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A Human Prostatic Epithelial Model of Hormonal Carcinogenesis[1]

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ABSTRACT

The effects of stromal and hormonal environment on the immortalized but nontumorigenic human prostatic epithelial cell line BPH-1 were investigated in an *in vivo* model. BPH-1 cells were recombined with rat urogenital sinus mesenchyme (UGM), and the tissue recombinants were grafted to the renal capsule of adult male athymic mouse hosts. BPH-1 + UGM recombinants formed solid branching epithelial cords with a well-defined basement membrane. The cords canalized to form ductal structures. The mesenchymal cells formed thick sheets of well-differentiated smooth muscle surrounding the epithelium, reinforcing the idea that the epithelium dictates the patterning of prostatic stromal cells. When hosts carrying BPH-1 + UGM tissue recombinants were exposed to testosterone propionate and 17-β-estradiol (T + E2), the tissue recombinants responded by forming invasive carcinomas, demonstrating mixed, predominantly squamous as well as adenocarcinomatous (small acinar and mucinous) differentiation. When either untreated or T + E2-treated hosts were castrated, epithelial apoptosis was observed in the grafts. When tumors were removed and regrafted to fresh hosts they grew rapidly. Tumors were serially regrafted through six generations. Histologically these tumors consisted largely of focally keratinizing squamous cell carcinoma with high-grade malignant cytological features. BPH-1 cells grown in the absence of UGM survived at the graft site but did not form tumors or organized structures. This behavior was not influenced by the presence or absence of T + E2 stimulation. These data show that an immortalized, nontumorigenic human prostatic epithelial cell line can undergo hormonal carcinogenesis in response to T + E2 stimulation. In addition, the data demonstrate that the stromal environment plays an important role in mediating hormonal carcinogenesis.

INTRODUCTION

One of the recognized deficiencies in the field of prostate cancer research is a lack of model systems that allow investigation of the phenomenon of prostatic carcinogenesis. Spontaneous and carcinogen-induced rat models of prostate cancer may be the most accurate in terms of their natural history but, historically, have been of limited use because of the low incidence and long latency of the tumors (1–6). These constraints of time and incidence make these models slow and costly to use. Recent technical advances have improved the efficiency of this methodology, but the time to form tumors remains a serious obstacle (7). We have demonstrated recently that mouse prostatic epithelium deficient in the *Rb*[3] gene is highly sensitive to hormonal carcinogenesis induced by T + E2 and can undergo a progression to cancer, which closely mimics the human disease (8). While rodent models are useful, it is desirable to use a human prostatic epithelium as the target cell type for hormonal carcinogens. We have reported previously that the human prostatic epithelial cell line BPH-1 can be induced to form adenocarcinomas when recombined with carcinoma-associated fibroblasts (9). This SV40T immortalized but nontumorigenic cell line is, thus, known to have the capacity to undergo malignant transformation when exposed to appropriate stimulation. SV40T expression is known to disrupt the *Rb* pathway (10). Therefore, it seemed reasonable to expect that the BPH-1 line may also be sensitive to hormonal carcinogens, thus providing an opportunity to examine hormonal carcinogenesis using a human prostatic epithelial cell as the target.

There are distinct androgenic responses in the prostate mediated through stromal and epithelial ARs. During development, epithelial proliferation and organization including ductal branching morphogenesis, canalization, and differentiation into basal and luminal cells is mediated by the mesenchymal ARs (11–13). For these processes, epithelial ARs are not required. Likewise, the adult androgens act upon stromal ARs [predominantly localized in the prostatic smooth muscle (14–17)] to maintain a fully differentiated growth-quiescent gland. Epithelial ARs are required for secretory function in the mature prostate (18).

The adult prostate is dependent upon the presence of testicular androgens to maintain secretory activity and a fully differentiated state. In the absence of androgenic stimulation the prostate undergoes a well-documented regression characterized by extensive epithelial apoptosis and regression and by a well-ordered dedifferentiation of the prostatic smooth muscle toward a more fibroblastic phenotype (19–24). Epithelial apoptosis in the prostate is a well-studied phenomenon, and many of the biochemical characteristics of the process have been described (25–29). Both epithelial and stromal cells apoptose in response to androgen ablation in the prostate (30). In nonmalignant tissues, prostatic epithelial apoptosis is attributable to a failure to occupy prostatic stromal ARs and is independent of the presence of epithelial ARs (31). Prostatic cell death as a result of androgen ablation has been a mainstay in the treatment of prostatic carcinoma for many years (32).

Estrogens also elicit effects on the prostate. These are presumed to be mediated through intraprostatic ERαs and βs (33, 34). The best characterized effect of estrogens on the adult prostate is the induction of squamous metaplasia (35). In addition, estrogens can act synergistically with androgens to induce benign prostatic hyperplasia in dogs (36). A combination of E2 and testosterone induces prostatic cancer in adult niobium rats (7, 37–40) and in Rb-deficient mouse prostatic epithelium (8).

ERα, the classic estrogen receptor, has consistently been reported in prostatic stroma in the rat (41, 42). ERβ, in contrast, is expressed at high levels in prostatic epithelium (43). Thus ERs are present in both the stromal and epithelial tissues of the prostate. It is notable that steroid autoradiographic studies of normal animals at different developmental stages have consistently demonstrated the binding of [^{3}H]estradiol in the prostatic stroma but not in the epithelium (44–46). Prostatic development appears to be normal in either ERα or ERβ

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[3] The abbreviations used are: UGM, urogenital sinus mesenchyme; T, testosterone propionate; E2, 17-β-estradiol; Rb, retinoblastoma; AR, androgen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; FBS, fetal bovine serum; PSA, prostate-specific antigen; PAP, prostatic acid phosphatase; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling; IGF, insulin-like growth factor; VP, ventral prostate.

knockout mice, suggesting that prostatic organogenesis is independent of signaling through either of these ERs (34, 47).

In this report, we characterize the response of the SV40 large T immortalized, nontumorigenic human prostatic epithelial cell line BPH-1 to recombination with rat UGM and to steroidal manipulation after grafting to intact male athymic rodent hosts. These studies explore aspects of the roles of androgens and estrogens during hormonal carcinogenesis in a tissue recombination model.

MATERIALS AND METHODS

Preparation and Processing of Grafts. Pregnant rats were obtained from Simonsen (Gilroy, CA). Rat UGM was prepared from 18-day embryonic fetuses (plug date denoted as day 0). Urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by tryptic digestion, as described previously (48). UGM was then additionally reduced to single cells by a 90-min digestion at 37°C with 187 units/ml collagenase (Life Technologies, Inc., Grand Island, NY). After digestion the cells were washed extensively with RPMI 1640 tissue culture. Viable cells were then counted using a hemacytometer, with viability determined by trypan blue exclusion.

BPH-1 cells (49) were from our own stocks. Cells were routinely maintained and passaged in RPMI 1640 with 5% FBS. BPH-1 cells were released from tissue culture plastic with trypsin, washed in growth medium containing 20% FBS, and viable cells were counted using trypan blue exclusion and a hemacytometer. Cell recombinants were prepared by mixing 100,000 epithelial (BPH-1) cells with 250,000 mesenchymal cells in suspension. For control grafts 350,000 cells of each cell type (BPH-1 or rUGM) were used. Cells were pelleted and resuspended in 50 μl of neutralized type 1 rat tail collagen prepared as described previously (50). The recombinants were allowed to set at 37°C for 15 min and were then covered with growth medium (RPMI 1640 + 5% FBS) containing testosterone (10^{-8} M) and cultured overnight. Recombinants were then grafted beneath the renal capsule of adult male athymic rats (Harlan, Indianapolis, IN) or adult male outbred athymic mice (Simonsen); the use of rat hosts was dictated by immunohistochemical analyses requiring the use of antibodies generated in mice. An illustrated tutorial for the subrenal capsule grafting technique can be viewed on the internet.[4] All of the animals were housed in the University of California San Francisco laboratory animal resource center with food and drinking water *ad libitum* under controlled conditions (12 h light, 12 h dark, and 20 ± 2°C).

Mice were treated hormonally by surgical implantation of a 1-cm Silastic capsule filled with testosterone and a 0.4-cm Silastic capsule filled with estradiol-17β (No. 602–305 Silastic tubing; 1.54-mm inside diameter × 3.18-mm outside diameter; Dow-Corning Corp., Midland MI). T and E2 were obtained from Sigma Chemical Co. (St. Louis, MO). Mice were hormonally treated for 4 months. Control animals received empty Silastic tubing.

Selected hosts (both T + E2-treated and untreated) were castrated at 4 months after grafting, and tissues were examined at 3 and 7 days after castration. In the case of the T + E2-treated animals, the hormone-containing implants were removed 3 days before castration.

Hosts were sacrificed by anesthetic overdose followed by cervical dislocation. Kidneys were excised, and grafts were dissected free of the host kidney, weighed, and then processed for immunohistochemistry.

To confirm whether grafts had undergone malignant transformation, small pieces of harvested grafts (15-mg wet weight) were implanted under the renal capsule or subcutaneously in nude mice. Hosts were sacrificed 1 month after grafting and the grafts excised, weighed, and processed for histology. In cases where growth had occurred, 15-mg tumor fragments were regrafted to fresh hosts, and the cycle was continued out to 6 regrafting generations.

Immunohistochemistry. Grafts were processed for paraffin sections. Samples were fixed in 10% neutral buffered formalin and processed to paraffin. Sections were cut on a microtome and mounted onto presialized microscope slides. Sections were dewaxed in Histoclear (National Diagnostic, Atlanta, GA) and hydrated in graded alcoholic solutions and distilled water. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 min followed by washing in PBS (pH 7.4). Five-percent normal goat or donkey serum in PBS (as appropriate) was applied to the sections for 30 min to bind nonspecific sites. The sections were then incubated with the primary antibodies overnight at 4°C or with nonimmune mouse IgG. In these experiments, rabbit polyclonal anti-AR antibody (PA1-111A) was purchased from Affinity BioReagents (Golden, CO). The anti-SV40T antibody PaB 101 was a generous gift from Dr. John Lehman (Department of Microbiology, Albany Medical College, Albany, NY). Anticytokeratin antibodies and all of the mouse monoclonals (LE41, LE61, LP34, and LL001 against keratins, 8, 18, 5, and 14, respectively) were generously provided by Dr. E. B. Lane, University of Dundee (Dundee, Scotland, United Kingdom). (Note that LP34 recognizes a broad spectrum of keratins; however, it most strongly reacts with keratin 5 and is basal cell-specific in the normal prostate). Antibodies raised against α-smooth muscle actin, desmin, myosin, laminin, vinculin, and vimentin were purchased from Sigma Chemical Co. Polyclonal rabbit antibodies raised against PSA and PAP were purchased from Dakopatts (Carpinteria, CA). Mouse anti-E-cadherin monoclonal antibody was purchased from Transduction Laboratories (San Diego, CA). Anti Ki67 was purchased from Immunotech (Westbrook, ME). Purified rabbit and mouse IgGs were obtained from Zymed Corp. (So. San Francisco, CA). Biotinylated antirabbit and antimouse IgG were obtained from Amersham International (Arlington Heights, IL). Biotinylated antigoat antibody was purchased from Sigma Chemical Co. Peroxidase-linked avidin/biotin complex reagents were obtained from Vector Laboratories (Foster City, CA). After incubation with primary antibody, sections were washed carefully with PBS and incubated with appropriate biotinylated secondary antimouse immunoglobulin-diluted with PBS at 1:200 for 30 min at room temperature. After incubation with the secondary antibody, sections were washed in PBS (three 10 min washes) and then incubated with avidin-biotin complex (Vector Laboratories) for 30 min at room temperature. After an additional 30 min of washing in PBS, immunoreactivity was visualized using 3,3′-diaminobenzidine in PBS and 0.03% H_2O_2. Sections were counterstained with hematoxylin and dehydrated in graded alcohols. Control sections were processed in parallel with mouse or rabbit nonimmune IgG (Dako) at the same concentration as the primary antibodies. Hoechst 33258 staining was performed as described previously (51).

Apoptotic Index. Apoptotic cells were identified by using the Oncor ApoTag kit (Intergen, Gaithersburg, MD). Briefly, sections were deparaffinized, rehydrated, and incubated in Oncor Protein Enzyme (20 μg/ml) for 15 min at room temperature. Sections were then incubated in working strength terminal deoxynucleotidyltransferase (TdT) enzyme buffer (76 μl reaction buffer and 32 μl TdT enzyme) for 1 h at 37°C, washed, and apoptotic bodies were labeled using antidigoxigenin-fluorescein. Sections were counterstained and mounted with Oncor Propidium Iodide/Antifade (Oncor). Apoptotic index was determined using fluorescence microscopy by counting the percentage of TUNEL-positive staining cells in the total epithelium. To avoid a counting bias microscope fields were selected using the tetramethylrhodamine isothiocyanate filter set (visualizing propidium iodide). The field was imaged and then the filters were switched to obtain an image of the TUNEL-stained nuclei. Staining with the Hoechst 33258 dye (Sigma Chemical Co.) was performed as described previously (51); this DNA stain intensely marks pyknotic nuclei and was used to confirm observations with the TUNEL system. Groups of data were compared by ANOVA $P < 0.05$ being considered significant.

Proliferative Index. Proliferating index was compiled by manually counting total epithelial nuclei and Ki67-expressing epithelial nuclei in microscopic fields. Proliferative indices were compiled using tissue from untreated hosts (which has a uniformly benign histology), and from testosterone and estradiol-treated hosts. In the case of treated hosts, samples were categorized histologically into three groups: benign solid cords, benign canalized ducts, and carcinoma. Ki67-labeling indices were calculated separately for each of these groups. Fifteen to 25 samples were examined/group using two fields/sample, counting a total of ~5000 nuclei/group. Groups were compared with ANOVA $P < 0.05$ being considered significant.

Determination of Cancer Incidence. Paraffin blocks containing grafts were completely sectioned and mounted on microscope slides. Approximately three serial sections/slide were mounted representing a total of 18 μm of tissue thickness. Each fifth slide was stained with H&E and examined microscopically. Samples were scored for the presence or absence of malignant histopathology. Malignancy was defined using both cytological and architectural criteria. Cytological features of malignancy included nuclear enlargement (nuclei 2 × normal), nuclear pleomorphism (2 × nuclear variability within groups), nuclear hyperchromasia, nuclear contour irregularity, and macro-

[4] http://mammary.nih.gov/tools/Cunha001/index.html.

nucleoli. Architectural criteria were characterized by haphazardly arranged small-to-medium-sized glandular structures and/or angulated and irregular tumor nests and trabeculae demonstrating destructive invasive growth. Invasion was defined by infiltration into normal adjacent host tissue and/or invasion into capsular fibrous tissue with desmoplastic tissue reaction. Glands demonstrating cytological features of malignancy but lacking features of destructive invasive growth were interpreted as dysplasia.

RESULTS

Gross Appearance. Control grafts of UGM and BPH-1 cells in the absence of the other cell type were small (<10 mg) at 4 months after grafting (Fig. 1 and Table 1). There was no statistical difference, as determined by the Student-Newman-Keuls test, between the wet weights of grafts of BPH-1 cells in untreated or T + E2-treated hosts. BPH-1 control grafts in untreated hosts have been examined after 1 year, and the small size is maintained. Recombinants composed of BPH-1 cells + rUGM cells were also small, typically ~40 mg at 1 month after grafting. rUGM + BPH-1 recombinants grown for several months demonstrated modest increases in size. Fig. 1 shows a BPH-1 + rUGM recombinant grown for 9 months *in vivo*. Even after this prolonged period the grafts had not grown beyond the immediate area of the kidney capsule and showed no sign of invasion into the host kidney. These grafts were each ~50 mg wet weight. In contrast, grafts of BPH-1 + rUGM tissue recombinants to hosts that were subsequently treated with T + E2 gave rise to significantly larger grafts (Table 1). Wet weights of the grafts 4 months after grafting are summarized in Table 1.

Histology. Histological analysis of the grafts showed profound differences between organization of both the epithelial and stromal components of the different graft types. BPH-1 cells grafted alone in either untreated or T + E2-treated hosts showed little organization, although occasional keratinized pearls were seen, as described previously (49). Whereas these grafts did not increase in size, the BPH-1 cells continued to divide actively within the grafts as demonstrated by Ki67 immunoreactivity as illustrated in Fig. 2. UGM grafts formed unorganized fibroblastic tissue (not illustrated). Most cells expressed vimentin; a minority also expressed α-smooth muscle actin. Other markers of smooth muscle differentiation were not detected.

In intact male hosts, the BPH-1 cells combined with rUGM organized into solid epithelial cords reminiscent of human fetal prostate (Fig. 3). The cords were surrounded by a distinct basement membrane, which was, in turn, surrounded by stromal cells forming dense sheets of α-smooth muscle actin, desmin, myosin, laminin, and vinculin but not vimentin. The identity of the BPH-1 cells was confirmed in all of the cases by immunostaining with antibodies against the SV40 large T antigen. Some epithelial structures became canalized with a well-defined lumen (Fig. 3). The epithelial cells within the cords and ducts exhibited membrane-localized expression of E-cadherin. Staining for ARs revealed expression in the UGM-derived smooth muscle cells but not in BPH-1 cells. Hoechst 33258 staining revealed that the stromal cells exhibited even nuclear staining characteristic of rat cells and not the punctate staining pattern associated with cells of mouse origin.

Table 1 *Incidence of tumors, as determined by histopathological examination, and wet weights of grafts 4 months after subrenal capsule implantation in intact male and testosterone and estradiol-treated male nude mouse hosts*

	Cancer incidence	Wet weight (mean ± SD)
Untreated host		
BPH-1 only	0/107 (0%)	3.91 ± 1.8 mg
UGM + BPH-1	1/218 (0.45%)	46.5 ± 34.6 mg
T + E2-treated host		
BPH-1 only	0/18 (0%)	8.0 ± 2.9 mg
BPH-1 + UGM	30/84 (35.7%)	151.5 ± 181.3 mg

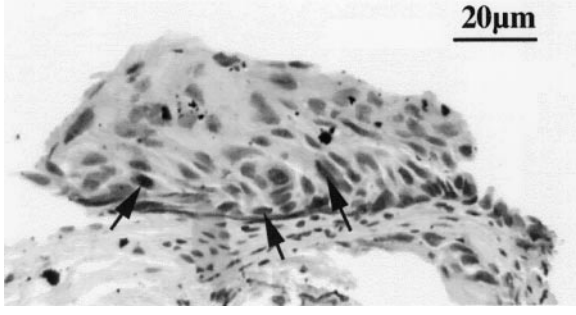

Fig. 2. Ki67 expression in a control graft of BPH-1 cells grafted to the kidney capsule of an intact male athymic mouse host in the absence of urogenital mesenchyme. Note the small cross-sectional area of the epithelial tissue. Note also that the cells continue to cycle (*arrows*).

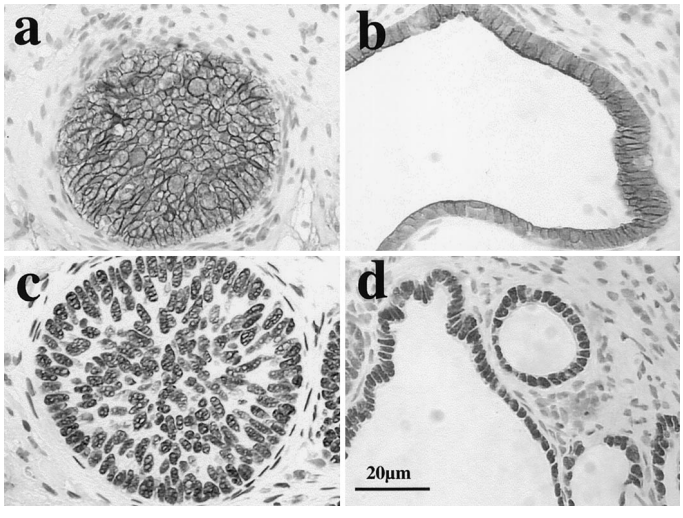

Fig. 3. Tissue recombinants composed of rat UGM + BPH-1 epithelial cells grafted to kidney capsule of an intact male athymic mouse host. Figure shows both solid epithelial cords (*a* and *c*) and fully canalized structures (*b* and *d*) seen in these grafts. Note that E-cadherin expression on cell membranes is maintained in the epithelial structures (*a*). In canalized structures luminal cell-specific cytokeratins, in this case keratin 8, were seen (*b*). SV40T expression was detected in all epithelial cells of both histological types (*c* and *d*).

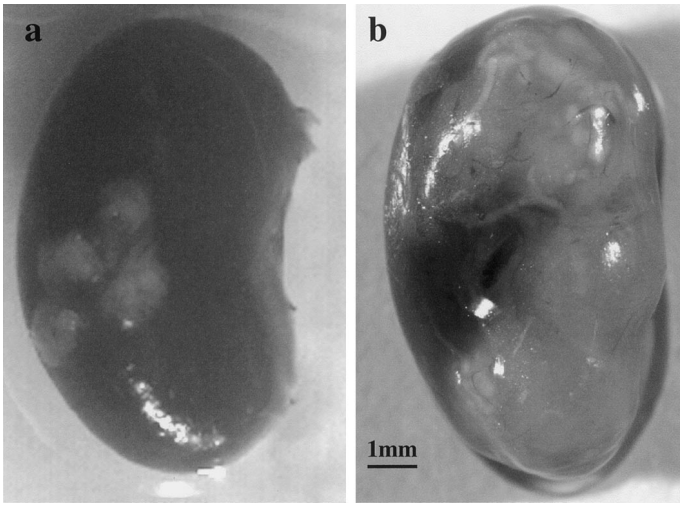

Fig. 1. Whole mount showing (*a*) four grafts of BPH-1 cells and (*b*) four tissue recombinants composed of BPH-1 epithelial cells and rat UGM after 9 months of growth under the kidney capsule of an intact male athymic mouse host. Note that the individual BPH-1 grafts are well defined and are whitish in color. The BPH-1 grafts remain very small, whereas the BPH-1 + rUGM tissue recombinants grow to a wet weight of ~40 mg each.

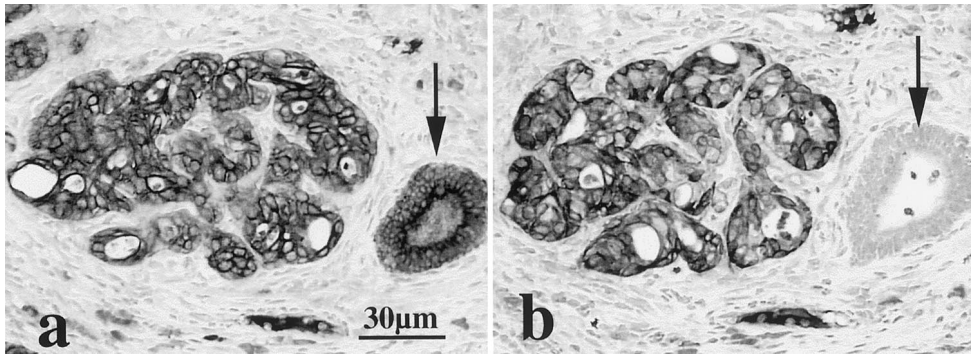

Fig. 4. Expression of keratins 8 (*a*) and 14 (*b*) in tissue recombinants composed of rat UGM + BPH-1 epithelial cells grafted to kidney capsule of an intact male athymic mouse host after 4 months of treatment with testosterone and estradiol. This figure demonstrates both malignant and benign histologies in the grafts. Note that the large area of cancer coexpresses the two cytokeratin markers, whereas the pathologically normal canalized duct expresses only cytokeratin 8 (*arrow*).

PSA and PAP were not detected by immunohistochemistry. The epithelial cords were much thicker than those seen in the human fetal prostate and seemed to become canalized by a process of cell death in the center rather than by an organized developmental process. Thus, many epithelial structures contained dead or dying cells within developing lumina. Examination of Ki67 immunoreactivity in these grafts revealed that the BPH-1 cells were still proliferating even 9 months after grafting. Examination of expression of cytokeratins in the epithelial structures (Fig. 4) revealed that in the solid epithelial cords most cells coexpressed both basal (5 and 14) and luminal (8 and 18) cytokeratins, mimicking cytokeratin expression in the fetal human prostate. However, in fully canalized ducts expression of keratins 5 and 14 was generally lost whereas luminal-type keratins were maintained.

When hosts carrying tissue recombinants were treated with testosterone and estradiol for 4 months, ~35% of the grafts underwent malignant transformation as determined by histopathological examination of tissue sections (Table 1). In these tumors a spectrum of malignant morphological features was seen. Some grafts developed only bulky expansion of the ductal structures by keratinizing squamous epithelium with severe cytological atypia ranging from features of high grade dysplasia to carcinoma *in situ*. Other specimens revealed microscopic foci of invasive high-grade carcinoma with squamous differentiation arising from the *in situ* component. More typically, bulky invasive tumors infiltrated the renal parenchyma entrapping host renal structures. Many tumors arose in direct continuity with benign prostatic ductular structures (Fig. 5). Some degree of mixed adenosquamous differentiation was invariably seen, though the squamous component was dominant in most tumors (Fig. 6). The glandular areas consisted largely of invasive small acinar glands and occasional cribriform structures (Fig. 6). These areas melded into the squamous areas with dense pink cytoplasm, intracellular bridges, and keratinization with focal keratin pearls. A few examples of predominantly small acinar adenocarcinoma were seen. All of the tumors demonstrated high-grade malignant cytological features including nuclear pleomorphism, anisonucleosis, nuclear hyperchromasia, nucleolar enlargement, and mitotic activity with rare apoptotic cells. Epithelial SV40T expression was maintained in the tumors. Tumors coexpressed basal and luminal-type cytokeratins. Tumorigenesis was associated with a loss of membranous expression of E-cadherin in the epithelial cells and with stromal changes. There was an exuberant desmoplastic response to the invasive tumor components in all of the cases. Smooth muscle differentiation, as characterized by expression of α-smooth muscle actin, was lost in the vicinity of the tumors.

Proliferative Index. On the basis of the counting of more than 17,000 individual tumor epithelial nuclei, the Ki67 labeling index averaged 15% in BPH-1 + rUGM tissue recombinants growing in untreated hosts and 32.7% in BPH-1 + rUGM tissue recombinants growing in testosterone + estradiol treated hosts (Fig. 7). Separating the analysis of different histologies (benign *versus* malignant and solid cords *versus* canalized ducts) in the treated groups revealed that both benign and malignant histologies proliferated at statistically similar rates (as determined by ANOVA analysis) but that this proliferative activity was significantly higher in hosts treated with testosterone + estradiol as compared with hormonally untreated hosts.

Apoptotic Response to Castration. The results of TUNEL staining are summarized in Table 2. Detection of pyknotic nuclei by Hoechst dye 33258 staining was consistent with these data, confirming that the TUNEL-stained cells were truly apoptotic and not artifactual. In BPH-1 + UGM tissue recombinants grown in intact hosts apoptotic activity was not detected (based upon counting 5000 blindly picked epithelial cells). It should be noted that occasional TUNEL-positive cells were seen, but by chance these were not present in the fields counted to compile this data set. Thus, there was an extremely low basal level of cell death under the steady state conditions in the intact male hosts. After castration there was a transient rise in epithelial apoptosis, which reached significance as determined by ANOVA analysis in the BPH-1 + UGM tissue recombinants grown in untreated hosts. This apoptotic activity declined by 7 days after castration. After removal of hormonal implants but before castration, epithelial apoptosis was seen in areas of both benign and malignant pathology in grafts from previously hormone-treated hosts. In areas of malignant pathology background levels of apoptosis were ~2.4% (Table 2). This was probably a result of the short period of time between removal of the T + E2 implants and castration of the hosts.

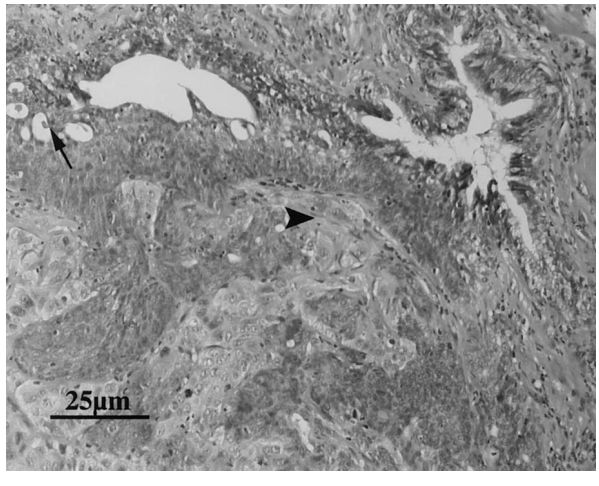

Fig. 5. Benign glandular morphology (*top right*) characterized large simple glands lined by an orderly arrangement of columnar epithelium with small uniform nuclei. Progression to dysplasia is present (*top middle*) characterized by disordered epithelium with nuclear enlargement within a large gland. Invasive malignant glands (*arrow*) and malignant squamous nests (*arrowhead*) arise in direct continuity with noninvasive glands (*bottom half*).

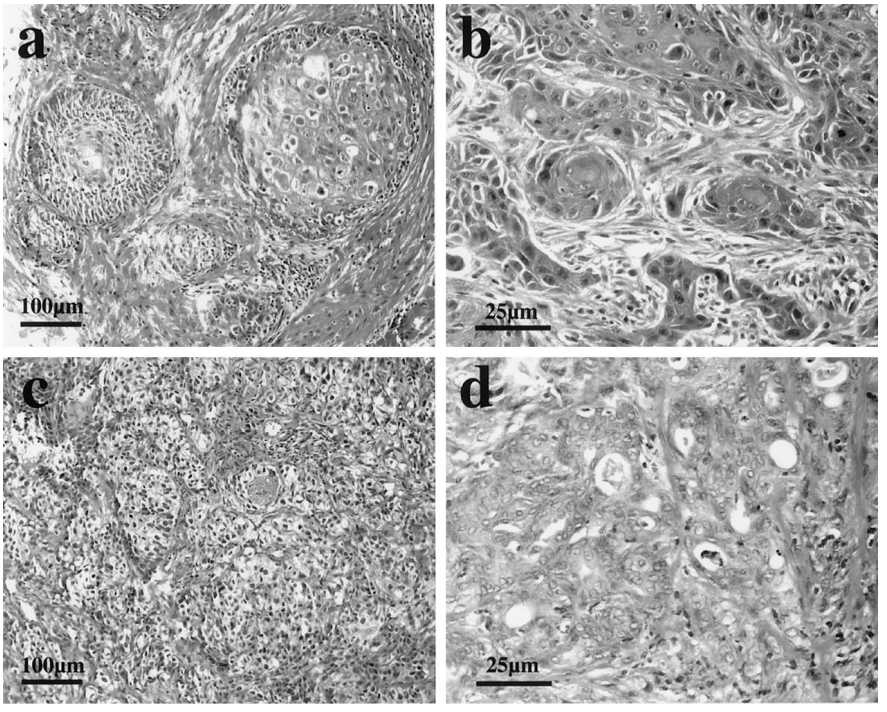

Fig. 6. Tissue recombinants composed of rat UGM + BPH-1 epithelial cells grafted to the kidney capsule of an intact male athymic mouse host after 4 months of treatment with testosterone and estradiol. *a* and *b*, invasive carcinoma with squamous differentiation demonstrating angulated infiltrative nests consisting of pleomorphic cells demonstrating anisonucleosis, coarse chromatin, irregular nuclear borders, prominent nucleoli, and dense, eosinophilic cytoplasm with distinct cytoplasmic borders (H&E stained). *c* and *d*, adenocarcinomatous component consisting of infiltrative glands with abortive glandular lumina and solid sheets with cribriform structures. The epithelium demonstrates nuclear pleomorphism with hyperchromasia and macronucleoli as well as amphophilic to clear, bubbly syncytial cytoplasm (H&E stained).

If this was the case, the baseline apoptotic rate may be too high, leading to the failure to observe a statistically significant increase (as determined by ANOVA) in apoptotic activity after castration of these hosts. Histopathologically, involution of tumors was observed at 3 and 7 days after castration (Fig. 8). At 3 days after castration, areas of cystic degeneration of the tumor were present, and apoptotic tumor epithelial cells were readily observed. Mitotic figures were readily identified. By 7 days after castration, the tumors had largely involuted, leaving only focal areas of residual carcinoma. Both apoptotic and mitotic activity were present in the residual tumor.

Confirmation of Malignant Transformation. To confirm that the different histopathological diagnoses were consistent with true independent malignant growth we tested the ability of tissues to grow independent of T + E2 stimulation. For this purpose, tissue fragments from five BPH-1 + UGM grafts from either T + E2-treated or untreated hosts were grafted to fresh athymic hosts. The T + E2-treated grafts were suspected to contain areas of malignant tissue on the basis of elevated wet weight. The untreated grafts were randomly selected, because these were all within a small wet weight range. BPH-1 + UGM grafts from untreated hosts did not significantly increase in size. In contrast, BPH-1 + UGM grafts from all of the T + E2-treated hosts grew rapidly. Subsequent regrafting of these tissues gave rise to progressively more rapidly growing tumors. In the sixth generation of regrafting, 15-mg fragments gave rise to tumors weighing 146 ± 45 mg over a 28-day period. Histologically these grafts were similar to the BPH-1 + UGM recombinants from which they were derived, consisting largely of keratinizing squamous cell carcinoma with high-grade malignant cytological features.

DISCUSSION

The present study demonstrates that a clonally derived immortalized human prostatic epithelial cell line (BPH-1), when recombined with rat UGM, can recapitulate some important aspects of human prostatic development and can be induced to undergo malignant transformation in response to stimulation by high levels of testosterone and estradiol.

Prostatic development requires androgenic stimulation of an inductive mesenchyme, which in turn interacts with an adjacent endodermally derived epithelium. Androgens act on mesenchymal ARs to stimulate epithelial proliferation and differentiation via a paracrine

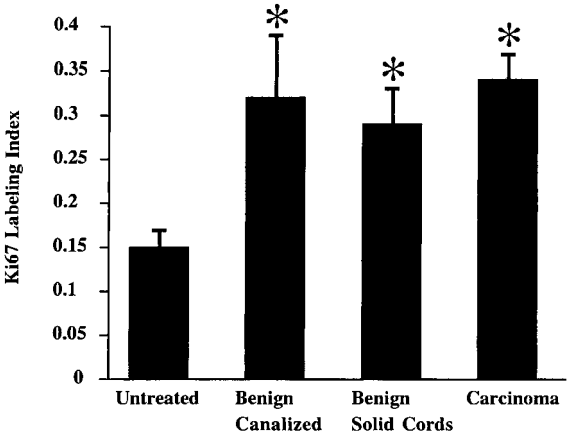

Fig. 7. Ki67 labeling index of epithelial cells from tissue recombinants composed of rat UGM + BPH-1 epithelial cells grafted to the kidney capsule of an intact male athymic mouse host either untreated or after 4 months of treatment with testosterone and estradiol. Ki67 labeling index was similar in benign *versus* malignant epithelium and was significantly elevated relative to untreated specimens as determined by analysis of variance (*, $P < 0.007$).

Table 2 *Apoptotic index, as determined by TUNEL staining in BPH-1 + rat urogenital mesenchyme grafts*

This table quantitates apoptotic activity in an area of benign histology in a previously untreated host and in an area of tumor in a previously T + E2-treated hosts, before and after castration of the host.

	% epithelial cells exhibiting TUNEL staining (cell count)	
Conditions	Benign tissue	Tumor
Control (intact host)	0% (0/5000)	2.39% (88/3668)
3 days after castration	3.23% (170/5275)	3.46% (130/3754)
7 days after castration	1.85% (98/5462)	3.33% (34/1021)

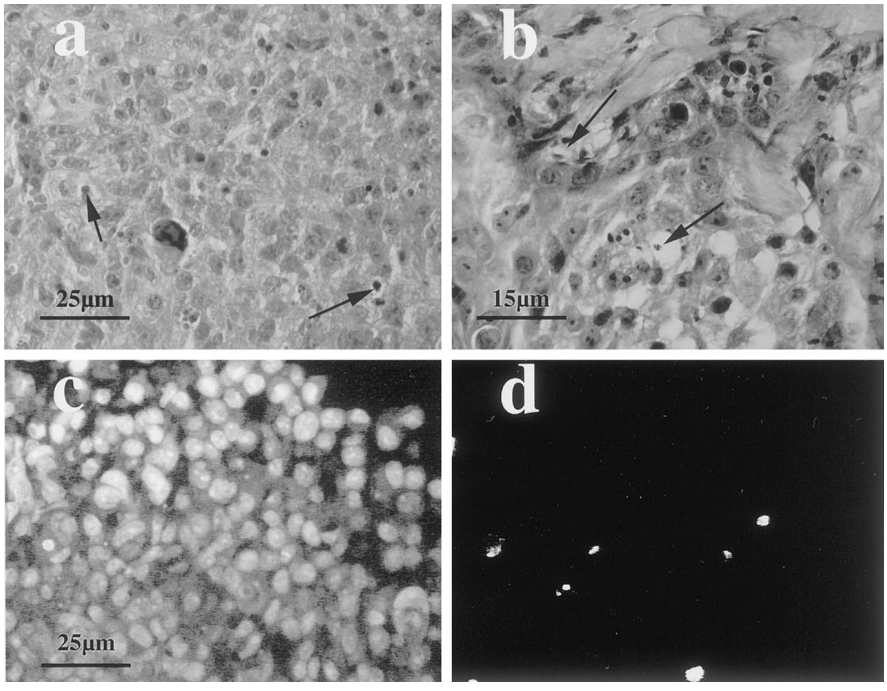

Fig. 8. Apoptotic activity in areas of adenocarcinoma after castration. H&E staining at 3 (*a*) and 7 (*b*) days after castration. Note that the graft is undergoing apoptosis and involution; apoptotic bodies are evident (*arrows*). Red and green channel fluorescence of a single field showing propidium iodide (*c*) and TUNEL staining (*d*) of an area of adenocarcinoma in a tissue recombinant 3 days after castration of the host.

mechanism (12, 13). The epithelium, in turn, acts upon the surrounding mesenchymal cells to induce the differentiation and patterning of smooth muscle (48).

In the developing prostate the epithelial cords are apparently homogeneous, expressing cytokeratins characteristic of both luminal and basal epithelial cells (52). These solid cords canalize giving rise to epithelial ducts lined with cytokeratin 8- and 18-expressing luminal cells underlain by cytokeratin 5- and 14-expressing basal epithelial cells. The solid cords produced by the BPH-1 cells in tissue recombinants with rUGM parallel this phenomenon, coexpressing keratins 5, 8, 14, and 18. When these cords canalized, characteristic basal cell cytokeratins were generally not seen, whereas keratins 8 and 18 are expressed. Tumors induced by T + E2 treatment generally coexpressed both luminal and basal-type cytokeratins. In tissue culture the BPH-1 cell line expresses cytokeratins 8 and 18, which are characteristic of luminal epithelium and not the basal cell cytokeratins 5 and 14 (49). These findings demonstrate that this epithelial cell line retains plasticity in its cytokeratin profile.

The present study confirms the idea that epithelium dictates differentiation and patterning of the stromal cells. This is consistent with our previous observations on similar tissue recombinants involving human prostatic epithelium plus rat UGM (48). In both cases rat UGM combined with human epithelium formed thick sheets of smooth muscle surrounding the epithelium, as compared with the thin smooth muscle sheaths normally seen in the rat prostate. This finding is also consistent with the observation that fetal rat gut endoderm can dictate the patterning of smooth muscle in subcutaneous graft sites (53). This suggests that the stromal and epithelial compartments of a gland are involved in a true reciprocal dialogue, apparently occurring over considerable distances, rather than a one-way transfer of instructions.

Castration leads to a rapid involution of prostatic epithelium by apoptosis as a result of failure to occupy stromal ARs (31). The BPH-1 cell line does not express ARs either in cell culture (49) or in tissue recombinants (9). AR expression can be induced in rodent and human bladder and ureteral and urethral epithelium by rUGM (54–56). The failure of BPH-1 cells to express ARs, thus, may be a result of their immortalization or a direct response to SV40T. These cells do contain an apparently normal X chromosome, so this failure to express ARs is probably not attributable to a loss of genetic material. BPH-1 cells grown in tissue recombinants with rUGM responded to androgen withdrawal by undergoing epithelial apoptosis, supporting the earlier observation that this response is not attributable to a failure to occupy epithelial ARs but rather because of failure to occupy stromal ARs. There are a number of potential mediators of prostatic epithelial apoptosis. The fas ligand/fas axis, for example, has been postulated to mediate prostatic epithelial apoptosis after androgen withdrawal (57, 58). It is known that some growth factors, for example, members of the transforming growth factor β family, can induce apoptosis in many tissues (59, 60) including the prostatic epithelium (61). Guenette and Tenniswood (62) have postulated that IGF signaling is necessary for cell survival in the adult prostate. However, Thomas *et al.* (63) suggest that loss of IGF signaling (as driven by increased expression of IGF-binding protein-5) is not a signal for apoptosis but rather follows the apoptotic events. Shabsigh *et al.* (64) have also suggested that the primary effect of castration is a loss of blood flow to the prostate and that the observation of epithelial apoptosis is a secondary event.

The levels of apoptosis achieved by the BPH-1 cells in these recombinants are somewhat modest as compared with the normal benchmark of the rat VP (65, 66). It should be noted that there is a wide variation in apoptotic rates between the various lobes of the rodent prostate and between the same lobes in different rodents (21, 31, 67–69). The rat VP is a somewhat unfortunate benchmark. Of all of the prostatic lobes in all of the species examined this undergoes the most regression in response to loss of androgenic stimulation. Approximately 90% of rat VP epithelial cells are lost, and the rat VP has the highest transient level of epithelial apoptosis. In contrast, androgen blockade in humans results in much lower levels of epithelial apoptosis. Although in humans the epithelial acini are severely reduced in number over a prolonged period, there is little over-reduction in prostatic mass (70). Thus, a 3–4% rate of apoptosis is a reasonable peak response of human prostatic epithelium to androgen withdrawal and is actually slightly higher than our own unpublished observations of human prostatic tissue grafted to athymic mice, which are subsequently castrated. It is significant that androgen ablation gave rise to epithelial apoptosis not only in benign structures but also in estab-

lished tumors. Because ARs were not expressed in the epithelial component of these tumors, this observation emphasizes the idea that interactions between the epithelial and stromal compartments play a key role in mediating tumor cell death (31) as well as in promoting tumor growth.

An important finding described herein is that a human prostatic epithelial cell line can be induced to undergo malignant transformation by treatment with testosterone and estradiol. E2 in combination with testosterone induces prostatic cancer in niobium rats at an incidence of ~20% at 1 year of treatment when hormones are applied in the classically described doses (37–40). Although more recent modifications (7) have increased this level of carcinogenesis, this model is still very slow and expensive to use. Pollard *et al.* (71, 72) demonstrated that testosterone alone can also induce prostate cancer in rats, whereas the nonaromatizable androgen dihydrotestosterone cannot. The implication of these studies is that the combined effects of testosterone and estrogens are required for prostatic carcinogenesis in rats.

The present model uses a human prostatic epithelial target cell, which can be considered to be genetically initiated by virtue of its immortalization by SV40T antigen. However, this cell line is consistently observed to be nontumorigenic in nude mouse hosts. Also BPH-1 cells grown in the absence of a prostatic stroma do not undergo malignant transformation even in the presence of T + E2 (present study). The definition of "nontumorigenic" used here is particularly rigorous and requires a formal demonstration that the grafted cells survive at the graft site for the duration of the test. Without identification of test cells in the graft site it is impossible to determine whether cells are truly nontumorigenic or have simply failed to survive transplantation. In the present model malignant transformation required both the presence of T + E2 and a steroid-responsive stromal environment. These observations emphasize the essential role of stroma in mediating hormonal carcinogenesis.

In intact adult males, androgens inhibit prostatic epithelial proliferation, maintaining a growth quiescent gland. In addition, androgens maintain secretory function and prevent apoptosis of both epithelial cells and cells of the adult prostatic vasculature (64, 73). However, androgens have been strongly implicated in prostatic carcinogenesis, because dogs and humans castrated early in life do not suffer from this disease. In contrast, when dogs are castrated in adulthood, they have an increased rate of prostate cancer (74). No equivalent human data set has been analyzed. It has long been speculated, based on changes associated with aging in humans and on animal model data, that alterations in the ratio of estrogens:androgens may play a role in the promotion of prostatic hyperplasia (36, 75, 76). Systemic high doses of estrogens inhibit testicular T synthesis by inhibiting the hypothalamic/pituitary system (77). However, in hormonal carcinogenesis elicited by silastic implants of T + E2, androgen levels are maintained. Treatment with estrogen alone elicits two clear effects on the prostate: estrogens induce epithelial proliferation, and they induce squamous differentiation (78–80). For promotion of carcinogenesis, the proliferative effect of E2 may be crucial. Induction of squamous metaplasia normally occurs in response to pharmacological estrogen levels, under conditions of "chemical castration" elicited via the hypothalamic/pituitary system. Estrogenic induction of squamous metaplasia may be protective in regard to prostatic carcinogenesis. This may be because whereas prostatic epithelial proliferation is stimulated, the proliferating cells (some with genomic alterations) are for the most part destined for terminal differentiation and sloughing into the luminal space. However, squamous cell malignancies are common in estrogen-rich environments such as the human cervix implying that squamous differentiation is not implicitly protective (81). Additionally, most *de novo* human prostatic carcinomas have distinctly secretory phenotypes demonstrating both glandular architectural features and expression of secretory proteins such as PSA and PAP. We would therefore consider that this tendency toward squamous differentiation is a limitation of this model system for some applications. In humans, pure squamous or mixed (adenosquamous) carcinoma of the prostate is exceptionally rare, suggesting that the development of a malignant phenotype *in vivo* may be dependent on gene(s) that are activated in epithelium with luminal differentiation. Therefore, if squamous differentiation does provide a protective effect in prostate, it may be related less to cell sloughing and more to the prevention of expression of the secretory phenotype. However, human tumors with adenosquamous differentiation are known to occur after hormonal manipulation, particularly after estrogen therapy (82, 83). This suggests the possibility of an alternative pathway of malignant progression. The combination of T + E2, maintains some level of prostatic glandular differentiation and may therefore be critical for the promotion of prostatic neoplasia. Thus, in the T + E2 model the most important function of T may be in inhibiting epithelial apoptosis. The BPH-1 cells used in this study do not express detectable ARs. Thus, the present study also demonstrates that the effect of androgens as hormonal carcinogens in this model is probably a paracrine effect possibly protecting the epithelial cells from apoptosis, whereas they sustain genetic damage from the presence of pharmacological doses of estrogens. Another aspect of the T + E2 carcinogenesis model system is the promotion of the growth of existing lesions. The T + E2 carcinogenesis model described here uses a target cell (BPH-1), which has already suffered a genetic insult. Thus, this model differs from the induction of tumors in genetically normal animals and may well mimic, in an exaggerated form, the promotion of spontaneously occurring human prostate tumors.

A number of studies have demonstrated that extremely high doses of sex steroids, in particular estrogens, may in themselves be genotoxic and, thus, capable of chemical modification of DNA required to initiate carcinogenesis. Metabolism of natural and synthetic estrogens generates free radicals, which are capable of DNA damage (84). Both diethylstilbestrol and estradiol have been shown to induce sister chromatid exchange (85–87). T + E2 treatment has been shown to increase DNA strand breakage as a result of free-radical action (88). Such initiating events would presumably act in a random or quasi-random manner to genetically damage cells in tissues where these compounds are concentrated as a result of nuclear binding to ER. This probably explains why T + E2 treatment has historically been found to affect androgen and estrogen target tissues such as the mammary gland, seminal vesicle, and prostate (89, 90). In the model described here, the BPH-1 cell line, which is the target of hormonal carcinogens, has clearly already undergone a major genetic insult, thus making these cells inherently more susceptible to malignant transformation in response to T + E2.

The truly malignant nature of the changes induced by T + E2 is emphasized by the transplantability of the tumors. This confirms the histopathological interpretation. Human pathology is based on many decades of observation and prognostication; thus, clinical pathologists can confidently predict the nature of a particular nuclear or cellular phenotype on the basis of thousands or perhaps millions of similar data. It is important to note that the examination of tissues from animal models does not have the same huge database. The use of novel model systems involving combinations of cells and hormones that do not occur in a natural setting is an additional level of complication for which data sets are extremely limited. Thus, we believe it is important for the users of such models to rigorously demonstrate that a phenotype that could routinely be described as malignant in a human patient does in fact fulfill important criteria of malignancy, such as independent tumor growth and invasive growth pattern.

In conclusion, the data presented here demonstrate that an immortalized nontumorigenic human prostatic epithelial cell line can recapitulate many of the critical aspects of prostatic development. We recognize that the failure of these cells to express ARs and classic human prostatic secretory markers such as PSA may be a limitation of this model for some applications, and as a result, parallel experiments with similar human prostatic epithelial lines that retain these markers are presently underway. Despite the lack of ARs, the BPH-1 line is susceptible to hormonal carcinogens when recombined with rUGM but not when grafted in the absence of mesenchymal cells. This underscores the key role that stromal cells can play in tumor progression. The tumors formed are transplantable and share many histological characteristics with human prostate cancer. We believe that this will be a useful model to investigate aspects of the roles of sex steroid hormones in the promotion of human prostate cancer.

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