA and protein levels were comparable with those expressed by EP156T cells at early passages (Fig. 3*A*).

Figure 3*C* shows that in the immortalized prostate stromal cell culture, PM151T, $p16^{INK4a}$ mRNA, and protein levels were significantly higher at early (p34 and p11) as opposed to later (p109 and p112) passages. $p16^{INK4a}$ expression in primary parental culture PM151 was very similar to early passage of hTERT-immortalized cells (Fig. 3*C*). Despite significant down-regulation, $p16^{INK4a}$ was still detectable at late passages by both QRT-PCR and Western blotting (Fig. 3*C*), in contrast to the immortalized epithelial-derived cells, in which $p16^{INK4a}$ expression was completely silenced by this stage. This suggests that complete abolition of $p16^{INK4a}$ is less crucial for the immortalization of stromal cells, which are derived from a heterogeneous population of cells of myofibroblast origin. It should be noted that changes in expression of $p16^{INK4a}$ in immortalization of fibroblastic-derived cells seem to be unpredictable. Some reports indicated total silencing of genes encoded by the *INK4a* locus (29), whereas others still detected $p16^{INK4a}$ expression after significant life span extension following introduction of hTERT (30, 31).

p53 pathway. In addition to its role as a tumor suppressor, p53 has been shown to induce growth arrest at senescence (27). To evaluate the activity of p53, we examined the $p21^{waf1}$ expression pattern, which is indicative of p53 function along the cell cycle and in senescent cultures (32). $p21^{waf1}$ is a p53 target gene that mediates cell cycle arrest by acting as a CDK inhibitor (27). Our expression analysis indicated fluctuations in $p21^{waf1}$ mRNA in both prostate epithelial (Fig. 3*D, left*) and stromal (Fig. 3*E, left*) cells along their life span. p53 and $p21^{waf1}$ protein levels remained largely unchanged along the culture of both EP156T (Fig. 3*D, right*) and PM151T (Fig. 3*E, right*) cells. Expression levels of $p21^{waf1}$ and p53 in primary cultures of both prostate epithelial cells and prostate stromal cells were comparable with their expression levels in the immortalized cultures (Fig. 3*D* and *E*).

To investigate whether the p53 genotoxic response pathway is also intact in the hTERT-immortalized cells, we induced DNA damage by cisplatin treatment and measured p53 levels. Both p53 and MDM2, its negative regulator (33), were induced in a dose-dependent manner 24 hours following treatment of primary and immortalized cells of early and late passage (Fig. 3*F*). Thus, neither telomerase introduction nor prolonged *in vitro* culture interfered with normal p53 function. These data suggest that the p53 pathway most likely remains intact in hTERT-immortalized cells.

Expression Pattern of Prostate-Specific Markers

Epithelial cell markers. To ensure that the immortalized prostate epithelial cells retain their specific cell type features, we compared their expression of cytokeratins, steroid receptors, and p63 with that of primary prostate epithelial cells. Prostate tumor cell lines were used as additional controls. The results are summarized in Table 1 and can be seen in Supplementary Fig. S1. We observed that immortalized epithelial cells exhibited an expression pattern that corresponds to a prostate basal epithelial cell phenotype, with high expression of basal cell markers (cytokeratins 14, 5, and 7 and p63) and very low expression of luminal cell markers (cytokeratins 8 and 18 and AR; Supplementary Fig. S1). Steroid receptor expression evaluated by Western blot analysis revealed expression of estrogen receptor-β in the immortalized epithelial cells. This was similar to primary epithelial cells and to LNCaP prostate tumor cells (Supplementary Fig. S1 *D*). It should be noted that the immortalized stromal cells also express the estrogen receptor-β. We also examined p63, a typical marker for basal epithelial cells (34), and found that p63 protein levels were high in immortalized epithelial cells at late passages (Supplementary Fig. S1*E*). The predominant isoform in the immortalized cells had the size of ΔNp63α, whereas an isoform migrating at the position of TAp63γ was also expressed but at significantly lower levels (Supplementary Fig. S1*F*, compare the right lane with the four lanes to the left). This agrees with the previous observation that p63ΔNα expression is characteristic of the prostate basal cell population (34). Interestingly, hTERT-immortalized epithelial cells expressed low levels of AR, and its levels were slightly increased at a later passage (Supplementary Fig. S1*G*). Based on these prostate-specific gene patterns, we conclude that the hTERT-immortalized epithelial cells mainly exhibit a basal cell phenotype.

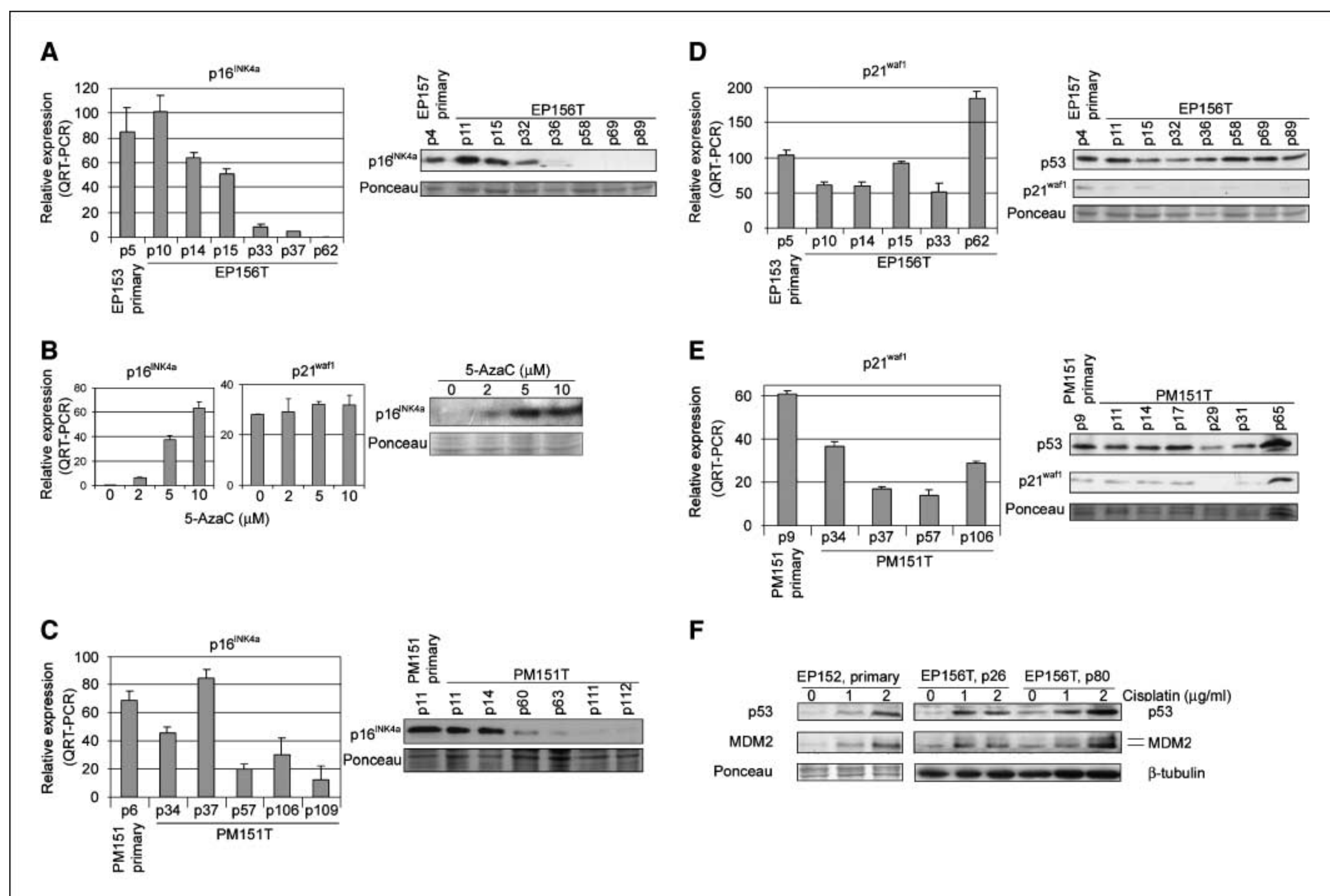

Figure 3. Expression pattern of *p16^{INK4a}*, *p21^{waf1}*, and *p53* genes in prostate-derived immortalized cells and activation of p53 in response to genotoxic stimulation. *A, left,* QRT-PCR analysis of p16^{INK4a} in primary prostate epithelial culture EP153 and in immortalized prostate epithelial cells EP156T. RNA samples were isolated from primary EP153 cells at passage 5 and from EP156T cells at progressive time points of the *in vitro* immortalization process. QRT-PCR measurements were done in duplicate and normalized to the GAPDH housekeeping control. *Right,* Western blot analysis of p16^{INK4a} in prostate epithelial immortalized culture EP156T at different passages and in primary prostate epithelial cells EP157 at passage 4. Protein lysates were prepared from the above cultures and analyzed for p16^{INK4a} expression. *B,* expression of p16^{INK4a} and p21^{waf1} following inhibition of DNA methylation by 5-aza-2′-deoxycytidine (*5-AzaC*). Subconfluent EP156T cells at passage 80 were treated for 5 days with increasing concentrations (2-10 μmol/L) of 5-aza-2′-deoxycytidine and analyzed for the expression of p16^{INK4a} and p21^{waf1} by QRT-PCR (*left*). p16^{INK4a} expression was also tested by Western blotting following the same treatment (*right*). *C,* QRT-PCR (*left*) and Western blot (*right*) analyses of p16^{INK4a} in primary (PM151) and immortalized (PM151T) prostate stromal cultures. For PM151 cells at passage 6 or passage 11 and for PM151T cells at progressive time points of hTERT-induced immortalization. *D, left,* QRT-PCR analysis of p21^{waf1} in primary prostate epithelial culture EP153 at passage 5 and in EP156T cells at progressive time points of the immortalization. *Right,* protein lysates were prepared from EP156T at progressive time points during *in vitro* culture and from primary EP157 cells at passage 4 and analyzed for p53 and p21^{waf1} expression by Western blotting. *E,* QRT-PCR analysis of p21^{waf1} at progressive time points of the immortalized PM151T (*left*). Western blot analysis for p53 and p21^{waf1} in the immortalized PM151T cells (*right*). Expression levels of p53 and p21^{waf1} in the primary parental culture PM151 at passage 9 are also presented. *F,* p53 induction following DNA damage in primary and immortalized prostate epithelial cells. Primary EP152, immortalized EP156T (passage 26) and immortalized EP156T (passage 80) were treated for 24 hours with 1 or 2 μg/mL cisplatin. Cell lysates were prepared and analyzed for p53 and MDM2 expression by Western blot. Ponceau staining or β-tubulin expression was used as a loading control in Western blot analyses. Passage numbers of primary and immortalized cells used to prepare protein and RNA extracts are indicated as "p4 . . . p112."

Table 1. Gene expression pattern of prostate epithelial markers in hTERT-immortalized epithelial (EP156T) and primary prostate epithelial cultures

Cell culture/marker	K7	K8	K14	K18	ER β	AR	p63
Primary prostate epithelial cells	++	++	+	+	++	ND	ND
EP156T, early passage	++	+	+	+	++	+	++
EP156T, late passage	ND	ND	+	−	ND	+	++

NOTE: ++, intermediate or high expression level; +, low expression level; −, no expression.
Abbreviation: ND, not determined.

Stromal cell markers. Smooth muscle differentiation is accompanied by transcriptional activation of the genes encoding contractile proteins, among which are smooth muscle-myosin heavy chain, smooth muscle α-actin, SM22, and calponin (35). This prompted us to test the expression of these genes in primary prostate stromal cells and in the hTERT-immortalized population. Using QRT-PCR, we found that hTERT-immortalized prostate stromal cells (PM151T) and primary parental culture (PM151) expressed smooth muscle α-actin, calponin, and SM22 at similar levels (Fig. 4*A*). In contrast to prostate stromal primary and immortalized cultures, calponin was not expressed in prostate epithelial cell line LNCaP (data not shown), and epithelial cytokeratins were not expressed in primary and immortalized stromal cells (Supplementary Fig. S1*B* and *C*). Recently discovered transcriptional activator of smooth muscle differentiation program, myocardin (35), was also expressed at comparable levels in primary and immortalized prostate smooth muscle cells (Fig. 4*A*). We further confirmed the expression of smooth muscle–specific proteins by Western blotting. Smooth muscle α-actin and smooth muscle-myosin heavy chain were highly expressed in the primary and the immortalized prostate stromal cultures (Fig. 4*A*). Using Western blot analysis, we found that primary and immortalized stromal cells exhibited similar levels of progesterone receptor, AR, and estrogen receptor-β (Fig. 4*B*). AR mRNA levels in prostate stromal immortalized cells were 10-fold lower in prostate primary and immortalized stromal cells than in LNCaP cells, as judged by QRT-PCR. There was no expression of AR in PC3 cell line. These findings support the conclusion that the immortalized cells retain typical features of cultured prostate smooth muscle cells.

Differentiation Potential of the Immortalized Prostate Cultures

Immortalized stromal cells. To further confirm that the immortalized stromal cultures indeed retain the functional properties of prostate smooth muscle cells, we subjected these immortalized cells, as well as primary stromal nonimmortalized cells, to TGF-β treatment to induce cell differentiation. Primary parental culture PM151 and hTERT-immortalized culture PM151T at early and at late passages were treated with 1 ng/mL TGF-β, and the levels of smooth muscle α-actin, SM22, and calponin were evaluated. As illustrated in Fig. 4*C*, 24 hours following treatment, a significant increase in the levels of these smooth muscle cells markers was observed in all cultures tested. In addition, we observed that 6 days following treatment, calponin protein levels were higher in the TGF-β-treated cultures of immortalized and primary cells (Fig. 4*C*). After 9 days, the level calponin in the nontreated culture was also increased (Fig. 4*C*), most likely as a result of cell density. An additional increment was noticed in the presence of TGF-β (Fig. 4*C*). Hence, the immortalized stromal cultures retained the ability to undergo muscle-specific differentiation.

Finally, cell functionality was evaluated by following the contractility of the immortalized smooth muscle cells. To that end, the cells were stimulated with 2 nmol/L Endothelin A, and cell contraction was followed under the microscope. As seen in Fig. 4*C*, cell contraction could be observed 1 hour following treatment.

Immortalized epithelial cells. In the normal prostate, the basal epithelial compartment is known to have a high proliferative potential and is believed to contain progenitor cells able to differentiate into secretory epithelia (36). Prostate basal epithelial cells can undergo partial differentiation *in vitro* (37) and can form glandular buds and branching structures in three-dimensional cultures (23, 38). To induce differentiation in three-dimensional cultures, cells grown from explant cultures were expanded for two passages, seeded on 2% Matrigel, and cultured under well-defined conditions in the presence of the synthetic androgen R1881. Under these conditions, single cells develop into spheroids that, upon immunohistochemical analysis, seem to have an architecture reminiscent of prostate secretory acini; thus, basal and luminal cell layers can be discriminated (23, 38). Hence, we refer to these spheroids as glandular buds. To study the putative multipotency of the immortalized prostate epithelial cells, we tested the EP156T cell culture for its clonogenic potential on Matrigel. Like primary prostate epithelial cells, the immortalized EP156T culture had the ability to form colonies in the three-dimensional culture system (Fig. 5). With increasing passage, the colonies seemed to become more compact (Fig. 5*A-C*). To assess whether these colonies have characteristics similar to the glandular buds that develop from primary epithelial cells, we analyzed frozen sections using antibodies against phenotypic markers that are known to discriminate prostate basal and luminal cells, including cytokeratin 5 (K5), cytokeratin 18 (K18), p63, AR, and PSA (39, 40). In the control structures, derived from primary prostate epithelial cells, the periphery of the bud was positive for p63 (Fig. 5*D*), whereas the central cell layer was positive for cytokeratin 18 (Fig. 5*H*). The whole structure was positive for cytokeratin 5 (Fig. 5*F*). AR was expressed in peripheral nuclei (Fig. 5*J*), and PSA was expressed at low levels in most cells (Fig. 5*L*), indicating that the cells differentiate into early and late progenitors but fail to terminally differentiate. Likewise, the EP156T-derived structures also had a peripheral cell layer positive for p63 (Fig. 5*E*), a luminal layer positive for cytokeratin 18 (Fig. 5*I*), and cytokeratin 5 (Fig. 5*G*) and PSA (Fig. 5*M*) in the whole structure. In contrast, AR was found in the cytoplasm of inner layer cells, and there was no lumen (Fig. 5*K*).

We conclude, therefore, that the EP156T cell population is multipotent and can give rise to more differentiated progeny that have the characteristics of the progenitor for exocrine PSA-producing prostate epithelial cells. It should be noted, however, that these immortalized cultures are unable to undergo cell differentiation to the full extent of normal primary cells. This suggests that immortalized EP156T cells have retained many, but not all, normal features.

Discussion

The goal of our study was to develop an experimental model suitable for deciphering the process of prostate carcinogenesis. Our working hypothesis was that the establishment of various types of prostate derived cell cultures would permit their subsequent transformation under controlled conditions *in vitro*. To immortalize prostate-derived epithelial and stromal cells, we used the hTERT approach, which successfully induced immortalization of both cultures while retaining many of their typical functional properties and specific markers.

Traditionally, viral agents known to inactivate the pRb/p16^{INK4a} and the p53 pathways have been used to immortalize different types of human cells, including prostate epithelial (7–10, 18, 41) and prostate stromal cells (41). Immortalized and tumor cells, generated *in vitro* from normal cultures by a cocktail of viral agents and cellular oncogenes, have provided important insights into the tumorigenic process (27, 42). In a recent study, prostate epithelial cells were immortalized using the SV40 early region products, and

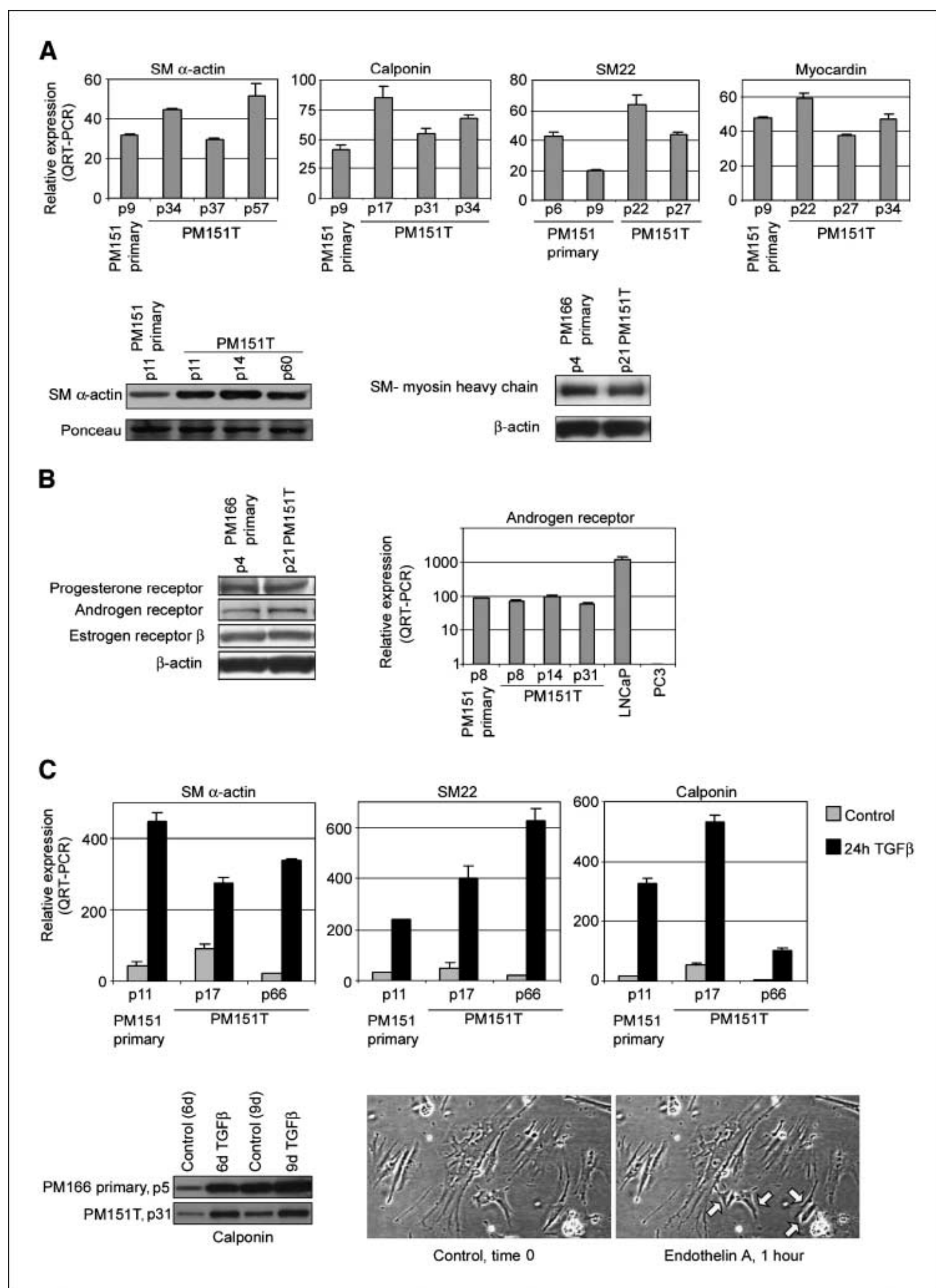

Figure 4. Expression pattern of smooth muscle (*SM*)–specific markers and steroid receptors and the differentiation potential of primary and hTERT-immortalized prostate stromal cells. *A,* expression pattern of smooth muscle–specific markers in primary (PM151 and PM166) and hTERT-immortalized (PM151T) prostate stromal cells. Smooth muscle α-actin, SM22, and calponin expression was analyzed by QRT-PCR (*top*). Expression of smooth muscle differentiation regulator myocardin was also examined by QRT-PCR (*top right*). Smooth muscle α-actin and smooth muscle-myosin heavy chain protein levels were analyzed by Western blot (*bottom*). *B,* protein levels of progesterone receptor, AR, and estrogen receptor-β, expressed in hTERT-immortalized stromal cells PM151T at passage 21 and in primary prostate stromal cells PM166 at passage 4 (*left*) were tested using Western blot analysis (*left*). AR expression in primary parental (PM151) cells and in their hTERT-immortalized derivatives (PM151T) was compared with its expression in androgen-dependent/sensitive cells LNCaP and androgen-independent cells PC3 by QRT-PCR. Relative expression values are presented using logarithmic scale (*right*). *C,* TGF-β-induced differentiation and Endothelin A–induced contraction. PM151 and PM151T cells were incubated without or with 1 ng/mL of TGF-β1 for 24 hours, and the expression of smooth muscle α-actin, SM22, and calponin was tested by QRT-PCR (*top*). Induction of calponin was tested in primary PM166 and in hTERT-immortalized PM151T cells following 6 and 9 days of TGF-β treatment using Western blot (*bottom left*). Endothelin A treatment induces contraction of hTERT-immortalized prostate epithelial cells. Cells were seeded in Matrigel-coated six-well plates. After 24 hours, they were stimulated to contract with 2 nmol/L Endothelin A under a microscope. The images show the cells immediately before adding Endothelin A and 1 hour thereafter. Contracted cells are indicated by *arrows* (*bottom right*).

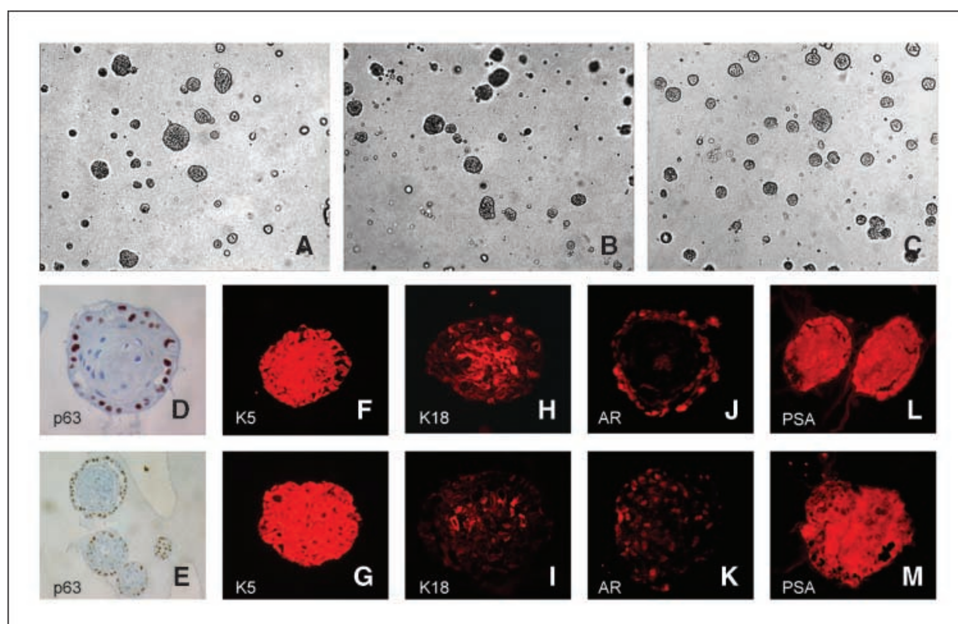

Figure 5. Differentiation of immortalized prostate epithelial cells in three-dimensional culture. *A-C,* formation of spheroids in a Matrigel three-dimensional cell culture of EP156T cells at passage 26 (*A*), 60 (*B*), and 89 (*C*). *D-M,* immunostaining for prostate epithelial markers differentially expressed in basal and luminal cell layers. *D, F, H, J,* and *L,* primary cells derived structures. The periphery of the bud is positive for p63 (*D*), whereas the whole structure is positive for K5 (*F*). Only the luminal cells are positive for K18 (*H*). AR stains the peripheral nuclei (*J*), and PSA is expressed at low levels in most cells (*L*), indicating that the cells differentiate into early and late progenitors but fail to terminally differentiate. Likewise, the EP156T derived structures (*E, G, I, K,* and *M*) also have a peripheral cell layer positive for p63 (*E*), whereas the whole structure is positive for K5 (*G*). The luminal layer is positive for K18 (*I*). Interestingly, the luminal layer contains positive nuclei for the AR (*K*), and there is no lumen formation. Similarly to primary culture, PSA is expressed at low levels in most cells of EP156T spheroids (*M*).

the authors addressed the role of overexpressed AR in differentiation and tumorigenicity of prostate epithelial cells (18). Another recent study used c-Myc to inactivate the pRb/p16^{INK4a} pathway and to induce immortalization of prostate epithelial cells (43). Complex alterations introduced by the use of viral and cellular oncogenes make it difficult to study the defined stepwise program that leads to malignant transformation and especially the premalignant steps. Therefore, to be able to investigate the detailed molecular network preceding the malignant transformation of the prostate derived cells, we felt that it was critical to develop a more moderate and stepwise system to transform these cells *in vitro*.

In our study, the only exogenous intervention used to immortalize prostate epithelial cells was hTERT introduction. In previous studies, viral oncogenes were used and, it was claimed that hTERT infection is insufficient to induce immortalization (18, 43, 44). It should be noted that although we did succeed in immortalizing prostate epithelial cells, in our hands, not every hTERT-infected culture attained immortalization. This can be explained by p16^{INK4a} up-regulation in growth-arrested cells (45) and the notion that p16^{INK4a} inactivation in cultured epithelial cells is a relatively rare event that enables cells to bypass senescence (46). Indeed, the nonimmortalized prostate epithelial hTERT-infected cultures expressed high p16^{INK4a} levels when they ceased growing (data not shown), whereas the immortalized cells lost p16^{INK4a} expression (Fig. 3*A*). We suggest that immortalization of prostate epithelial cells is critically dependent on the inactivation of p16^{INK4a} in addition to active telomerase expression. This agrees with earlier findings reported by Jarrard et. al., which show that pRb/p16^{INK4a} pathway alterations are required for bypassing senescence in human prostate epithelial cells (17). Thus, our approach is different from others, because we minimized forced genetic modifications, instead selecting for more naturally occurring changes that arise during the establishment of immortalized cells.

p16^{INK4a} silencing by DNA methylation often occurs during immortalization of human cells (27). However, this study is the first to show the activation of this mechanism in prostate epithelial cells *in vitro*. p16^{INK4a} promoter methylation causes the inactivation of this gene in a considerable fraction of prostate tumors (47). On the other hand, in breast tissue samples, *p16^{INK4a}* gene promoter methylation occurs in individual cells of histologically normal specimens, and these cells were proposed to be early progenitors of cancer (46). Likewise, telomerase activation was shown to occur at early stages of prostate cancer formation (48). It is tempting to speculate that both spontaneous p16^{INK4a} silencing and telomerase activation represent premalignant events, which are inherent in our *in vitro* system.

Both the epithelial and the stromal prostate-derived cells represent promising models for studying further transformation by well-known and accepted genetic modulations. The immortalized epithelial cells seem to have an overtly normal phenotype. This is manifested by the fact that they expressed cytokeratins 5, 6, 7, 8, 14, and 18 at varying levels, p63, estrogen receptor-β, and low levels of AR. In addition, they can differentiate in three-dimensional cultures into structures that closely resemble the spheroids obtained from primary cells. The immortalized smooth muscle cells express the progesterone receptor, the estrogen receptor-β, AR, smooth muscle-myosin heavy chain, calponin, SM22, smooth muscle α-actin, and myocardin. Moreover, they were able to differentiate and up-regulate several markers upon TGF-β treatment and to contract upon endothelin A treatment under *in vitro* conditions. Based on these observations, we propose that these cell cultures will be useful for the investigation of the physiology of normal prostate. In addition, these cultures may serve as an accurate model for the study in a well-defined manner of malignant transformation *in vitro*.

It should be noted that others also reported on the immortalization of prostate epithelial cells using hTERT (49, 50). However, those cells seem to exhibit rather transformed phenotype and thus cannot substitute for our immortalized prostate epithelial cells. Yasunaga et al. (49) report on the immortalization of prostate cancer cells using hTERT (49). In the study of Gu et al. (50), despite the conclusion made by a pathologist that the cells of origin (RC-165N) are from normal tissue, karyotype analysis indicated that the immortalized epithelial cells exhibited an aneuploid genome (50). Moreover, the immortalized RC-165N/hTERT cells exhibit a morphology, which is indistinguishable of transformed epithelial

cells, whereas our immortalized EP156T cells at early passage are morphologically similar to primary normal prostate epithelial cells. Thus, the originality of our study is in that we have immortalized apparently normal prostate epithelial cells and characterized the immortalization process.

The immortalized cultures described here were derived from the transition zone of the prostate. This zone gives rise to a minority (about 20%) of human prostate adenocarcinomas, whereas the majority of such tumors arise from the peripheral zone of the prostate (51). It is presently unclear whether the tumors arising from the two zones have identical biology. Therefore, it will be of interest to establish in the future additional cultures derived from the peripheral zone and compare their behavior to that of the transition zone–derived cultures presented in our study. Moreover, because the cultures described here were obtained from prostate cancer patients, we can not formally exclude the presence of a minor component of transformed cells within the culture. However, all available data strongly suggest that the cultures maintain a stable phenotype with features characteristic of normal prostate tissue, arguing against the possibility that such minor component, if at all present, gains gradual prevalence in the culture owing to a selective growth advantage. Thus, by all tested criteria, these cultures indeed seem to represent bona fide nontransformed prostate cells, whose further genetic manipulation can delineate relevant steps in prostate cancer progression.

The substantial role attributed to stromal-epithelial interactions in cancer suggests that an *in vitro* system to study such interactions is urgently needed. *In vivo* studies have shown that malignant and normal epithelial growth is fine tuned by stromal input (52). However, *in vitro* systems are required to better resolve the molecular nature of the stromal influence on epithelial growth in the prostate. Cocultures of hTERT-immortalized smooth muscle cells with epithelial prostate cells may be used to reconstitute stromal-epithelial interactions *in vitro*, thereby mimicking the physiologic microenvironment. In the future, we aim to establish cocultures of the immortalized stromal and epithelial prostate cells and, in this context, to study specific tumorigenic alterations and their effect on prostate cell malignancy.

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