Rescue of Embryonic Epithelium Reveals That the Homozygous Deletion of the Retinoblastoma Gene Confers Growth Factor Independence and Immortality but Does Not Influence Epithelial Differentiation or Tissue Morphogenesis*

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The ability to rescue viable prostate precursor tissue from retinoblastoma-deficient (Rb−/−) fetal mice has allowed for the isolation and characterization of the first Rb−/− prostate epithelial cell line. This cell line, designated Rb−/−/PrE, was utilized for experiments examining the consequences of Rb loss on an epithelial population. These findings demonstrated that Rb deletion has no discernible effect on prostatic histodifferentiation in Rb−/−/PrE cultures. When Rb−/−/PrE cells were recombined with embryonic rat urogenital mesenchyme and implanted into athymic male nude mouse hosts, the recombinants developed into fully differentiated and morphologically normal prostate tissue. The Rb−/−/PrE phenotype was characterized by serum independence in culture and immortality in vivo, when compared with wild type controls. Cell cycle analysis revealed elevated S phase DNA content accompanied by increased expression of cyclin E1 and proliferating cell nuclear antigen. Rb−/−/PrE cultures also exhibited a diminished ability to growth arrest under high density culture conditions. We believe that the development of Rb−/− prostate tissue and cell lines has provided a unique experimental platform with which to investigate the consequences of Rb deletion in epithelial cells under various physiological conditions. Additionally, the development of this technology will allow similar studies in other tissues and cell populations rescued from Rb−/− fetuses.

The Rb1 gene product mediates numerous cellular functions including cell cycle regulation (1, 2), maintenance of chromosomal integrity (3, 4), cellular differentiation (5–7), and the survival of epithelial cells (8–11). The Rb gene encodes a phosphoprotein (pRb) that regulates the transition between G1 and S phases of the cell cycle by transducing growth-inhibitory signals that arrest cells in G1 (12–14). Functional regulation of pRb is cell cycle-dependent, being strictly controlled by the activity of cyclin-dependent kinases that regulate the state of pRb phosphorylation. Dephosphorylated pRb inhibits the transcription of genes via its interaction with members of the E2F family of transcription factors. As the cell approaches the G1/S border, cyclin D-ckd4/6 and cyclin E-ckd2 complexes sequentially phosphorylate pRb. These events lead to the release of E2F and subsequent activation of E2F-regulated genes, such as c-myc, cyclin E, PCNA, and DNA polymerase, that are required for entry and activation of the S phase.

The control of G1/S transit provides a dogmatic view of pRb function, as cell cycle regulation is vastly more complex than the simple scheme provided here. The overlapping function of two structurally related family members, p107 and p130, represents an interactive mechanism in which pRb, p107, and p130 share the ability to regulate different members of the E2F family and thus a variety of target genes (13, 15, 16). Although structurally similar, there is growing evidence supporting distinct cellular functions for each Rb family member. All three proteins are differentially expressed during mouse development (17), and their ability to initiate growth arrest is cell type-specific (18). In addition, these proteins preferentially associate with different E2F family members. Although pRb interacts with E2F1–4, there is apparent redundancy in the regulation of both E2F4 and E2F5 by p107 and p130 (reviewed in Ref. 19). The similarities and differences between these proteins are also apparent in mice carrying single or compound knockouts of Rb, p107 and p130. When the Rb gene is deleted through targeted disruption, the embryos die at 13 days of gestation from defective development of erythroid and neuronal tissues (9–11). In stark contrast, targeted disruption of either p107 or p130 does not result in an obvious phenotype, and the mice remain viable (20).

Members of the Rb family are believed to play active roles in tissue development by regulating a postmitotic state required for cellular differentiation (5, 20, 21). The normal differentiation and development of the prostate gland are critically dependent upon androgenic steroids which, following binding to murine, prostate-specific dorsal lateral protein marker; MEFs, mouse embryonic fibroblasts.
the androgen receptor, transactivate or repress a number of transcriptional targets, including cell cycle regulatory genes. It was recently demonstrated (8, 22–24) that pRb is activated during androgen-stimulated epithelial proliferation and during androgen ablation-induced apoptosis. The pRb protein has also been shown to function as a transcriptional co-activator of the androgen receptor (25). Taken together, these results tentatively position Rb as a central mediator of androgen action controlling the differentiation, growth, and death of prostate epithelium; however, this hypothesis has not been tested.

Cell culture on models currently available to study epithelial physiology have a limited scope of relevance with regard to the pRb pathway. Many transformed prostate cell lines, such as DU145, already exhibit nonfunctional pRb due to mutation (26, 27). Viral oncogenes are common tools used to immortalize cells in culture or study cell cycle regulatory mechanisms. The viral oncogenes SV-40 large T antigen, adenosivirus E1A and E1B, and human papilloma virus E6 and E7 target and inactivate the pocket protein family members (pRb, p107, and p130) as well as p53 (28–30). The use of viral oncogenes has provided substantial insight into the function of the Rb family members and their roles in regulating cell cycle, cell growth, and differentiation; however, viral oncogenes are promiscuous in their interactions with other cellular proteins and promote genomic instability making interpretation of these experimental models difficult. The chromosomal imbalances directly influenced by viral oncogenes have been identified as either random or nonrandom genetic events and include gross chromosomal translocations (31–33). Therefore, models that specifically target and inactivate Rb, which minimize complicating genetic alterations inherent with viral oncogenes, might provide novel insight into the physiologic role of Rb in epithelial cells.

The homozygous deletion of the Rb gene results in embryonic lethality due to a variety of developmental abnormalities (9–11). The embryonic lethality of the Rb knockout mutation has prevented the development of Rb−/− epithelial cell lines, and thus many of the functional aspects of pRb have not been independently characterized in this cellular population. Recently, the application of tissue recombination, using fetal tissue rescued from embryonic mice (34). To our knowledge, this is the first in vitro model to allow for the study of targeted Rb deletion on an epithelial population and provides a unique experimental platform with which to investigate the physiologic consequences of Rb deletion on the regulation of cell cycle, differentiation, cell survival, and carcinogenesis.

MATERIALS AND METHODS

PCR Genotyping for Rb−/−—Tissues and Cell Lines—Heterozygous Rb+/− mice were purchased from The Jackson Laboratories (Bar Harbor, ME). To increase litter size and thus the chances of Rb−/− offspring, the heterozygotes were crossed to CD1 mice. The genetic identity of the offspring was confirmed by PCR genotyping to identify the presence of the neomycin selection cassette that was used to disrupt the Rb gene. The Rb heterozygotes were crossed anthe fuses, and the prostatic ductal tips were then recombined as described previously (34). The Rb status of tissue grafts was determined by PCR analysis. Control (The Jackson Laboratories) and experimental DNA samples were amplified using wild type- and mutant-specific primers and analyzed on a 2% agarose gels containing ethidium bromide. The protocol for PCR cycling conditions was obtained from The Jackson Laboratory technical support (micetech@jax.org). PCR primers (The Jackson Laboratories) used for genotyping tissues and cell lines were as follows: Rb knockout allele, forward 5′-AAT TGC GGC CGC ATC TTC ATC GC-3′ (dMR205) and reverse 5′-GAA GAA CGA GAT CAG CAG CAG-3′ (dMR207); Rb wild type allele, forward 5′-AAT TGC GCC CGC ATC TGC ATC TTC ATC GC-3′ (dMR205) and reverse 5′-GCC ATG TTC TGC GGT CCC TAG-3′ (dMR206) (10).

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Serial Recombination of Rb−/− Prostate Tissues—Serial tissue recombination was used to assess the ability of the Rb−/− epithelial cells to undergo multiple rounds of growth and generate immortalized tissue. A ductal tip of ~300 μm was micro-dissected from rescued Rb−/− prostatic tissue (34), recombined with normal rat urogenital mesenchyme (rUGM), and grafted beneath the renal capsule of an intact male athymic mouse host. After 1 month of growth, the host was sacrificed, and the graft was retrieved. The resultant 40 mg of prostatic tissue (38) was again micro-dissected, and another 300-μm ductal tip was recombinated with fresh rUGM and grafted into a second athymic host, resulting in a “second generation” graft. This recombination protocol, repeated 8 times, resulted in ~13 epithelial population doublings for each round of recombination and re-grafting as estimated by the number of epithelial cells from the tissue weight using the Coffey equation 1g = 10^6 cells (39).

Generation of Wild type PrE and Rb−/− PrE Cells—Rb−/− prostatic grafts were established in nude mice, and then the ductal fragments were recombined with rUGM as described previously (34). A portion of each excised graft was fixed for histologic examination, and the rest were utilized to create Rb−/− PrE epithelial cultures. The tissue was minced with a scalpel and forces and plated onto a tissue culture dish coated in a collagen substrate in a minimal volume of medium to allow for attachment of the tissue to the matrix. These tissues were grown in RPMI 1640 (BioWhittaker). Media were supplemented with ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, Collaborative Research), BPE (10 μg/ml bovine pituitary extract, Sigma), epidermal growth factor (10 μg/ml, Collaborative Research), cholera toxin (1.0 μg/ml, Sigma), amphotericin B (250 μg/ml, fungizone, Invitrogen), doxorubicin (5 μg/ml, Sigma), 200 mg/ml-glutamine (Invitrogen), and 100 units/ml penicillin G and 100 units/ml streptomycin (BioWhittaker). This formulation supports the growth of epithelial cells while retarding the growth of the fibroblast cells. Approximately 10–14 days after plating, the tissue pieces were removed from the cultures, and selection was performed with 200 μg/ml G418 (BioWhittaker). Once large areas of epithelial cells became established, cells were passaged 1.3 by trypsinization. Between passage 5 and passage 10, cultures were gradually switched to a medium containing only 5% FBS, l-glutamine (Invitrogen), and 100 units/ml penicillin G and 100 units/ml streptomycin (Invitrogen) in RPMI 1640 termed “5% FBS growth medium.”

Wild type PrE cultures were generated from prostates excised from 6-week-old strain-matched male CD1 mice (Harlan Laboratories) following euthanasia with CO2. Prostate tissues were minced and plated in the defined BPE-containing culture medium described above. These cultures were maintained identical to the Rb−/− PrE described here without the addition of G418.

The wild type control cell line, termed PrE, utilized for comparison herein, spontaneously immortalized in culture and was therefore utilized as a control for spontaneous immortalization of mouse prostatic epithelial cultures.

Species Determination of Cells Utilizing Hoechst 33258 Staining—Mouse Rb−/− PrE cells were grown on Falcon chamber slides to 70% confluence and fixed with 100% ethanol on ice for 5 min before washing with two changes of cold PBS. Fixed cells were then stained with Hoechst 33258 dye (5 μg/ml, Sigma) for 1 min at room temperature. Following staining, cultures were again washed three times in cold PBS, wet-mounted (Biomedica Corp.), and photographed using a Zeiss Axioskop fluorescent microscope to confirm that the cells were of mouse origin (40).

Long Term Serum-free Growth Analysis—Wild type and Rb−/− PrE cells were cultured on 60-mm culture dishes containing 5% FBS growth medium. Three days after plating (termed “Day 0”), culture media were changed to serum-free media consisting of RPMI 1640 (BioWhittaker), 100 units/ml penicillin G, and 100 units/ml streptomycin (Invitrogen) without l-glutamine. Cultures were fed with the aforementioned serum-free medium every 3 days and counted at the indicated times, where each time point is the average and S.D. of triplicate dishes. Viable cell counts were analyzed by trypan blue exclusion. Photographs were taken on a Nikon Diaphot 200 with a Nikon digital camera.

Serial Re-grafting of Rb−/− PrE Cells by Cellular Recombination—At passage 21, Rb−/− PrE cells were utilized to generate prostate grafts via cellular recombination. To prepare grafts, 2.5 × 10^6 urogenital mesenchymal and 1 × 10^5 Rb−/− PrE cells were combined in a collagen matrix as described previously (41). These grafts were then trans-

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planted beneath the renal capsule of an adult, male nude mouse host (Charles River Laboratories) and grown for 1 month. Host animals were then sacrificed, and the grafts were harvested, subjected to fixation, and evaluated utilizing immunohistochemical techniques.

**Immunohistochemistry**—Tissue sections were deparaffinized in Histoclear (National Diagnostic) and hydrated in graded alcoholic solutions and PBS. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 min and washed in PBS prior to staining. Immunocytochemical staining for expression of cytokeratins CK8, CK14, and CK18 in Rb+/− PrE cells was performed as described previously (34). Cells were grown on chamber slides (Falcon) coated with 5 μg/ml fibronectin prior to fixation with 100% ethanol for 5 min on ice. Cells were then washed with PBS, and glucose oxidase substrate was employed in conjunction with the mouse and rabbit staining kits from Vector Laboratories. Staining for the AR and estrogen receptor α (ERα), nDLP, and p63 was also repeated with the re-grafted Rb+/− PrE cells (passage 20). Following growth in nude mouse hosts, the re-grafted tissue was harvested and subjected to the staining procedure as described previously (34, 42).

**Western Blot, Antibodies, and Protein Analysis**—Primary antibodies were obtained as follows: anti-E-cadherin (Transduction Laboratories, C20820), anti-Rb (Pharmingen, 14001A), anti-p107 (Transduction Laboratories, R27020), anti-AR (Santa Cruz Biotechnology, SC-816), anti-estrogen receptors α and β (Santa Cruz Biotechnology, SC-542 and SC-8974, respectively), anti-cyclin D1 (Santa Cruz Biotechnology, SC-8396), anti-cyclin E1 (Santa Cruz Biotechnology, SC-481), anti-PCNA (Santa Cruz Biotechnology, SC-9857), anti-actin (Santa Cruz Biotechnology, SC-1615), and high molecular weight pan-cytokeratin (20622, Dako). Horseradish peroxidase-conjugated secondary antibodies were obtained as follows: donkey anti-mouse (Amresco, E974), goat anti-rabbit (Bio-Rad), and donkey anti-goat (Bio-Rad). For protein analysis, cultured cells were lysed on ice in 50 mM Tris, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, and protease inhibitors (40 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 50 μg/ml aprotinin, 200 μM sodium orthovanadate). Following centrifugation, the supernatants were collected, quantitated using a Bradford microtiter assay, and denatured with a reducing 2× sample loading buffer for 5 min at 100 °C. All proteins were then separated on Tris/glycine precast NOVEX gels and analyzed utilizing the ECL detection system (Amersham Biosciences) as described previously (43).

**Growth Kinetics of PrE and Rb+/− PrE Cells**—Cultures of PrE and Rb+/− PrE were maintained in RPMI 1640 (BioWhittaker) containing 5% dextran-coated, charcoal stripped FBS, termed “5% CCS growth medium,” and compared with cultures grown in 5% FBS growth medium. For these experiments, PrE and Rb+/− PrE cells were plated at a density of 5 × 10⁴ cells into 100-mm culture dishes, and viability and cell number were assessed via trypan blue (Invitrogen) exclusion on various days after plating.

**Flow Cytometric Evaluations**—PrE and Rb+/− PrE (1 × 10⁶, passages 15–20) were plated into 100-mm dishes and analyzed 4 days later to determine log phase cell cycle profiles. To ascertain growth arrest in confluent cultures, cells were plated at a higher density (2 × 10⁶ cells per dish) and retained in culture for a total of 15 days. Serum-containing medium was replaced every 3 days on the long term cultures. Cells were harvested by trypsinization, fixed, and stained with 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI). Prior to examination of cells by flow cytometry, 1 × 10⁶ cells were collected by trypsinization, centrifuged, and reconstituted with 800 μl of PBS (without calcium or magnesium). One drop of an internal trout DNA control (Reiss Enterprise, 1007) was added to each sample. Then, 3.5 ml of cold 100% ethanol was added dropwise while mixing for fixation. Samples were then incubated for 1 h on ice prior to centrifugation and re-hydrated with 1 ml of PBS for 15 min on ice. Again, all samples were centrifuged and reconstituted with 1.5 ml of DAPI staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% Nonidet P-40). A final concentration of 1.0 μg/ml DAPI (Molecular Probes, D-1306) was utilized for flow cytometric evaluations. Flow cytometry was carried out at the University of Michigan Flow Cytometry Core facility using BD Biosciences FACSVantage SE model 127. Data were acquired to 10⁵ events per sample. MultiCycle software (Phoenix Flow Systems, San Diego, CA) was utilized to estimate the percentage of cells in G₁, S, and G₂/M phases of the cell cycle populations.

**Spectral Karyotype (SKY) Analysis and Comparative Genomic Hybridization (CGH)**—Metaphase chromosomes from cultures of wild type PrE and Rb+/− PrE were obtained by mitotic shake off after 1 h of colcemide (1 μg/ml) treatment. Slides were hybridized with SKY kits, prepared from flow-sorted chromosomes, and detected 72 h later as described previously (44). Images of 10–15 metaphase cells were acquired using a DMRXA microscope (Leica, Wetzlar, Germany) equipped with a custom-designed SKY-3 optical filter (Chroma Technology, Brattleboro, VT), a spectral cube, and a charge-coupled device camera (Hamamatsu, Bridgewater, NJ). Analysis was performed with SkyView software (Applied Spectral Imaging, Ltd., Migdal Haemek, Israel) as described elsewhere (45). For CGH analysis, DNA was prepared under high salt conditions. Biotin-labeled DNA was derived from PrE and Rb+/− PrE cultures and co-precipitated with digoxigenin-labeled reference DNA obtained from sex- and strain-matched Rb+/+ and/or Rb+/+ mice. DNA was hybridized to sex-matched normal murine (C57) lymphocyte metaphase chromosomes and detected, and images were acquired with Q-CGH software (Leica Imaging Systems, Cambridge, UK) (46).
RESULTS

Genotype and Phenotype of PrE and Rb−/−PrE Cells—Because the Rb gene was disrupted by the insertion of the neomycin resistance cassette (47), the use of cellular recombination (34), employing neomycin-sensitive, wild type uUGM, and neomycin-resistant Rb−/−PrE mouse epithelium allowed for the specific selection of Rb−/−PrE from the wild type stroma. Following selection on neomycin for several weeks, PCR genotyping revealed that the Rb−/−PrE cells exhibited only the larger 420-bp mutant PCR product compared with the 400-bp wild type PCR product or the mixed PCR products of the heterozygous control cells (Fig. 1A). Western analysis of Rb−/−PrE cells revealed complete loss of pRb protein expression while maintaining E-cadherin (epithelial-cadherin) protein expression (Fig. 1B), confirming the Rb−/− genotype and epithelial lineage of the Rb−/−PrE cells. Rb−/−PrE cultures growing in 5% FBS growth medium were photographed at subconfluent and confluent densities utilizing phase-contrast microscopy (Fig. 1C). Staining Rb−/−PrE cells with Hoechst 33258 dye revealed homogeneous, punctate nuclear patterns characteristic of mouse cells, whereas control rat epithelial cells exhibited a non-punctate staining pattern, confirming that Rb−/−PrE cells were not derived from rat tissues (Fig. 1C).

Analysis of Rb−/−PrE Survival and Growth in the Absence of Serum—Rb appears to play a paradoxical role in the regulation of cell survival, as evidence has emerged supporting both pro- and anti-apoptotic functions of the protein (4). Wild type PrE and Rb−/−PrE cells cultured beyond passage 8 are maintained in media supplemented with 5% FBS. We wanted to determine whether Rb−/−PrE cells could continue to proliferate in the absence of serum containing growth factors and to determine whether the loss of pRb would compromise survival. Viable cell counts of PrE and Rb−/−PrE cultures were documented under serum-free conditions (Fig. 2A). Rb−/−PrE cells remained viable and continued to proliferate for more than 50 days in serum-free media, whereas the wild type PrE cells ceased to proliferate and exhibited a marked loss in viability. These results suggest that the Rb−/−PrE cultures can circumvent cell death programs in the absence of growth factors.

We rationalized that the loss of Rb would allow cells to override growth-restricting or apoptotic mechanisms that may result in immortalization. We could demonstrate that the Rb−/−PrE epithelial cells did not senesce and continued to proliferate for more than 120 passages (Fig. 2B). These results are in contrast to previous studies of Rb−/− mouse embryonic fibroblasts in which these cells senesced at early passage (19, 48). Even though Rb−/−PrE cells are immortal in culture, spontaneous immortalization of murine cells has been reported (49), and we have observed the spontaneous immortalization of approximately half of our wild type PrE cultures (data not shown). To determine that the immortalization of Rb−/−PrE cells resulted from artifactual effects of cell culturing or was a specific outcome of Rb disruption, we examined the immortalization of Rb−/−PrE cells in recombinant tissue grafts in vivo. PrE and Rb−/−PrE grafts were subjected to multiple rounds of serial tissue recombination with uUGM in male athymic-mouse hosts. Serial regrafting was repeated for up to seven additional passages in vivo, for a total of eight in vivo passages. Each in vivo passage (to expand epithelial cell growth) represents ∼13 population doublings. The wild type grafts only proliferated...
and survived through three rounds of serial regrafting. In contrast, the Rb−/− PrE grafts survived eight rounds of recombination in vivo and were still viable at the termination of the experiment (Fig. 2C).

The Loss of Rb Does Not Influence Prostatic Histodifferentiation and Morphogenesis in Vitro and in Vivo—Rb is believed to play an active role in tissue development by regulating a postmitotic state required for cellular differentiation, including androgen-mediated differentiation of prostate epithelium (reviewed in Ref. 50). Morphologic examination and E-cadherin expression in Fig. 1 confirmed the epithelial lineage of the mouse Rb−/− PrE cells. To examine more closely the state of Rb−/− PrE prostatic differentiation in culture cells, we examined a number of prostatic epithelial markers. Cytokeratin expression was assessed in Rb−/− PrE cells revealing low expression of the basal epithelial marker, cytokeratin 14, and high expression of the luminal epithelial marker, cytokeratin 18 (Fig. 3, A and B, respectively), indicating a mixed epithelial population dominated by a luminal phenotype. Western blot analysis of Rb−/− PrE cultures at early and later passage also revealed strong androgen receptor (AR) and estrogen receptor β (ERβ) expression (Fig. 3C), whereas the estrogen receptor α immunoreactivity was negative (data not shown). These results demonstrated that the Rb−/− PrE cells continued to express prostate-specific markers even after many passages in culture.

To determine whether Rb−/− PrE cells could recapitulate prostate histodifferentiation and morphogenesis in vivo, the Rb−/− PrE (passage 20) and wild type PrE (passage 20) were recombined with rUGM and grafted into intact male athymic host mice. Hematoxylin and eosin staining revealed that the wild type rUGM+PrE grafts (Fig. 4A) and the rUGM+Rb−/− PrE grafts (Fig. 4B) were indistinguishable. Both grafts exhibited normal prostate glandular morphology and stained positively for AR and for the murine prostate-specific dorsal lateral protein marker (mDLP), confirming the murine prostate lineage of this graft (Fig. 4, C and D). It has been reported previously (42) that p63, a homologue of p53, is expressed in basal epithelium of glandular tissue, such as the prostate gland, and that p63 is required for prostatic development. Staining with a p63 antibody revealed strong staining in a population of basal cells just beneath the luminal component in both wild type and Rb−/− grafts (Fig. 4, E and F). These findings confirm the ability of Rb−/− PrE cells to recapitulate normal prostatic histodifferentiation and morphogenesis in vivo.

Rb−/− PrE Cells Retain Intact p107/p130 and p53/p21 Pathways—Beyond the established role of pRb in cell cycle arrest, the involvement of p107 and p130 in the inhibition of cell growth has also been documented in several cell types (18). In normal cells the levels of p130 and p107 change dramatically during the cell cycle. When cells are engaged in cycle and moving through S phase, the expression of p130 is low; however, when cells are forced to exit the cell cycle by serum withdrawal or contact inhibition, p130 protein accumulates rapidly (13, 15). The expression of p107 is modulated in an opposing manner to p130. p107 is present at high levels in cycling cells, and like pRB is hyperphosphorylated; however, when cells exit the cycle in response to serum withdrawal or contact inhibition, p107 is rapidly dephosphorylated and protein levels decrease. To determine whether the immortalized, serum-independent phenotype of Rb−/− PrE cells resulted exclusively from Rb loss and not from anionic loss of p107 or p130, we examined the expression of these proteins in control PrE and Rb−/− PrE cells in either subconfluent, serum-free, or high density culture conditions. In subconfluent cultures, the hyper- and hypophosphorylated forms of p107 were detected in the control PrE and Rb−/− PrE cells. Western blot analysis of both PrE and Rb−/− PrE cells revealed that p107 was dephosphorylated, and protein levels were dramatically reduced in serum-free and high density cultures (Fig. 5A). From the same lysates, levels of p130 were slightly elevated in untreated PrE cells compared with Rb−/− PrE but accumulated to the same extent in both cell lines under serum-free and high density culture conditions (Fig. 5B). These results demonstrated that p107 and p130 respond normally to G1 growth arrest signals in Rb−/− PrE cells and wild type PrE control cultures.

Physiologic exit from the cell cycle and the induction of growth arrest have been linked to p53 and p53-dependent cell cycle inhibitors p19ARF and p21Cip1 (51). Cell cycle exit in response to non-physiologic signals such as treatment with ionizing radiation and DNA-intercalating agents have also been shown to be regulated by the p53-dependent activation of p21. To confirm that the immortalizing effects observed in Rb−/− PrE cells was a direct result of Rb loss and not a result of auxillary loss of p53 and/or p21, the expression and functionality of these proteins in Rb−/− PrE cells were examined. Western blot analysis of Rb−/− PrE cells demonstrated that induction of p53 protein was comparable with those observed in wild type cells following UV irradiation (Fig. 5C). Levels of p21 were also induced following UV irradiation in both Rb−/− PrE and wild type cells (Fig. 5D). These results suggest that the p53 pathway is functional in the Rb−/− PrE cultures.

Growth Kinetics of PrE and Rb−/− PrE Cultures—To compare growth characteristics of PrE to Rb−/− PrE cells, proliferation studies were performed on passages 20–22 of these two cell lines. At the indicated times, the cells were trypsinized and counted, and the viability was assessed by trypan blue exclusion assay. Fig. 6 is a representative experiment demonstrating PrE and Rb−/− PrE growth kinetics. The results demonstrate...
that the 5% FBS growth medium supported the growth of both cell lines. Additionally, there was a 2-fold enhanced proliferation rate of the Rb−/−PrE cells over the control PrE cells. To compare the growth kinetics in the absence of hormones and steroids, we performed this same experiment with cells cultured in dextran-coated, charcoal-stripped 5% serum growth medium (5% dextran-coated, charcoal stripped (DCC)). We found that under this culture condition, both cell lines grew equally well in the presence or absence of steroids.

Analysis of Cell Cycle Re-entry in Serum-free Synchronized 

PrE and Rb−/−PrE Cells—We next wanted to investigate the mechanism(s) that may be responsible for the increased growth kinetics of the Rb−/−PrE cells. The expression levels of cell cycle regulatory proteins were assessed in synchronized wild type and Rb−/−PrE cells as these cells re-entered the cell cycle following serum starvation. At the indicated times, the cells were harvested and analyzed for protein expression of cyclin D1, cyclin E1, PCNA, and actin. PrE cells exhibited slightly higher levels of cyclin D1 expression compared with the Rb−/−PrE cells; however, cyclin D1 increased in both lines as they reenter the cell cycle (Fig. 7). In contrast, expression of cyclin E1 and PCNA is significantly elevated in Rb−/−PrE compared with the wild type control. Both PCNA and cyclin E1 have been found in complex with E2F transcription factors at the G1/S phase border of the cell cycle and are believed to play a critical role in the activation of several S phase-specific proteins (52, 53). Therefore, the loss of Rb and liberation of E2F1 likely promotes the expression of E2F target genes, such as cyclin E1 and PCNA, that in turn drive the cells into DNA synthesis, resulting in the enhanced growth kinetics profile, as seen in the Rb−/−PrE cells.

Rb−/−PrE Cells Have an Increased DNA Ploidy—To understand the functional consequences of Rb loss on cell cycle regulation in prostate epithelial cells, we evaluated the potential of these cultures to undergo G1 arrest under high density culture conditions in the presence of serum growth factors. PrE and Rb−/−PrE cultures were grown in 5% FBS and analyzed at subconfluent and high density culture conditions by flow cytometry. At subconfluent culture conditions, there was no significant difference in the distribution of cells in the various phases of the cell cycle between PrE and Rb−/−PrE cells. When PrE and Rb−/−PrE cultures were maintained at high density (15 days) in 5% FBS growth medium, some differences were noted in the DNA ploidy (Fig. 8, C and D). The was an increase in DNA content of Rb−/−PrE cells compared with wild type PrE cells as indicated by the increases of the mean G1 value of 124 versus 194 units in the Rb−/−PrE cells. However, in high density cultures, the distribution of cells in G1 is similar, with PrE and Rb−/− exhibiting 61 and 70% G1, respectively. In multiple experiments, we noted differences in the
distribution of cells in S phase, where the Rb−/−PrE cultures had ~2-fold higher S phase content than wild type PrE cells. Therefore the data shown in Fig. 8 revealed that whereas both PrE and Rb−/−PrE cells have similar G1 distribution, the Rb−/−PrE cells have increased DNA content (ploidy) as compared with wild type.

Karyotyping of Rb−/−PrE Cell Line—To determine the extent of chromosomal abnormalities that may have resulted following Rb loss, chromosomal integrity of the Rb−/−PrE cells was analyzed by spectral karyotyping (SKY). As shown (Fig. 9A), metaphase chromosomes from two cell lines representing an early passage (passage 14) and a later passage (passage 25) were obtained by mitotic shake off and subjected to SKY analysis. Both passages were hypertriploid with gains corresponding to 4–6 copies of chromosome 19 in all cells and a recurring loss of chromosome 4 (2 copies of chromosome 4 in 60% of cells). The Rb−/−PrE cells also revealed recurring but lower level gains of chromosome 15, as well as a rare translocation involving chromosomes Y and 17. The ISCN karyotype of the passage 14 Rb−/−PrE cells showed the following: 59−70 XY, +X[10], +Y[9], der(Y)t(Y;17)(B1;D1) [2], −4[6], +5[5], +8[3], +9[5], +10[5], +11[4], +15[10], +16[4], +17[3], +18[3], +19<x>2[10] (cp10). The ISCN karyotype of 10 cells of the passage 25 Rb−/−PrE was similarly hypertriploid and had an ISCN karyotype as follows: 61−74 XY, +X[8], +Y[6], −4[6], +5[5], −6[3], +7[2], +8[5], +9[6], −10[8], −11[6], +12[5], +13[4], +14[3], −15[3], +15[3], +16[4], +19<x>2[10] (cp10). No cells were karyotypically identical, and each cell exhibited an average of 8 chromosomal gains or losses from a modal number of 3.

To assess the above changes on the DNA level, CGH was performed with DNA extracted from Rb−/−PrE cell lines at passages 10, 20, and 40, and control DNA was extracted from strain-matched wild type mouse DNA. Results showed that all three Rb−/−PrE DNA samples from passages 10, 20, and 40 shared loss of chromosome 4 and gains of chromosome 19, although by passage 40 there was significant amplification of chromosome 19 as well as one copy gains of chromosomes 6, 11, and 15. These data strongly suggest that the homozygous loss of Rb resulted in chromosomal changes including the loss of mouse chromosome 4 and significant gains of chromosome 19 over the wild type control (Fig. 9B and data not shown).

**DISCUSSION**

Until now, the lethal nature of the Rb knockout precluded the establishment of Rb−/−epithelial cell lines, where definitive experiments to investigate physiological roles for Rb in specific epithelial populations could be performed. The current study describes the successful establishment of an Rb−/−prostate epithelial cell line that was rescued from fetal urogenital precursor tissue. The resultant cell line, termed Rb−/−PrE, was utilized for the physiologic examination of Rb deletion in a specific epithelial population.

Historically, the use of transforming oncogenes, such as E1a, E6/E7, and large T antigen, have been useful to address Rb function; however, these reagents do not exclusively target Rb and promote such genomic instability that experimental interpretation is difficult. The chromosomal imbalances directly influenced by viral oncogenes have been identified as predominantly random genetic events (31–33). One of the central objectives of this study was to delineate and characterize the physiological function of pRb in a prostate epithelial population, if possible, with minimal complications of genetic instability inherent in tumor cells and cells transformed with viral oncogenes. Therefore, the state of chromosomal integrity was essential in the characterization of the Rb−/−PrE line.

SKY analysis revealed that the deletion of Rb in prostate epithelium gave rise to aberrations that are consistent with immortalization. Aneuploidy in the Rb−/−PrE cells character-
Figure 9. **SKY analysis of Rb−/−PrE cells.** A, metaphase analysis of passage 40 Rb−/−PrE cell line by spectral karyotyping. Arrows show 6 copies of chromosome 19 in an otherwise near triploid cell line (arrows). Asterisk shows recurrent translocation between Y and 17 seen in 20% of cells. B, CGH analysis showing gains of chromosome 19 and loss of chromosome 4 in passage 40 Rb−/−PrE cells. Computed profiles of chromosome 4 and 19 show the degree of loss or gain, respectively, compared with sex- and age-matched +/− DNA. Mode value is black line, and gain and loss of one copy are depicted as fine green and red lines, respectively. Blue line represents the DNA profile of sample. The green line indicates gains, and the red line indicates loss, shown next to idiom.

Numerous studies (9–11) have suggested that pRb plays an essential role in embryonic development, and the deletion of Rb in a variety of models resulted in marked abnormalities in the differentiation of specific cell types (5–7). In this study, we have demonstrated that despite the lack of pRb protein expression, Rb−/−PrE cells continue to express markers of terminally differentiated prostatic epithelium and that these cells are fully capable of recapitulating normal prostatic morphogenesis *in vivo*, complete with expression of prostate-specific secretory proteins. It has been suggested that cellular differentiation can be divided into the following three general steps: cell cycle exit, protection from apoptosis, and tissue-specific gene expression (58). Our findings suggest that the loss of Rb does override the ability of Rb−/−PrE cells to growth arrest. A role for Rb disruption in immortalization is more strongly suggested by the *in vivo* experiments demonstrating that only the Rb−/−PrE grafts and not wild type grafts were immortal. The Rb−/−PrE cells were also able to survive in serum-free media, a condition that eventually induces cell death in the wild type controls. Finally, we found that Rb loss does not affect the ability of prostate epithelium to undergo normal prostatic histodifferentiation and recapitulate prostate morphogenesis. This is in agreement with another study demonstrating that the loss of Rb did not adversely affect the normal development of murine mammary gland (37).

The Rb gene product regulates the transition between G1 and S phases in the cell cycle and functions in transducing growth-inhibitory signals that arrest cells in G1. Deletion of Rb in a variety of cancer cell types has been associated with deregulated cell cycle control and endoreduplication (59). When PrE and Rb−/−PrE cells were analyzed for differences in expression of cell cycle regulatory proteins, enhanced expression of cyclin E1 in conjunction with increased PCNA levels was noted in the Rb−/−PrE cells, whereas cyclin D1 levels were reduced. Increased expression of cyclin E and PCNA in the Rb−/−PrE cells was likely due to the liberation of E2F and subsequent activation of transcription. The finding that cyclin D1 was repressed in the Rb−/−PrE cultures might be explained by an independent transcriptional mechanism by which E2F-1 and SP1 cooperate to repress cyclin D1 transcription at specific sites in the cyclin D1 promoter (60). The observation that E2F may regulate such opposing outcomes of different cell cycle regulatory targets suggests a clear dissociation of these two pathways in prostate epithelium. The Rb−/−PrE cells are more active in DNA synthesis, and this may be attributed to the increased cell growth kinetics and loss of growth arrest.

The loss of chromosome 4 is a recurring event in both the wild type PrE cells and Rb−/−PrE cells and, at a threshold level, may result in part from unrelated events due to culturing. However, evidence does exist that suggests a selective pressure may, in part, be caused by this Rb null genotype. Although the Rb−/−PrE cells are not completely immune to genetic alterations, the chromosomal changes described here are minimal and reflect a specific increase in chromosome 19. The Rb−/−PrE cells are not prone to the more frequent genetic translocations attained in cells associated with p53 mutation tumorigenesis, viral-oncogenic transformation. The finding is that the Rb−/−PrE line, although susceptible to chromosomal gain, does not exhibit chromosomal rearrangement or translocation due to an intact p53 repair mechanism.
potential of these cells. These results support the hypothesis that the loss of Rb does result in a more proliferative and aneuploid phenotype and contribute to alterations in cell cycle regulatory proteins possibly through a constitutively active E2F1. A downstream effect of this enhanced proliferative activity may result in the compression of the G2/M phase as noted in the Rb−/−PrE cultures at high density. This experiment provides an excellent example of the regulatory control provided by pRb at the G1/S border where, in the absence of pRb, E2F is free to activate genes such as cyclin E1 and PCNA that drive DNA synthesis. These findings suggest that the loss of Rb on a specific epithelial population may circumvent growth inhibitory constraints that support the immortalized phenotype and promote epithelial survival in the absence of growth factors.

Cellular senescence has been associated with a reduction in telomere length. This hypothesis was supported by studies demonstrating a direct link between limited cell division and progressive telomeric shortening. It was also postulated that maintenance of telomere length by telomerase might override senescence. Such a causal role for telomerase in cellular senescence has been demonstrated by transfection of the human telomerase reverse transcriptase cDNA into normal cells resulting in their ability to bypass senescence and prolong the life span in vitro (61). We found that telomerase activity was elevated in both Rb−/−PrE, but because telomerase activity was also elevated in the spontaneously immortalized control wild type PrE cells, it is impossible to conclude that there is a specific regulatory role for Rb in telomerase expression. These results do suggest, however, that telomerase activity may be important in the immortalization of mouse epithelial cells.

The similarities and differences between pRb, p130, and p107 are apparent in mice carrying single or compound knockouts of the corresponding genes. When the Rb gene is deleted through homologous recombination, embryos die at 13 days of gestation from defective development of erythroid and neuronal tissues (9–11). In stark contrast, targeted disruption of either p107 or p130 is not lethal and does not result in an obvious phenotype (20, 62). Recent studies (19, 48) have demonstrated that mouse embryonic fibroblasts (MEFs), harboring a triple knockout of pRb, p107, and p130, are resistant to G1 arrest signals and do not undergo senescence in culture. These cells also exhibit some features of transformed cells, such as focal proliferation on monolayers and anchorage-independent growth; however, individual mutants do not undergo transformation. As with the Rb−/− fibroblasts, Rb−/− prostate epithelial cells do not exhibit characteristics of transformation. Although mutation of individual Rb family members in MEFs resulted in minor alterations in both cell cycle regulation and DNA damage response, these cells still remained sensitive to G1 arrest signals and subsequently entered into senescence. In general, the cell cycle studies of Rb−/−PrE epithelial cells support those findings in MEF cells, in that Rb deletion resulted in only minor changes in cell cycle regulation. Taken together, these results suggest that cell cycle regulation and cellular transformation of Rb−/−MEFs and Rb−/−PrEs are not attained by a single mutation of the Rb gene and is likely influenced by the simultaneous disruption of all three pocket protein family members. In contrast to the findings in Rb−/−MEFs, our results suggested that the loss of Rb does support the immortalization of prostate epithelium, particularly in vivo.

The Rb gene encodes a key regulatory component of the cell cycle that is frequently disrupted in many human cancers, including adenocarcinoma of the prostate gland (reviewed in Ref. 50). In a recently published model of mouse prostate cancer (34), the use of Rb−/−PrE prostatic tissue and its response to hormonal carcinogenesis were described. In that study, Rb−/− prostatic tissue was highly susceptible to hormone-induced malignant transformation. This model has drawn much interest due to the recapitulation of several key features of human prostate cancer, namely in its progression from dysplasia to carcinoma accompanied by the loss of the basal epithelium. The role of pRb in human neoplasia, including prostate carcinoma, has been the subject of rigorous investigation for a number of years; however, the specific function of pRb in the etiology of prostate tumorigenesis has yet to be determined.

If Rb inactivation has a role in epithelial transformation, the following question occurs. Does the inactivation of Rb influence an immortalized phenotype by overriding growth inhibitory or apoptotic mechanisms or does the inactivation of Rb result in alterations that lead to transformation? We hypothesized that the specific loss of Rb in an epithelial population would disrupt the G1 restriction point, allowing these cells to bypass senescence. Our data demonstrated that Rb−/−PrE cells were immortal in vitro and in vivo but were not transformed. The degree of cellular transformation was determined by the inability of Rb−/−PrE cells to form colonies in soft agar, invade through biological membranes, or to induce tumors when injected subcutaneously into nude mice, compared with transformed prostate cancer cells (data not shown). Thus, the loss of Rb may be an event that is required for the immortalization of epithelial cells as other growth-inhibitory pathways, such as p53/p21 and p107/p130, appear intact. It remains to be determined what additional alterations are required on the Rb−/− background to promote full transformation of these cells. Identifying these events in the context of Rb loss would be invaluable in elucidating the sequence of molecular events leading to epithelial transformation.

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Rescue of Embryonic Epithelium Reveals That the Homozygous Deletion of the Retinoblastoma Gene Confers Growth Factor Independence and Immortality but Does Not Influence Epithelial Differentiation or Tissue Morphogenesis

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