Estrogen-Initiated Transformation of Prostate Epithelium Derived from Normal Human Prostate Stem-Progenitor Cells

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The present study sought to determine whether estrogens with testosterone support are sufficient to transform the normal human prostate epithelium and promote progression to invasive adenocarcinoma using a novel chimeric prostate model. Adult prostate stem/early progenitor cells were isolated from normal human prostates through prostasphere formation in three-dimensional culture. The stem/early progenitor cell status and clonality of prostasphere cells was confirmed by immunocytochemistry and Hoechst staining. Normal prostate progenitor cells were found to express estrogen receptor $\alpha$, estrogen receptor $\beta$, and G protein-coupled receptor 30 mRNA and protein and were responsive to 1 nM estradiol-17$\beta$ with increased numbers and prostasphere size, implicating them as direct estrogen targets. Recombinants of human prostate progenitor cells with rat urogenital sinus mesenchyme formed chimeric prostate tissue in vivo under the renal capsule of nude mice. Cytodifferentiation of human prostate progenitor cells in chimeric tissues was confirmed by immunohistochemistry using epithelial cell markers (p63, cytokeratin 8/18, and androgen receptor), whereas human origin and functional differentiation were confirmed by expression of human nuclear antigen and prostate-specific antigen, respectively. Once mature tissues formed, the hosts were exposed to elevated testosterone and estradiol-17$\beta$ for 1–4 months, and prostate pathology was longitudinally monitored. Induction of prostate cancer in the human stem/progenitor cell-generated prostatic tissue was observed over time, progressing from normal histology to epithelial hyperplasia, prostate intraepithelial neoplasia, and prostate cancer with local renal invasion. These findings provide the first direct evidence that human prostate progenitor cells are estrogen targets and that estradiol in an androgen-supported milieu is a carcinogen for human prostate epithelium. (Endocrinology 152: 2150–2163, 2011)

Prostate cancer is the most common noncutaneous cancer and the second leading cause of cancer deaths in North American men. Increasing evidence indicates that in addition to androgens, estrogens play key roles in prostate carcinogenesis and progression; however, the mechanisms are not fully understood (1-5). Progress toward understanding the role of sex steroids in the etiology of human prostate cancer has been hindered by the lack of a suitable laboratory research model (6). The only nonhuman mammals known to develop prostate cancer naturally are primates and dogs (7); however, both models are expensive and limited by long tumor latencies. Experimental rodent models have been developed and used extensively to elucidate the mechanisms of prostate carcinogenesis and in-

Abbreviations: AR, Androgen receptor; CK, cytokeratin; 2D, two dimensional; 3D, three dimensional; E$_2$, estradiol-17$\beta$; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPR, G protein-coupled receptor; H&E, hematoxylin and eosin; HG, high grade; ICC, immunocytochemistry; IHC, immunohistochemistry; PRN, prostate intraepithelial neoplasia; PR, progesterone receptor; PrEC, prostate epithelial cell; PrEGM, Prostate Epithelial Cell Growth Medium; PRL, prolactin; PSA, prostate-specific antigen; SQM, squamous metaplasia; T, testosterone; UGM, urogenital sinus mesenchyme.
include transgenic mouse (8, 9) and rat prostate cancers (10), spontaneous and chemically inducible Lobund-Wistar rat prostate cancer (11, 12), and hormone-inducible Dunning (13) and Noble rat prostate cancers (14). These models, however, are limited by their nonhuman origin, which restricts their direct application to humans. Prostate cancer models of human origin consist of primary cell or tissue slice cultures (15), multiple cancer cell lines used in vitro and in vivo as grafts (16, 17), as well as cancer tissue xenografts (18). However, these cells and tissues are derived from established prostate cancers that hamper their use in the investigation of initiating events and early transformation. To investigate these early steps in prostate carcinogenesis from normal cells to transformed epithelium, a model using normal human prostate cells is required. Currently, there is an unanswered need for such a model with transformation and malignant progression driven by steroid hormones.

Adult stem cells are found in adult tissues and act as a repair system by maintaining the normal turnover of the regenerative organ (19–21). Based on the stem cell hypothesis of cancer development, malignant tumors may originate from transformation of resident normal tissue stem cells that both self-renew and differentiate into abnormal progeny that continuously seed tumor growth (22, 23). Recent evidence indicates a special role of steroid hormones in the control of normal mammary stem cell function (24, 25) that implicates stem cells as the potential target during hormonal carcinogenesis.

The prostate gland is a ductal structure composed of basal, luminal, and a small number of neuroendocrine epithelial cells surrounded by stromal fibroblasts and smooth muscle cells. Adult prostate stem cells have recently been identified in human and rodent prostate tissue (22, 23, 26–28). This rare cell type self-renews and has potential to differentiate into the three epithelial cell types, an essential characteristic of a stem cell (29). Another characteristic of adult prostate stem/progenitor cells is their unique ability to form spheroids when cultured in an anchorage-independent, three-dimensional (3D) matrigel system where differentiated cells fail to survive (30–32). The prostate regenerating abilities of adult prostate stem cells isolated from rodents, patient samples, and human cancer cell lines have been documented by several laboratories using recombination with rat urogenital sinus mesenchyme (UGM) followed by in vivo growth as renal grafts in immunocompromised mice (26, 27, 33, 34). Taylor et al. (35) similarly reported the formation of human prostate-like tissues from human embryonic stem cells when mixed with rat UGM using a renal graft approach. However, an in vivo model of prostate tissue regeneration using normal human prostate adult stem cells as the starting material has not been reported to date.

In the current study, we first developed a method to isolate stem/progenitor cells from normal human prostates and generate normal human prostate-like tissue in vivo when combined with inductive rat UGM using a renal graft approach in nude mice. Prostatic hormonal carcinogenesis induced by combined testosterone (T) and estradiol-17β (E2) treatment for several months has been well established in both mouse (36, 37) and rat models (38, 39). Using this approach, we exposed the grafted mice containing chimeric human-rat prostate structures to elevated T+E2 via sc pellets. Over a 1- to 4-month-exposure period, the human prostate-like structures developed progressive disease from atypical hyperplasia to prostate intraepithelial neoplasia (PIN) and high-grade (HG) prostate cancer with local invasion. We have thus developed the first model of hormone-induced human prostate cancer initiation and progression from normal human prostate cells into full malignancy.

Materials and Methods

Animals
Timed pregnant Sprague Dawley rats were purchased from Harlan (Indianapolis, IN) and male nude mice from Charles River (Wilmington, MA). Animals were fed standard chow ad libitum. All studies were approved by the Institutional Animal Care Committee.

Cell and prostasphere cultures
Primary human prostate epithelial cells (PrEC) were obtained from normal healthy donors between 20 and 30 yr of age (Lonz Walker, Walkersville, Inc., Walkersville, MD) and maintained at 37 C in fibronectin-coated flasks using Prostate Epithelial Cell Growth Medium (PrEGM) (Lonza). Cells were passaged once and frozen for subsequent 3D cultures. The epithelial cultures lacked contamination by other cell types as revealed by immunohistochemistry (IHC) for p63 and vimentin (data not shown). LNCaP, PC3, and DU145 cells were cultured as described (5).

Prostasphere culture conditions were modifications of published protocols (33, 40, 41). Briefly, 1 x 10^5 PrEC cells were resuspended in 1:1 matrigel (BD Biosciences, Bedford, MA)/PrEGM (Lonza), plated in 12-well plates with 1 ml PrEGM, and cultured at 37 C in 5% CO2. Medium was replenished every 3 d, and prostasphere formation and growth were monitored by real-time imaging using a Zeiss Axiovert 200 inverted microscope with automated X-Y-Z stage and an Axiocam (Carl Zeiss MicroImaging, Inc., Thornwood, NY). For tissue recombination, matrigel was digested in dispase (1 mg/ml; Invitrogen, Carlsbad, CA) for 30 min. Spheres were resuspended in type I collagenase (190 U/ml) for 45 min, washed, resuspended in 0.05% trypsin/EDTA for 10 min, passed 5–10 times through a 27-gauge needle, filtered (40 μm), and cells counted.

Generation of chimeric prostate-like structures through cell-tissue recombination and renal grafts
The urogenital sinus from embryonic d-17 rat fetuses was removed, digested, and UGM separated from urogenital epithe-
overnight on 1% agar with PrEGM in 5% CO₂ at 37 C. Re- 
mixed with fluorescent microscopy.

fixed, mounted in propidium iodide, and H342 signal was mon-

3D culture for 4–7 d. Prostaspheres were 

 Autoradiography of the cells, tissue type, and differentiation of the grafts 

indicated in chimeric tissues through T+E₂ treatment of the host. Mice 

were implanted with sc pellets containing 2.5 mg of T and 2.5 mg of 

sustained steroid delivery for 1–4 months. This 10:1 ratio 

promoted prostate cancer in recombinants with BPH-1 cells (37). 

One month after initiation of T+E₂, several specimens were biop-

sied and grafts returned for an additional 1–3 months of T+E₂. At 

killing, blood was collected, and grafts were processed for histologic 
examination as described (43). Serial sections for each tissue were 

examined by two board-certified pathologists and classified for 

prostate pathology according to criteria established by the Armed 

Forces Institute of Pathology. Control treatments included 13 mice 

given T implants alone for 3 months.

Results

Induction of the cells, tissue type, and differentiation status of the grafts 

as previously described (43). ICC staining was performed on 

prostaspheres grown overnight on chamber slides to permit limited 

cell outgrowth and improve reagent penetration. Spheres 

were fixed in acetone/methanol (1:1) and immunostained as de-

scribed (43). Antibodies used are provided in Supplemental Ta-

ble 1, published on The Endocrine Society’s Journals Online web 

site at http://endo.endojournals.org. Confocal imaging was con-

ducted on a Zeiss LSM 510 META Confocal Microscope.

Hoechst exclusion assay and lineage labeling

A Hoechst exclusion assay was performed to confirm stem/ 

progenitor cell status. Day-7 prostaspheres or two-dimensional 

(2D) PrEC cells were stained with 0.5 μg/ml Hoechst 33342 

(H342; Sigma-Aldrich, Inc., St. Louis, MO) in Hanks’ balanced 
salt solution buffer for 30 min at 37 C, washed, fixed, and imaged 

as described. As parallel controls, cells in separate wells were 
treated with verapamil (50 μM; Sigma-Aldrich, Inc.), an ABCG2 
transporter inhibitor, for 10 min before H342 staining.

To assess prostasphere clonality, single PrEC cell suspensions 

were stained with high concentration H342 (5 μg/ml) in PrEGM 

for 45 min, washed, and mixed with equal amounts of unstained 

PrEC cells followed by 3D culture for 4–7 d. Prostaspheres were 

fixed, mounted in propidium iodide, and H342 signal was mon-
tored using fluorescent microscopy.

Radioimmunoassay

Serum T and E₂ levels were measured using murine RIA kits 

(TKT2 for T, TKE21 for E₂; Siemens Medical Solutions Diagnos-
tics, Los Angeles, CA) at the Ligand Assay and Analysis Core Lab-

ery, Center for Research in Reproduction, University of Vir-

ginia (Charlottesville, VA). For T, sensitivity was 0.1 ng/ml, and the 

intra- and interassay coefficients of variance were 3.9 and 7.8%, 

respectively. For E₂, sensitivity was 10 pg/ml, and the intra- 

and interassay coefficients of variance were 4.4 and 7.8%, respecti-

Quantitative real-time RT-PCR

Total RNA was isolated using TRIZol Reagent (Invitrogen).

cDNA were synthesized using reverse transcriptase and random 

hexamers (Clontech Laboratories, Mountain View, CA). PCR in 

SYBR GreenER PCR Master-Mix (Invitrogen) were carried out 

using 7900HT Fast Real-time PCR System (Applied Biosystems, 

Carlsbad, CA). Primer sequences are provided in Supplemental 

Table 2. Individual mRNA levels were normalized to glyceral-

dehyde-3-phosphate dehydrogenase (GAPDH).

Statistic analysis

Data were analyzed using InStat ver. 3 (GraphPad Software, 

Inc., San Diego, CA) using Student’s t test or ANOVA as appro-

priate, followed by post hoc tests. Values are expressed as mean ± 

SEM, and P < 0.05 was considered significant.
mixed Hoechst-stained and -unstained cells were observed, indicating that prostaspheres are derived from single progenitor cells with self-renewal ability.

Previous work using flow cytometry showed that only epithelial cells with stem cell markers have the capacity to survive and form prostaspheres in 3D culture (32). To confirm that prostaspheres in the present study consist of stem/progenitor cell populations, d-7 prostaspheres were immunolabeled with stem cell markers, including Nanog, Trop2, CD49f, SSEA4, and ABCG2 (27, 44, 45). As shown in Fig. 2A, prostasphere cells were all positive for several combinations of these markers, indicating a relatively homogenous population of stem and early-stage progenitor cells. Further confirmation was obtained by Hoechst exclusion assay that examines the unique ability of stem/progenitor cells to exclude low concentration Hoechst dye due to functional ABCG2 transporter proteins expressed in these cells. Prostaspheres incubated in 0.5 μg/ml Hoechst were negative, a property that was lost upon preincubation with verapamil, an ABCG2 inhibitor (Fig. 2B). In contrast, parental PrEC cells retained Hoechst in the absence and presence of verapamil. Together, these findings confirm the stem/progenitor cell nature of the human d-7 prostasphere cells.

**Prostasphere steroid receptor expression and response to E2**

To determine whether isolated adult prostate progenitor cells have potential to respond to steroids, we exami...
ined the steroid receptor status of d-7 prostasphere cells by quantitative real-time RT-PCR and ICC. Steroid receptor mRNA levels are shown in Fig. 3A with expression normalized to LNCaP levels. Although prostate progenitor cells expressed androgen receptor (AR) mRNA at limit of detection levels and were AR negative at the protein level, they were positive for all known estrogen receptors (ER), including ERα, ERβ1, and G protein-coupled receptor (GPR)30. Importantly, the ER mRNA expression levels were markedly higher in normal progenitor cells relative to LNCaP cells with 6-fold higher ERβ1, 15-fold higher GPR30, and 125-fold higher ERα expression. In comparison, undifferentiated progenitor cells more closely resembled steroid receptor profiles in the androgen-independent cell lines PC3 and DU145 with elevated ER expression, minimal progesterone receptor (PR), and no AR mRNA. ER and GPR30 protein expression was confirmed by immunofluorescent staining. Although ERβ localized to the nucleus, ERα was found in the nucleus and cytoplasm whereas GPR30 localized to the membrane and cytoplasm in the majority of the progenitor cells (Fig. 3B). These findings suggest that normal human prostate progenitor cells have potential to respond to estrogen ligands.

To directly test this possibility, prostaspheres were exposed to 1 nM E2 for 7 days as they formed and expanded in culture. E2 increased both the number of prostaspheres that formed by d-7 as well as their size, indicated by a greater proportion of prostaspheres more than 80 μm in diameter (Fig. 3C). Thus the present results demonstrate that normal human prostate progenitor cells are responsive to estrogens with increased rates of self-renewal.

**In vitro differentiation of normal prostaspheres**

With continued prostasphere culture through d-10, the cells began to differentiate, forming double-layered prostaspheres 100–150 μm in diameter (Fig. 4A). To further characterize these cells, d-10 prostaspheres were immunolabeled for basal and luminal epithelial cell markers. Peripheral cells (outer layer) were positive for p63, a basal cell marker (Fig. 4B), whereas the innermost cells were p63-negative. In contrast, cells toward the spheroid center were positive for cytokeratin (CK)8 and NKX3.1 (Fig. 4C) indicating that interior prostasphere cells were differentiating toward a luminal cell phenotype. To further analyze the differentiation capacity for prostasphere cells in vitro, cultures were continued through d-30. During this period, prostaspheres frequently expanded laterally, forming branch-like structures (Fig. 4, D and E) with cross-sections exhibiting lumen-like formation in the prostasphere centers (Fig. 4F).

Steroid receptor profiles of d-30 prostaspheres were measured and compared with expression levels in d-7 prostaspheres and to parental PrEC cells grown in 2D culture. In comparison with d-7 progenitor cells, d-30 prostaspheres expressed increased levels of AR and higher levels of all ER and PR (Fig. 4G). Furthermore, d-30
prostaspheres expressed prostate-specific antigen (PSA) mRNA documenting functional differentiation of luminal cells was initiated in vitro (Fig. 4G). Importantly, the steroid receptor profiles and PSA expression levels of the d-30 prostaspheres were nearly identical to their parental PrEC. Together, these results demonstrate that the normal adult prostate stem/progenitor cells isolated by prostasphere assay can differentiate into functional basal and luminal epithelial cells in vitro.

**Regeneration of normal prostate-like tissue from human prostate stem/progenitor cells in vivo**

We investigated the differentiation and regenerative abilities of normal adult prostate progenitor cells by using an in vivo cell/tissue recombination and renal graft model (Supplemental Fig. 2). Day-7 prostasphere cells were used as the source of human prostate progenitor cells because characterizations confirmed their primary stem/progenitor status at this time point. By varying the number of progenitor cells used in the recombinants from 100 to 10,000 cells with a constant amount of UGM from a single embryo, renal grafts of increasing size were obtained after 4 wk of in vivo growth (Fig. 5A). Consequently, approximately 3000 progenitor cells were used for each graft in subsequent experiments. Histological examination of 4-wk graft sections by hematoxylin and eosin (H&E) staining showed formation of mature prostate-like structures with secretory material in the lumens (Fig. 5B). Immunofluorescent staining showed p63 expression in basal cells (Fig. 5C) and CK8/18 (Fig. 5C) and AR (Fig. 5D) in adluminal cells demonstrating cytodifferentiation of prostate progenitor cells into basal and luminal epithelial cells in vivo. The human origin and functional differentiation of the epithelium in chimeric grafts were confirmed by expression of human nuclear antigen (Fig. 5E) and secretion of PSA (Fig. 5F), a human-specific secretory product. All prostate grafts were confirmed as histologically normal by a board-certified pathologist.

**Induction of epithelial hyperplasia, metaplasia, and carcinogenesis in normal chimeric prostate grafts with T+E2 exposure**

To induce progressive hormonal carcinogenesis in normal human prostate cells, nude mice bearing chimeric...
prostate grafts were treated with elevated T+E2 over a 1- to 4-month period after the recombinant tissue had fully formed under the renal capsule. In control grafts without steroid pellets for 1–3 months or with T alone for 3 months, the chimeric tissues were histologically normal with no evidence of any prostatic lesion. Subcutaneous pellets of 25 mg of T and 2.5 mg of E2 produced serum steroid levels of 25.4 ± 2.1 ng/ml T and 675 ± 43 pg/ml E2, which is 5- to 9-fold higher than levels measured in untreated male mice as well as human males. A total of 45 grafts were collected at the time of killing after 1–4 months of hormone treatment and histologically examined by a pathologist for evidence of prostate lesions (Table 1). For renal grafts treated with hormones beyond 1 month (n = 35), an aseptic open biopsy was performed at 1, 2, or 3 months after hormone initiation, and the graft was returned to the abdominal cavity for continued growth, thus permitting direct assessment of lesion progression over time (Fig. 6, A and B). One month after initiation of T+E2 treatment, 50% of specimens were graded as normal, whereas 50% contained epithelial hyperplasia and/or squamous metaplasia (SQM) (Fig. 6, C and D). By 2 months of T+E2 treatment and beyond, none of the chimeric prostate grafts were classified as normal, whereas 74% contained SQM, a hallmark lesion of estrogen exposure (Table 1) (46–48). Between 2 and 4 months of treatment with steroids, hyperplasia of the human epithelium, at times atypical, increased from 22% at 2 months to 50% at 3 months and 80% at 4 months with a total incidence of 43% in all examined grafts. HG PIN lesions were first observed in the human epithelium after 2 months of hormone exposure as evidenced by prominent nucleioli (visible at low magnification), nuclear enlargement, hyperchromasia, and overlapping epithelial cells (Fig. 6E). PIN incidence progressively increased over time from 29% at 2 months to 40% at 4 months with an overall HG PIN incidence over 2–4 months of 31%. Several foci of PIN lesions were seen in most grafts when they appeared and were not considered isolated. Importantly, several grafts that contained HG PIN were classified as normal or hyperplastic epithelium in biopsies from prior months (Fig. 6).

Classification of prostate cancer was assigned to four chimeric grafts collected at 2, 3, and 4 months of T+E2 treatment for an incidence of 11% over the 2- to 4-month period. Two cases at 2 and 3 months were characterized as local microinvasion of neoplastic cells based on nuclear morphology, prominent nucleioli at low magnification, loss of CK14 positive basal cells in neoplastic acini, and presence of CK8/18 positive luminal cells within the stromal region (Fig. 7, A and B). Malignant progression to poorly differentiated adenocarcinoma with invasion to the adjacent kidney paranchyma was observed in two separate chimeric grafts between 2 and 4 months of hormone exposure (Fig. 7, C–H). Earlier biopsies of these grafts had revealed only epithelial hyperplasia or PIN, thus providing evidence of malignant progression over time. Signs of malignancy included irregular small to medium size glandular

FIG. 4. In vitro differentiation of normal prostaspheres with extended culture. A, By d-10 of culture, the cells located in the prostasphere center began to differentiate, forming a double-layered structure of 100–150 μm in diameter. B, Immunofluorescent labeling of d-10 prostaspheres with p63 (green) and 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (blue) shows that the basal cells (aqua-green) are located in the outer layer, whereas the more centrally located cells are p63 negative. The green signal in the compacted prostasphere center was nonspecific and appeared when IgG was substituted for primary antibody (inset). C, Immunofluorescent labeling of d-10 prostaspheres with CK8 (green), NKX3.1 (red-purple), and DAPI nuclear stain (blue). The positively stained cells are located toward the prostasphere center and represent the differentiated cells. D–F, Prostaspheres cultured through d-30 began to grow laterally, forming ductal-like structures (D and E) with lumen formation (F). Scale bar, 50 μm (A–F). G, Steroid receptor and PSA expression levels in d-7 and d-30 prostaspheres (PS) and in parental PrEC grown in 2D culture as measured by real-time RT-PCR. Data were normalized to GAPDH in each sample and expressed relative to d-7 prostasphere levels set as 1. Day-30 prostasphere cells and the primary epithelial cell cultures from human prostate expressed AR, higher levels of all ER and PR, as well as PSA when compared with the undifferentiated d-7 prostasphere cells.
structures, abortive glandular lumens, back-to-back lumens, infiltrative glands, and loss of the basement membrane and a basal cell layer (Fig. 7, C–E). Immunostaining of the specimens for epithelial-specific CK8/18 and CK14 documented loss of basal cells in the human cancer and kidney invasion of the malignant epithelial cells (Fig. 7F). Immunostaining for human nuclear antigen (Fig. 7G) and PSA (Fig. 7H) in the cancerous regions of the grafts confirmed the human origin of the cancerous cells.

**Discussion**

In the present study, we report three major findings that provide an increased understanding of human prostate progenitor cell differentiation and hormone-driven cancer initiation and progression. We have shown for the first time 1) the efficacy of culturing stem/progenitor cells from normal human prostates using a prostasphere assay and the ability to derive normal humanized prostate-like tissues in vivo using a chimeric cell-tissue recombinant renal graft approach; 2) that normal human prostate stem/early progenitor cells express high levels of ER, including ERα, ERβ, and GPR30, and exhibit a proliferative response to 1 nM E2; and 3) that elevated E2 levels in a T-supported milieu can initiate prostate carcinogenesis and promote early progression to adenocarcinoma with an invasive phenotype.

E2 has been classified as a carcinogen by the International Agency for Research on Cancer based on findings in women (49). Although there is accumulating evidence to suggest a central role for estrogens in prostate cancer, direct evidence that estrogens initiate

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<th>TABLE 1. Incidence of prostate pathology in human prostate epithelium of chimeric grafts after 1–4 months of T+E2 treatment</th>
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Cumulative incidence 2–4

| 35 | 0% (0/35) | 74% (26/35) | 43% (15/35) | 31% (11/35) | 11% (4/35) |

Results show diagnosis of entire graft at final autopsy.

* Some specimens contained multiple diagnoses.
prostate cancer in humans has been elusive. A rising $E_2:T$ ratio in aging men (50), association of polymorphisms in estrogen metabolizing genes and urine hydroxy-estrone ratios with higher prostate cancer risk (51, 52), progressive increases in aromatase expression in primary prostate

![Image of prostate biopsy](image1)

FIG. 6. Graft biopsy after 1 and 2 months of $T+E_2$ exposure revealed SQM and epithelial hyperplasia progressing to HG PIN over time within the same chimeric prostate graft. A, Open biopsy through ligature permitted removal of a portion of the renal graft, which was then returned to the abdominal cavity for continued growth and progression. B, The same graft was removed after 2 months of hormone treatment revealing the continued growth of the chimeric tissue. C, At 1 month of $T+E_2$ exposure, extensive epithelial hyperplasia with infolding of ducts was observed throughout the chimeric graft. D, SQM was frequently observed after 1 month and beyond of $T+E_2$ treatment. E, Histologic examination of the graft shown in B revealed areas of HG PIN with piling and overlapping epithelial cells, nuclear enlargement, hyperchromasia, and prominence of nucleoli. Scale bar, $50 \mu m$.

![Image of prostate biopsy](image2)

FIG. 7. Prostate cancer in chimeric prostate renal grafts induced by $T+E_2$ treatment for 2–4 months. A, H&E section of graft exposed to $T+E_2$ for 2 months reveals neoplastic epithelium with enlarged nuclei and prominent nucleoli and their local invasion into the underlying stromal region (cells highlighted with arrowheads). B, CK8/18 immunostained acini shown in A confirms the local invasion of neoplastic cells into the stroma. C and D, Within 2 months of elevated $T+E_2$ exposure, full malignancy was induced in another chimeric renal graft as evidenced by irregular small to medium size glandular structures, abortive glandular lumen, back-to-back lumens, infiltrative glands, and loss of the basement membrane and basal cell layer. Multiple lesions of this type were observed within the graft. E and F, Adjacent sections of a chimeric prostate graft after 4 months of $T+E_2$ treatment with H&E stain (E) and immunofluorescent labeling with CK8/18 (red) and CK14 (green) (F). Staining showed heterogeneous glandular structures with mixture of normal glands, PIN lesions, and carcinoma as well as invasion into kidney. G, Immunostaining with antibody specific to human nuclear antigen (pink nuclei, arrowheads) identifies the human origin of infiltrating epithelial cells (CK8/18+, red) within the stroma. H, Immunolabeling for PSA (red) confirms the human identity of the cancerous ducts and infiltrating cells (arrows) in the grafted tissue. Scale bar, $50 \mu m$.

prostate cancer in humans has been elusive. A rising $E_2:T$ ratio in aging men (50), association of polymorphisms in estrogen metabolizing genes and urine hydroxy-estrone ratios with higher prostate cancer risk (51, 52), progressive increases in aromatase expression in primary prostate...
cancers to metastatic prostate cancers (53), and marked alterations in ER expression with cancer progression (3, 5) support the hypothesis that estrogens are involved in the etiology and progression of this disease. Multiple studies over the past several decades using animal models have provided strong evidence of a carcinogenic role for estrogens in the prostate (13, 14, 54, 55), but whether this is directly applicable to the human prostate has not been clarified. To address this issue, we developed an experimental in vivo model using normal human prostate progenitor cells grown as a chimeric prostatic tissue. This novel system permitted the direct assessment of whether E2 was capable of initiating and promoting carcinogenesis in the normal human prostate epithelium. Previous experiments using a tissue recombinant system with BPH-1 cells that are immortalized with large T-antigen and contain chromosomal abnormalities (56) had shown that elevated T+E2 was capable of promoting these preinitiated, yet benign cells into invasive cancers, thus indicating that estrogens can function as a cancer promoter in human prostate epithelium (36, 37). By starting with normal primary PrEC from young, disease-free organ donors, the present study demonstrates for the first time that E2 in a T-supported milieu is sufficient to initiate human PrEC transformation and promote the development of prostatic adenocarcinoma to a locally invasive phenotype. Support that estrogens are the culprit steroid in prostatic hormonal carcinogenesis comes from control grafts without T+E2 or with T implants alone that showed no evidence of prostate pathology after 3 months of growth. There are a number of possible mechanisms through which estrogens may initiate prostate carcinogenesis and promote progression, and dissection of specific pathways will be directly addressed in future studies using this model. The most obvious mechanism is through direct ER-mediated pathways in prostate epithelium that expresses ERα, ERβ, and GPR30 (3, 57, 58). Augmented signaling through these receptors has been shown to disrupt homeostasis and perturb cell proliferative and apoptotic pathways in a number of estrogen-target tissues and cancers, such as the breast and uterus. Alternatively, because rat UGM cells and some mature rat prostate stromal cells express ERα, but not ERβ (59, 60), it is possible that ERα-mediated signaling in prostatic stroma may contribute to carcinogenesis in the epithelium through paracrine-mediated factors. For example, we have previously shown that early-life estrogens up-regulate stromal Fgf10 expression in rat dorsolateral prostate lobes (61), and paracrine stimulation of murine basal/stem cells by stromal Fgf10 was recently shown to drive adenocarcinoma in a cell recombination model (62). There is also evidence for genotoxic effects of estrogens in several cancers, including the prostate, through formation of catechol estrogens and induction of oxidative stress pathways (63, 64), and it is possible that the elevated estrogen levels used in this study are capable of action through these mechanisms. It deserves mention that increased circulating E2 stimulates the release of pituitary prolactin (PRL) and estrogen effects in prostate dysgenesis, including inflammatory responses may be mediated, in part, through elevation in circulating PRL (65–67). Prostatic, i.e. autocrine, PRL production acting through signal transducer and activator of transcription 5 has been shown to promote human prostate cancer (68) and stimulate basal/stem cell proliferation and prostate carcinogenesis in a novel transgenic murine model (69). Although these data support a role for elevated PRL in prostate carcinogenesis, it is important to note that local PRL production is not under estrogenic regulation.

A critical aspect of the present dataset is the evidence that human prostate stem and early progenitor cells are specific targets for estrogen action in the prostate. The d-7 prostasphere progenitor cells expressed robust levels of estrogen signaling molecules, including ERα, ERβ, and GPR30, that activate common and distinct signaling cascades when liganded by E2. Localization of ERβ in the nucleus, ERα in the nucleus and cytoplasm, and GPR30 in the membrane and cytoplasm provides the progenitor cells with multiple opportunities for responding to estrogens, including rapid membrane-initiated estrogen signaling as well as the classical nuclear transcription factor pathways. That these signaling pathways are functional is supported by their response to 1 nM E2 with increased recruitment of resident stem cells to form prostaspheres as well as increased proliferation producing larger prostaspheres. These findings raise the intriguing possibility that stem and early progenitor cell populations in prostate tissues might be susceptible targets of elevated E2 during the induction of hormonal carcinogenesis. This is particularly appealing in light of the recent evidence that transformation of prostate stem cells is sufficient for prostate cancer initiation in rodent and human models (62, 70). If cancers are seeded by transformed stem cells as the stem cell theory for cancer development posits, an increased number of stem and progenitor cells in response to chronic and/or elevated estrogens would increase prostate cancer risk by the sheer presence of more cells available for transformation. Furthermore, it is possible that elevated estrogens, acting through ER signaling pathways in the adult prostate progenitor cells, may directly reprogram or transform these cells, thus rendering them with tumor initiating capacity. Evidence in support of this comes from our studies in rodent models, where developmental estrogen expo-
tures reprogram the prostate, leading to increased basal cell numbers and differentiation defects of the adult epithelium that predispose to dysplasia (71). These ER-dependent responses are mediated through immediate changes in gene expression as well as life-long epigenetic modifications that imprint the developing gland (39, 72, 73).

It is accepted that adult stem cells are involved in normal tissue replenishment, whereas cancer stem cells can support growth and progression of malignancies. Although conventional therapies for prostate cancer eradicate the majority of tumor cells, most patients with advanced prostate cancer progress to androgen-independent, metastatic disease that remains incurable by current treatment strategies. Recent evidence suggests that prostate cancer stem cells, which are AR negative and resistant to androgen/AR-based therapies, may be the underlying cause of disease relapse (74). The therapeutic challenges for prostate cancer may thus involve screening and selecting chemotherapeutic agents that target cancer stem cells. The present findings that human prostate progenitor cells express high levels of ER but no AR and respond to estrogens with increased proliferation sheds a new light on the therapeutic potential for selective ER modulators in the treatment of prostate cancer (75). Although the present studies focused on ER expression in normal prostate progenitor cells, there is a high probability for continued ER expression or perhaps amplified ER levels in diseased prostate stem cells making them likely targets for specific ER antagonists.

The present study documents the efficacy of obtaining prostaspheres containing prostate stem and progenitor cells from normal human prostate specimens that are able to differentiate in vitro as well as reconstitute prostate-like tissue in vivo using the renal graft approach. This approach has previously been used for prostate cancer specimens, benign regions of diseased prostate samples with known field effects (76) as well as from established prostate cancer cell lines (30, 32, 77, 78). That this can be done with relative ease from normal donor prostate specimens provides an opportunity for examining the range of processes during normal human prostate development as well as disease induction and treatment. Although the isolation of prostate stem cells is far more specific using flow cytometry (27, 28, 31), this process results in a relative low cell yield that limits its applications. Witte and co-workers (32), using both approaches simultaneously, have shown that prostate stem cells that express Trop2, CD44, and CD49f markers preferentially exhibited sphere-forming capacity in a 3D culture system. The clear advantage of the latter approach is the expansion of stem cell numbers through self-renewal in culture. Immunolabeling of d-7 prostasphere cells from normal specimens in the present study with multiple stem cell markers revealed that they were committed stem/early progenitor cells that had not begun differentiation into cell lineages. As documented previously in prostate cancer patient samples and cell lines (30, 33, 77, 79), the normal prostaspheres here were capable of cytodifferentiation and functional differentiation in vitro to basal and luminal cell bilayers with lateral outgrowths and lumens. Together, these results indicate that the normal adult prostaspheres cells have multipotent differentiation ability. Through the use of the prostate cell-tissue recombinant model, the prostasphere cells from normal human prostate epithelium consistently and reproducibly reconstituted a normal and functional chimeric prostate-like tissue as evidenced by histologic criteria and secretion of PSA. In more than 50 grafts using prostaspheres from multiple organ donors, no instances of prostate pathology of any type have been detected. This model not only affords us the importance of using normal cells of human origin but also allows the in vitro manipulation of stem cells before grafting with resultant impacts observed in vivo using the model presented herein. Although concern has been expressed with regards to the epithelial cell of origin in cross-species recombinants in the murine kidney (80), immunostaining for human nuclear antigen and PSA confirmed the human origin of the prostate epithelium in both the normal as well as malignant tissues in the present studies.

In summary, we document the efficacy of deriving large numbers of prostate stem/early progenitor cells from normal adult prostate glands and using these to generate normal humanized prostate-like tissue in vivo. With these in vitro and in vivo models, we demonstrate that estrogens in an androgen-supported milieu are capable of prostate cancer initiation and progression to adenocarcinoma. Robust expression of ERα, ERβ, and GPR30 in the human prostate progenitor cells and their proliferative response to E2 indicates that prostate stem cells and early progenitor cells may be direct targets for estrogen-induced carcinogenesis. Detailing conversion and progression of normal prostate stem/progenitor cells to cancerous cells in response to steroids will enable a thorough understanding of prostate carcinogenesis, which may lead to a more targeted means of developing future therapeutics.

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