

Prostate Cancer Cells with Stem Cell Characteristics Reconstitute the Original Human Tumor *In vivo*

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Abstract

Cancer may arise from a cancer stem/progenitor cell that shares characteristics with its normal counterpart. We report the reconstitution of the original human prostate cancer specimen from epithelial cell lines (termed HPET for human prostate epithelial/hTERT) derived from this sample. These tumors can be described in terms of Gleason score, a classification not applied to any of the transgenic mouse models currently developed to mimic human disease. Immunohistochemical and Western blot analyses indicate that they do not express androgen receptor or p63, similar to that reported for prostate stem cells. These cell lines also express embryonic stem markers (Oct4, Nanog, and Sox2) as well as early progenitor cell markers (CD44 and Nestin) *in vitro*. Clonally derived HPET cells reconstitute the original human tumor *in vivo* and differentiate into the three prostate epithelial cell lineages, indicating that they arise from a common stem/progenitor cell. Serial transplantation experiments reconstitute the tumors, suggesting that a fraction of parental or clonally derived HPET cells have self-renewal potential. Thus, this model may enhance our understanding of human tumor development and provide a mechanism for studying cancer stem/progenitor cells in differentiation, tumorigenesis, preclinical testing, and the development of drug resistance. [Cancer Res 2007;67(10):4807–15]

Introduction

Cancer stem cells are thought to arise from a multipotent stem cell that has accumulated genetic alterations during its long life span (1). An alternative hypothesis suggests that normal stem cell differentiation may be arrested during cell determination and differentiation, thereby affecting the development of malignant potential (2). Cancer stem/progenitor cells may exhibit characteristics similar to normal stem cells. For example, stem cells express the protein telomerase, which maintains telomere length and facilitates continuous cell division (3). They divide and give rise to multiple progenitor cell types that down-regulate telomerase activity during gestation (4) and early cell passage *in vitro* (5). Thus, loss of telomerase activity seems to limit the mitotic capacity of progenitor cells. Roy et al. showed that hTERT-immortalized progenitor cells from human fetal spinal cord sustained telomerase

activity and continued to give rise to mature spinal interneurons and motor neurons, indicating that hTERT overexpression permitted generation of progenitor lines able to give rise to phenotypically restricted neurons (3). hTERT has also been successfully used to immortalize human prostate epithelial (HPE) cells from primary tumors to generate RC-58T/hTERT and 957E/hTERT cell lines (6, 7). Recently, Litvinov et al. reported that the 957E/hTERT cell line contained prostate stem cells (8). Thus, although immortalization using hTERT is not equivalent to cancer stem cell isolation, it can be used to extend the mitotic capacity of progenitor cells and immortalize cells with stem cell characteristics.

Cancer stem cells share similar properties of self-renewal and differentiation as well as a similar phenotype with adult stem cells isolated from the same tissue (1, 2). The most compelling evidence of the existence of prostate stem cells in the basal cell compartment is derived from the mouse castration model where androgen withdrawal results in glandular involution and apoptosis in ~90% of epithelial cells but leaves the basal cell layer intact (9). Androgen replacement restores basal and luminal epithelial cells, inducing proliferation and differentiation (9). Slow-cycling cells retaining bromodeoxyuridine labeling following androgen withdrawal/replacement experiments have been identified in both basal and luminal cell compartments, implying that prostate stem cells are not restricted to one epithelial cell compartment (10). However, direct evidence of a putative prostate cancer stem cell has not been reported.

We routinely culture primary HPE cells to study the role of the androgen receptor (AR) in the development of androgen-independent prostate cancer (11). To facilitate these studies, we attempted to derive HPET (where T represents hTERT) cell lines through the stable integration of *hTERT* into these primary cells. Here, we report the characterization of these cell lines. Surprisingly, they exhibit stem cell characteristics, expressing embryonic stem cell markers, including Oct4, Nanog, and Sox-2, in addition to the early progenitor cell markers CD133, CD44, and nestin. HPET cells do not express p63 and AR, similar to other reports on prostate stem cells (6, 7). Most importantly, clonally derived HPET cells are capable of reconstituting the original prostate tumor from which they were derived and retain the ability to differentiate into basal, luminal, and neuroendocrine epithelial cell types of the prostate *in vivo*. Therefore, we present the first direct evidence for the existence of a putative prostate cancer stem cell.

Materials and Methods

hTERT construct and Lentiviral particles. The 3.5-kb *EcoRI-SalI* fragment from pBABE-PURO-*hTERT* (provided by Dr. Judith Campisi, Lawrence Berkeley National Laboratory, Berkeley, CA) was subcloned into the *EcoRI* and *SalI* sites of pLenti-EGFP (Invitrogen/Life Technologies) to create pLenti-*hTERT-EGFP*. Viral particles were produced by transfecting 293 FT cells with 3 μ g pLenti-*EGFP* expression plasmid DNA and 9 μ g ViraPower packaging mix (Invitrogen/Life Technologies) per 10-cm plate.

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

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Supernatants were collected 48 h after transfection, filter sterilized, and stored at -80°C . All procedures were done as recommended by Vanderbilt University safety regulations for Lentivirus usage.

Establishment of HPET cell lines. Radical retropubic prostatectomy specimens were obtained in compliance with the laws and institutional guidelines approved by the Institutional Review Board Committee of Vanderbilt University. Prostate tissues were processed, and HPE cells were cultured and maintained as described previously (12). Briefly, punch biopsies of prostate tissue were minced; tissue fragments were plated on Primaria plates (Falcon); and HPE cells were cultured in epithelial cell-selective medium (RPMI 1640 supplemented with 2.5% charcoal-stripped, heat-inactivated fetal bovine serum; 20 mmol/L HEPES buffer; 100 units/mL penicillin; 100 mg/mL streptomycin; 0.25 mg/mL amphotericin B; 50 mg/mL gentamicin; 56 mg/mL bovine pituitary extract; $1 \times$ insulin-transferrin-selenium; 10 ng/mL epidermal growth factor; and 50 ng/mL cholera toxin) and maintained in 5% CO_2 at 37°C .

The parental HPET cell line was established by transducing HPE cells (passage 2) with supernatant containing pLenti-*hTERT-EGFP* particles and culturing them in selection medium containing 10 $\mu\text{g}/\text{mL}$ blasticidin. The HPET-1, HPET-5, HPET-11, and HPET-13 clonal lines were established by serial dilution and verified visually by at least two individuals. Telomerase activity was determined as described (see Fig. 1C; ref. 13).

Anchorage-independent growth assays. Clonogenicity generation assay were done as previously described with minor modifications (14). Briefly, subconfluent cultures were harvested and plated in triplicate 35-mm wells of six-well plates in 2 mL medium with 0.5% low gelling temperature agarose at a concentration of 5×10^3 cells per mL on a 2-mL base layer of 1.0% agarose in medium. Cells were fed additional medium weekly. After 4 weeks, colonies with >40 cells were counted under an optical microscope. For spheroid generation assay, cells were plated on ultralow adhesion tissue culture dishes (Corning) at the density of 2×10^4 per mL in epithelial cell-selective medium without serum. Pictures were taken at 3 days after plating.

Cell differentiation on Matrigel. HPET and HPET1 cells were trypsinized, and 2.5×10^5 cells were mixed in 50 μL of 50% (v/v) epithelial medium and Matrigel (BD Biosciences; ref. 15). The mixture solidified in a six-well Petri dish at 37°C and then detached from the dish. Four milliliters of epithelial medium were added.

Immunofluorescence microscopy. Primary cultured cells were harvested by trypsinization and attached onto glass slides (VWR) by incubation at 37°C overnight. Slides were fixed in 4% paraformaldehyde in PBS for 15 min, washed thrice in PBS, and permeabilized for 5 min on ice in PBS + 0.1% Triton X-100. Slides were blocked in PBS + 3% bovine serum albumin + 3% donkey serum for 30 min at room temperature. The slides were incubated with primary antibodies for 1 h at room temperature and subsequently incubated with Alexa Fluor 594- and/or Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. Then, cells were washed in PBS and mounted using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole to counterstain nuclei (Vector Laboratories). Images were captured with a Leica fluorescence microscope equipped with a digital camera (Vanderbilt University Medical Center Immunohistochemistry Core Laboratory). To show specificity of staining, the following controls were included: omission of either the primary antibody or the secondary antibodies.

Reverse transcription-PCR. Total cellular RNA from HPET, HPET-1, HPET-5, HPET-11, HPET-13, and LNCaP cell lines was extracted using TRI Reagent (Sigma) according to the manufacturer's instructions. Two micrograms of total RNA for all cell lines were used to generate cDNA (RT-PCR Access System, Promega). cDNA was amplified using 1 μL of the reverse transcriptase reaction products in 25 μL with 10 pmol of the primers for 35 cycles. The PCR products were analyzed by electrophoresis on 1.5% agarose gels. Primers are listed in Supplementary Table S1. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control in all reactions.

Western blot analysis. Cells were extracted with ice-cold $1 \times$ lysis buffer (Promega) and a cocktail of protease and phosphatase inhibitors (Sigma). Lysates were then centrifuged for 10 min at $14,000 \times g$ at 4°C ; 20 μg protein from each supernatant was subjected to 4% to 20% SDS-PAGE and transferred to polyvinylidene difluoride and blocked and probed overnight

at 4°C with primary antibody. Peroxidase-conjugated secondary antibody was added at a 1:10,000 dilution and developed with enhanced chemiluminescence.

Immunohistochemical analysis. Tissues were fixed in 10% buffered formalin overnight followed by transfer to 50% alcohol. The paraffin-embedded tissues were sectioned (5 μm). Sections were deparaffinized and rehydrated in ethanol solutions. After antigen unmasking by boiling in 10 mmol/L sodium citrate buffer (pH 6.0) for 20 min, the sections were treated with 3% hydrogen peroxide for 5 min. The following detection and visualization procedures were done according to manufacturer's protocol (Vector Laboratories). AR, p63, green fluorescent protein (GFP), Ki-67, CD44, Nestin (Santa Cruz), cytokeratin 8 (CK8), CK18, prostatic acid phosphatase (PAP; Sigma), E-cadherin, Synaptophysin (BD Transduction Laboratories), 34 β E12 (DAKO), and hMT (Chemicon) were used. Negative control slides were done without primary antibodies.

Tissue recombination assay. All animals were housed in pathogen-free units at Vanderbilt University Medical Center, and all procedures were done in compliance with Institutional Animal Care and Use Committee regulations. Rat urogenital sinus mesenchyme (rUGM) was prepared from 18-day embryonic fetuses of pregnant Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.). Dissection and separation of urogenital sinus epithelium and UGM were done as previously described (16). HPET and HPET-5 cells (1×10^5) were then recombined with rUGM (2×10^5) in neutralized type I rat tail collagen gels. Tissue recombinants were grafted beneath the renal capsule of adult homozygous severe combined immunodeficient (SCID) male nude mice (Charles River Laboratories) as described. Grafts were harvested at 12 weeks. The number of tumor grafts examined was detailed in Supplementary Table S2.

Results

hTERT overexpression immortalizes HPE cells. To immortalize HPE cells, we cultured primary HPE cells from human Gleason grade 8 to 9 prostate cancer specimens under conditions favoring epithelial cell growth (12). HPE cells (passage 2) were transduced with pLenti-*hTERT-EGFP* particles and cultured in selection medium containing 10 $\mu\text{g}/\text{mL}$ blasticidin to establish the parental hTERT-expressing (HPET) cell line. HPE cells expressing hTERT and GFP (labeled as HPET cells) were already evident after 1 week in selection medium containing 10 $\mu\text{g}/\text{mL}$ blasticidin (Fig. 1A and B). They grew as adherent cells with epithelial cell morphology (Fig. 1D). HPET cells have successfully been cultured over a 1-year period with no evidence of decreased proliferative capacity. The original HPE cell culture exhibited measurable telomerase activity, and this activity was increased in *hTERT*-immortalized parental HPET and clonal HPET-1, HPET-5, HPET-11, and HPET-13 cell lines (Fig. 1C). The levels of hTERT activity in the parental HPET and clonal cells lines seemed indistinguishable. Clonally derived HPET cell lines were established by serial dilution. Six 96-well plates were seeded at one to a few cells per well, and 55 wells contained single cells. Despite increased hTERT expression, only four single cell clones (labeled HPET-1, HPET-5, and HPET-11 and HPET-13) survived and expanded to over one million cells. These clonally derived cells exhibited typical cobblestone morphology seen in epithelial cell cultures (Fig. 1D). All remaining clones and single cells underwent senescence within 4 to 6 weeks.

Parental HPET and clonal HPET cells do not express AR or p63. In agreement with previous reports on putative prostate stem/progenitor cells (17, 18), parental and clonal HPET cells do not express AR protein (Fig. 2A and B), or secretory proteins such as prostate-specific antigen (PSA) and PAP (data not shown). AR is not observed even when cells are cultured in the presence of androgen (data not shown). Data obtained from HPET-5 cells are representative of that observed in the HPET-1, HPET-5, HPET-11,

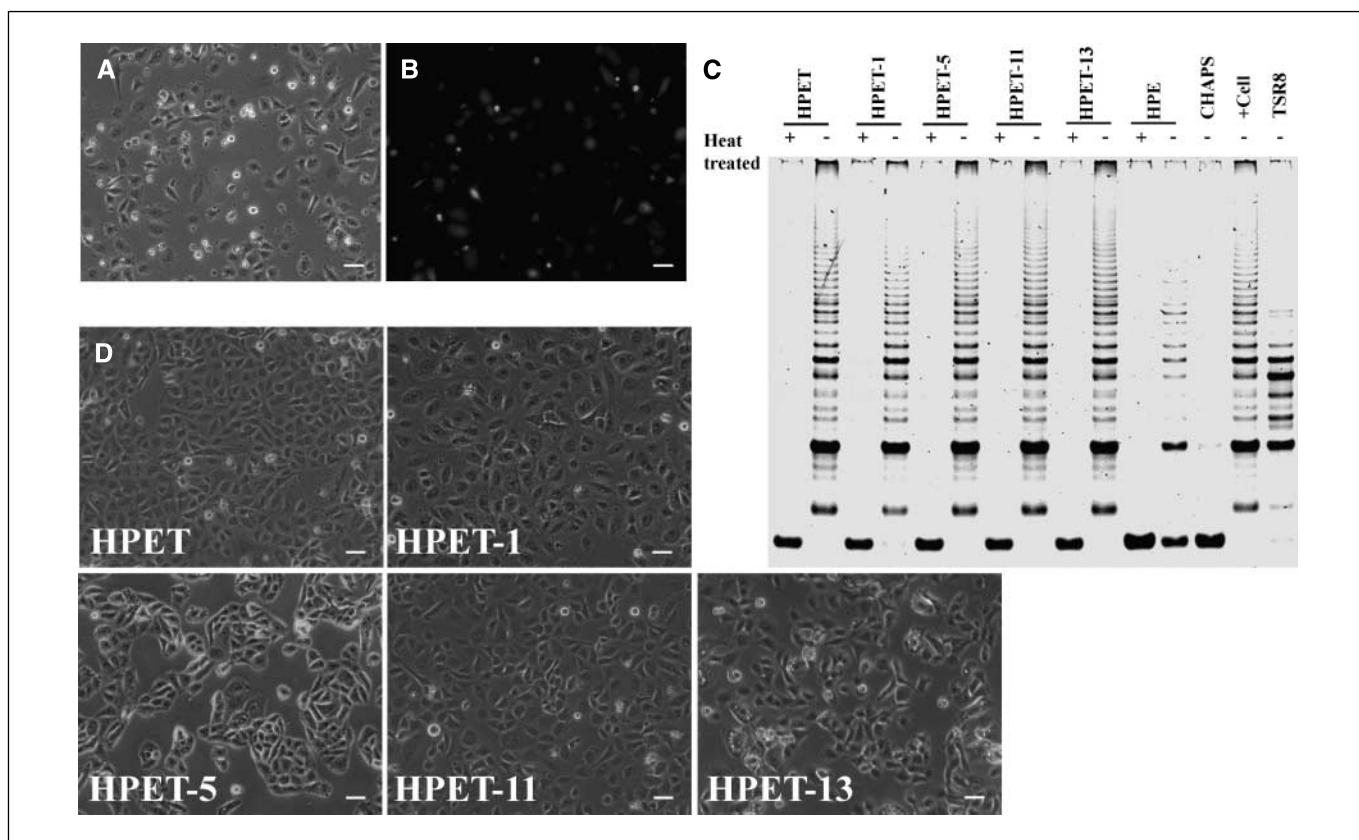


Figure 1. Generation of the HPET and HPET-5 cell lines. *A*, primary HPE cells infected with virus. *B*, confirmation of transduction using GFP expression. *C*, telomerase activity in cell lysates from parental HPET cells; clonal HPET-1, HPET-5, HPET-11, and HPET-13 cells; and original non-immortalized HPE cells. Cells were analyzed for telomerase activity by TRAP assay according to the manufacturer's instructions (TRAPeze, Chemicon). Telomerase activity was assayed from 2 μ g of cell extract including telomerase-positive control cells provided in the TRAPeze kit. Lysis buffer alone and 2 μ g of protein extract derived from all samples was heat treated at 85°C for 5 min (which inactivates telomerase) and were used as negative controls. TSR8 DNA template provided in the TRAPeze kit served as a PCR-positive control. *D*, representative micrographs of established parental HPET and clonal HPET-1, HPET-5, HPET-11, and HPET-13 cell lines as indicated. Bar, 50 μ m.

and HPET-13 clonal lines. Less than 1% HPET cells express minimal levels of the basal cell marker p63, whereas p63 is absent in the clonal HPET cell lines (Fig. 2*A*). CK8 and CK18 are expressed at low levels in a small number of HPET and HPET-5 cells, indicating limited luminal differentiation in culture. Together, these findings suggest that HPET and HPET-5 cells are not derived from a p63-expressing basal cell.

In contrast to previous reports on putative mammary and prostate stem cells (17), we do not observe Hoechst 33342 dye exclusion in either parental or clonal HPET cells (data not shown). This supports a recent report on the generation of a functional mammary gland from a single stem cell (19), suggesting that Hoechst 33342 dye exclusion is not representative of all stem/progenitor cells. HPET and clonal HPET cells were subsequently screened with a panel of stem cell markers to determine their molecular profile.

Parental HPET and clonal HPET-5 cells express embryonic stem cell and early progenitor cell markers and exhibit other stem cell-like properties. As anticipated, expression levels of embryonic stem cell and prostate progenitor markers varied between the parental and clonal HPET cell lines because they were derived from different clones (Fig. 2*B–D*). However, all lines express mRNA for the embryonic stem cell markers Nanog, Oct4, and Sox2 that regulate pluripotency and self-renewal in mouse

and human embryonic stem cells (20–22). Early prostate progenitor cell markers, such as CD44 (23), CD133 (24, 25), Nestin (26), and the receptor tyrosine kinase c-kit (27), are also expressed (Fig. 2*C*). CD44 and Nestin expression in HPET and HPET-5 cells was confirmed by immunocytochemical and Western blot analysis (Fig. 2*B* and *D*). Thus, HPET and HPET-5 cells express both stem cell and early progenitor cell markers.

To test the progenitor capacity of (AR⁻)p63⁻CD44⁺Nestin⁺ HPET and HPET-5 cells, we determined their ability to form spheroids and reconstitute prostatic glandular structure *in vitro*. Typically, both cell types form spheroids in low adherence culture (Fig. 3*A*). HPET cells form large and small colonies and exhibit a 3-fold higher colony frequency (Fig. 3*C* and *D*), indicating that the parental line is not clonal. Clonal HPET-5 cells only form uniformly small colonies. When cultured on Matrigel (Fig. 3*B*), both form glandular-like structures with lumens; however, the branched structures of HPET-5 cells seem larger and more complex. These findings indicate that both parental and clonal HPET cells possess stem cell-like characteristics.

HPET cells of single-cell origin recapitulate the original human tumor with multipotent differentiation. To determine whether HPET cells recapitulate the original prostate tumor *in vivo*, they were recombined with inductive rat embryonic mesenchyme and grafted under the renal capsule of male SCID mice (Fig. 4*A*

and *B*). HPET cells form tumors in 3 months with an average size of $6 \times 6 \times 3$ mm. The human origin of the tumor is confirmed by GFP staining and human-specific mitochondrial protein hMT (28) expression (Fig. 4*C*). Importantly, histopathologic and immunohistochemical analyses indicate that HPET tumors are nearly identical to the original human prostate cancer biopsy from which they were derived (Fig. 5*A* and Fig. 6*A*). They show typical heterogeneity observed in human prostate cancer and are classified as Gleason 4 + 4. Gleason 5 patterns like those observed in the original tumor (Gleason 5 + 4) are also present (Fig. 6*A*).

Immunohistochemical analysis indicates that AR is re-expressed in most cells, although some tumor foci are AR⁻, similar to that observed in advanced prostate adenocarcinomas (29). HPET tumors also re-express the differentiated protein PAP, although PSA was not observed (data not shown). All tumors exhibit a high proliferative index as seen by Ki67 staining. Significantly, the three prostate epithelial cell lineages are represented (Fig. 5*A* and *D*). Luminal epithelial cells are identified by their high levels of E-cadherin and CK8/18 expression. In regions where cribriform high-grade prostatic intraepithelial

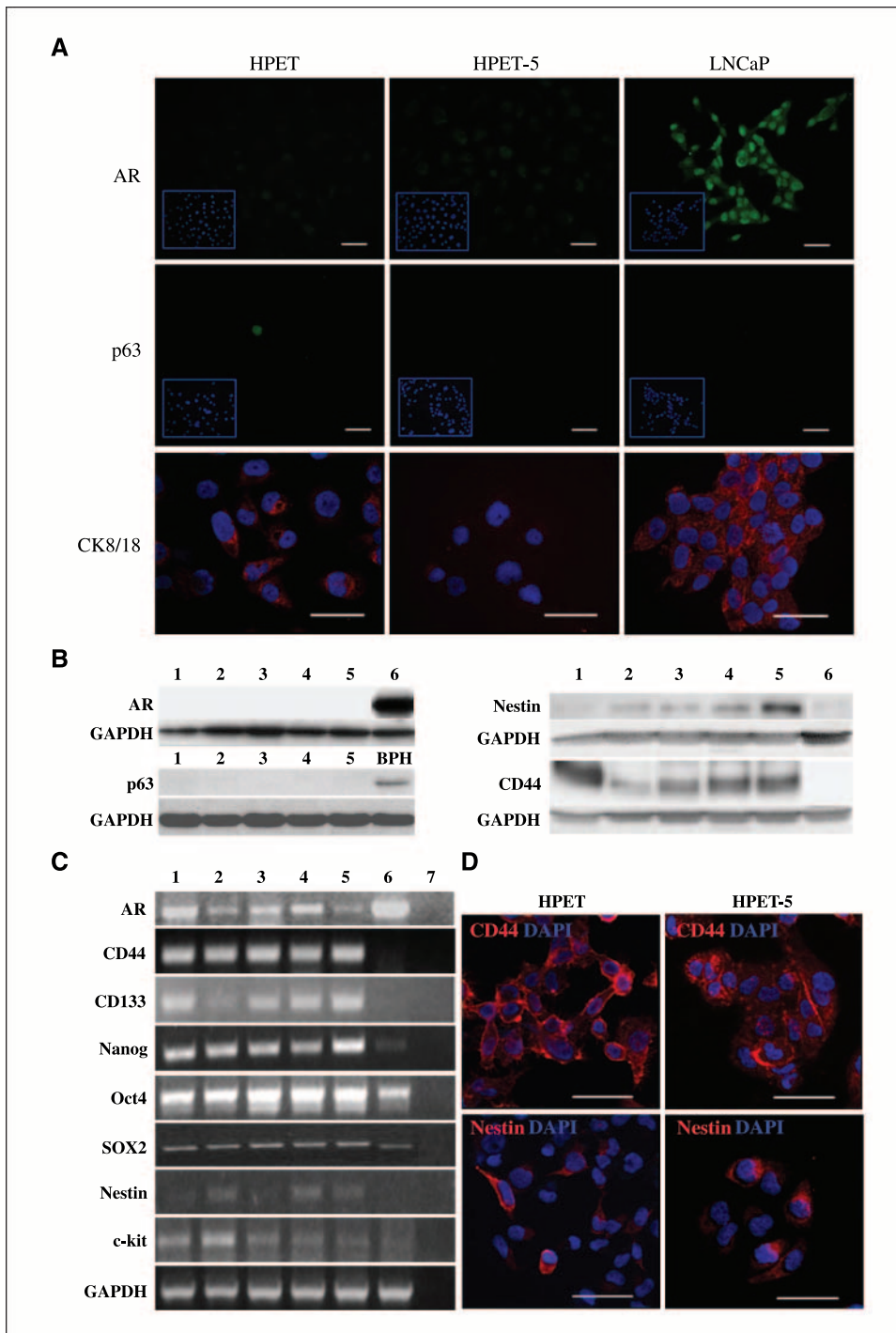
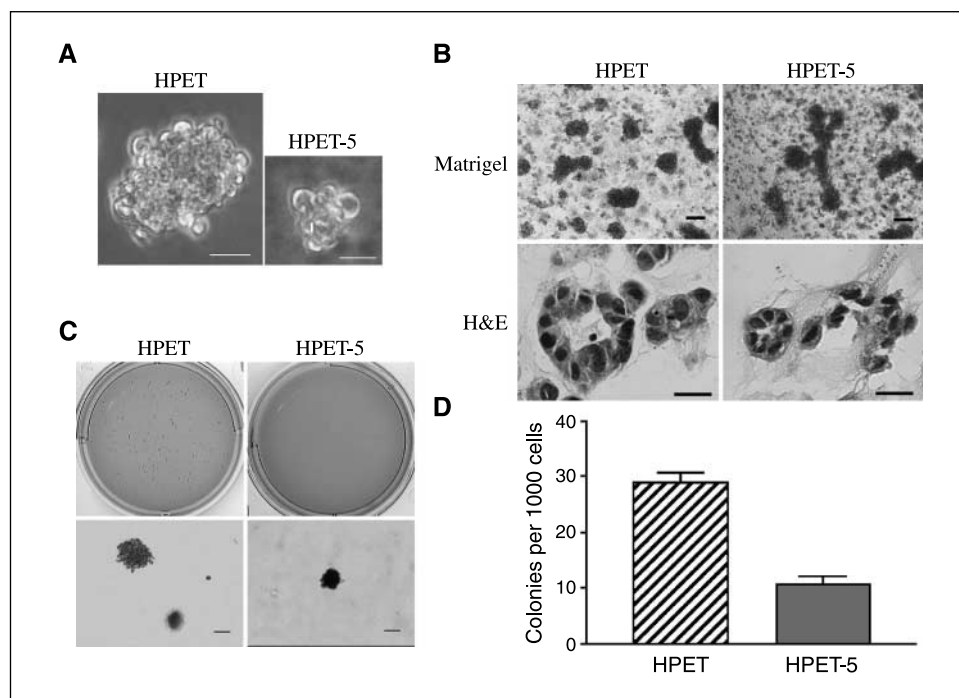


Figure 2. Characterization of AR, p63, cytokeratin, and stem cell markers in parental HPET and clonal HPET-5 cells. *A*, immunofluorescence staining with anti-AR, anti-p63, and anti-CK8/18 antibodies specific to the human proteins. The prostate cancer cell line LNCaP served as a control. *B*, Western blot analysis for AR, p63, Nestin, and CD44. Lane 1, parental HPET cell line; lanes 2 to 5, clonal cell lines HPET-1, HPET-5, HPET-11, and HPET-13, respectively. LNCaP cells (lane 6) are used as positive controls except for p63 expression where human benign prostatic hyperplasia-1 (BPH) cells serve as positive control. *C*, expression of embryonic stem cell and early progenitor cell markers. Primers are listed in Supplementary Table S1. Controls: LNCaP cells (lane 6); no template in the reverse transcription-PCR reaction (lane 7). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the internal control. *D*, CD44 and Nestin expression by immunofluorescence staining with anti-CD44 and anti-Nestin antibodies. Bar, 50 μ m.

Figure 3. Characterization of the progenitor capacity of (AR⁻)p63⁻CD44⁺Nestin⁺ HPET and HPET-5 cells *in vitro*. **A**, multicellular spheroid formation on low-adherence culture plates. Bar, 50 μ m. **B**, colonies and branching structures of (AR⁻)p63⁻CD44⁺Nestin⁺ HPET and HPET-5 cells on Matrigel. Representative H&E-stained sections (*bottom*). Bar, 200 μ m (*top*) and 20 μ m (*bottom*). **C**, colony-forming ability on agar. Bar, 100 μ m. **D**, histogram of Fig. 2C. Columns, mean ($n = 3$); bars, SE.



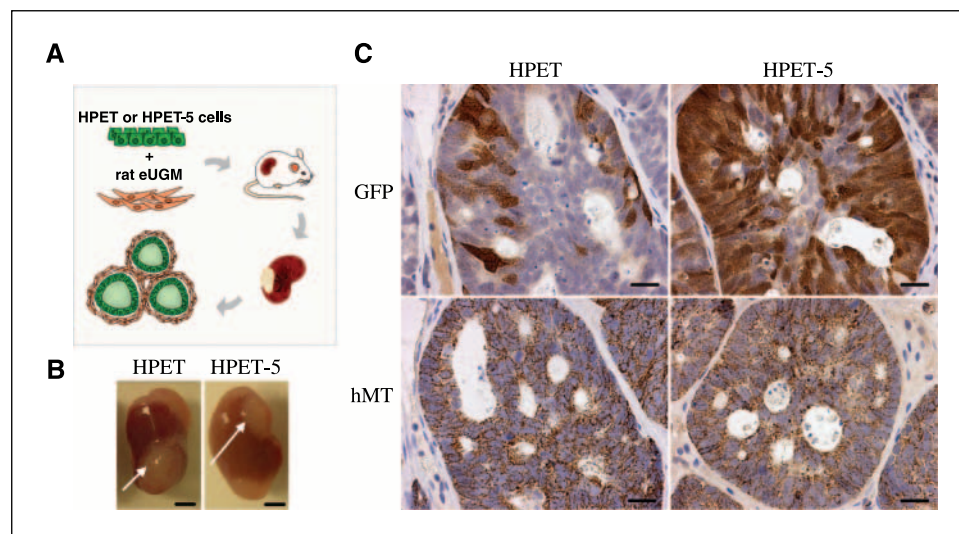
neoplasia are present, basal epithelial cells expressing p63 and 34 β E12 are still observed surrounding the more glandular structures in the tumor grafts as well as in the original human tumor. Other cells express the neuroendocrine cell marker synaptophysin similar to that observed in the original human tumor. Thus, HPET cells recapitulate the histopathology of the original prostate adenocarcinoma *in vivo*.

The observation that HPET cells are AR⁻ and p63⁻ suggests that they have stem cell-like characteristics. Alternatively, they could contain prostate cancer stem/progenitor cells. To pursue this further, clonal lines were analyzed in the tissue recombination assay to determine whether they could reconstitute the original tumor *in vivo*. Similar to parental HPET line, HPET clones, derived from a single cell and represented by HPET-5,

recapitulate the histopathology of the original prostate adenocarcinoma *in vivo* and differentiate into the three prostate epithelial cell lineages *in vivo* (Fig. 5B). Thus, it seems that these three cell lineages arise from a common (AR⁻)p63⁻CD44⁺Nestin⁺ stem/progenitor cell.

Within most tumors, there likely exists a subpopulation of cancer stem cells that continue to potentiate tumorigenesis. As seen in Fig. 5C, CD44 expression occurs at the cell membrane in both basal and luminal-like cells. Nestin expression is observed in a few cells within the basal and luminal cell compartments, suggesting that prostate cancer stem/progenitor cells are not restricted to the basal cell compartment. These findings concur with the observation that slow-cycling/stem cells are present in both basal and luminal cell compartments (10). Serial transplantation experiments were done

Figure 4. Single-cell origin (AR⁻)p63⁻CD44⁺Nestin⁺ HPET-5 cells can regenerate prostate glandular structure *in vivo*. **A**, diagrammatic representation of the tissue recombination assay. *rat eUGM*, rat day 18 embryonic urogenital mesenchyme. **B**, prostate tumor grafts under the capsule of SCID mouse kidneys. Bar, 2 mm. **C**, GFP and human mitochondrial protein (*hMT*) expression in prostate epithelial cells. Number of tumor grafts examined was detailed in Supplementary Table S2. Bar, 20 μ m.



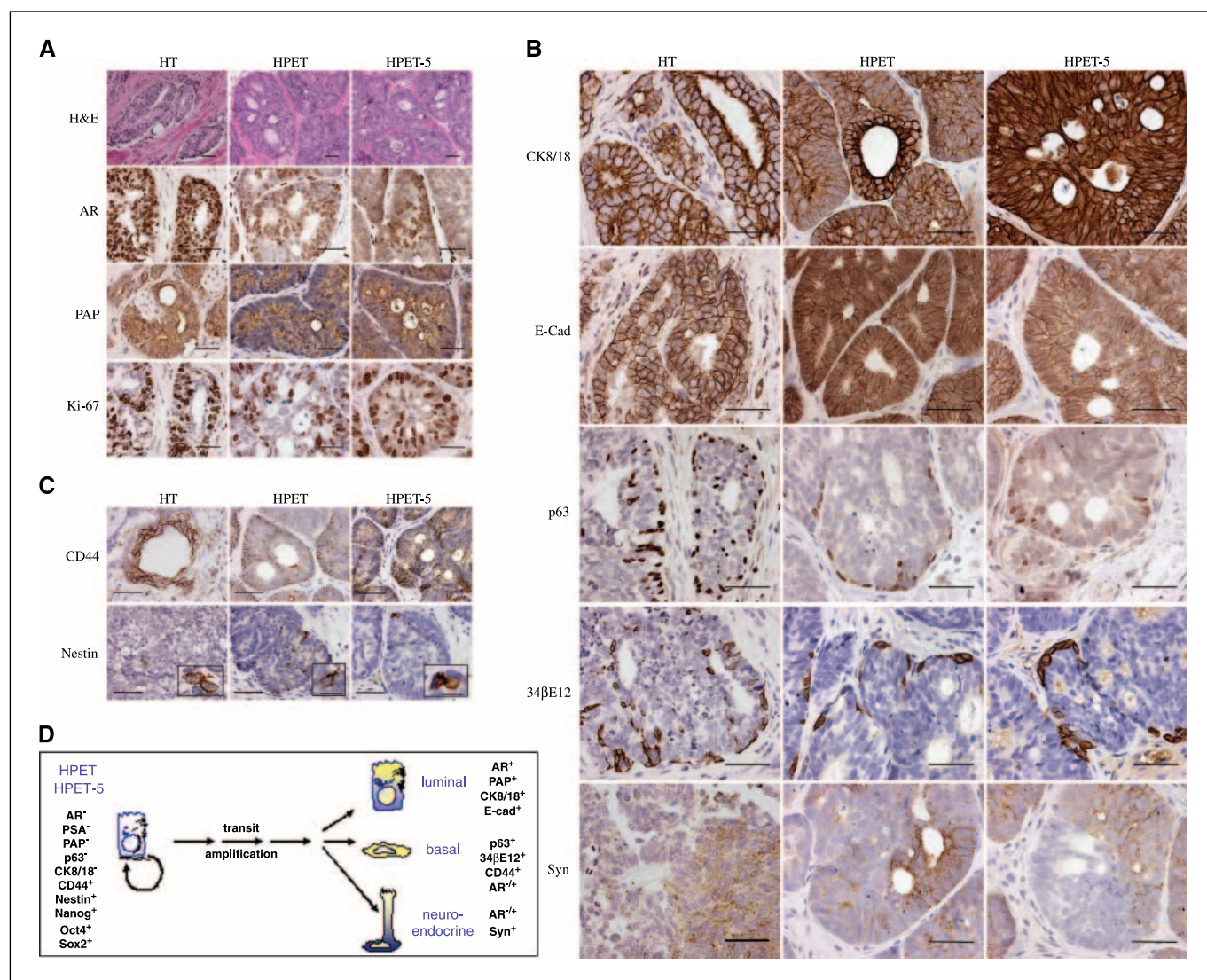


Figure 5. (AR⁻)p63⁻CD44⁺Nestin⁺ HPET-5 cells differentiate into the epithelial cell lineages of the prostate and recapitulate the original human tumor (HT). *A*, similar to that of the original HT specimen, epithelial cells in HPET and HPET-5 tumors re-expressed AR and PAP. The high proliferative index in the human tumor (as seen by anti-Ki-67 staining) was reproduced in HPET and HPET-5 tumors. *Bar*, 50 μ m. *B*, epithelial cell differentiation in tumors derived from single-cell origin (AR⁻)p63⁻CD44⁺Nestin⁺ HPET-5 cells. Staining with anti-CK8/18 and anti-E-cadherin antibodies identified luminal epithelial cells. p63- and 34 β E12-positive cells depicted basal epithelial cells surrounding glandular structures. Prostate tumors often express neuroendocrine factors such as synaptophysin (Syn). HPET and HPET-5 cells expressing synaptophysin were also observed. *Bar*, 50 μ m. *C*, identification of cells that retain CD44 and Nestin expression. Both stem/progenitor cell markers were expressed in the human tumor and HPET and HPET-5 tumors. *Bar*, 50 μ m. Inserts highlight their staining pattern. *Bar*, 10 μ m. *D*, diagrammatic summary of the immunohistochemical analyses representing the differentiation of HPET and HPET-5 cells to luminal cells, basal cells, and neuroendocrine cells.

to determine whether a fraction of HPET and HPET-5 tumor cells have growth potential similar to that of prostate stem cells. HPET and HPET-5 tumors are regenerated through each cycle, reconstituting the original tumor and retain small subpopulations of cells that express CD44 and Nestin (Fig. 6B), providing functional evidence for the property of self-renewal.

Discussion

Similar to normal organs arising from normal stem cells, tumors could be viewed as aberrant organs composed of heterogeneous cell populations arising from cancer cells that have acquired the capability for indefinite proliferation (1). Indeed, the clonal origin of many tumors suggests that tumorigenic cells undergo processes analogous to self-renewal and differentiation, generating self-

renewing cancer stem cell populations as well as cancer cells with limited or no proliferative potential (1). The HPET clonal lines support these observations in that they reconstitute the original tumor, differentiate into basal, luminal, and neuroendocrine epithelial cell lineages of the prostate (Fig. 4) and retain their capacity for proliferation through serial transplantation (Fig. 6).

In the HPET and HPET-5 cell model, AR and p63 are absent until glandular formation, when they are re-expressed in the appropriate cell type *in vivo*. The prostate stem cell is thought to survive in an androgen-deprived environment and does not express AR (30). HPET and HPET-5 cells cultured in the absence or presence of androgen do not express AR. *In vivo*, however, AR is expressed in prostatic epithelium, suggesting that AR expression depends on microenvironmental factors and correlates with cellular differentiation. AR has been associated with differentiation. Berger et al.

(31) showed that with the introduction of AR, HPE cell (PrEC) lines immortalized with *hTERT* and transformed by SV40 large/small T antigen (PrEC LHS-AR) or *H-ras* (PrEC LHSR-AR) underwent partial differentiation into a luminal phenotype *in vitro* and *in vivo*. In a different study, HPE cells immortalized with *hTERT* differentiated into glandular buds in three-dimensional culture on Matrigel (32). These structures consisted of a peripheral layer of p63-positive cells surrounding luminal cells expressing low levels of AR and PSA (32).

The origin of the prostate stem cell is still under debate. The p63 null mouse model (33, 34) suggests that epithelial development does not occur in the absence of p63, which is highly expressed in basal or progenitor layers of many epithelial tissues (35). However, prostatic buds only appear on embryonic day 17.5 and are underdeveloped at the time *p63*^{-/-} mice die perinatally. Signoretti and Loda show that the whole prostatic epithelium is derived from p63⁺ ROSA26 embryonic stem cells injected into *p63*^{-/-} blastocysts in blastocyst complementation experiments (36). In contrast, using renal grafting to rescue *p63*^{-/-} urogenital sinus and allow differentiation, Kurita et al. show that in the absence of p63-expressing basal cells, luminal secretory and neuroendocrine epithelial cells still develop and are able to regenerate after castration (37). In our study, clonal HPET-5 cells do not express

p63; yet, differentiation and glandular formation occur and p63 is re-expressed in basal prostatic cells. A significant difference between our study and the *p63*^{-/-} mouse model is that although HPET p63 expression does not occur, the endogenous *p63* gene is still present. Under the appropriate stimuli *in vivo*, p63 protein is re-expressed in the basal cell type.

Another basal cell marker is 34βE12. This marker is clinically used to differentiate prostate cancer lesions from benign prostatic glands. Although rare, 34βE12-positive cells have been detected in prostatic adenocarcinoma (38–40), indicating that prostate cancer cells can exhibit characteristics attributed to a basal cell phenotype. Our study provides the first direct evidence that prostate cancer stem/progenitor-like cells from single cell origin differentiate to luminal and neuroendocrine epithelial cells as well as into basal cells *in vivo*, although these represent <0.1% of tumor cells. In analyzing 34βE12 expression in HPET and HPET-5 tumors, most if not all staining occurred where limited glandular organization was still evident. The same pattern was observed in the original human prostate cancer specimen. Alternatively, although basal cells are primarily lost in adenocarcinoma, prostate cancer stem cells likely have not lost the ability to differentiate to p63- or 34βE12-positive cells. This may occur in the appropriate microenvironment.

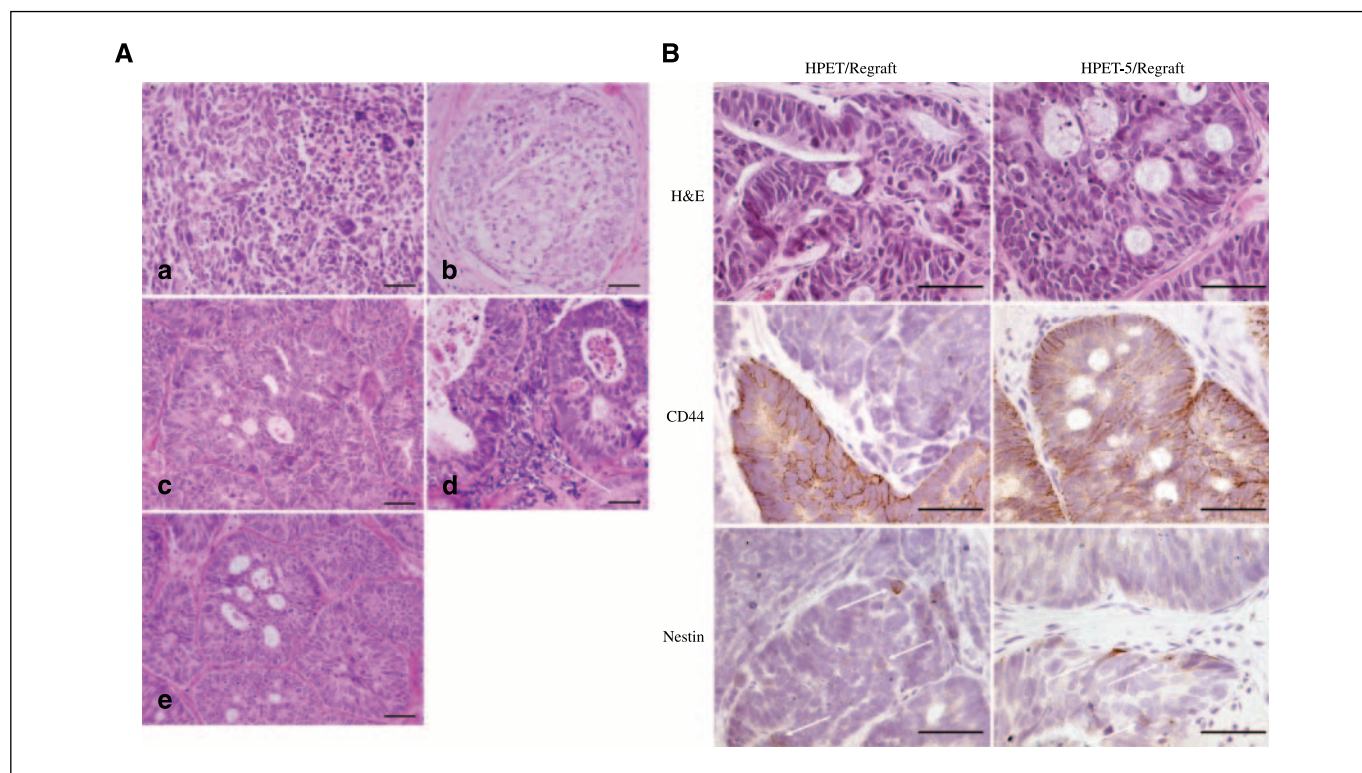


Figure 6. Histopathologic comparison of the original human tumor specimen with HPET and HPET-5 tumor grafts. *A*, *a*, original human tumor specimen. H&E section of a radical prostatectomy whole-mount human specimen showing prostatic adenocarcinoma (Gleason grade 5 + 4 = 9) with neuroendocrine features. Within the higher Gleason pattern, there are nuclei with "salt and pepper" chromatin and lack of prominent nucleoli suggestive of neuroendocrine differentiation. No lymphovascular invasion is identified within these whole mount sections. *b*, human tumor containing Gleason pattern 4. *c*, HPET tumor graft. Prostatic adenocarcinoma Gleason score 4 + 4 = 8 with abundant mitotic figures and apoptotic bodies. This tumor has abundant cribriforming patterns with salt and pepper nuclei similar to the human tumor with a paucity of prominent nucleoli. There are focal areas of marked nuclear atypia that are also found in the human tumor. Sparse stroma is present around glands with small, thin-walled vessels within stroma. *d*, small HPET focus containing Gleason pattern 5 (white arrow). *e*, HPET-5 tumor graft. Prostatic adenocarcinoma, Gleason grade 4 + 4 = 8 with neuroendocrine features, abundant mitotic figures and apoptosis. No Gleason pattern 5 is found. There is cytologic atypia and sparse stroma around glands with small, thin-walled vessels within stroma. *B*, HPET and HPET-5 tumors are regenerated upon serial transplantation. Serial transplantation was carried out by combining a small fragment of HPET or HPET-5 tumor tissue from the original graft with embryonic rUGM and grafting them under the kidney capsule of SCID mice. Histologically, the regenerated tumors recapitulated the original tumors and retained small subpopulations of cells expressing CD44 and Nestin. Bar, 50 μ m.

Histopathologic analyses indicate that HPET- and HPET-5-derived tumors reconstitute the Gleason score of the original human tumor biopsy specimen *in vivo*. This model is unique in that for the first time, pathologic evaluations and outcomes in response to therapeutic treatment can be based on Gleason score. Gleason score is a composite number composed of two cancer patterns recognized by their architectural arrangement and is the most frequently used grading system for human prostate cancer. The combination of these patterns is a powerful prognostic indicator of outcome as well as of biochemical failure for disease recurrence (41). Gleason score also influences treatment and the ability to predict the probability of lymph node metastasis (41). This scoring system is not directly applicable to the transgenic mouse models and is reviewed in detail elsewhere (42).

HPET and HPET-5 cells express the embryonic stem cell markers Oct4, Nanog, and Sox2 and the early progenitor cell markers CD44, Nestin, CD133, and c-kit. Transcription factors seem to act as molecular switches that control cell fate during development. The POU transcription factor Oct4 is expressed in the oocyte, declines during the first two divisions, is re-expressed at the four- to eight-cell stage and becomes restricted to the inner cell mass (ICM) of the embryo (43). Oct4 maintains pluripotency, whereas up-regulation of Oct4 expression results in differentiation to primitive endoderm and mesoderm and down-regulation induces loss of pluripotency and dedifferentiation into trophoblast (22). Nanog expression also occurs at the eight-cell morula stage and in ICM (44). Increased Nanog expression prevents differentiation, and this may act to restrict the differentiation-inducing potential of Oct4 (44, 45). Both Oct4 and Nanog expression is down-regulated as embryogenesis progresses, and neither are expressed in normal adult tissues (22, 44). Their role in maintaining HPET cell pluripotency remains to be established.

A third member in this network of transcriptional factors is Sox2. Sox2 is expressed with Oct4 and Nanog; however, it is not restricted to multipotent embryonic cell lineages (46). Sox2 expression is also present in developing brain, neural tube, sensory placodes, branchial arches, and gut endoderm and continues to be expressed in adult

tissues such as the ovary (46). Interestingly, Sox2 seems to bind the promoter of the *Nestin* gene to regulate its expression in neural primordium (47). However, Nestin is not restricted to neural stem cells, being expressed in embryonic stem-derived progenitor cells that can develop into neuroectodermal, endodermal, and mesodermal lineages (26). Prostate cancer stem cells seem to express CD133 (30). CD133, initially isolated from neuroepithelial cells, is also found in hematopoietic and endothelial progenitor cells (48). An intriguing hypothesis is that neural crest is the source of all adult stem cells in peripheral tissues (49), and that differentiation across germ layers to many organs depends upon microenvironment (50). Although HPET cells express Nestin and CD133, it is uncertain whether prostatic HPET cells originate from neural crest.

The clonal HPET cancer stem/progenitor cell lines with embryonic stem cell and early progenitor cell characteristics provide the first *in vivo* model that recapitulates the original Gleason score of the tumor from which they were derived. Thus, this model may enhance our understanding of human tumor development and provide a mechanism for preclinical testing of diagnostic, treatment, and prevention strategies for cancer patients. Furthermore, they provide cell lines critical to performing in-depth mechanistic studies on the function of cancer stem cells and epithelial cell differentiation. Because many epithelial cancers seem to arise from cancer stem cells and often exhibit similar characteristics, knowledge generated by the HPET cell lines will likely be applicable to other epithelial cancers.

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