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Mouse prostate cancer cell lines established from primary and post-castration recurrent tumors

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Abstract

The clinical course of prostate cancer is grouped into two broad phases. The first phase, which is the growth of the androgen-dependent cancer (AD-Ca) responds well to androgen depletion treatment while the second phase, that could be termed as androgen depletion-independent cancer (ADI-Ca) does not. We used two separate prostate tumors, one AD-Ca and one ADI-Ca from the conditional *Pten* deletion mouse model to generate from each a pair of cell lines. The AD-Ca cell lines (E2 and E4) and the ADI-Ca cell lines (cE1 and cE2) display bi-allelic deletion at the *Pten* gene locus, an event which is specific for the prostate epithelium for this mouse model, and a fairly similar level of expression of the androgen receptor (AR). The ADI-Ca cell lines (cE series) grow well in the absence of androgen, display increased AR transcription under androgen-deprived environment, and retain the sensitivity to increased proliferation when androgen is supplemented. The AD-Ca cell lines (E series) grow slowly in the absence of androgen, and, unlike cE cells, do not show increased AR expression when maintained in the absence of androgen. The detection of epithelial cell markers, such as CK8, CK14, CK18 and E-cadherin in the cE series is conforming with the polygonal epithelial morphology of these cells in culture. The E cells also present mostly polygonal-shaped morphology with a small percent of cells with fibroblastoid morphology, and produce little or very low levels of cytokeratins, but increased levels of vimentin, Twist and Slug, the markers known to be associated with epithelial-mesenchymal transition. Each of the cell lines, when inoculated subcutaneously into male or female NOD.SCID mice induced tumors within eight weeks with 100% incidence. Histopathological examinations of the tumor sections, however, led to noticeable biological differences. The cE series engenders adenocarcinomas, particularly in male hosts, and the E series induces sarcomatoid carcinomas (positively stained for CK8 and AR as well as vimentin expression) in either male or female hosts. These new cell lines are promising models for the elucidation of the androgen metabolism and their role in prostate cancer.

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Keywords

Conditional *Pten*-deletion mouse model of prostate adenocarcinoma; mouse prostate cancer cell lines; androgen-dependent and androgen depletion-independent prostate cancer; prostate sarcomatoid carcinoma

Introduction

Androgen depletion has been a gold standard therapy in the clinical management of prostate cancer with localized or metastasized disease (1,2). Because androgen is necessary to maintain the function of the prostate gland as well as for the initial growth of the prostate tumors, this therapy of the androgen-dependent cancer (AD-Ca) rapidly leads to the shrinkage of the tumors. Unfortunately, within a period of two years, most patients do, however, manifest a relapse resulting in recurrent tumor, which may be best described (3) as androgen depletion-independent cancer (ADI-Ca). How prostate cancer cells survive and proliferate under the low-androgen environment remains unclear, although multiple mechanisms, such as, increased level of expression of androgen receptor (AR) in the cancer cells, mutations or post-translational modifications of AR that broaden its ligand specificity, bypassing of the androgen-AR signaling to an extent through utilization of alternative pathways, etc., have been implicated (4–7).

There is currently a strong interest in the application of the knowledge gained from the analyses of human prostate cancer in modeling this disease in mice. Several mouse models of prostate cancer have been developed based on genetic alternations which are found frequently in human prostate cancer in order to recapitulate its natural course of initiation and progression (8–13). Among these models, the conditional *Pten* deletion mouse model (*cPten*^{-/-}) demonstrates a short latency for the development of adenocarcinoma (14). A refined (*cPten*^{-/-L}) model further increases the utility of the conditional *Pten* deletion model by combining it with a conditional luciferase expression, which is mediated by the same *Cre/LoxP* regulation system that is activated in the prostate epithelial cells (15). In this model, the progression of tumor development and growth can be longitudinally monitored in living animals using bioluminescence imaging (BLI) technology. Importantly, the tumor regression after castration and then relapse can also be similarly monitored in these animals.

For studies of the mechanisms of cancer, cancer cell lines are powerful tools. A limited number of human prostate cancer lines have been established from primary or metastatic cancers (16–19), and there is indeed a dearth of parallel cell lines developed from both AD-Ca and ADI-Ca phases. Mouse models of prostate cancer can readily provide tissue materials for establishing cell lines from different phases of the disease progression. However, only a few murine prostate cancer cell lines have been derived from the primary tumors to date (20–22), and none from the ADI-Ca phase. Here, we report establishment and characterization of four prostate cancer cell lines from the *cPten*^{-/-L} mouse model, two derived from an AD-Ca and the other two from a separate ADI-Ca.

Material and Methods

Animals

Mice with prostate epithelium-specific inactivation of *Pten* coincidental with luciferase reporter gene activation, as well as castration experiments after tumor growth were as described before (15). All mice were maintained under identical conditions and animal experimentation was conducted using the standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Generation of cell lines from *cPten*^{-/-} L prostate cancer model

Freshly collected prostate tumor tissues were minced with crossed scalpels (size 11 blades), transferred to a 50 ml tube and incubated in 5 ml of a digestion medium at 37 °C overnight on a rotator (37°C, 140 rpm). The digestion medium contained DMEM/F12 (Invitrogen), 10% fetal bovine serum (FBS; Gemini), collagenase (1 mg/ml), hyaluronidase (1 mg/ml) and DNase I (1 µg/ml). Collagenase, hyaluronidase and DNase I were purchased from Sigma-Aldrich, St. Louis, MO. After low speed centrifugation, single cells and cell clumps were collected and subjected to treatment with 0.05% Trypsin-EDTA for 10 min at 37 °C and sequentially passed through 100 µm and 40 µm cell strainers (BD Bioscience). The preparation was then cultured in a maintenance medium that contained DMEM, 10% FBS, 25 µg/mL bovine pituitary extract (Invitrogen), 5 µg/mL insulin (Sigma-Aldrich) and 6 ng/mL recombinant human epidermal growth factor (rhEGF) (Invitrogen). When cell colonies with epithelial cell morphology were observed, cells were trypsinized, diluted with culture medium to obtain a concentration of approximately one cell/ 100µL, and seeded in 96-well plates (100µL/ well). After culturing overnight at 37°C, each well was carefully examined under the bright field in microscopy. Wells containing single cells with the epithelial morphology were marked, and the culture medium in such wells was changed every three days. Expanded cell populations derived from these colonies were subjected to further characterization.

Quantitative RT- PCR

Total cellular RNA (1 µg), extracted by RNAqueous-4PCR Kit (Ambion) was reverse-transcribed by random hexamers using qScript™ cDNA Synthesis Kit (Quanta), and the reverse transcription reaction (1µL) was then subjected to PCR amplification using FastStart Universal SYBR Green Master (Roche). PCR signals were recorded and analyzed in Stratagene MX3000P qPCR system with MxPro software (Stratagene; v4.01). Primer sets are listed in Table 1.

PCR genotyping

DNA extracted from cell lines were used as templates for PCR reactions using specific primer sets for *Pten* and *Cre*. These primer sets are also listed in Table 1.

Western blot analysis

The whole-cell lysates were prepared as described (23). The antibodies used in this Western blots were anti-AR (1:100; Santa Cruz) and anti-actin (1:1000; Santa Cruz).

Assays for growth in serum-free medium

For testing the efficiency of growth of these cell lines in an androgen-free environment, a modified serum-free medium (SFM) was used (24). It was composed of DMEM/F-12 supplemented with 0.5% glucose (Sigma), and 2mM glutamine, 10g/ml insulin, 5.5 g/ml transferrin, 6.5 ng/ml selenium, and 6 ng/ml rhEGF (all from Invitrogen). Initially, cells (5×10^4) were plated in 6-well plates in the maintenance medium for overnight, washed two times with 1×PBS, and then cultured in SFM for 5 days. The culture medium was changed every 2 days, and the cell proliferation rate was determined at those time points by cell counting. SFM containing 1 or 5 nM of methyltrienolone (R1881) (Perkin Elmer), a synthetic androgen agonist, was utilized to determine the effect of androgen on cell proliferation.

Tumorigenicity assays

Cells (1×10^6) were mixed with 100 µL Matrigel (BD bioscience) / maintenance medium at a ratio of 1:1 and then inoculated subcutaneously into NOD.SCID mice of 8–12 weeks of age. Grafts were collected surgically at eight weeks post-inoculation from the euthanized animals.

Immunostainings

Paraffin sections (5 μ m) of tissues fixed in 4% PFA were stained for either H&E or immunohistochemistry (IHC) or immunofluorescence (IF), following the published protocol (15). Antigen retrieval for IHC was conducted by boiling the slides in 10 mM citrate buffer, pH 6.0 for 15 min, and then cooling down to room temperature. The sections were allowed to react with primary antibodies against androgen receptor (AR; 1:200; Santa Cruz), CK8 (1:100; TROMA-1 antibody; Developmental Studies Hybridoma Bank, University of Iowa), Vimentin (1: 50; Cell Signaling) or Ki67 (1:200; Vector Laboratories) for overnight at 4°C, followed by incubation with biotinylated secondary antibodies (1:200) against rabbit or rat IgG (Vector Laboratories) for 30 min, and then stained using the ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine (Dako) as substrate. IF analysis of the parallel sections was done by using primary antibodies against CK8, E-cadherin (1:100; Cell Signaling), N-cadherin (1:200; Abcam), and α -smooth muscle actin (SMA) (Cy3-conjugated; 1:200; Sigma-Aldrich). FITC conjugated secondary antibodies against rabbit or rat IgG (1:80; Sigma-Aldrich) were then applied. After staining, sections were mounted with medium containing DAPI for the labeling the nuclei.

Karyotyping

Analysis of karyotypes was performed essentially as described in (25). Briefly, actively growing cells were cultured in the presence of 0.05 μ g / ml colcemid (Invitrogen) for 4 hours, harvested after trypsin dissociation and collected in 15-ml conical centrifuge tubes. Cell pellets were suspended in 1.5 ml of fresh growth medium, 10 ml of a 37°C potassium chloride solution (0.075 M) was slowly added followed by 17 minutes incubation at 37°C. Then, two to three drops of ice-cold fixative (methanol: glacial acetic acid = 3:1) were mixed into the cell suspension. After immediate centrifugation, the fixation step with 10 ml ice-cold fresh fixative was repeated three times. Finally, the cell pellets were suspended in 1 to 0.5 ml fixative and chromosome spreads were made by dropping 70 μ l onto glass slides. Slides stained with Giemsa dye were counted using a 100 $\times$ or a 40 $\times$ oil immersion objective. At least 25 spreads were counted for each cell line. A statistical analysis was performed with InStat 3.05 (GraphPad).

Statistical analysis

The results of cell growth and marker analyses were evaluated as the mean $\pm$ SE of at least two different experiments performed in triplicate. Statistical calculations were done with Microsoft Excel analysis tools. Differences between individual groups were analyzed by independent *t* test. *P* values of <0.05 were considered statistically significant.

Results

Characterization of the new murine prostate tumor cell lines

We first isolated cells from an AD-Ca tissue from an intact mouse of 8 months of age. After culturing of single cells in 96-well plates, five colonies from different wells were obtained and cultured to enlarge the population, two from which, named E2 and E4, were selected for further characterization. By using the same method, cell lines were derived from an ADI-Ca tumor collected from a 13 month-old male mouse, which was castrated at the age of 8 months. Initial decrease followed by increase in BLI signals were monitored in this mouse for the growth of the recurrent tumor during the 5 months of observation after castration. From this recurrent tumor at the primary site, 3 single cell colonies were produced, two of which were named cE1 and cE2, and subjected to further characterization. As illustrated in Fig. 1A, E2 and E4 cells appeared to display generally a polygonal shaped morphology along with the presence of a small fraction of cells (<5%) that assumed a flattened and elongated shape (indicated by

arrows). The cE1, cE2 cells mostly exhibited polygonal shapes but apparently with increased intracellular adhesion as these cells tend to grow in clumps in the culture.

To determine the *Pten* gene status in these four cell lines, a genotyping analysis was performed. Genomic DNA extracted from these cell line cells were subjected to PCR reactions with a primer set specific for the exon 5 region of the *Pten* floxed allele. This primer set was able to amplify a 1 kb-long DNA fragment in the absence of DNA recombination. However, in the event of Cre-mediated recombination at the floxed site, there should not be any amplified product. Genomic DNA extracted from the tail tissue of *cPten*^{-/-}L mice and their normal counterparts were used as controls for the PCR products. The sizes were expected to be 1.0 kb and 0.9 kb for the floxed allele and the wild-type allele, respectively. As illustrated in Fig. 1B, such appropriate bands were detected in the tail DNA collected from the *cPten*^{-/-}L and normal mice, with no evidence for such products from any of the DNAs isolated from the cell lines. The results implied homozygous deletion of the floxed *Pten* allele in each of the epithelial cell lines. A similar analysis with the DNA isolated from the cancer-associated fibroblasts (CAFs) from the same mouse model showed the presence of only unrecombined floxed allele, reconfirming the prostate epithelium-specificity of the *Cre-loxP* system in the model (23). The results supported an epithelial origin for each of the four cell lines established. The retention of the *Cre* gene in the cell lines was also confirmed by PCR analysis (Fig. 1C). When the cell lines were subjected to karyotyping analysis, some individual differences became apparent. As shown in Table 2, E2, E4 and cE2 appeared near tetraploid, whereas cE1 was clearly aneuploid.

For determining the characteristics of molecular expressions, we used western blot to analyze AR and real-time PCR technique for mRNAs for basal cell markers (CK5, p63, CK14), luminal cell markers (CK18, CK8), neuroendocrine phenotype markers (26–28), namely chromogranin A (CgA) and synaptophysin (Synp), and the so-called prostate stem cell antigen, PSCA (29), a marker for intermediate epithelial cell differentiation. The values obtained from each PCR reaction was normalized to that of β -actin. As shown in Fig. 1D, the level of AR expression was fairly similar in all of these cell lines. Each of the cell lines expressed only very low levels of CK5, p63, CgA and PSCA, and a barely detectable level of Synp (Fig. 1E). The cE1 and cE2 cells, but not E2, E4 cells, displayed significant levels of CK8, CK14 and CK18 (Fig. 1F).

We next examined these cell lines for the effect of androgen on the proliferation rate. In SFM, E2, E4 cells grew slowly. However, the proliferation rate for cE1, cE2 cells under identical conditions was higher displaying an approximately 5-fold increase in the cell number after 5-day culture (Fig 2A). The cell proliferation rate of E2, E4 cells in SFM could be increased by about 1.2 to 1.7 fold by the addition of 1 to 5 nM concentration of androgen (R1881), as illustrated for the E4 cells in Fig. 2B. The response of cE1, cE2 cells to the same levels of androgen was higher displaying a 3.0- and 3.5- fold increase in the proliferation rate (Fig. 2C–D). To determine the effect of androgen on the AR expression in these cell lines, we cultured the cells in a medium composed of DMEM and 10% charcoal stripped serum (CSS) up to 18 days. RNA extracted from the cells cultured either in the maintenance medium or CSS medium were examined using real-time PCR with primers specific to AR. As illustrated in Fig. 2E, a significant (2.0- to 2.5-fold; $P < 0.01$) increase of AR transcripts was detected in cE series cell lines cultured in CSS medium relative to cells in maintenance medium. However, In contrast to cE cells, there was no striking increase in the AR expression level between E cells cultured in the maintenance and the CSS medium; rather a slight decrease was observed.

Tumorigenicity of the cell lines

For testing the tumorigenicity of the cell lines we used grafting on both male and female NOD.SCID recipients to examine the influence of gender on the intake of grafts and tumor histology. Mice were euthanized after 8 weeks later, and grafts were collected, weighed, and

tissue sections prepared. Our data demonstrated that the tumor incidence for all cell lines was 100% (Table 3), and, in general, grafts formed in male were larger than those formed in female. When the tumors formed in the males from different cell lines were compared, the tumor weights appeared to be similar for the E2, cE1, and cE2 cells, while the grafts from E4 weighed 3-fold higher; in females, E series grafts weighed 3- to 18-fold more than the cE series grafts.

When the grafts from the male mice were examined for pathology, a major difference between the cell lines from AD-Ca and ADI-Ca series was noted. As illustrated in Fig. 3A, H & E staining of E2, E4 grafts demonstrated spindle cell neoplasms. Since neoplastic cells expressed not only AR, but also vimentin and CK8, these neoplasms were diagnosed as sarcomatoid carcinoma (30) as per the “Pathological Classification of Prostate Lesions in Genetically Engineered Mice” (31). Distinct from the E2, E4 grafts (Fig. 3A, upper part), cE1, cE2 grafts demonstrated the histology of adenocarcinoma with presentation of glandular structures composed of round-shaped neoplastic epithelial cells expressing AR and CK8 (Fig. 3B), and with such epithelial cells that had penetrated into the stromal layers. Vimentin-positive cells could be detected in the stromal regions, but not inside the epithelial layers. Sections from E2, E4 grafts from the female mice also demonstrated histopathology of sarcomatoid carcinoma, except that the percentage of CK8⁺ cells was higher than that in the E2, E4 grafts in male mice (Fig. 3A). While cE1, cE2 cells were also able to form tumors in the female mice, the morphology of glandular structures in these grafts was not as clear as those observed in the male. The IHC staining demonstrated that cE1, cE2 grafts in male had positive nuclear staining for AR. In contrast, their counterparts in the female displayed mostly a diffuse cytoplasmic AR staining. Ki67, the cell proliferation marker, positive cells were detected in all grafts (Fig. 3C).

Analysis for EMT markers

Suspecting that E2, E4 cells might have undergone some degree of EMT conversion in cultures, we further tested for the expression levels of certain EMT markers in all cell lines by using real-time PCR. As illustrated in Fig. 4A, while E-cadherin expression was present in cE1 and cE2 cells, it was practically absent in E2 and E4 cells; N-cadherin levels were very low in all cell lines. E2, E4 cells appeared to produce significantly more vimentin than the cE1, cE2 cells. Expression of Twist, Slug and Snail, the known transcriptional repressors for EMT (28–31) was also increased in the E2, E4 cells relative to the cE1, cE2 cells (Fig. 4B). A few markers were also examined at the protein level using the grafts formed *in vivo*. Tissue sections adjacent to those used for H&E and IHC staining, were analyzed by IF using antibodies against CK8, E-cadherin, N-cadherin and α -SMA. As illustrated in Fig. 4C–E, cells that stained for α -SMA did not appear to simultaneously express CK8, E-cadherin, or N-cadherin on sections from cE1 and cE2 grafts grown either in male or female mice. However, many such cells (arrows) were detected in the sections from E2 and E4 grafts.

Discussion

The importance of this report is that it describes isolation and properties of new tumorigenic cell lines derived from two different tumors developed in the *cPten*^{-/-}L mouse model. These cell lines add to the limited repertoire of cell lines currently available for the study of prostate cancer. Of the four cell lines we describe here, E2, E4 were derived from an AD-Ca, and cE1, cE2 from ADI-Ca of a separate mouse. While each of the cell lines is strong in AR production, the two series vary in their growth rates, levels of expression of certain cell markers, and sensitivity to androgen exposure. In general, E2, E4 cells, expressing low levels of basal cell markers (CK5 and p63) but practically no luminal cell markers, show a requirement for androgen for optimal growth in cell cultures. E2 and E4 cells appear to express a few EMT markers, namely vimentin, Twist, Slug and Snail, at levels significantly higher than the cE series. The cE1, cE2 cells, which express significantly higher levels of two luminal cell markers

(CK8 and CK18) as well as a basal cell marker (CK14) than the E2 and E4 cells, can grow well in the absence of androgen, and at the same time retaining the capacity to produce increased AR transcripts when deprived of androgen, and to increase proliferation when androgen is supplemented. Since AR expression is similar in both series, the difference in the androgen sensitivity is likely to be regulated by mechanisms much more complex than simply by the levels of AR. It has been reported that AR remains critical for cell-cycle progression of human prostate cancer cell lines that are not dependent on androgen for growth (32,33), and the loss of PTEN function in a set of mouse neoplastic epithelial cell lines enhances androgen independence (21). In the *Pten*-deleted cell lines we have generated, a clear picture of the effect of androgen on cell growth is also not derived. We find that the two cell lines obtained from the androgen-dependent phase of the mouse prostate tumor can slowly proliferate for a limited time under androgen deprivation, and the cell lines from the ADI phase of the tumor show the ability to grow significantly more efficiently under such an environment while retaining the potential of increased proliferation when exposed to androgen. Since the study was performed using the E and cE lines from two different mice, it is, however, unclear whether the cE cells represent an evolution from an androgen-dependent state correlating with a linear progression of the disease, or an entirely independent phenomenon. To address this issue, it would be necessary to analyze samples of the same tumor and derived cell lines from a single animal at successive stages of progression from AD-Ca to ADI-Ca.

Another interesting observation we made is that these two series of cell lines drastically differ in their ability to induce tumor types in the *in vivo* assays. E series cell lines formed sarcomatoid carcinoma in both male and female mice, whereas cE series cell lines formed adenocarcinoma in males but undifferentiated tumors in the female (Fig. 3B). Consistent with the detection of reduced cell-cell adhesion properties as well the presence of a small fraction of cells with elongated and flattened shapes in the *in vitro* cultures of the E series, the real-time PCR results from these cells also indicate an increased levels of some EMT markers in these cell lines, as stated above. The possibility that the E series cells might have acquired a degree of EMT is supported by the demonstration that spindle-shaped cells co-expressing both epithelial and mesenchymal markers could be readily detected in the tumors formed *in vivo* from these cells. No such evidence was obtained with the cE cell lines (Fig. 4 C–E). In the tumors induced by the cE series, however, a major influence of the gender of the host animal was clearly evident. Tumors induced in the males by the cE cells present distinct glandular structures with the presence of CK8 positive cells both inside and outside the lumen, whereas, there is a dense distribution of CK8 positive cells in the undifferentiated tumors formed in the females, especially in cE2 grafts (Fig. 3B). These results suggest that the cE cells retain the capacity to generate lesions akin to prostatic adenocarcinoma, and this property appears to be dependent on the levels of male hormones. In this connection, the AR staining in the tumor sections of both E and cE cell lines that are formed in the male mice is found to be located mostly in the nuclei, but are detected primarily in the cytoplasm of the tumor cells in the grafts formed in the female mice, where the circulating androgen levels are expected to be low. Thus, a regulation by androgen appears to be more prominent in the formation of adenocarcinomas from the cE1 and cE2 cells, and less for the histopathological phenotype of the tumors induced by E2 and E4 cells. Induction of sarcomatoid carcinoma by the E series, however, remains quite intriguing. First of all, sarcomatoid carcinomas of the prostate are rare both in humans and mice (31). Second, contrary to our expectation, these cell lines from the relatively early phase of the prostate tumorigenesis appear to be less differentiated than those from the late ADI-Ca phase. It is, thus, unclear whether the isolation of E2 and E4 cells is related to clonal variations that may not be representative of the majority of the cancer cells but are coincidentally selected in the cultures or their presence in the tumors may have a hitherto unknown biological implication in tumorigenesis induced by *Pten* gene activation in the prostate epithelium. Isolation and characterization of additional neoplastic cell clones from the AD-Ca phase will be necessary for further evaluation of these issues.

In summary, we have generated two pairs of new mouse prostate cancer cell lines with homozygous deletion of the *Pten* tumor suppressor gene from two different prostate tumors: one pair of cell lines from an androgen-dependent tumor and the other pair from an androgen depletion-independent (recurrent) tumor. The particular cell lines, derived from spontaneous tumor models of a similar genetic background, display properties that are distinctive. Each of two cell lines derived from the recurrent tumor grows well in the absence of androgen, displays the ability to increase AR transcription when deprived of androgen, maintains sensitivity to androgen levels for increased proliferation, and can produce adenocarcinomas in male NOD.SCID mice. The pair of cell lines obtained from the androgen-dependent phase requires androgen for optimal growth, exhibits certain EMT-like properties *in vitro*, and can induce sarcomatoid carcinomas displaying a hybrid epithelial-mesenchymal cell phenotype *in vivo* when transplanted in either male or female hosts. These cell lines would likely to be important resources to explore the mechanisms in prostate cancer progression and manifestation of the diverse histopathological phenotypes.

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Figure 1.

Characteristics of the E2, E4 and cE1, cE2 cell lines. A, comparison the morphology of cells under the bright field in microscopy. While all cell lines appear to display polygonal-shaped cells that are distinctive of epithelial cells, E2 or E4 cells also manifest a small amount of cells with elongated morphology, as indicated by arrows. Bar, 100 μ m. B. PCR genotyping of the cell lines using *Pten*-specific primers. (a: normal mouse tail tissue; b: *cPten*^{-/-} L mouse tail tissue). C. PCR detection of the *Cre* gene in the cell lines. D. Analysis of AR expression in the mouse cell lines by western blots; human prostate cancer cell lines used as controls were: PC3, negative; LNCaP, positive. E,F. Comparative real-time PCR analysis of marker gene expression including basal (CK5, p63, CK14), luminal (CK18, CK8), neuroendocrine cell markers (CgA, Syph), and PSCA.

Figure 2.

The comparison of proliferation rates of the cell lines in cultures. A. Growth rates in serum-free-medium (SFM). The differences in the number of cells counted at day 5 were significant between the E and cE series ($P < 0.01$). B. Illustration of the effect of low concentrations of androgen (R1881) on the growth of E4 cells in SFM (similar results were also obtained with E2 cells). The growth rate was significantly increased ($P < 0.05$) in the presence of 5 nM, but not 1 nM R1881. C. Effect of similar concentrations of androgen on the growth of cE1, and cE2 cells (D). At day 5, the stimulation by androgen was significant ($P < 0.01$) with both cell lines. E. Analyses of the AR expression levels in E and cE series cultured in the maintenance or an androgen-free medium derived by using charcoal-stripped serum in the place of normal serum (CSS). The differences between cE1 and cE1-CSS and between cE2 and cE2-CSS were significant ($P < 0.01$).

C **Figure 3.**

Microanatomic analyses of tumors induced by the cell lines in male or female in NOD.SCID mice. A. H&E and IHC staining of sections of E2 and E4 grafts for AR, CK8 and vimentin. A higher magnification of an area marked by the arrow is shown in upper-left corner of each section. *Bar*, 100 μ m. B. Similar staining experiments with sections from cE1 and cE2 grafts. *Bar*, 100 μ m. C. IHC staining of the grafts for Ki67. *Bar*, 100 μ m.

A **B**

C

CK8 / α -SMA

cE

E

Male

Female

Figure 4.

Analysis for the expression of EMT-related markers in the cell lines. A. Relative expression of E-cadherin, N-cadherin and vimentin, and B, that of Twist, Snail and Slug as determined by real-time PCR. The difference in the level of expression of each of vimentin, Twist, Snail or Slug in the E series relative to the cE series was significant ($P < 0.01$). C–E. Illustrations of co-immunofluorescence results when tumor grafts from the E and cE cell lines were stained red for α -SMA and green for either CK8(C), E-cadherin (D) or N-cadherin (E). White arrows indicate cells with co-expression (yellow). Bar, 50 μ m

Table 1

Primer sets used in real-time PCR and genotyping*

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
CK5	ACCTTCGAAACACCAACGAC	TTGGCACACTGCTTCTTGAC
p63	GAAGGCAGATGAAGACAGCA	GGAAGTCATCTGGATTCCGT
AR	AACCAACCAGATTCTTTGC	ATTAGTGAAGACCGCCAAC
CK14	GACTTCCGGACCAAGTTTGA	CCTTGAGGCTCTCAATCTGC
CK8	ATCGAGATCACCACCTACCG	TGAAGCCAGGGCTAGTGAGT
CK18	ACTCCGCAAGGTGGTAGATG	GCCTCGATTCTGTCTCCAG
PSCA	GCTGCTACTCTGACCTGTGC	TTCACAATCGGGCTATGGTA
CgA	GGGAGCTGGAACATAAGCAG	TGTCCTCCCATTCTCTGGAC
Syph	CTTTGTGAAGGTGCTGCAAT	GTCTTGTGGCACAATCCAC
E-cadherin	TCCAGGAACCTCCGTGATG	GGGTAACCTCTCTCGGTCCAG
N-cadherin	AGCCTGGGACGTATGTGATG	ATGTTGGGTGAAGGTGTGCT
Vimentin	CAAGTCCAAGTTTGCTGACCT	TCTTCCATCTCACGCATCTG
Twist	CTCGGACAAGCTGAGCAAG	ACGGAAGAAGGCGTAGCTGAG
Snail	ACCCACACTGGTGAGAAGC	GACCAAGGCTGGAAGGAGTC
Slug	CACAGTTATTATTCCCCATATCT	GCAGTCTCTCCTCTTCGTCA
β -actin [†]	AGTGTGACGTTGACATCCGT	CTTGCTGATCCACATCTGCT
Pten	AAGCAAGCACTCTGCGAAACTG	GATTGTCATCTTCACTTAGCCATTGGT
Cre	GATCCTGGCAATTTCGGCTAT	TTGCCTGCATTACCGGTCGAT

* Most of the primers were as described (21) except for Twist (34) and those we designed for E-cadherin, N-cadherin, vimentin, Snail, Slug, *Pten*, and *Cre*.

[†] For use as a control gene in real-time PCR.

Table 2

Representative karyotypes of the cell lines

Cell Line	Chromosome Number	Sample Number
E2	Near 4N (79.3±14.9)	40
E4	Near 4N (76.1±23)	31
cE1	Near 3N (63.8±15.1)	26
cE2	Near 4N (78.6±29.5)	25

Table 3

The comparison of the incidence and weights of the tumors formed

Host	Cell Line	Incidence (%)	Tumor Weight (g)
Male	E2	4/4 (100)	0.25 ±0.24
Male	E4	4/4 (100)	0.80±0.54
Male	cE1	4/4 (100)	0.25±0.08
Male	cE2	4/4 (100)	0.26±0.07
Female	E2	4/4 (100)	0.17±0.03
Female	E4	8/8 (100)	0.56±0.15
Female	cE1	8/8 (100)	0.05±0.01
Female	cE2	4/4 (100)	0.03±0.02