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Sex Hormone-induced Carcinogenesis in Rb-deficient Prostate Tissue[1]

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ABSTRACT

The retinoblastoma (Rb) gene product is a prototypic tumor suppressor. Mice lacking the *Rb* gene are not viable and die *in utero* at ~13 days of gestation. In this study, we have rescued $Rb^{-/-}$ prostates by grafting pelvic organ rudiments from $Rb^{-/-}$ mouse embryos under the renal capsule of adult male nude mouse hosts. Grafts of embryonic pelvic organs developed into functional prostatic tissue. Some of the prostatic tissue generated was further used to construct chimeric prostatic tissue recombinants by combining wild-type rat urogenital mesenchyme (rUGM) with $Rb^{-/-}$ and $Rb^{+/+}$ prostatic epithelium (PRE). The tissue recombinants were grown as subcapsular renal grafts and treated from the time of grafting with Silastic capsules containing 25 mg of testosterone plus 2.5 mg of estradiol. During 5–8 weeks of hormone treatment, rUGM+$Rb^{+/+}$PRE tissue recombinants developed prostatic hyperplasia, whereas PRE in rUGM+$Rb^{-/-}$PRE tissue recombinants developed hyperplasia, atypical hyperplasia, and carcinoma. During carcinogenesis in rUGM+$Rb^{-/-}$PRE tissue recombinants, prostatic epithelial cells of the basal lineage disappeared, whereas the luminal cells underwent carcinogenesis. Epithelial E-cadherin almost totally disappeared. In all cases, epithelial PCNA labeling was elevated in tissue recombinants containing $Rb^{-/-}$ *versus* $Rb^{+/+}$ epithelium. These epithelial changes were associated with almost total loss of smooth muscle cells in the stroma. In contrast, in untreated hosts rUGM+$Rb^{+/+}$PRE tissue recombinants developed normally, and rUGM+$Rb^{-/-}$PRE tissue recombinants developed mild epithelial hyperplasia. The results of this study demonstrate that $Rb^{-/-}$ prostatic tissue can be rescued from embryonic lethal mice and used to test its susceptibility to hormonal carcinogenesis. Deletion of the *Rb* gene predisposes prostatic epithelium to hyperplasia and increases proliferative activity. Susceptibility to hormonal carcinogenesis in response to exogenous testosterone + estradiol is manifested in the progression from atypical hyperplasia to carcinoma. Thus, these findings demonstrate that the absence of the *Rb* tumor suppressor gene may predispose prostatic epithelial cells to carcinogenesis. Rescue of organs from $Rb^{-/-}$ embryos not only provides an opportunity to analyze the *Rb* gene pathway in the development and progression of prostate cancer but also provides an opportunity for specifically evaluating the role of the Rb pathway in development and carcinogenesis in other organs, such as the mammary gland and colon. Because rUGM greatly stimulates prostatic epithelial proliferation, the tissue recombinant model is a particularly useful tool for assessing the functional role of other genes in prostatic carcinogenesis through use of the appropriate transgenic or gene knockout mice.

INTRODUCTION

Spontaneous prostatic carcinogenesis in humans and in rodents is an extremely long process. Thus, one of the goals of all models is to truncate the long latent period so as to attain efficiency and cost effectiveness. To achieve prostatic carcinogenesis in a timely fashion, highly abnormal conditions are often used. These include the use of hormones at pharmacological doses, treatment with chemical carcinogens, and the targeting of potent viral oncogenes to the PRE[3] and/or stroma. Thus, all models can be criticized for using abnormal conditions to elicit carcinogenesis in a timely fashion. The model presented here is no exception because it uses a combination of hormones at pharmacological doses and a defined genetic defect (Rb−/−) as a starting point. The issue for models of human diseases is not whether they precisely mimic the natural history of the disease in all respects, but whether the model advances our understanding of the disease process.

The major *in vivo* models of prostatic carcinogenesis are: (*a*) those models in which prostate cancer can be induced, or its incidence increased, by chemical and/or hormonal carcinogens. Examples include the Noble and Lobund-Wistar rat prostatic carcinoma models. These models replicate many of the early phases of carcinogenesis. Their main drawbacks are long latency and low incidence of cancer (1–5); (*b*) transgenic mouse models of prostate cancer. These include the TRAMP (6) and the LPB-Tag or "LADY" series (7), which use the probasin promoter to target expression of SV40-T antigen to the luminal prostatic epithelial cells. The basal prostatic epithelial cells apparently do not express SV40-T antigen in these models. These transgenic mice develop prostatic cancer with very high efficiency. Their tumors are androgen responsive and they metastasize; (*c*) prostatic reconstruction models of prostatic cancer. The prostatic reconstruction model (8) uses the use of tissue recombinants composed of UGM and UGE grafted beneath the renal capsule of athymic mouse hosts. Thompson *et al.* (8) used viruses to introduce activated oncogenes into UGM and UGE of the developing prostate to generate prostatic tissue expressing oncogenes in the prostatic epithelium or stroma.

Each of the above models is contrived and uses highly abnormal approaches. Each model has, however, significantly advanced the field of prostatic carcinogenesis, despite this inherent artificiality. The newly established model described in the present communication also has it own degree of artificiality but is valuable because prostatic carcinogenesis is elicited at high efficiency and with low latency in response to treatment with T and E2. This model uses aspects of the hormone-induced tumor models, genetic modification, and tissue recombination models.

Prostatic carcinogenesis is a multistep process involving both genetic alterations to the epithelial cells such as activation of oncogenes (9–12), inactivation of tumor suppressor genes (13, 14), as well as perturbation of stromal-epithelial interactions (15–18). The involvement of multiple oncogenes and tumor suppressor genes in carcinogenesis has been demonstrated for many types of carcinomas (19, 20). Alterations in tumor suppressor genes such as the *RB* gene have been suggested to play a role in the development of prostate cancer (13, 21–23). The *RB* gene is located on human chromosome 13 and encodes a M_r 110,000 nuclear protein involved in cell cycle control and other processes (24). The importance of the *RB* gene in tumorigenesis was originally recognized in familial retinoblastoma and subsequently the involvement of RB has been de-

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[3] The abbreviations used are: PRE, prostatic epithelium; Rb, retinoblastoma gene product; UGM, rat urogenital sinus mesenchyme; rUGM, rat UGM; UGE, urogenital sinus epithelium; T, testosterone propionate; E2, estradiol; PCNA, proliferating cell nuclear antigen; ER, estrogen receptor.

scribed in a number of other human neoplasias including bladder (25), breast (26–28), and lung cancer (29–31). In human prostate cancer, *RB* gene mutations have been reported in 16.4% of primary human prostatic cancers, which suggested that inactivation of *RB* may be an important event in at least a subset of prostatic carcinomas (32–34). Moreover, it was reported that 27% of human prostatic adenocarcinomas have lost one *RB* allele (35). The relatively high frequency of this occurrence suggests that *RB* may be involved in the development of prostatic lesions.

To study Rb function, gene targeting has been used to inactivate Rb in mice (36–38). Mice homozygous for Rb disruption ($Rb^{-/-}$) die at about 13 days of gestation, several days before the prostate forms. Thus, it appears problematic to study prostatic carcinogenesis in $Rb^{-/-}$ mice. To solve this problem, we have generated $Rb^{-/-}$ prostatic tissues by grafting embryonic pelvic viscera beneath the renal capsule of male nude mice. Embryonic prostatic rudiments grafted under the renal capsule grow substantially, undergo prostatic morphogenesis, and produce prostatic secretory proteins (39).

It is well established that UGM can induce prostatic epithelial growth, ductal branching morphogenesis, and cytodifferentiation in a variety of embryonic and adult epithelia (39–42). In particular, we have shown that embryonic rat or mouse UGM can induce small (100–300 μm) segments of prostatic ducts (PRE) to form as much as 50–60 mg wet weight of prostatic tissue after 1 month of growth in nude mouse hosts (42, 43). The method involves grafting UGM + PRE tissue recombinants under the renal capsule of intact male nude mice (42). An important feature of the UGM + PRE tissue recombinant system is the marked stimulation of epithelial proliferation, because the original 300-μm ductal segment containing about 5,000 epithelial cells yields about 20,000,000 prostatic epithelial cells during 1 month of *in vivo* growth (42).

E2 in combination with testosterone is an effective method of inducing prostatic cancer in adult Noble rats (2, 44–48). The starting point of the carcinogenic process in the model described here is a single genetic defect, the homozygous deletion of Rb in combination with an established model of hormonal carcinogenesis. Because the *RB* gene is perturbed in only a relatively small percentage of human prostate cancers, it should be stressed that the present study uses Rb-knockout prostatic tissue, not because of a role of RB in human prostate cancer but rather because of the enhanced susceptibility of this tissue to undergo further genetic change leading to prostatic carcinogenesis.

MATERIALS AND METHODS

Rescue of Rb Tissues and Genotyping. Heterozygous (Rb+/−) male and female mice were mated. At 12 days of gestation (plug day denoted as day 0), mothers were sacrificed, and fetuses were removed. Fetuses were laid on their backs and opened along the midline from the diaphragm to the pubic symphysis. Pelvic visceral rudiments were dissected as a single unit and grafted beneath the renal capsule of intact male athymic mouse hosts (49).[4] The Rb status of the fetuses was determined by PCR, as described previously (37). After 1 month of growth, fully developed prostatic ductal structures were identified grossly within grafts harvested from the renal graft site. Such fresh prostatic ductal tissue was cut into small ductal segments for recombination with rUGM.

Characterization of *in Vivo* Rescued Prostatic Tissue. The prostatic phenotype of tissues within pelvic visceral grafts was confirmed by histological and immunohistochemical staining using a panel of antibodies against known prostatic markers as outlined below.

Tissue Separation, Recombination, and Grafting. Pregnant Sprague Dawley rats were obtained from Charles River (Wilmington, MA). rUGM was prepared from 18-day embryonic fetuses. For this purpose, urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components after tryptic digestion and mechanical separation, as described previously (49). $Rb^{+/+}$ and $Rb^{-/-}$ prostatic ductal segments were cut into small (200–500 μm) pieces and placed on top of rUGM in dishes containing a nutrient agar medium, as described previously (43, 50). After 24 h, the tissue recombinants were grafted underneath the renal capsule.

Induction of Carcinogenesis. Athymic male mouse hosts (Charles River) bearing rUGM + $Rb^{+/+}$PRE and rUGM + $Rb^{-/-}$PRE tissue recombinants were treated hormonally at the time of grafting by surgical implantation of a 1-cm Silastic capsule filled with 25 mg of T and a 0.4-cm Silastic capsule filled with 2.5 mg of 17β-estradiol. The Silastic tubing (Dow-Corning Co., Midland, MI) had an inside diameter of 1.54 mm and an outside diameter of 3.18 mm. E2 and T were obtained from Sigma Chemical Co. (St. Louis, MO). Hosts were hormonally treated for 5 or 8 weeks, beginning at the time of grafting. The control group received empty Silastic capsules. All animals were housed in the University of California San Francisco Animal Care Facility with food and drinking water *ad libitum* under controlled lighting conditions (12 h light, 12 h dark).

Histopathological Grading and Hoechst 33258 Dye Staining. Host animals were sacrificed at 5 or 8 weeks after implantation of T + E2 capsules by cervical dislocation. The tissue recombinants were carefully dissected from the host kidney, fixed in 10% neutral buffered formalin, and embedded in paraffin. Six-μm sections were stained with H&E to determine the histopathology of the prostatic tissue recombinants, which were also stained with Hoechst dye 33258 (CalBiochem, La Jolla, CA) to confirm that the epithelium was of mouse origin and the stroma of rat origin (51). Slides were examined histologically from serially sectioned tissue recombinants to determine the incidence of prostatic lesions. Every 15th section was examined (a separation of 90 μm). Approximately five sections per tissue recombinant were examined, dependent upon the size of the harvested graft. Slides were scored to determine the presence of normal, hyperplasia, atypical hyperplasia, or carcinoma.

Immunohistochemical Staining. Tissue sections were deparaffinized in Histoclear (National Diagnostic, Atlanta, GA) and hydrated in graded alcoholic solutions and PBS. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 min, followed by washing in PBS. Normal goat serum 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-androgen receptor antibody was purchased from Affinity BioReagents (Golden, CO). Mouse monoclonal anti-cytokeratin 14 (CK14, LL001) and monoclonal anti-cytokeratin 8 (CK8, LE41) were generously provided by Dr. E. B. Lane (University of Dundee, Dundee, Scotland). Mouse anti-PCNA monoclonal antibody was purchased from PharMingen (San Diego, CA), and the mouse anti-smooth muscle α-actin monoclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-mouse dorsolateral prostate secretion (mDLP; Ref. 52) was used at a dilution of 1:5000. Rabbit anti-mouse seminal vesicle secretion (53) was also used for immunohistochemical staining at a dilution of 1:5000, as described previously (52). Mouse anti-E-cadherin monoclonal antibody was purchased from Transduction Laboratories (San Diego, CA). Antibodies against Rb and related family members (P107 and P130) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After careful washing, the sections were incubated in biotinylated secondary immunoglobulin of appropriate species specificity (Sigma; 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 for 30 min at room temperature. After the last PBS wash, the sections were developed for about 1–5 min using 3,3-diaminobenzidine in PBS and 0.03% H_2O_2. Sections were counterstained with hematoxylin and dehydrated in alcohol. Control sections were processed in parallel with mouse nonimmune IgG at the same concentration as the primary antibodies.

Generation of PCNA Labeling Indices. After sectioning and staining, multiple grafts (range, 6–12) were examined to determine the percentage of epithelial cells showing positive immunoreactivity against PCNA. For each individual graft, the percentage of expressing cells was counted in six separate, low magnification microscopic fields. Mean and SE were calculated for each group. Differences were analyzed statistically by ANOVA.

RESULTS

Rescue of $Rb^{-/-}$ Prostate Tissue. Mature prostatic tissue was identified grossly as ductal masses in grafts of embryonic pelvic visceral rudiments from 12 day $Rb^{+/+}$ and $Rb^{-/-}$ embryos grown for

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

1 month in male athymic mouse hosts (Fig. 1*a*). Histologically, the ductal tissue from both wild-type and knockout sources resembled prostate and stained with anti-mDLP antisera (Fig. 1*b*), which reacts with mouse prostatic secretory proteins (52). Cytoplasmic and luminal staining of prostatic secretion was observed. Cytokeratin 14 was visualized in basal epithelial cells, whereas luminal cells expressed cytokeratin 8. Androgen receptors were detected by immunohistochemistry in both epithelial and stromal cells of the $Rb^{+/+}$ and $Rb^{-/-}$ rescued prostatic tissue (Fig. 1*c*). Histological analysis of the grafted embryonic pelvic viscera also revealed bladder, colonic, and seminal vesicle tissue. The seminal vesicle tissue did not stain with mDLP antisera but stained with anti-mSV antisera (not illustrated). As expected, Rb protein could not be detected in either wild type or Rb-knockout tissue. P130 was expressed in nuclei of all epithelial cells. P107 was detected in nuclei of a subpopulation of epithelial cells. Both $Rb^{-/-}$ and $Rb^{+/+}$ grafts gave similar results. Stromal expression of P130 was extremely low in the wild-type grafts, whereas in the Rb-knockout tissue, ~40% of the stromal nuclei were stained. Expression of P107 and P130 was not affected by hormonal treatment or type of histopathological lesion. On the basis of immunocytochemical observations, the absence of Rb was not compensated by increased expression of P107 and P130.

rUGM+mPRE Tissue Recombinants: General Observations. As expected, the rUGM+$Rb^{-/-}$PRE and rUGM+$Rb^{+/+}$PRE tissue recombinants increased many fold in size and formed large (34.6 ± 2.4 mg; $n = 37$) masses of well-differentiated prostatic ductal tissue when grown for 5–8 weeks in untreated male hosts. In the rUGM+$Rb^{+/+}$PRE and rUGM+$Rb^{-/-}$PRE tissue recombinants, the epithelial cells were of mouse origin (Fig. 1*d*), as determined by nuclear staining patterns with Hoechst dye 33258, whereas the stroma was predominantly rat in origin (except for host mouse vasculature). The ductal epithelium of these tissue recombinants exhibited immunostaining with anti-mDLP and androgen receptors as described above (not illustrated). Epithelial PCNA labeling, a measure of proliferation, was consistently and significantly elevated in all tissue recombinants containing $Rb^{-/-}$ *versus* $Rb^{+/+}$ epithelium, irrespective of hormone treatment (Table 1). Average epithelial PCNA labeling values within the $Rb^{-/-}$ *versus* $Rb^{+/+}$ groups did not differ significantly at the time of harvest between different hormone treatment groups.

rUGM+$Rb^{+/+}$PRE Tissue Recombinants Grown in Untreated Hosts. As reported previously, prostatic tissues from the rUGM+$Rb^{+/+}$PRE tissue recombinants in untreated control hosts (implanted with empty Silastic capsules) consisted of normal prostatic ductal-acinar tissue lined by a simple columnar epithelium surrounded by stromal components (Fig. 2*a*). In such epithelial ducts, the cytokeratin 14-positive basal epithelial cells formed a discontinuous layer underneath the tall columnar luminal epithelial cells (Fig. 2*b*). Ductal lumina were lined by tall columnar luminal cells expressing cytokeratin 8 (Fig. 2*c*). E-cadherin was expressed along adjacent epithelial cell membranes (Fig. 2*d*), and α-actin-positive smooth muscle cells (Fig. 2*e*) surrounded the epithelial ducts in rUGM+$Rb^{+/+}$PRE tissue recombinants. The smooth muscle layer was in intimate association with the epithelial basement membrane. Occasional foci of epithelial hyperplasia were also seen in these grafts (Table 2). The epithelial proliferation rate was low as judged by PCNA staining (Fig. 2*f*). Rb was not detected by immunohistochemistry, P130 was detected in all epithelial cells and a very low number of stromal cells, and P107 was detected in a minor population of epithelial cells and in a low number of stromal cells.

rUGM+$Rb^{+/+}$PRE Tissue Recombinants Grown in T + E2-treated Hosts. In rUGM+$Rb^{+/+}$PRE tissue recombinants grown in T + E2-treated hosts, most of the ductal-acinar tissue was lined by a simple columnar epithelium. The stroma was densely cellular. Some glands were lined by a multilayered epithelium that frequently formed back-to-back glands (Fig. 3*a*). Such hyperplastic epithelial ducts exhibited an increased density of basal epithelial cells expressing cytokeratin 14 (Fig. 3*b*) in comparison with the untreated control group (compare with Fig. 2*b*). The ductal lumina were lined by tall columnar luminal cells expressing cytokeratin 8 (Fig. 3*c*). E-cadherin was expressed along adjacent epithelial cell membranes (Fig. 3*d*). The induced prostatic tissue contained α-actin-positive smooth muscle cells in intimate association with the ductal epithelium (Fig. 3*e*), similar to that of rUGM+$Rb^{+/+}$PRE tissue recombinants grown in

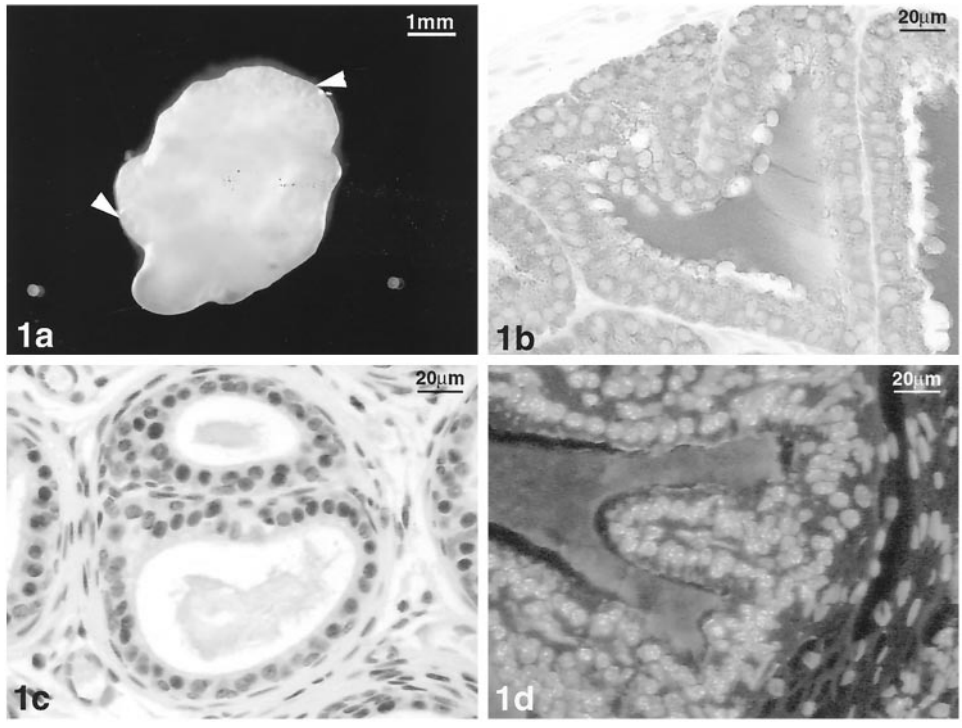

Fig. 1. Rescued $Rb^{-/-}$ tissues and a rUGM+$Rb^{-/-}$PRE tissue recombinant grown in male nude mouse hosts. *a*, whole mount of rescued $Rb^{-/-}$ prostate tissue demonstrating the appearance of prostatic ducts (*arrowheads*). *b*, the rescued $Rb^{-/-}$ mouse prostate stained with anti-mDLP. Note strong staining of cytoplasm and luminal secretion. *c*, section of a rescued $Rb^{-/-}$ mouse prostate stained with anti-androgen receptor. Note androgen receptor staining of both epithelial and stromal cells. *d*, rUGM+$Rb^{-/-}$PRE tissue recombinant stained with Hoechst dye 33258. Epithelial nuclei have many bright intranuclear spots indicative of murine cells, whereas stromal cell nuclei are homogeneously stained, indicative of rat cells.

Table 1 *PCNA labeling in rUGM + PRE tissue recombinants treated with or without T + E2*

Epithelium	Treatment	Grafts	% expressing PCNA	SE
Rb+/+	Untreated	12	34.0[a]	2.1
Rb−/−	Untreated	11	61.5[b]	2.7
Rb+/+	T + E2	6	23.1[a]	3.4
Rb−/−	T + E2	8	55.7[b]	6.4

[a–b] Values for groups a and b differ significantly as judged by ANOVA.

untreated hosts. Foci of epithelial hyperplasia were also seen in these grafts (see Table 2). The cribiform pattern in the ducts of the rUGM+$Rb^{+/+}$PRE tissue recombinants suggests a rather extensive hyperplasia. Epithelial proliferation as judged by PCNA staining is shown in Fig. 3*f*. Expression of Rb, P130, and P107 was identical to the untreated group.

rUGM+$Rb^{-/-}$PRE Tissue Recombinants Grown in Untreated Control Hosts. Prostatic histodifferentiation was normal in rUGM+$Rb^{-/-}$PRE tissue recombinants after 5 or 8 weeks of growth in control hosts implanted with empty Silastic capsules. The prostatic glands were lined with columnar, cytokeratin 8-positive luminal epithelial cells and were underlain with occasional cytokeratin 14-positive basal cells. In ~40% of grafts, regions of hyperplasia were also seen; these were characterized by a more or less continuous layer of cytokeratin 14-positive basal cells (Fig. 4, *a–c*). E-cadherin was expressed along adjacent epithelial cell membranes (Fig. 4*d*). The stroma contained α-actin-positive smooth muscle cells in intimate association with the prostatic ducts (Fig. 4*e*). PCNA labeling was elevated in the $Rb^{-/-}$ epithelium (Fig. 4*f*) relative to that of wild-type epithelium in rUGM+$Rb^{+/+}$PRE tissue recombinants grown in control hosts. Thus, the two features unique to the untreated rUGM+$Rb^{-/-}$PRE tissue recombinants were expansion of the basal epithelial cell compartment to a continuous layer and a distinct elevation in PCNA labeling in PRE.

rUGM+$Rb^{-/-}$PRE Tissue Recombinants Grown in T + E2-treated Hosts. Focal areas of prostatic hyperplasia, atypical hyperplasia, and carcinoma were observed in rUGM+$Rb^{-/-}$PRE tissue recombinants grown in T + E2-treated hosts, although normal prostatic tissue was also observed in these grafts (incidence described in Table 2).

In rUGM+$Rb^{-/-}$PRE tissue recombinants treated with T + E2, focal areas of prostatic hyperplasia were observed as ducts lined with multiple layers of polarized epithelial cells. Numerous papillary projections were present in the hyperplastic ducts (Fig. 5*a*). In addition, there were minor pleomorphic nuclear changes with occasional mitotic figures in the epithelial ducts and in the stroma. Luminal epithelial cells continued to express cytokeratin 8 (not illustrated). The continuous layer of cytokeratin 14-positive basal cells showed focal areas of stratification (Fig. 5*b*). The hyperplastic epithelium retained a normal cellular polarity, and

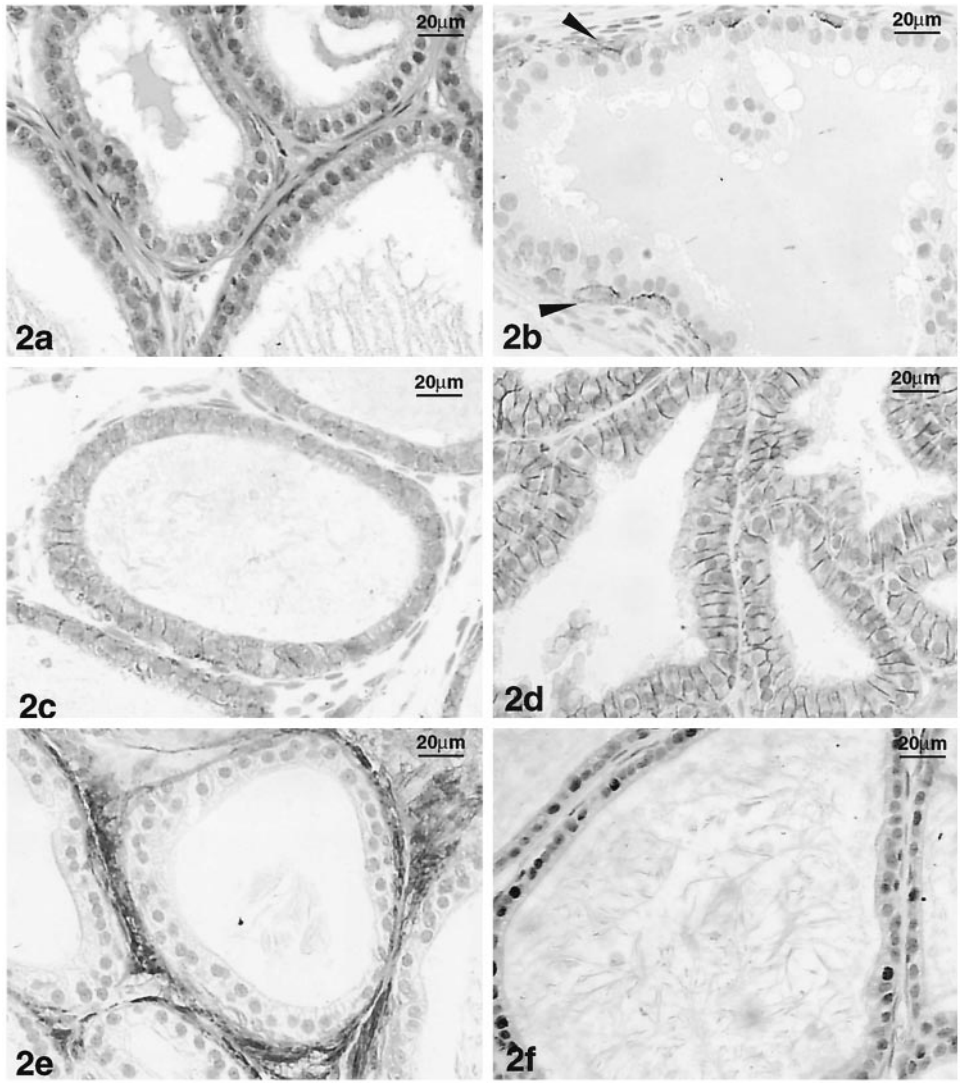

Fig. 2. rUGM+$Rb^{+/+}$PRE tissue recombinants grown for 8 weeks in intact untreated male hosts. *a*, the glandular tissue in rUGM+$Rb^{+/+}$PRE tissue recombinants resembles prostatic tissue (H&E stain). *b*, immunostaining with anti-cytokeratin 14 reveals a discontinuous layer of basal epithelial cells (*arrowheads*). *c*, luminal cells are immunostained with anti-cytokeratin 8. *d*, immunostaining with anti-E-cadherin reveals E-cadherin along epithelial cell membranes. *e*, smooth muscle cells in intimate association with the epithelial ducts are immunostained with anti-α-actin. *f*, immunostaining with anti-PCNA reveals a low percentage of labeled epithelial cells.

Table 2 *Pathological grading in rUGM + PRE tissue recombinants treated with or without T + E2 prepared with wild-type or Rb−/− prostatic epithelium*

Incidence of the various histotypes was determined by examining multiple sections from each tissue recombinant. The presence of any given histology found and the number of recombinants examined are indicated.

Type of prostatic epithelium	Treatment	Normal	Hyperplasia	Atypical hyperplasia	Cancer
Rb+/+	Untreated	22/22	10/22	0/22	0/22
Rb+/+	T + E2	13/13	13/13	0/13	0/13
Rb−/−	Untreated	20/20	8/20	0/20	0/20
Rb−/−	T + E2	16/16	16/16	10/16	4/16

E-cadherin was expressed along adjacent epithelial cell membranes (Fig. 5*c*). The stroma surrounding the hyperplastic epithelium expressed smooth muscle α-actin (Fig. 5*d*). Epithelial proliferation rate was high as judged by PCNA labeling (not illustrated).

Prostatic hyperplasia with cytological atypia was also observed focally in rUGM+$Rb^{-/-}$PRE tissue recombinants grown in T + E2-treated hosts. Such atypical hyperplastic lesions were characterized by elevated epithelial proliferation and stratification with variable degrees of tissue disorganization and cytological atypia (Fig. 6*a*). The $Rb^{-/-}$ epithelial cells of rUGM+$Rb^{-/-}$PRE tissue recombinants showed nuclear crowding, enlargement, and pleomorphism (Fig. 6*a*) with a high proliferation rate as judged by PCNA staining (not illustrated). Some mitotic figures were observed. In large ducts, several atypical hyperplastic foci were sometimes seen within a single duct, although not all ducts exhibited atypical hyperplastic changes. The atypical hyperplastic epithelial cells were cytokeratin 8 positive (not illustrated). Cytokeratin 14-positive basal epithelial cells were rarely detected (Fig. 6*b*). E-cadherin was abnormal and variable. In some areas, E-cadherin was expressed weakly along adjacent epithelial cell membranes, whereas in other areas, E-cadherin was undetectable or cytoplasmic (Fig. 6*c*). Atypical hyperplastic prostatic ducts were surrounded by a disorganized incomplete α-actin-positive smooth muscle layer, which was separated by variable distances from the epithelial basement membrane by an unstained connective tissue containing extracellular matrix and unstained cells (presumably fibroblasts; Fig. 6*d*).

Prostatic carcinoma *in situ* and adenocarcinoma were also observed focally in T + E2-treated rUGM+$Rb^{-/-}$PRE tissue recombinants 5–8 weeks after implantation of hormone pellets (Fig. 7*a*). Disorganized cribriform patterns were observed containing tumor cells having large pleomorphic nuclei with prominent nucleoli. Neoplastic cells filled entire

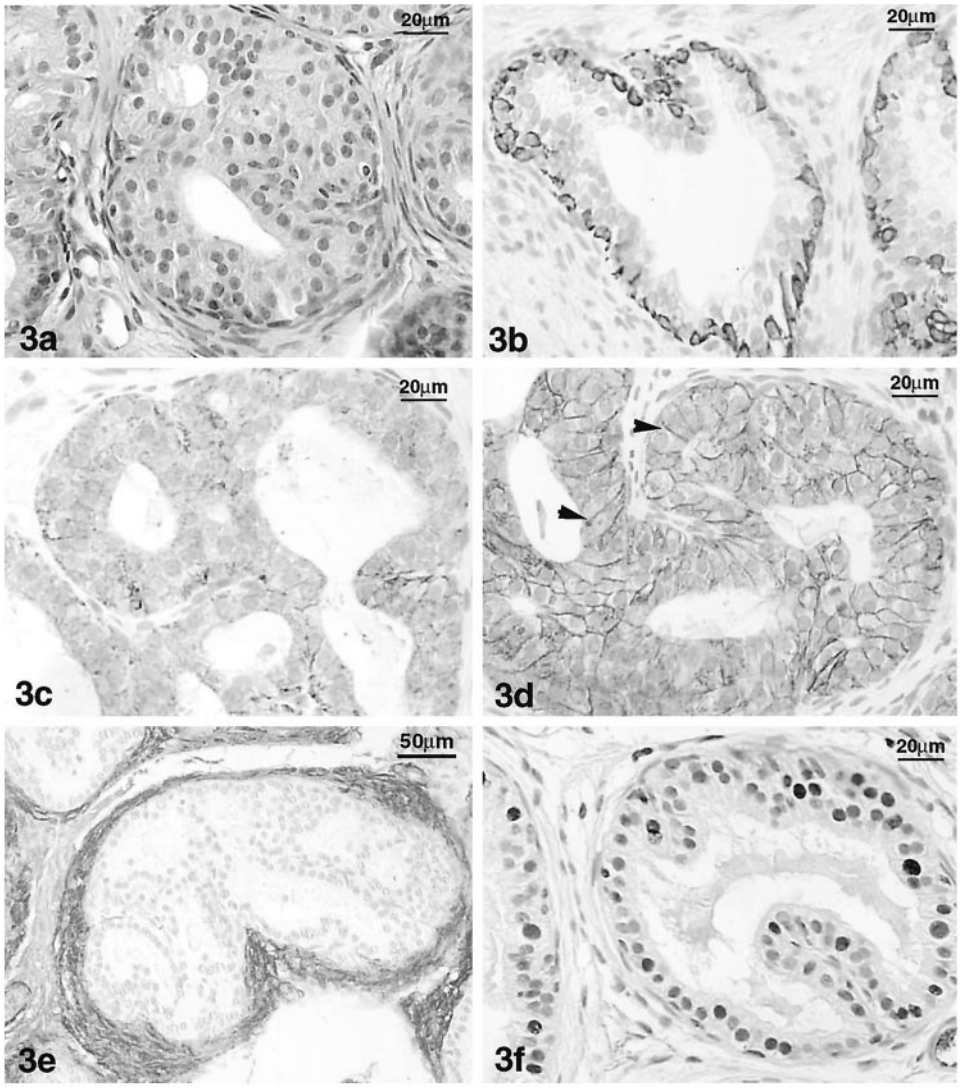

Fig. 3. Hyperplastic prostatic tissue in rUGM+$Rb^{+/+}$PRE tissue recombinants grown for 8 weeks in T + E2-treated hosts. *a*, a hyperplastic multilayered epithelium has developed with minor pleomorphic nuclear changes. *b*, immunostaining with anti-cytokeratin 14 reveals a nearly continuous layer of basal epithelial cells underlying the hyperplastic epithelium. *c*, use of anti-cytokeratin 8 demonstrates that the luminal cells express this characteristic marker. *d*, anti-E-cadherin immunohistochemistry shows a normal epithelial membrane staining pattern (*arrowheads*). *e*, smooth muscle cells in intimate association with the epithelial ducts are immunostained with anti-α-actin. *f*, immunostaining with anti-PCNA.

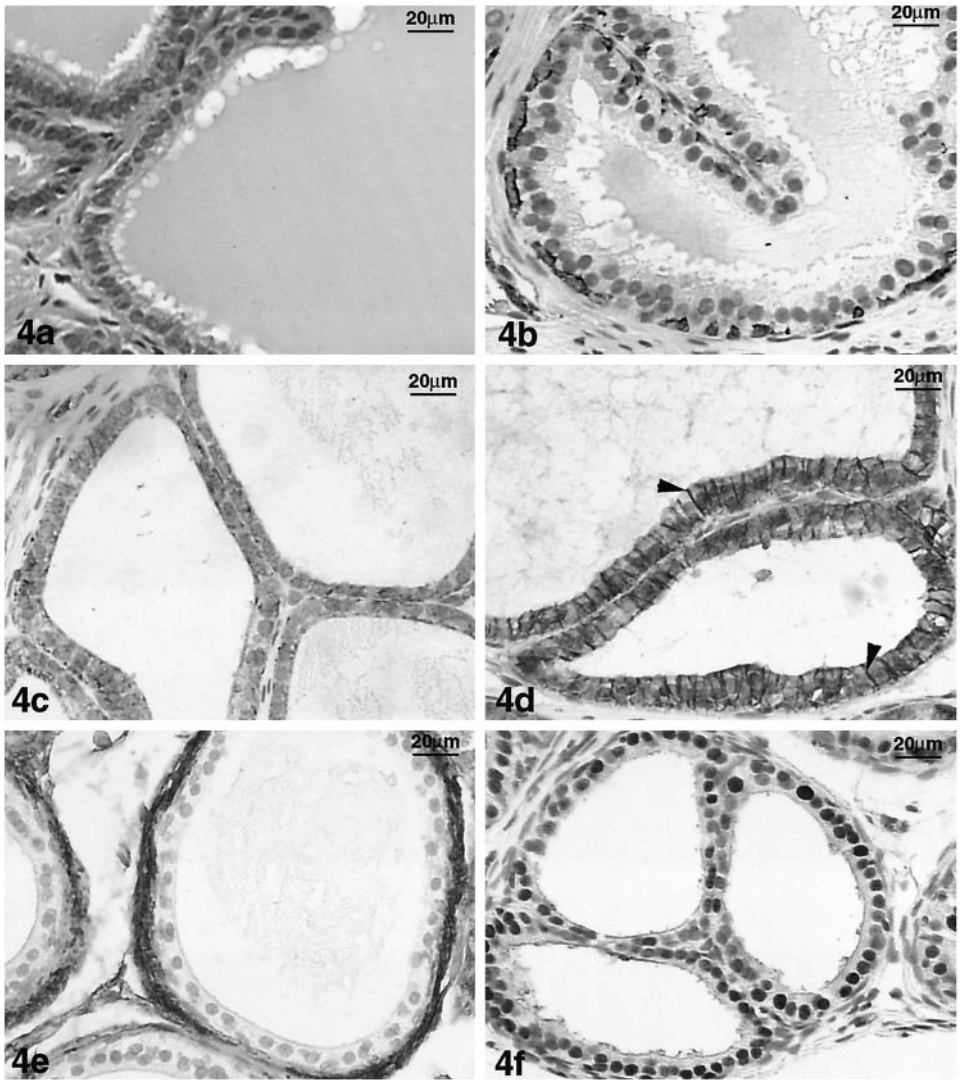

Fig. 4. rUGM+Rb$^{-/-}$PRE tissue recombinants grown for 8 weeks in untreated hosts. *a*, rUGM+Rb$^{-/-}$PRE prostate tissue stained with H&E reveals normal prostatic histodifferentiation. *b*, anti-cytokeratin 14 immunocytochemistry reveals an almost continuous layer of basal epithelial cells. *c*, anti-cytokeratin 8 immunocytochemistry shows a normal luminal epithelial layer. *d*, anti-E-cadherin immunohistochemistry. Normal staining of epithelial membranes is seen (*arrowheads*). *e*, immunostaining with anti-α-actin. Smooth muscle cells are in intimate association with the epithelial ducts. *f*, immunostaining with anti-PCNA reveals high labeling of the Rb$^{-/-}$ epithelium.

ducts and invaded the interacinar connective tissue or invaded adjacent acini, resulting in the formation of tumor masses composed of small back-to-back glands (Fig. 7*a*). Mitotic figures were detected in some areas, and abnormal mitoses were occasionally observed. Cytokeratin 14-positive basal cells were not detected or were present in reduced numbers (Fig. 7*b*) and instead the carcinoma cells expressed the luminal cell marker, cytokeratin 8 (Fig. 7*c*). E-cadherin expression was abnormal, and in most areas E-cadherin was undetectable (Fig. 7*d*). In carcinomatous areas, few α-actin-positive smooth muscle cells were observed (Fig. 7*e*). Epithelial proliferation rate was high in carcinomas based upon PCNA staining (Fig. 7*f*). Thus, areas interpreted as malignant exhibited nuclear pleomorphism, elevated epithelial proliferation, increased mitoses and abnormal mitoses, loss of basal epithelial cells, loss of prostatic smooth muscle, reduction or loss of E-cadherin staining, and perturbation in ductal organization and epithelial polarity. Rb was not detected in any Rb$-/-$ tissue. P130 and P 107 expression mirrored that seen in the tissue recombinants that used wild-type epithelium. It was noted that the expression patterns of these proteins were consistent across the whole range of histological phenotypes from normal to cancer. Thus, at least at the level of immunohistochemical detection, there was no apparent regulation of these proteins in relation to tumor progression.

The incidence of the various histological phenotypes in all of the tissue recombinants is summarized in Table 2.

DISCUSSION

Despite the fact that prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in men (54), its etiology is poorly understood. Actions of estrogens on the prostate are believed to be mediated via ER-α and/or ER-β (55). Estrogen appears to play an important role in the pathobiology of the prostate, although normal development of the prostate occurs in mice null for ER-α or ER-β (56–58). Exogenous estrogens given during the perinatal period elicit permanent abnormalities in prostatic growth (59–62), differentiation (63), function (64), androgen metabolism (65), and expression of androgen receptors (66, 67) and may lead to prostatic cancer (68–70). In adulthood, chronic treatment with estrogen acts synergistically with androgen to induce benign prostatic hyperplasia in dogs (71).

E2 in combination with T given at a pharmacological level is an effective method of inducing prostatic cancer in adult Noble rats (46–48). This rat model of prostatic cancer generally has a long latency and low tumor incidence, although new hormonal treatment protocols have partially addressed these problems (2). The model of prostate cancer presented in this report combines hormonal induction of prostate cancer (2), enhanced epithelial proliferation elicited by tissue recombination with rUGM (42), and use of Rb$^{-/-}$ mouse

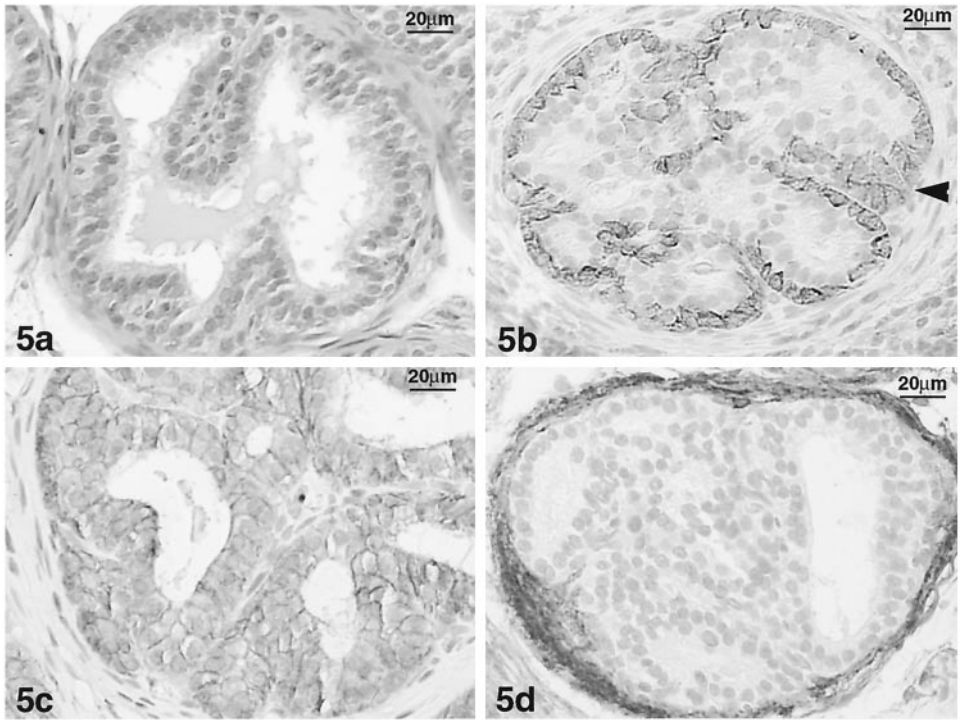

Fig. 5. Focal areas of prostatic epithelial hyperplasia in rUGM+$Rb^{-/-}$PRE tissue recombinants grown for 8 weeks in T + E2-treated hosts. *a*, the PRE of rUGM+$Rb^{-/-}$PRE tissue recombinants treated with T + E2 is stratified and has numerous papillary projections (H&E stain). *b*, anti-cytokeratin 14 immunocytochemistry reveals an almost continuous layer of basal epithelial cells and areas of stratification of basal cells (*arrowhead*). *c*, anti-E-cadherin immunohistochemistry demonstrates normal epithelial membrane staining (*arrowheads*). *d*, immunostaining with anti-α-actin. Smooth muscle cells in intimate association with the epithelial ducts are demonstrated.

prostatic epithelium generated through rescue of embryonic tissues from $Rb^{-/-}$ embryos. In this way, the starting point of the carcinogenic process is a single genetic defect, homozygous deletion of Rb.

The rescue of $Rb^{-/-}$ prostatic epithelium from embryonic lethal embryos was achieved by grafting embryonic pelvic organs at 12 days of gestation, 1 day before the expected demise of $Rb^{-/-}$ embryos (37). The grafts develop a range of tissues (prostate, bladder, colon, and seminal vesicle tissue), from which the prostatic tissue can be easily recognized grossly using a dissecting microscope because of its ductal morphology. Such "rescued" prostatic tissue exhibits prostatic ductal morphology and expresses androgen receptors and mouse prostatic secretory proteins and is organized in a manner characteristic of wild-type prostate.

Multifocal areas of prostatic hyperplasia, atypical hyperplasia, and carcinoma were observed in all rUGM+$Rb^{-/-}$PRE tissue recombinants grown in T + E2-treated hosts, whereas in untreated rUGM+$Rb^{-/-}$PRE tissue recombinants, the most severe lesion observed was simple basal cell hyperplasia. Carcinomatous areas in T + E2-treated rUGM+$Rb^{-/-}$PRE tissue recombinants exhibited nuclear pleomorphism, elevated epithelial proliferation, increased mi-

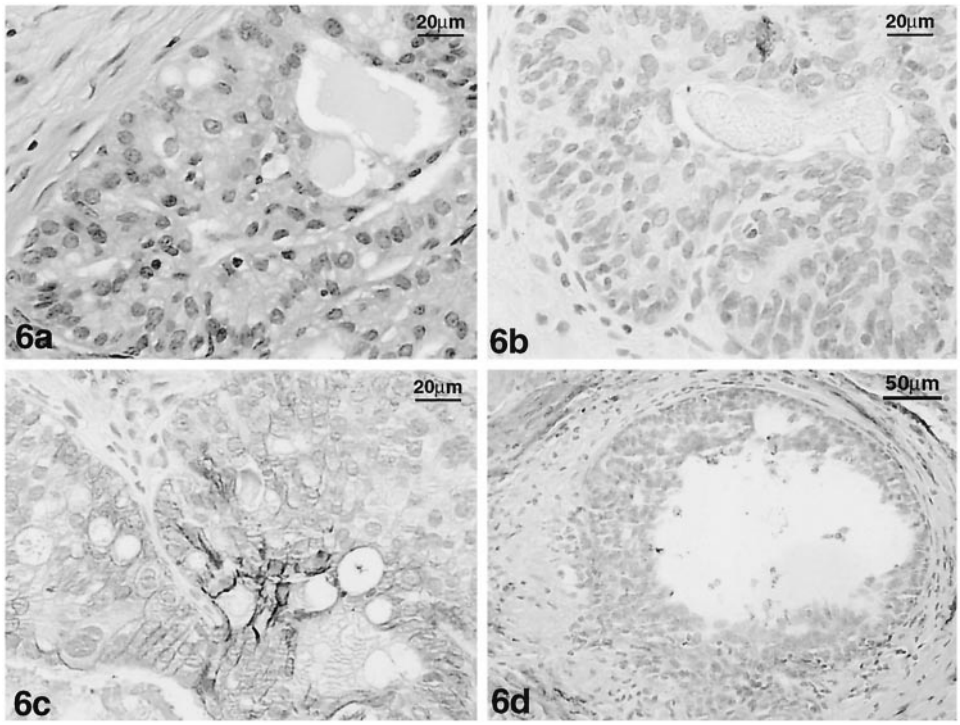

Fig. 6. Focal areas of prostatic atypical hyperplasia in rUGM+$Rb^{-/-}$PRE tissue recombinants grown for 8 weeks in T + E2-treated hosts. *a*, the $Rb^{-/-}$ PRE is stratified with variable degrees of tissue disorganization and cytological atypia (H&E stain). *b*, anti-cytokeratin 14 immunocytochemistry reveals an almost complete absence of basal epithelial cells. *c*, anti-E-cadherin immunohistochemistry shows an abnormal pattern of epithelial staining with reduction or absence of staining in some areas. *d*, immunostaining with anti-α-actin reveals a reduction in smooth muscle cells. Smooth muscle cells are separated from the epithelium by a thick layer of unstained connective tissue.

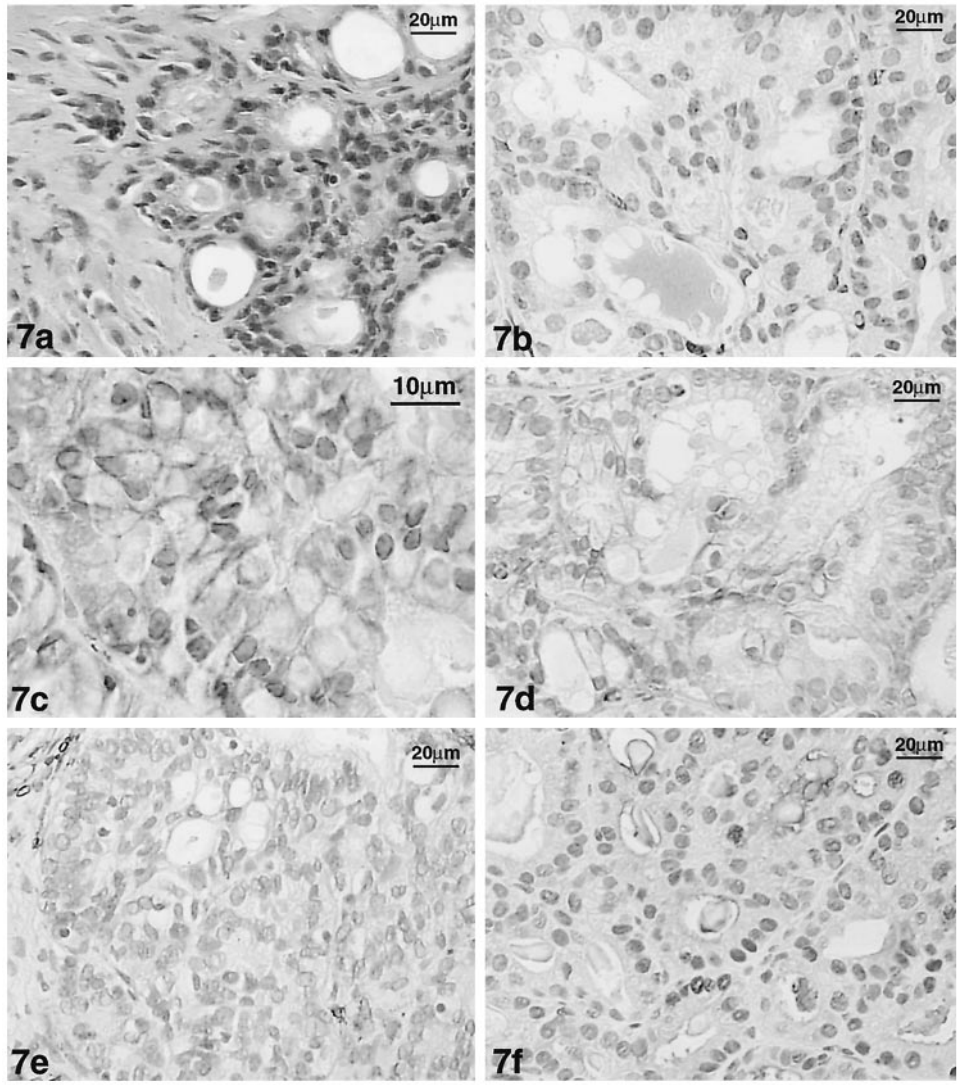

Fig. 7. Focal areas of prostatic adenocarcinoma in rUGM+Rb$^{-/-}$PRE tissue recombinants grown for 8 weeks in T + E2-treated hosts. *a*, tumor mass composed of undifferentiated epithelial cells that developed in a rUGM+Rb$^{-/-}$PRE tissue recombinant grown for 8 weeks in T + E2-treated host (H&E stain). *b*, anti-cytokeratin 14 immunocytochemistry reveals an almost complete absence of basal epithelial cells. *c*, anti-cytokeratin 8 immunocytochemistry demonstrates that the tumor mass is composed of cytokeratin 8-positive cells. *d*, anti-E-cadherin immunohistochemistry. There is an almost complete absence of membrane staining. *e*, immunostaining with anti-α-actin. Smooth muscle cells are almost completely absent. *f*, immunostaining with anti-PCNA reveals high labeling of the Rb$^{-/-}$ epithelium.

toses and abnormal mitoses, loss of basal epithelial cells, complete loss of prostatic smooth muscle, loss of membrane staining for E-cadherin, and perturbation in ductal organization and epithelial polarity. Epithelial proliferation was generally elevated in rUGM+Rb$^{-/-}$PRE *versus* rUGM+Rb$^{+/+}$PRE tissue recombinants, especially in hyperplastic, atypical hyperplastic, and carcinomatous regions. This elevation in PCNA staining is consistent with the role of Rb as a cell cycle check point modulator (72, 73). Studies in progress indicate that the carcinomas described are transplantable, and moreover, the atypical hyperplasias progress to cancer when serially grafted to new hosts.

The present model resembles human prostate cancer in that it involves change in the histodifferentiation of both the epithelium and the stroma. Normal prostatic epithelium, rescued Rb$^{-/-}$ and Rb$^{+/+}$ prostatic epithelium, and prostatic epithelium of untreated rUGM+Rb$^{-/-}$PRE and rUGM+Rb$^{+/+}$PRE tissue recombinants contains a discontinuous layer of cytokeratin 14-positive basal cells underlying the cytokeratin 8-positive luminal epithelial cells. The prostatic hyperplasias that developed in a response to T + E2 treatment contained epithelium having a nearly continuous layer of basal cells, which in some cases was stratified. Prostatic carcinomas in T + E2-treated rUGM+Rb$^{-/-}$PRE tissue recombinants were composed exclusively of cytokeratin 8-positive epithelial cells as is also the case for most human prostate cancers (74). The cytokeratin 14-positive basal cells almost completely disappeared during the carcinogenic process in T + E2-treated rUGM+Rb$^{-/-}$PRE tissue recombinants. This means that carcinomatous clones probably arose from the cytokeratin 8-positive luminal epithelial cells, although other interpretations are possible.

The progressive architectural derangements in the prostatic epithelium of T + E2-treated rUGM+Rb$^{-/-}$PRE tissue recombinants were manifested in loss of epithelial polarity, epithelial crowding, stratification, and invasiveness. Such changes were associated with perturbation or loss in the membrane expression of E-cadherin. E-cadherin is a M_r 120,000 transmembrane glycoprotein involved in epithelial cell adhesion (75, 76). A functional E-cadherin system is required for maintenance of normal epithelial morphology. E-cadherin is localized on adjacent cell membranes of normal epithelial cells (including PRE) and in various highly differentiated "noninvasive" carcinoma cells (including prostate cancer). In contrast, E-cadherin expression is lower or entirely lacking in poorly differentiated invasive carcinoma cells (75–79). Patients with prostatic tumors negative for E-cadherin or having abnormal patterns of E-cadherin expression have a worse prognosis than patients having prostatic tumors with normal expression of E-cadherin (80). The loss of portions of chromosome 16, which is fairly common in prostate cancer, may result in deletion of all or part of the *E-cadherin* gene, which maps to 16q21 (81). The loss of membrane expression of E-cadherin in carcinomas observed in

T + E2-treated rUGM+Rb$^{-/-}$PRE tissue recombinants is presumably related to the disorganization of ductal architecture.

Stromal histodifferentiation was coordinately perturbed in T + E2-treated rUGM+Rb$^{-/-}$PRE tissue recombinants with the loss of the smooth muscle layer that is normally in intimate contact with the ductal basement membrane. Thus, during the carcinogenic process induced by T + E2, the normal homeostatic interaction of prostatic epithelium with smooth muscle has become an interaction of abnormal prostatic epithelium with a predominantly fibroblastic stroma. This abnormal fibroblastic stroma, which emerged during the carcinogenic process, has been shown to profoundly affect carcinogenic progression through altering epithelial differentiation, proliferation, and apoptosis as described recently (18, 82). Stromal alteration has been reported during carcinogenesis in many organs. Carcinoma-associated fibroblasts show a spectrum of differences from their normal counterparts (83–91). The importance of the stromal reaction in malignant breast tumors has been stressed recently (92). Likewise, the importance of stromal change and the role of carcinoma-associated fibroblasts have been demonstrated in prostatic carcinogenesis and tumorigenesis (8, 10, 18, 93, 94). Thus, the pathway to prostatic carcinogenesis appears to entail progressive and concomitant changes in both epithelial and stromal elements with resultant abnormalities in homeostatic communication between abnormal stromal and a genetically altered PRE. Such abnormal cell-cell interactions promote malignant progression (18).

The model described in this report of prostatic carcinogenesis can be extended to the analysis of carcinogenesis in other organs such as the mammary gland, salivary gland, pancreas, and colon. The rescue of organs from embryonic lethal mouse embryos can be achieved for virtually any organ rudiment, provided the embryo survives to at least 12 days of gestation. For prostate and mammary gland, small ductal fragments can be induced to proliferate extensively when combined with UGM in the case of the prostate (42, 43) or grafted into a cleared fat pad in the case of mammary tissue (95). Although not tested, it is likely that similar methods could be developed in which recombination of embryonic homologous mesenchyme with adult epithelium stimulates proliferation of homologous adult epithelial cells. Use of rescued organs from Rb−/− embryos in carcinogenic studies does not necessarily imply a role of the *Rb* gene in carcinogenesis of a particular organ. Instead, the use of Rb−/− tissues provides a well-defined genetic model that rapidly recapitulates the sorts of genetic and phenotypic changes seen in progression of carcinogenesis. Thus, it is quite likely that comparable new models of experimental carcinogenesis could be devised using tissues from embryonic lethal embryos with well-defined genetic defects, specifically those implicated in human prostate cancer. In this regard, it is also technically possible to use tissues from embryonic lethal embryos with either single or double mutants in this assay.

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