

Rescue and Isolation of *Rb*-deficient Prostate Epithelium by Tissue Recombination

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Abstract

The ability to rescue viable prostate precursor tissue from *Rb*^{-/-} fetal mice has allowed for the generation of *Rb*^{-/-} prostate tissue and *Rb*^{-/-} prostate epithelial cell lines. Herein, we provide a protocol for the rescue of urogenital precursor tissue from mouse embryos harboring the lethal *Rb*^{-/-} mutation. The rescued precursors can matured as subrenal capsule grafts in athymic mice. Subsequently prostatic tissue can be used as a source for *Rb*^{-/-} epithelium in a tissue recombination protocol for the generation of chimeric prostate grafts in athymic male mouse hosts. We have also provided a detailed description for isolating and propagating the *Rb*^{-/-} epithelium from such tissue recombinants as established cell lines. Methods for characterizing the grafts and cell lines by determining the retention of prostate-specific epithelial expression markers, including cytokeratins, the androgen receptor, estrogen receptor β and the dorsolateral prostatic secretory protein (mDLP) are given.

Key Words: Retinoblastoma (*Rb*); primary culture; development; genotyping; tissue recombination (TR); prostate; epithelium; differentiation; immortalization.

1. Introduction

Prostate carcinogenesis is a multistep process involving the perturbation of normal stromal-epithelial interactions (1–3) and genetic alterations of the epithelium resulting in activation of oncogenes (4–7) and inactivation of tumor-suppressor

genes (8,9). The involvement of multiple oncogenes and tumor-suppressor genes in carcinogenesis has been demonstrated in many types of human carcinomas (10,11). Alterations in tumor-suppressor genes such as the retinoblastoma (*Rb*) gene have been suggested to play a role in the development of human prostate cancer (8,12–14). The *Rb* gene encodes a 110 kDa phosphoprotein (pRb) that regulates the transition between G1 and S phases in the cell cycle by transducing growth-inhibitory signals that arrest cells in G1 (15). 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. The growth inhibitory function of pRb is attained through signals exerted at the level of gene transcription in association with the E2F family of transcription factors. As the cell approaches the G1-S border, pRb can be sequentially phosphorylated (inactivated) by cyclin D/cdk4/6 and cyclin E/cdk2 complexes, leading to the release of E2F and subsequent activation of E2F-regulated genes that are required for S-phase entry (16). The importance of the *Rb* gene in tumorigenesis was originally recognized in familial retinoblastoma and subsequently the involvement of *Rb* has been described in many human cancers including bladder (17), breast (18–20), and lung cancer (21–23). In human prostate cancer, estimates of the frequency of *Rb* gene mutations and deletions vary widely covering a range from 1–50% of cancer cases (24–32). To some extent this disparity may be a result of *Rb* alterations being infrequent in early human prostate cancer and becoming more common as the disease progresses. However, a review of the literature still shows disparities between estimates at apparently matched disease stages. It is clear though that a subset of human prostate cancer does contain changes at the *Rb* gene and protein levels. The function of *Rb* and its role in human carcinogenesis has been the subject of vigorous investigation for a number of years, however, the specific role *Rb* plays in the etiology of prostate cancer has yet to be determined.

A major obstacle to the investigation of *Rb* in carcinogenesis has been the lethality of the homozygous *Rb* knockout in mice. Mice homozygous for *Rb* disruption (*Rb*–/–) die at 13 d of gestation, several days before the prostate forms. The cause of death, disruption of erythropoiesis and neurogenesis, is unrelated to many of the tumors that could usefully be studied using these animals. At first sight, it would appear problematic to study prostatic carcinogenesis in mice that die before prostatic tissue forms. We have recently overcome this obstacle and have been able to circumvent the lethal phenotype through the employment of tissue rescue and recombination technology (33). Tissue rescue involves grafting organs, or organ precursors, beneath the renal capsule of athymic rodent hosts where they can undergo development to form the tissues of interest. Tissue recombination allows amplification of specific epithelial cell populations from the rescued tissues. This procedure has enabled the isolation of *Rb*–/– prostate

tissues and cells. In this model, pelvic visceral rudiments of E12 *Rb*^{-/-} embryos were grown as subrenal capsule grafts in adult male nude mouse hosts. Following a month of development, prostatic tissue was microdissected and characterized. *Rb*^{-/-} prostatic epithelial cells were then expanded by recombining prostatic ductal tips from the microdissected tissue with rat urogenital sinus mesenchyme and regrafting the resultant recombinants to new male athymic mouse hosts (33). These grafts have been shown to retain multiple molecular markers of prostate epithelium as well as sensitivity to hormones. In the current study, we describe the isolation and characterization of a *Rb*^{-/-} prostate epithelial cell line derived from rescued prostate *Rb*^{-/-} tissue. Thus, these models are the first to allow for the continuous study of targeted *Rb* deletion in a specific nonchimeric organ and cell lines past E12.5 of embryonic development. The *Rb*^{-/-}PrE cell line also provides an excellent experimental platform with which to investigate, for the first time, the physiological consequences of the specific deletion of *Rb* in an epithelial population.

2. Materials

2.1. Tissue Rescue

1. Dissecting instruments (all from Fine Scientific Tools).
2. Large scissors 14054-13.
3. Small scissors 14060-09.
4. Vanna scissors 15100-09.
5. Large forceps 11021-12.
6. Medium forceps 11027-12.
7. No. 3 forceps 11231-30.
8. No. 5 forceps 11252-30.
9. Dissecting scope and light source.
10. 100- and 30-mm Petri dishes—bacteriological dishes are fine for this. They are cheaper than tissue-culture coated plates.
11. Microconcavity slides—These are an off-catalog item from Fisher NC 9583502.
12. Hanks Balanced Salt Solution (HBSS).
13. Syringes Tuberculin type with attached needles (Beckton-Dickenson 309625). Alternatively, 1-mL syringes and 25-gage needles.
14. Sterile Pasteur pipets.
15. Bunsen burner.
16. Blue (1 mL) pipet tips.
17. Calcium/magnesium-free HBSS.
18. Silastic tubing (Fisher Scientific 11-18915G). Now marketed as “laboratory tubing.”
19. Anesthetic: Avertin for Mouse Anesthesia
Stock Solution: 25 g 2-, 2-, 2-Tribromoethanol (Aldrich T4, 840-2), 15.5 mL tert-Amyl alcohol (Aldrich 24, 048-6).

To prepare avertin stock, mix and warm to 40°C to dissolve solid. Do not heat. Tribromoethanol is light sensitive, so cover with foil. When completely dissolved wrap in foil and store at 4°C. If the tribromoethanol recrystallizes, warm again to redissolve. Stock is good for many months.

To make final solution, mix 19.75 mL HBSS with 0.25 mL of avertin stock (you can also use any growth medium, but phenol (ϕ) red is needed to monitor pH). The mixture will have to be gently warmed and stirred to dissolve the stock (45°C in a water bath is fine). Do not heat excessively as the alcohols will vaporize, and the mixture will not work. Heating should be the minimum required to achieve solution. If the mixture becomes acid (yellow), it has been heated too much and should be discarded. When dissolved, store at 4°C. This working stock is good for up to 14 d.

To use, inject intraperitoneally. Dosage is 0.02 mL/g body weight (a 25 g mouse gets 0.5 mL, a 30-g mouse gets 0.6 mL). Mice should go down in 2–3 min and will remain asleep for 30–40 min. Check for response to paw squeezing. Individual responses to anesthetic agents do vary slightly, if necessary administer extra anesthetic (0.05–0.1 mL).

Animal Hosts to receive tissue recombinants.

1. CD1 Nude (Charles River) Ideally, use hosts around 60 d of age. Animals less than 45 d old are rather small for this work.
2. Sterile gauze swabs—(Johnson and Johnson 2318).
3. Betadine—Purdue Frederick.
4. 70% ethanol.
5. Sutures—For preference, #3 silk with a small curved needle. The use of silk provides a visual confirmation that a kidney has been grafted. However, please note that some IACUCs will not approve the use of nonresorbable sutures. (Ethicon Inc.)
6. Wound clipper and clips. (Beckton Dickenson—Clipper 427630, Clips 427631.)
7. Heating pad (Gaymar T/Pump TP-500).

Analgesia: These surgeries are well tolerated and historically analgesia has not been used routinely. However, recently, IACUCs have been insisting upon the use of analgesic agents. For these purposes, the approved protocol is:

1. Drug: Buprenorphine (Reckitt & Colman—NDC 12496-0757-1).
2. Dosage: 0.01–0.05 mg/kg.
3. Route: subcutaneously.
4. Frequency: Once at surgery, additionally as needed.

2.2. Tissue Recombination

1. Pregnant rats: For urogenital sinus dissection.
2. Timed pregnant—plug date is d 0.

3. Rats 18 d gestation. (Outbred strains such as Sprague-Dawley are preferred as these produce larger litters than inbred strains)
4. Agar plates: 1% agar (Difco) 5 mL; 2X DME/H16 3.8 mL; Serum (fetal bovine) 1 mL.
5. Trypsin—Sigma or Difco 1:250 (Sigma T-4799).
6. Make a 10 mg/mL solution in calcium/magnesium-free HBSS.
7. DNase type 1—Sigma (DN-25).

2.3. Primary Cultures

1. RPMI-1640, BioWhittaker.
2. Insulin, transferrin, selenium (ITS) (Collaborative Research).
3. Bovine pituitary extract (BPE) (Sigma).
4. Epidermal growth factor (EGF) (Collaborative Research).
5. Cholera Toxin (Sigma).
6. Fungizone (Gibco).
7. Dexamethasone (Sigma).
8. L-glutamine, penicillin G, and streptomycin (Gibco).
9. G418 neomycin (Gibco).
10. PCR primers (Jackson Laboratories, Bar Harbor, MA).
 - a. *Rb* wild-type forward 5'-AAT TGC GGC CGC ATC TGC ATC TTT ATC GC-3' (oIMR025).
 - b. *Rb* knockout reverse 5'-GAA GAA CGA GAT CAG CAG-3' (oIMR027).
 - c. *Rb* wild-type allele reverse 5'-CCC ATG TTC GGT CCC TAG-3' (oIMR026).

2.4. Characterization of *Rb*–/–PrE Cells

1. pRb antibody 14001A (PharMingen).
2. Donkey antimouse peroxidase conjugated IgG E974 (Amresco).
3. Protease inhibitors; PMSF, leupeptin, aprotinin, sodium orthovanadate.
4. Hoechst 33258 dye (Sigma).
5. Androgen receptor antibody sc-816 and estrogen receptor β antibody sc-8974 Santa Cruz.

3. Methods

3.1. Tissue Rescue

1. Heterozygous (*Rb*+/-) male and female mice can be purchased from the Jackson Laboratory (Bar Harbor, ME) and mated. At 12 d of gestation (plug day denoted as d 0), mothers are sacrificed and fetuses removed and placed under a dissecting microscope where the pelvic visceral rudiments will be removed. This mass of tissue will include the cloaca, and other adjacent organs. This tissue is then grafted beneath the renal capsule of intact male athymic mouse hosts (34).
2. Surgery to the renal capsule is somewhat demanding to learn, but is extremely efficient in terms of graft success. There are two sources of training in the subrenal capsule grafting method that may be of interest. The first is the NIH mammary gland

website, see <http://mammary.nih.gov/tools/mousework/Cunha001/index.html>. The second source of information is a DVD of surgical techniques that resulted from a training course entitled "Techniques in Modeling Human Prostate Cancer in Mice," held at The Jackson Laboratory, Bar Harbor, ME, and supported by the NCI Mouse Models of Human Cancer Consortium. This DVD is available from The Jackson Laboratory.

3. The status of the *Rb* gene in the fetuses will be determined by PCR (see specific protocol in **Subheading 3.4.**, step 1). After 1 mo of growth, the tissue masses are removed from the renal capsule of the nude mouse hosts and the various structures teased apart under a dissecting microscope. The tissues that develop from these grafts include rectum, prostate, and urinary bladder, as well as other closely associated organs, such as seminal vesicles and genital tubercle. The two major glandular structures found are the seminal vesicles and prostate that are easily distinguished by their characteristic structures and by the color of the secretions that they contain. Prostatic ductal structures can be identified grossly within grafts by their glandular features (see **Fig. 1**). Some of this tissue can then be fixed for further immunohistochemical analysis or dissected into small ductal segments for recombination with rat urogenital mesenchyme (rUGM).
4. Although morphologically distinct, the prostatic phenotype of tissues within pelvic visceral grafts should be confirmed by histological and immunohistochemical staining. Tissues can be fixed in 10% formalin overnight, embedded in paraffin, and sectioned on a microtome. Tissue sections can be deparaffinized in Histoclear (National Diagnostic, Atlanta, GA) and hydrated in graded alcoholic solutions and distilled water. Endogenous peroxidase activity should be blocked with 0.5% hydrogen peroxide in methanol for 30 min followed by washing in phosphate-buffered saline (PBS) pH 7.4. Normal goat serum is usually 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.
5. To confirm the histological lineage of the grafts, a number of tissue- and species-specific antigens can be examined by immunohistochemistry. For prostate grafts, antibodies that recognize the mouse dorsolateral prostate secretory protein (mDLP) and mouse seminal vesicle secretory protein can be used at 1:1500 and 1:5000, respectively (**35**), will distinguish between these two tissue components. The employment of antiandrogen-receptor antibody (PA1-111A, 1:100 Affinity BioReagents, Golden, CO) is also useful in determining prostate specific lineage. Monoclonal antibodies specific for mouse cytokeratin 14 and cytokeratin 8 can be used to distinguish basal epithelial cells, which should stain positive for cytokeratin 14 (see **Fig. 2a**), whereas the luminal epithelium should express cytokeratin 8 (see **Fig. 2b**). Mouse anti-PCNA monoclonal antibody (PC-10, 1:200, PharMingen, San Diego, CA) can also be used to determine the epithelial proliferation rate in *Rb*-/- tissues, which has previously been shown to be higher in the knockout cells (**33**). Using the antismooth muscle α -actin monoclonal antibody (A-2547, 1:500, Sigma, St. Louis, MO) and the anti-E-cadherin monoclonal antibody (C20820, 1:200, Transduction Laboratories, San Diego, CA), can be useful in determining if the recombined grafts

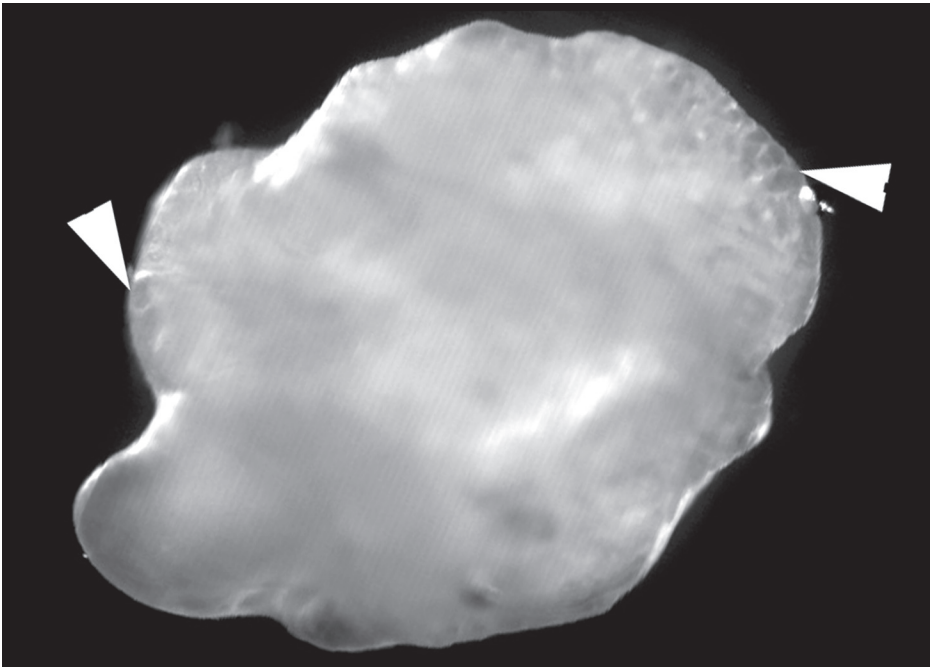


Fig. 1. *Rb*^{-/-} prostatic tissue following rescue and 28 d of growth in an athymic male host. Gross appearance of the *Rb*^{-/-} graft shows a mass of tissue that includes prostate ductal structures (arrowheads).

have achieved normal differentiation and histological architecture. E-cadherin should be observed as strong membranous staining along adjacent epithelial cells (see Fig. 2c). Actin-positive smooth muscle cells (see Fig. 2d) should also surround the epithelial ducts and exhibit an intimate association with the epithelial basement membrane. Following all primary antibody incubations, the sections should be washed carefully and then subjected to a secondary incubation in biotinylated goat antimouse immunoglobulin (diluted with PBS at 1:200, Sigma, St. Louis, MO).

6. After incubation with the secondary antibody, sections are then washed in PBS (three 10-min washes), and incubated with avidin-biotin complex for 30 min at room temperature. After the last PBS wash, the sections should be developed for about 1–5 min using 3, 3-diaminobenzidine (DAB) in PBS and 0.03% H₂O₂. Sections can then be counterstained with hematoxylin, and dehydrated in alcohol. Control sections can be processed in parallel with mouse nonimmune IgG at the same concentration as the primary antibodies.

3.2. Tissue Recombination

1. UGM is prepared from 18-d embryonic Sprague-Dawley rat fetuses (plug date denoted as d 0). For this purpose, urogenital sinuses should be dissected from fetuses and

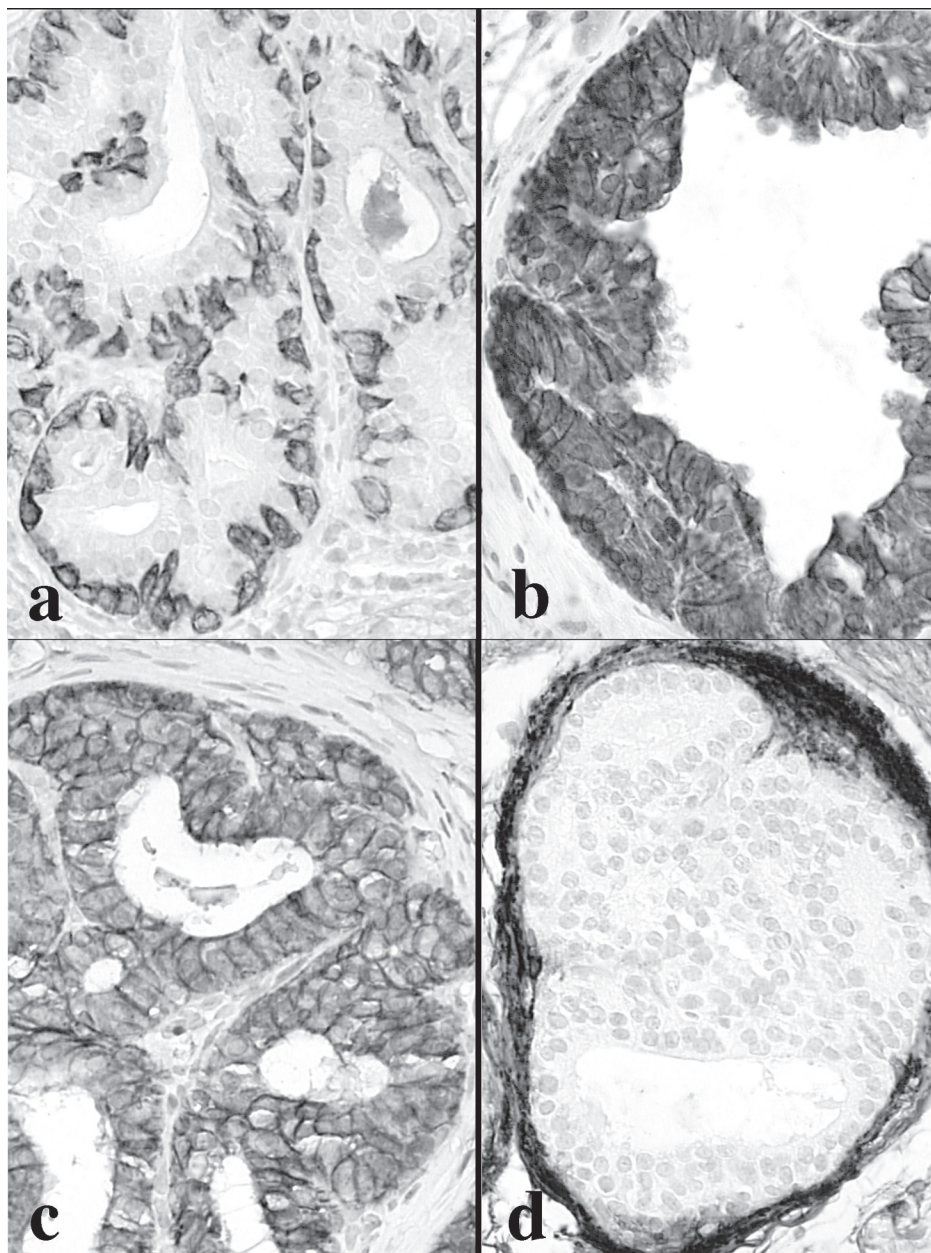


Fig. 2. Rescued *Rb*^{-/-} tissues retains the expression of cell specific antigens. When microdissected and stained these glandular structures express cytokeratin 14 (a), cytokeratin 8 (b), E-cadherin (c) and muscle α -actin (d).

separated into epithelial and mesenchymal components following tryptic digestion and mechanical separation. The dissection of the embryonic urogenital sinus is too lengthy to be discussed in detail here. The specific protocol and diagrammatic depiction of the dissection including the location of cuts and the appearance of the various products are illustrated in **ref. 34**.

2. Following dissection, the urogenital sinuses should be submerged in a 10 mg/mL solution of 1:250 trypsin in calcium/magnesium-free Hanks solution for approx 75 min either on ice or at 4°C. Tryptic digestion is then terminated by washing the sinuses three times in medium containing 10% FBS. The epithelial and mesenchymal tissue layers are separated mechanically using no. 5 forceps and a hypodermic needle.
3. Tissue recombinants are prepared by placing *Rb*+/+ and *Rb*-/- prostatic ductal segments (cut into small 200–500 µm pieces) on top of rUGM in dishes containing nutrient agar: 1% agar (Difco), 2X DME/H16, and FBS. Details for this part of the procedure can be found in **ref. 36**.
4. After 24 h, the tissue recombinants were grafted underneath the renal capsule of intact male athymic mouse hosts. Following 1 mo of growth, the hosts were sacrificed and the grafts harvested. Pieces of graft were fixed for immunohistochemical characterization. The remainder of the grafts can be used as a source material for the generation of cell cultures. The tissue recombination protocol is shown schematically in **Fig. 3**.

3.3. Primary Isolation and Establishment of *Rb*-/- Epithelial Cells

1. To begin the isolation of *Rb*-/- prostate epithelial cells, recombined prostate grafts were excised and cut into small (approx 1 mm³) pieces. A portion of each excised graft should be fixed in formalin for histological examination to confirm prostatic phenotype. The remaining samples will be utilized in the preparation of primary epithelial cultures.
2. The tissue should be minced further with a scalpel and forceps and plated onto tissue-culture plastic (Falcon dishes) or on collagen substrate in a minimal volume of medium to allow for attachment of cells and tissue to the matrix.
3. The culture media should consist of either DMEM (#12-604F BioWhittaker) or RPMI-1640 (#12-702F BioWhittaker). Media should be supplemented with ITS ([5 µg/mL] insulin, [5 µg/mL] transferrin, [5 ng/mL] selenium, #40351 Collaborative Research), BPE ([10 µg/mL] bovine pituitary extract, #P1167 Sigma), EGF ([10 µg/mL] Epidermal Growth Factor, #40001 Collaborative Research), Cholera Toxin ([0.01 µg/mL to 1.0 µg/mL], #C-8052 Sigma), amphotericin B ([250 µg/mL], Fungizone #15295-017 Gibco), Dexamethasone ([5 µM], #D-2915 Sigma), [200 mM] L-glutamine, 100 U/mL penicillin G, and 100 U/mL streptomycin (#25030-081, #15140-148, respectively, Gibco). This formulation supports the growth of epithelial cells while retarding the growth of fibroblast cells.
4. Approximately 2 wk after plating, individual cells will be present radiating out from the tissue pieces. At this point the cells can be removed from the cultures and neomycin selection initiated. At the start, the cells should be selected with 100 µg/mL

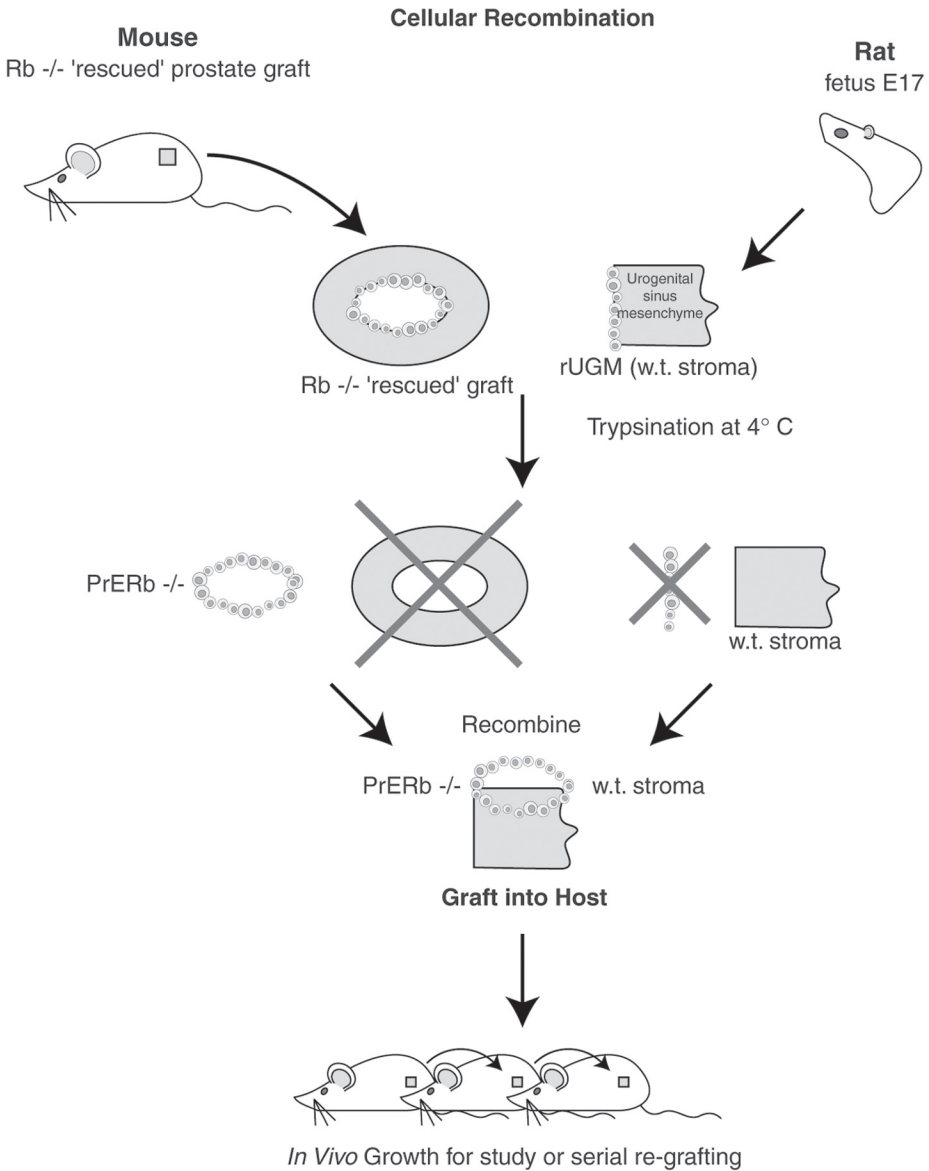


Fig. 3. Schematic of cellular recombination using rUGM and *Rb*-/- prostate epithelium. *Rb*-/- epithelium (*Rb*-/-PrE) isolated from mouse *Rb*-/- rescued prostate tissue are recombined with wild-type rat urogenital sinus mesenchyme (wt stroma) derived from 18-d embryonic rats and grafted beneath the renal capsule of male nude mouse hosts.

G418 (Gibco) and the concentration gradually increased to 200 µg/mL. However the optimal concentration for selection may need to be determined empirically.

5. When large areas of epithelium become established the cells can then be passaged 1:3 by trypsinization and plated into 100-mm dishes under the same neomycin selective pressure (200 µg/mL). At passage 4, the cultures should be completely void of fibroblast cells. At passage 5, 2.5% FBS can be added to the culture medium in addition to the aforementioned growth factors and supplements. At passage 10, the cultures can be weaned off all growth factors and switched to RPMI-1640 supplemented with 5% FBS and 200 µg/mL G418. Cultures can be maintained by splitting once per week at a 1:6 dilution.

3.4. Characterization of *Rb*^{-/-} Cell Lines

1. The genetic status of wild-type or *Rb*^{-/-} grafts and cell lines should be determined by PCR. Preparations of control DNA are commercially available from the Jackson Laboratory and DNA from *Rb*^{-/-} cell lines or tissues can be extracted following standard DNA isolation protocols. Confirmation of the *Rb*^{-/-} genotype during cell isolation or in vivo studies is necessary to assure that the correct cells are employed during the various stages of the experimental protocol. Since the *Rb* gene was disrupted by insertion of the neomycin selection cassette, its presence is indicative of the *Rb*^{-/-} mutation. To distinguish the *Rb*^{-/-} mutation in cultured cells and tissue, the following DNA primers should be used in PCR genotyping. The reverse neomycin-specific primer (oIMR027) binds to its respective sequence in the neomycin resistance cassette and the forward wild-type *Rb* primer (oIMR025) binds to its respective sequence in the remaining *Rb* gene. These primers will amplify a 420 bp product from the mutant *Rb*^{-/-} alleles on a 2.0% agarose gel. The same (oIMR025) primer will be used with another reverse primer (oIMR026) specific for wild-type *Rb* that detects wild-type *Rb* sequence. This primer set will amplify a slightly smaller 400 bp product from the wild-type *Rb*^{+/+} alleles on a 2.0% agarose gel. These primers are also commercially available from the Jackson Laboratory. As shown in **Fig. 4**, PCRs employing these primer sets reveal the larger 420 bp product from DNA isolated from *Rb*^{-/-} cells compared to the 400 bp wild-type PCR product or the mixed PCR products if heterozygous cells are used.
2. Although PCR genotyping should give a clear indication as to the genetic status of *Rb*^{-/-} cells, it is prudent to confirm the loss of *Rb* by examining the expression of the *Rb* gene product, pRb 110 kDa. The pRb-specific antibody 14001A (PharMingen, Torre Pines, CA) is a monoclonal antibody that recognizes both the hyper- and hypophosphorylated forms of *Rb* and works well for Western blot analysis. The donkey antimouse peroxidase conjugated IgG E974 (Amresco) was used as a secondary antibody for the anti-Rb primary. For protein analysis, cultured cells can be lysed on ice in 300 µL of 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EGTA, and protease inhibitors were added fresh: 40 µM PMSF, 5 µg/mL leupeptin, 50 µg/mL aprotinin, 200 µM sodium orthovanadate. Following centrifugation at 15,000g for 5 min in a Eppendorf-style centrifuge, the supernatants were collected, quantitated using a Bradford microtiter assay, and prepared in a reducing

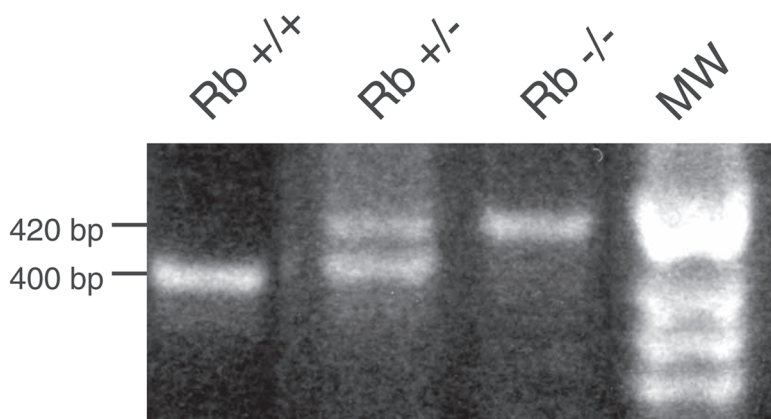


Fig. 4. Verification of *Rb*^{-/-}PrE genotype. Primer sets 025-026 (*Rb*^{+/+}) and 025-027 (*Rb*^{-/-}) amplified only wild-type (wt) alleles (400 bp) from wild-type control cells (*Rb*^{+/+}). The same primer sets amplified wild-type (400 bp) and mutant alleles (420 bp) from heterozygous control cells (*Rb*^{+/-}). Only the mutant allele (420 bp) was amplified with these primer sets from *Rb*^{-/-}PrE cells (*Rb*^{-/-}).

protein sample-loading buffer for electrophoresis. All proteins will then be separated on 6% or Tris/Glycine precast NOVEX gels and analyzed using the ECL (Amersham) detection system. The resulting Western analysis of *Rb*^{-/-} prostate epithelial cells should reveal complete loss of pRb protein expression confirming the *Rb*^{-/-} genotype. As a positive control for the pRb protein and antibody, it is recommended to run the identical amount of lysate from wildtype cells.

3. The use of cellular recombination, employing neomycin-sensitive wild-type rUGM and neomycin-resistant *Rb*^{-/-} mouse epithelium should allow for the specific selection of *Rb*^{-/-} from the wild-type rat stroma or contaminating rat epithelium, however, the absence of rat cells in these cultures should be confirmed by Hoechst 33258 staining. Hoechst staining is widely accepted as a rapid and highly specific method to distinguish mouse cells from contaminating rat cells. Fixed cells should be stained with Hoechst 33258 dye (Sigma, St. Louis, MO) at 5 μ g/mL for 1 min at room temperature. Following staining, cultures will be washed three times in PBS, wet mounted, and examined microscopically to confirm that the cells are of mouse origin. Following staining, cells can be examined and photographed using a fluorescent microscope. Examples of Hoechst-stained *Rb*^{-/-} murine and rat epithelium are shown in **Fig. 5**. Microscopic examination should reveal punctate nuclear patterns in the *Rb*^{-/-} cultures characteristic of mouse cells (see **Fig. 5A**), while control rat epithelial cells exhibited a homogeneous, nonpunctate staining pattern (see **Fig. 5B**).
4. The pRb protein is thought to regulate a postmitotic state required for cellular differentiation and tissue development. However, our initial examination of *Rb*^{-/-}

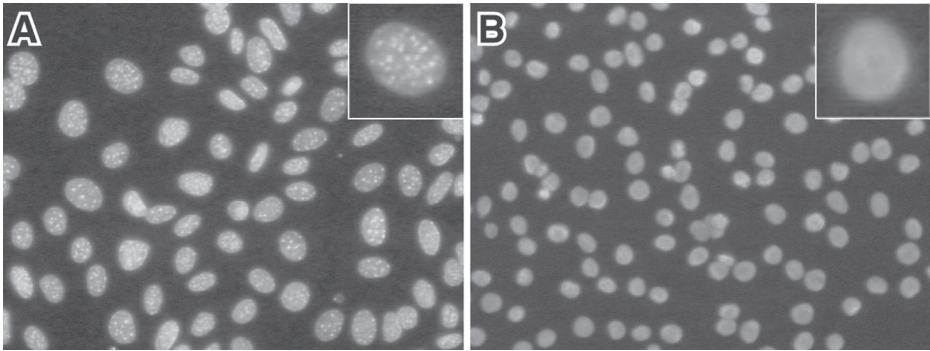


Fig. 5. In vitro characterization of the *Rb*^{-/-}PrE cell line. The punctate staining pattern of the Hoechst 33258 stain is evident in mouse *Rb*^{-/-}PrE cells (A), although absent in control rat epithelium (B).

prostate epithelial cells revealed normal epithelial morphology. Several steps should be taken at this point in the culturing of *Rb*^{-/-} cells to determine their state of differentiation. Initially, simple examination of cellular morphology can reveal much about the cell line in question. Does the phenotype reflect a large, flattened morphology indicative of basal cells or is the phenotype consistent with spindle-shaped luminal cells? The exact delineation of epithelial lineage will require a molecular assessment of the expression of both epithelial and tissue-specific markers. In the case of *Rb*^{-/-} prostate epithelium, a cytokeratin profile can be performed using antibodies directed against cytokeratins 14 and 8 as described for fixed tissue in **Subheading 3**. Immunohistochemistry is an efficient application of these antibodies in cultured cells, allowing for both the evaluation of specific cytokeratins coupled to morphologic profile. As shown in **Fig. 6A,B**, the morphology of the *Rb*^{-/-} cultures suggests a mixed epithelial phenotype. Using cytokeratin-specific antibodies in conjunction with cell morphology revealed large, flattened cells that were strongly positive for cytokeratin 14 expression, suggestive of a basal epithelial phenotype (see **Fig. 6C**). The predominant spindle-shaped cells, expressing cytokeratin 8 are likely of luminal epithelium origin (see **Fig. 6D**). Expression of prostate-specific antigens, such as the androgen receptor (sc-816 Santa Cruz, Santa Cruz, CA), estrogen receptor β (sc-8974 Santa Cruz) or mouse dorsal lateral protein (35) can also be examined by Western blot analysis (see protocol above). Western blotting of *Rb*^{-/-} cultures and tissues are useful when quantitative analysis of proteins is desired.

5. All indications from experiments examining differentiation markers of cultured *Rb*^{-/-} prostate epithelial cells demonstrated that these cells have not undergone any dramatic dedifferentiation as a result of *Rb* loss. Although somewhat involved, examining the ability of *Rb*^{-/-} cells to differentiate and develop into normal prostate tissue, following multiple passages in culture, is the best confirmatory test to determine the role of *Rb* in cellular differentiation and tissue morphogenesis. We

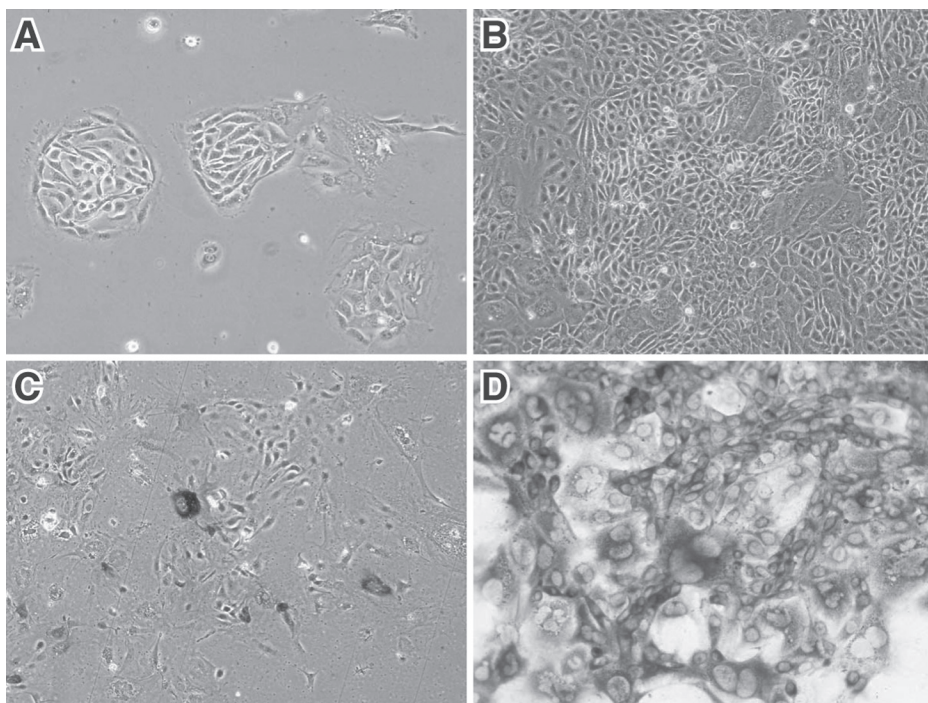


Fig. 6. In vitro characterization of the $Rb^{-/-}$ PrE cell line. Phase-contrast microscopy of subconfluent (A) and confluent (B) cultures of $Rb^{-/-}$ PrE. $Rb^{-/-}$ PrE cultures immunostained with antibodies against cytokeratin 14 (C) and cytokeratin 8 (D).

have determined that $Rb^{-/-}$ PrE cells could recapitulate prostate tissue *in vivo*. These same experiments can be achieved by combining $Rb^{-/-}$ PrE (passage 15) with rUGM, grafted to intact male athymic mouse hosts for 8 wk at which time the $Rb^{-/-}$ genotype can be confirmed by PCR. The prostate grafts are microdissected and used in a second round of recombination and regrafting in a male athymic host to produce a second-generation graft. Prostatic histodifferentiation of these serial regrafts can then be compared to wild-type grafts. As shown in Fig. 7, microscopic examination revealed normal prostate glandular morphology. In addition the expression of prostate-specific antigens, such as the androgen receptor and mouse dorsal lateral protein can be examined to confirm the murine prostate lineage of the grafts.

3.5. Summary

Due to the lack of a viable *Rb* knockout, experiments to examine the physiologic function for *Rb* in the prostate gland have not previously been accomplished. The technology outlined in this chapter describes innovative models

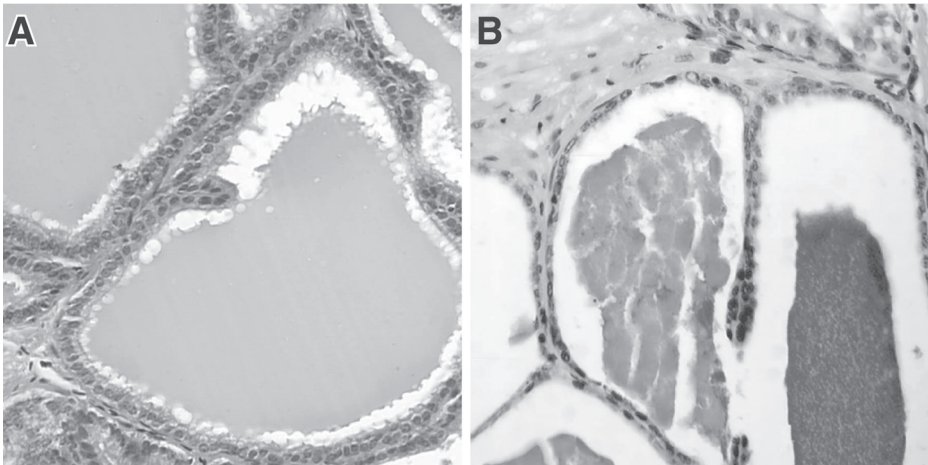


Fig. 7. *Rb*^{-/-}PrE cells recapitulate prostatic histodifferentiation in vivo. Wild-type *Rb*^{+/+} (A) and passage 15 *Rb*^{-/-}PrE cells (B) form normal prostate ductal architecture in the tissue recombination.

employing tissue and cellular recombination to analyze homozygous deletion of the *Rb* gene in prostate tissue grafts and cell lines. These models are the first to allow for the continuous study of targeted *Rb* deletion in specific non-chimeric organs past E12.5 of embryonic development and provide excellent experimental platforms with which to investigate the physiological consequences of *Rb* deletion on an epithelial population. Although more conventional methods using transforming oncogenes have undoubtedly been useful in *Rb* research, these reagents do not specifically target *Rb* and result in such severe genetic instability that mechanistic interpretation becomes difficult. These models will be used to generate data on many aspects of *Rb* function. They have the potential to reveal novel functions and downstream targets of *Rb* and to elucidate the role of *Rb* in epithelial signaling, differentiation and death. Similar approaches could also be applied to a range of late embryonic or perinatal-lethal gene mutations and knockout mice.

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