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pRb-Independent Growth Arrest and Transcriptional Regulation of E2F Target Genes[1]

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Abstract

The retinoblastoma tumor suppressor (pRb) has traditionally been studied as a negative regulator of cell cycle progression through its interactions with the E2F family of transcription factors. Utilizing prostate epithelial cell lines established from *Rb*$^{+/+}$ and *Rb*$^{-/-}$ prostate tissues, we previously demonstrated that *Rb*$^{-/-}$ epithelial cells were not transformed and retained the ability to differentiate *in vivo* despite the lack of pRb. To further study the effects of pRb loss in an epithelial cell population, we utilized oligonucleotide microarrays to identify any pRb-dependent transcriptional regulation during serum depletion–induced growth arrest. These studies identified 120 unique transcripts regulated by growth arrest in *Rb*$^{+/+}$ cells. In these wild-type cells, the majority (80%) of altered transcripts were downregulated, including 40 previously identified E2F target genes. Although the transcriptional repression of E2F target genes is characteristic of pRb pocket protein family activity, further analysis revealed that, compared to *Rb*$^{+/+}$ cells, *Rb*$^{-/-}$ cells exhibited a nearly identical response for all transcripts including those of E2F target genes. These findings demonstrate that pRb is not strictly required for the vast majority of transcriptional alterations associated with growth arrest.

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Introduction

Inactivation of the *Rb* gene has been implicated in the development of many human cancers, including prostate cancer [1]. The protein product (pRb) of this gene is a nuclear phosphoprotein associated with G_1/S phase cell cycle transition [2–4], cellular differentiation [5,6], senescence [7], and apoptosis [8,9]. The molecular mechanism underlying pRb function appears to be considerably complex as pRb interacts with a medley of cellular proteins (see Ref. [10] for a review). Despite these many diverse interactions, pRb has traditionally been viewed as a repressor of E2F transcription factors and thereby a transducer of growth-inhibitory signals that result in a G_1 phase growth arrest.

The E2F transcription factor family consists of seven proteins (E2Fs 1–7) and their heterodimeric binding partners DP1 and DP2 (see Ref. [11] for review). E2Fs 1–5 contain conserved transactivation and Rb pocket protein interaction domains; however, despite this fact, E2F4 and E2F5 primarily exhibit features of transcriptional repressors being found predominantly in complex with pocket proteins and chromatin-modifying enzymes during G_0/G_1 when E2F target genes are repressed [12]. E2Fs 1–3, on the other hand, are effective transcriptional activators [13–15]. Through an intricate interplay of cell cycle–regulated activation and repression, the E2F family mediates the transcription of an extensive list of genes, many of which are required for DNA replication and cell cycle progression (i.e., cyclin E, Cdc2, DNA Pol A2, and topoisomerase 2A). Recently, microarray studies have delineated additional roles for E2Fs in the regulation of many genes involved in other phases of the cell cycle (i.e., G_2/M) [16,17].

Regulation of E2F activity is not only maintained by pRb, but also by the other pocket protein family members p107 and p130. The three pocket protein family members share a common pocket domain that interacts with proteins containing an LXCXE recognition motif [10]. Despite their similarity, the different pocket proteins exhibit specificity in their binding to various E2F family members. In general, the activating E2Fs (E2Fs 1–3) are principally regulated by pRb, E2F5 is bound by p130, and E2F4 can be found interacting with pRb, p107, or p130 [18]. E2F6 and E2F7 do not contain the pocket protein interaction domain and have not been shown to bind any of the pocket proteins [19].

Although pocket protein function has been studied through multiple approaches, homologous recombination at individual pocket protein loci has provided significant insights into pRb, p107, and p130 function. To date, knockout studies have been

Abbreviations: *Rb*, retinoblastoma tumor suppressor gene; pRb, retinoblastoma protein; wtPrE, wild-type prostate epithelium; Rb$^{-/-}$PrE, *Rb*$^{-/-}$ prostate epithelium; MEF, murine embryonic fibroblast; DAPI, 4′,6-diamidino-2-phenylindole; BrdU, bromo-deoxyuridine

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performed for the *Rb1*, *Rbl1* (p107), and *Rbl2* (p130) loci, both individually and in a wide variety of combinations in mice [20–27]. Homozygous inactivation of *Rb* led to embryonic lethality at E13.5 due to defects in the central nervous system and erythropoiesis. However, studies with chimeric mice and heterozygotes ($Rb^{+/-}$) suggested that pRb is only necessary for the development of specific cell lineages and promotes tumors only in the pituitary and thyroid glands following loss of heterozygosity of the wild-type *Rb* allele [21,25,27,28]. Mice lacking either p107 or p130 did not exhibit overt phenotypes [22,24]. However, combinatorial deletions of pocket proteins ($p107^{-/-}Rb^{-/-}$, $p130^{-/-}Rb^{-/-}$, or $p107^{-/-}$ $p130^{-/-}$) resulted in earlier embryonic lethality than the single $Rb^{-/-}$ mutant and an overall increased severity of phenotypes, suggesting that compensation may occur among the pocket protein family members when one of the three members is absent [24,26,29]. Studies with murine embryonic fibroblasts (MEFs) generated from these knockout animals also suggested the existence of compensatory mechanisms. Loss of pRb, p107, or p130 alone failed to cause major differences in cell cycle profiles of these cultures compared to wild-type MEFs [30–32]. However, a triple knockout of pRb, p107, and p130 together evoked convincing loss of G_1 growth arrest function and immortalization [31,32].

Until recently, studies with $Rb^{-/-}$ samples have been primarily limited to MEFs due to the early embryonic lethality of the *Rb* mutation. To study the effect of homozygous loss of *Rb* in a mature tissue, Wang et al. [33] rescued viable urogenital tissue from E11 $Rb^{-/-}$ embryos through implantation of pelvic visceral rudiments into nude mice followed by tissue recombination with rat urogenital mesenchyme for continued expansion of these tissues. Consistent with the role of pRb as a tumor suppressor, prostate tissue lacking pRb exhibited significantly increased sensitivity to estradiol and testosterone-induced tumorigenesis compared to wild-type tissues [33].

Recently, we have generated $Rb^{-/-}$ prostate epithelial cell lines (termed $Rb^{-/-}$PrE) from $Rb^{-/-}$ prostatic tissues rescued by tissue recombination [34]. Although these $Rb^{-/-}$ prostate epithelial cells have the ability to survive for extended periods of time under low-serum conditions, they remain capable of inducing a G_1 growth arrest when grown to confluence. Therefore, we hypothesized that although $Rb^{-/-}$PrE exhibited considerable similarity to wild-type prostate epithelial cells (wtPrE) in several regards, differences that distinguish $Rb^{-/-}$PrE from wtPrE may exist at the molecular level. To assess the gene expression profiles of $Rb^{+/+}$ and $Rb^{-/-}$ prostate epithelial cells, we have utilized microarray analysis in an attempt to identify critical differences that exist between cells of these two genotypes.

Materials and Methods

Cell Culture and Treatments

wtPrE ($Rb^{+/+}$) and $Rb^{-/-}$PrE prostate epithelial cell lines have been previously described [34]. Both cell lines were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, and penicillin/streptomycin. For cell cycle analyses, cell lines were plated into 100-mm dishes and allowed to adhere for 24 hours. Cells were then washed once with phosphate-buffered saline (PBS) prior to the addition of RPMI 1640 with no serum supplement. Twenty-four or 48 hours posttreatment, cells were harvested for viable cell counts with trypan blue, cell cycle analysis, or RNA/protein isolation as described below.

Flow Cytometry

A total of 1×10^6 wtPrE or $Rb^{-/-}$PrE cells was grown in the presence or absence of serum for 48 hours, as described above. Following treatment, cells were harvested by trypsinization and fixed with 80% ethanol in PBS for 1 hour on ice. Cells were then rehydrated in PBS for 15 minutes and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride (Molecular Probes, Eugene, OR) for 15 minutes at room temperature. DAPI-stained cells were then detected on a FACSVantage SE Model 127 (BD Biosciences, San Jose, CA) and analyzed for cell cycle distribution with the MultiCycle software (Phoenix Flow Systems, San Diego, CA) by the University of Michigan's Flow Cytometry Core facility.

Western Blot Analysis

wtPrE and $Rb^{-/-}$PrE cells were harvested by mechanical disruption with cell scrapers followed by gentle centrifugation. Cell pellets were then lysed in appropriate volumes of lysis buffer [50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EGTA, 100 μg/ml phenylmethanesulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 1.0 mM sodium orthovanidate] for 1 hour on ice. Cellular debris was then removed by centrifugation, and supernatants were collected and quantitated using a Bradford protein assay (BioRad). Equal amounts of protein were then separated on precast Tris–glycine SDS polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked for 1 hour at room temperature in TBST [10 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Tween 20] containing 10% nonfat dry milk before being probed overnight at 4°C with primary antibodies diluted in 2.5% milk/TBST. Following three washes in 2.5% milk/TBST, membranes were incubated for 1 hour at room temperature with appropriate horseradish peroxidase–conjugated secondary antibodies. Following a second set of three washes in 2.5% milk/TBST, membranes were developed with ECL detection reagents (Amersham Pharmacia Biotech). Primary antibodies were obtained as follows: pRb (no. 554136; Pharmingen, San Diego, CA), p107 (sc-318; Santa Cruz Biotechnology, Santa Cruz, CA), and p130 (R27020; Transduction Laboratories, Lexington, KY).

RNA Isolation and Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 3×10^6 cells utilizing Qiagen's RNeasy Mini kit. Following quantification, 2 μg of total RNA was combined with 2.5 μM oligo $(dT)_{20}$ primers,

cDNA synthesis buffer (Invitrogen), 5 μM DTT, 40 U of RNase OUT (Invitrogen), 1 mM dNTP mix, and 15 U of Thermoscript Reverse Transcriptase (Invitrogen) to reverse-transcribe mRNA for 1 hour at 55°C. Total cDNA was then utilized in standard PCR reactions with gene-specific primers. To ensure that samples were within the linear range of amplification when analyzed, aliquots were removed from each reaction following PCR cycles 25, 28, 31, and 34, and electrophoresed on 1.2% agarose gels containing ethidium bromide. A 59°C annealing temperature was utilized for all PCR reactions. Primers for each gene are as follows: actin: forward 5′-gctcttttccagccttcctt-3′/reverse 5′-tgatccacatctgctggaag-3′; Ccnb1rs1: forward 5′-ggttgataatccctctccaagc-3′/reverse 5′-cattcttagccaggtgctgc-3′; Cdc2a: forward 5′-ccgtcgtaacctgttgagtaac-3′/reverse 5′-gacaggaagagagccaacgg-3′; Cdc25c: forward 5′-gccttgacctttcgaatcttag-3′/reverse 5′-cagaggaacacaatgatgactc-3′; c-Fos: forward 5′-ccaagtgccggaatcggagg-3′/reverse 5′-gcttcagggtaggtgaagaca-3′; c-Myb: forward 5′-ctataacgacgaagaccctgag-3′/reverse 5′-gaatggtgtgggagttcgag-3′; Cycb2: forward 5′-gacgtatgcgtgccatcctg-3′/reverse 5′-cttcaggagtctgctgctggc-3′; DNA primase p46: forward 5′-gatgacgtaaggagatgctgc-3′/reverse 5′-cgataggcacagaaatccgac-3′; Fen1: forward 5′-gagtctgtggagctgaagtgg-3′/reverse 5′-cacttggaggcacaggtcag-3′; follistatin: forward 5′-gttccaacatcacctggaagg-3′/reverse 5′-ggaaagctgtagtcctggtc-3′; HPRT: forward 5′-cagtacagccccaaaatggt-3′/reverse 5′-ttactaggcagatggccaca-3′; Ifi203: forward 5′-gcctccagaatcctcataagtc-3′/reverse 5′-ctgacattggtgagtcgtcca-3′; Ki67: forward 5′-gaagggcactctccgctcag-3′/reverse 5′-ggttggttcctcctgccagt-3′; Mcmd: forward 5′-gaccaaggcatttaccagaac-3′/reverse 5′-attgatgtctccacggatgtg-3′; Nupr1: forward 5′-gaggatgaagatggaatcctgg-3′/reverse 5′-ctgcttcttgctcccatcttg-3′; Plk1: forward 5′-gtatgtacgggggcgctttc-3′/reverse 5′-gtcacacagctgatacccaag-3′; proliferin: forward 5′-ccatgtgtgcaatgaggaatgg-3′/reverse 5′-gtctaggcagctgatcatgcc-3′; Rrm2: forward 5′-cgccgagctggaaagtaaag-3′/reverse 5′-gacaattcatggtgtagccag-3′; tenascin C: forward 5′-cagccattgccactgtggacag-3′/reverse 5′-gtctccaaacccagcagcatag-3′; and thymidylate synthase: forward 5′-gcaggcacgatacagcctgag-3′/reverse 5′-cactgaaagcactctaaacagc-3′.

Microarray and Data Analysis

Total RNA from wtPrE and $Rb^{-/-}$PrE cells cultured in the presence or absence of 5% FBS for 48 hours was obtained with Qiagen's RNeasy Mini kit according to the manufacturer's protocol. Two 100-mm culture dishes of each cell line were pooled to generate each sample and each condition was analyzed by microarray analysis a minimum of two times (nine arrays total: 3 wtPrE + serum, 2 wtPrE − serum, 2 $Rb^{-/-}$PrE + serum, and 2 $Rb^{-/-}$PrE − serum). RNA quantity and quality were assessed with Agilent (Palo Alto, CA) RNA 6000 Nano Eukaryote total RNA LabChips run on an Agilent 2100 BioAnalyzer at the University of Michigan's Cancer Center DNA Microarray Core facility. Fragmented biotin-labeled complementary RNA was generated from 10 μg of total RNA and hybridized to Affymetrix (Santa Clara, CA) Murine Genome U74Av2 Arrays according to the manufacturer's suggested protocol at the University of Michigan's Cancer Center DNA Microarray Core facility. These Affymetrix GeneChips are capable of measuring the expression levels of 12,422 sequences including ~6000 functionally characterized sequences from the mouse UniGene database (build 74) and ~6000 expressed sequence tag clusters. Arrays were scanned with an Affymetrix GeneChip Scanner and the resulting .cel files were analyzed with dChip (www.dchip.org) to generate expression values for each probe set and 90% confidence intervals for sample comparisons. Probe sets with expression values ≤50 were not considered to be significantly above background signal and were therefore not considered further. Probe sets exhibiting a ≥2.0 lower bound of the fold change confidence interval following serum withdrawal were considered to be significant. Microarray data are available online at NCBI Gene Expression Omnibus (http://www. ncbi.nlm.nih.gov/geo/) accession no. GSE934.

Results

$Rb^{-/-}$PrE Cells Retain the Ability to Perform Growth Arrest Under Serum-Depleted Conditions

Transduction of growth-inhibitory signals, which result in growth arrest in the G_1 phase of the cell cycle, is believed to be the primary role of pRb [35]. To investigate the role of pRb in the regulation of serum withdrawal–induced growth arrest, we employed murine epithelial cell lines established from $Rb^{+/+}$ or $Rb^{-/-}$ prostate tissues [34]. We have previously confirmed the genotypes of all cell lines utilized in this study by PCR and Western blot analysis [34].

As expected, the proliferation rates of wtPrE and $Rb^{-/-}$PrE were identical under log-phase growth conditions in the presence of serum when pRb was inactivated by cyclin dependent kinase–mediated phosphorylation (Figure 1*A*). When serum was removed from the culture media, $Rb^{+/+}$ cells growth-arrested rapidly with minimal increase in cell number after 24 and 48 hours. Surprisingly, the growth rate of $Rb^{-/-}$ cell lines was also dramatically decreased when serum was removed from cultures for the same period of time (Figure 1*A*). To examine the cell cycle distribution of wtPrE and $Rb^{-/-}$PrE under these short-term serum-free culture conditions, cells were stained with DAPI as a measure of DNA content and subjected to flow cytometry. In agreement with the aforementioned growth kinetics data, cell cycle analysis revealed very similar profiles for both wild-type and knockout cell lines (Figure 1*B*). When serum was removed from the culture media, both cell lines exhibited consistent 10% to 15% increases in G_1 phase populations and corresponding 10% to 15% decreases in S phase populations (Figure 1*B*).

Considering the surprising similarity between the growth kinetics and cell cycle profiles of $Rb^{+/+}$ and $Rb^{-/-}$ prostate cell lines, we examined the response of the pocket protein family members, pRb, p107, and p130, in both cell lines following serum depletion. As expected, pRb transitions from

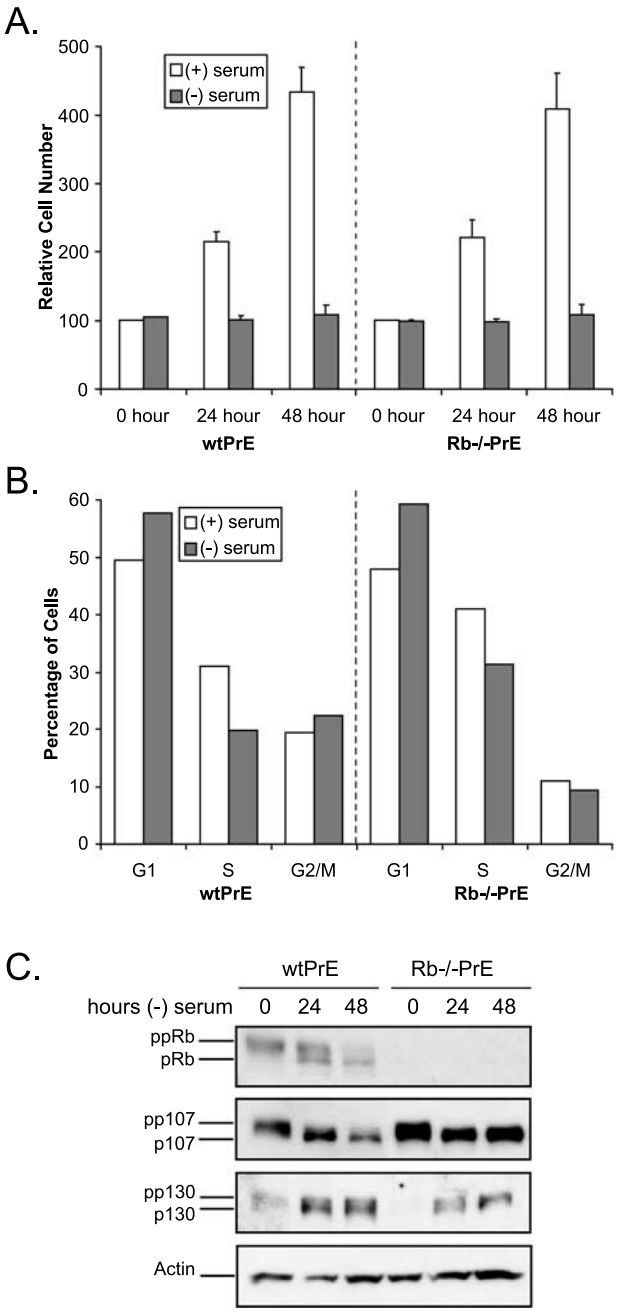

Figure 1. *Growth kinetics and cell cycle analysis of wtPrE and $Rb^{-/-}$PrE. $Rb^{+/+}$ and $Rb^{-/-}$ cell lines were cultured in the presence or absence of 5% FBS supplement and analyzed by (A) trypan blue staining to determine viable cell number at 0, 24, and 48 hours; (B) DAPI staining to determine cell cycle populations at 48 hours; and (C) Western blot analysis for pRb, p107, and p130 at 0, 24, and 48 hours.*

a hyperphosphorylated inactive state to a dephosphorylated active state over a 48-hour period of serum-free culture (Figure 1*C*). Consistent with previous findings [34,36], wtPrE cells exhibited decreased levels and potent dephosphorylation of p107, but increased levels of p130 following serum withdrawal. Although the $Rb^{-/-}$PrE cells did not express detectable levels of pRb due to the insertion of stop codons in the third exon of the *Rb1* gene [21], they continue to express p107 and p130. These two pocket proteins exhibited identical growth arrest–induced changes as those observed in the pRb-containing wtPrE cells (Figure 1*C*). Levels of p107 protein were approximately two-fold higher in $Rb^{-/-}$ compared to $Rb^{+/+}$ cells. Taken together, these findings suggest that similar growth arrest mechanisms exist in both cell lines independent of *Rb* gene and protein status.

The Loss of pRb Does Not Alter the Transcription of Cell Cycle–Regulated Genes

The most prominently studied mechanism by which pRb regulates cell cycle arrest is regulation of transcription through interaction with the E2F family of transcription factors. Therefore, a global inspection of gene expression alterations induced by serum depletion and growth arrest was performed with 12.4K Affymetrix GeneChip arrays. Wild-type and $Rb^{-/-}$PrE cells were cultured for 48 hours in the presence or absence of 5% FBS prior to RNA extraction. This timepoint was selected as pRb and p107 were ~50% dephosphorylated after 24 hours and nearly 100% dephosphorylated by 48 hours in wtPrE cells cultured in the absence of serum. A minimum of two biologic replicates was performed for each cell line/treatment combination (wtPrE + serum, wtPrE − serum, $Rb^{-/-}$PrE + serum, and $Rb^{-/-}$PrE − serum). Data from the scanned arrays were converted to gene expression values, and 90% confidence intervals were generated for growth-arrested *versus* control comparisons with dChip software. Genes with a signal intensity ≥50 and a lower bound of the confidence interval ≥2.0 were considered to be significant. These analytical criteria identified 120 unique affected transcripts, of which 24 (20%) transcripts were reproducibly upregulated and 96 (80%) transcripts were consistently downregulated during growth arrest in wtPrE (Table 1).

Clustering of the genes based on the functional roles of their protein products revealed that a large proportion of the transcripts (38 of 85 genes with known function) was previously characterized as playing roles in cell cycle progression or being cell cycle–regulated [37–39]. These genes included many involved in DNA replication (e.g., Rrm2, Rrm1, Top2a, Mcm3, Mcm5, and Pole2) and mitosis and cell division (e.g., Plk1, CycB1, CycB2, Rab6kifl, Kif8, Kif4, Cdc2a, and Cdc25c). Additionally, several genes involved in the regulation of transcription (e.g., c-Fos), chromatin structure (e.g., Ezh2), DNA repair (e.g., Rad51), and cytoskeletal structure and adhesion (e.g., claudin 1 and tenascin C) were also identified as being altered following serum depletion.

Interestingly, when the gene expression alterations induced in wtPrE and $Rb^{-/-}$PrE cells by serum depletion were compared (Figure 2), patterns of repressed and activated genes were highly conserved in the $Rb^{-/-}$PrE cells despite the absence of pRb. To quantify the similarity for each transcript between the two cell lines, a ratio was generated by dividing the average change in gene expression following serum depletion observed in the wtPrE cell line by the average change in gene expression observed in the $Rb^{-/-}$PrE cell line (Table 1). Of the 120 unique transcripts, only 14 exhibited a ≥2.0-fold difference between the two cell lines and, of these, only six transcripts exhibited a ≥2.5-fold

Table 1. Comparison of Gene Expression Alterations in wtPrE and $Rb^{-/-}$PrE Cells Following Serum Withdrawal.

Accession	Locus Link	Gene	wtT/wtU*	KOT/KOU	Fold Change	Repressed by p16/pRb†	Bound or Regulated by E2Fs	Cell Cycle–Regulated
Cell cycle–related (41 unique transcripts)								
(A) DNA replication/chromosomal assembly/S phase progression (19 unique transcripts)								
M14223	20135	Ribonucleotide reductase M2 (Rrm2)‡	−5.69	−5.25	1.08	a,b	c,d	c
M12848	17863	Myeloblastosis oncogene (*c-Myb*)‡	−5.08	−3.15	1.61			
U01915	21973	Topoisomerase (DNA) II alpha (Top2a)	−4.74	−5.44	−1.15	a	c,d,f,g	i
X62154	17215	Minichromosome maintenance–deficient (Mcm3)‡	−4.27	−1.62	*2.64*	a	c,g	c,i,j
AJ223087	23834	Cell division cycle 6 homolog (Cdc6)	−3.73	−5.00	−1.34	g		
X77731	13178	Deoxycytidine kinase (Dck)	−3.58	−2.63	1.36	b		
AJ003132	27214	Activator of S phase kinase (Ask)	−3.33	−3.39	−1.02	d,g		
L26320	14156	Flap structure–specific endonuclease 1 (Fen1)‡	−3.11	−2.33	1.33	a,b	c,d,f,g	c
AA929330	17215	Minichromosome maintenance–deficient (Mcm3)‡	−2.95	−1.66	1.78	a	c,g	c,i,j
D26090	17218	Minichromosome maintenance–deficient 5 (Mcm5)	−2.9	−2.02	1.44	a	d,g	c
K02927	20133	Ribonucleotide reductase M1 (Rrm1)	−2.82	−2.25	1.25	a,b	d,g	c
M33988		Histone H2A.1 (H2afa)	−2.81	−3.14	−1.12			
AA681520	57441	Geminin (Gmnn)	−2.79	−2.60	1.07	a		
J04620	19075	DNA primase, p49 subunit (Prim1)‡	−2.63	−1.33	1.98	a	c,d,f,g	
AF036898	18974	DNA polymerase epsilon, subunit 2 (Pole2)	−2.55	−1.97	1.29			
M13352	22171	Thymidylate synthase (Tyms, TS)‡	−2.35	−2.70	−1.15	a	f	
X77731	13178	Deoxycytidine kinase (Dck)	−2.32	−2.22	1.05	b		
X75483	12428	Cyclin A2 (Ccna2)	−2.23	−2.51	−1.13	a	c,g	c,i,j
X60980	21877	Thymidine kinase 1 (Tk1)	−2.23	−2.38	−1.07	a	c,d,g	
AU044050	22171	Thymidylate synthase (Tyms, TS)‡	−2.15	−2.37	−1.10	a	c	
AB025409	54124	CDC28 protein kinase 1 (Cks1)	−2.14	−2.34	−1.09	g	i,j	
X16495		Histone H2A	−1.67	−2.63	−1.57			
K02245	18811	Proliferin‡	1.08	−4.01	*−4.33*			
(B) Mitosis/cell division (19 unique transcripts)								
U01063	18817	Polo-like kinase homolog (Plk1)‡	−6.20	−6.79	−1.10	a	g	c,i,j
X82786	17345	Antigen identified by monoclonal antibody Ki 67‡	−6.01	−7.53	−1.25	a	f,g,c	c,i
X64713	12429	Cyclin B1, related sequence 1 (Ccnb1-rs1, CycB1)‡	−5.82	−5.94	−1.02	a	c,d	c,i,j
AB013819	11799	Baculoviral IAP repeat-containing 5 (Survivin)	−5.66	−9.68	−1.71			
L11316	13605	Epithelial cell–transforming sequence (*ect*) 2 oncogene	−5.60	−4.40	1.27			c
AF002823	12235	Budding uninhibited by benz-1 homolog (Bub1a)	−4.53	−5.39	−1.19	a	c,d	
X66032	12442	Cyclin B2 (Ccnb2, Cycb2)‡	−4.46	−5.33	−1.20	a	c	
AV309347	17345	Antigen identified by monoclonal antibody Ki 67‡	−4.35	−7.47	−1.72	a	c,f,g	c,i
Y09632	19348	Rab6, kinesin-like (Rab6kifl)	−4.29	−6.04	−1.41			c
U80932	20878	Serine/threonine kinase 6 (Stk6, Ark1, Ayk1)	−4.17	−4.47	−1.07			c
D21099	20877	Serine/threonine kinase 5 (Stk5, Aim1, Stk-1)	−3.90	−4.99	−1.28		d,f	c
AW061324	107995	Cell division cycle 20 homolog (Cdc20)	−3.89	−4.02	−1.03	a		c,i,j
AJ223293	16587	Kinesin-like 1 (Knsl1, Kif8, Kifl1)	−3.70	−5.35	−1.45	a	i,j	
L29480	20873	Serine/threonine kinase 18 (Sak)	−3.54	−3.92	−1.11		d,f	
L16926	12532	Cell division cycle 25 homolog C (Cdc25c)‡	−3.43	−3.85	−1.12			c
D12646	16571	Kinesin heavy chain member 4 (Kif4, Kns4)	−3.21	−4.32	−1.35			c
M38724	12534	Cell division cycle 2 homolog A (Cdc2a)‡	−3.18	−3.28	−1.03	a	c,f,g	c,i,j
U83902	56150	MAD2-like 1 (Mad2l1)	−3.16	−3.54	−1.12		g	
AF013166	18005	NIMA (never in mitosis gene a)–related kinase 2 (Nek2)	−2.85	−3.59	−1.26		g	i
AV059766	19348	Rab6, kinesin-like (Rab6kifl)	−2.40	−3.40	−1.42			c
AA823653	16319	Inner centromere protein (Incenp)	−2.28	−3.02	−1.32	a		
(C) Miscellaneous cell cycle–related genes (three unique transcripts)								
AW209238	21335	Transforming, acidic coiled coil containing protein 3	−4.11	−5.47	−1.33			
AF062378	12316	Calmodulin binding protein 1 (Calmbp1, Sha1)	−2.40	−3.50	−1.46			
D87326	14841	Germ cell–specific gene 2 (Gsg2, Haspin)	−2.13	−2.25	−1.06			
Transcription/chromatin structure/DNA repair (eight unique transcripts)								
X67668	15352	High-mobility group box 2 (Hmgb2)	−4.13	−2.74	1.51	a,b	c,f	c
U25691	15201	Helicase, lymphoid-specific (Hells)	−3.39	−2.17	1.56			
U52951	14056	Enhancer of zeste homolog 2 (Ezh2)	−2.63	−1.82	1.45	a,b	c,d,e	
D13803	19361	RAD51 homolog (Rad51)	−2.40	−3.09	−1.29	a	c,d,g,h	c
AA896295	60411	SoxLZ/Sox6 leucine zipper binding protein in testis	−2.33	−2.37	−1.02			
AI852641	56312	Nuclear protein 1 (Nupr1, p8)‡	1.20	2.88	*2.40*			
X67083	13198	DNA damage-inducible transcript 3 (Ddit3, Gadd153)	2.19	2.54	1.16			
V00727	14281	FBJ osteosarcoma oncogene (c-Fos)‡	3.25	1.39	*2.34*			

(continued on next page)

Table 1. (*continued*)

Accession	Locus Link	Gene	wtT/wtU*	KOT/KOU	Fold Change	Repressed by p16/pRb†	Bound or Regulated by E2Fs	Cell Cycle–Regulated
Signal transduction (four unique transcripts)								
U94828	19734	Regulator of G-protein signaling 16 (Rgs16)	−3.62	−3.48	1.04			
AV349152	19734	Regulator of G-protein signaling 16 (Rgs16)	−3.25	−4.07	−1.25			
M90388	19260	Protein tyrosine phosphatase, nonreceptor type 8	−2.42	−1.22	1.98			
M32490	16007	Cysteine-rich protein 61 (Cyr61)	−1.50	−2.83	−1.89			
AF031127		inositol trisphosphate receptor type 2	1.77	2.26	1.28			
Cytoskeleton/adhesion/extracellular matrix/cell–cell signaling (four unique transcripts)								
AF072127	12737	Claudin 1 (Cldn1)‡	−3.04	−3.09	−1.02			
AV230686	21923	Tenascin C (Tnc)‡	−2.37	−5.42	*−2.29*			
U52524	15117	Hyaluronan synthase 2 (Has2)	−1.77	−2.60	−1.47			i
X56304	21923	Tenascin C (Tnc)‡	−1.18	−4.56	*−3.86*			
M81445	14619	Gap junction membrane channel protein beta 2 (Gjb2)	2.01	2.64	1.31			
Immune response (seven unique transcripts)								
X04653	110454	Lymphocyte antigen 6 complex, locus A (Ly6a)	−2.54	−1.38	1.84			
AF022371	15950	Interferon-activated gene 203 (Ifi203)‡	1.26	2.59	*2.06*			
Y07812	17153	mal, T cell differentiation protein (Mal, Mpv17)	1.51	2.58	1.71			
AF000236	12778	Chemokine orphan receptor 1 (Cmkor1, Rdc1)	2.45	2.43	−1.01		d	
Y00629	15040	Histocompatibility 2, T region locus 23 (H2-T23)	2.45	2.85	1.16		d	
AF068182	17060	Lymphocyte antigen 57 (B-cell linker, Blnk)	2.87	1.34	*−2.14*			
AF068182	17060	Lymphocyte antigen 57 (B-cell linker, Blnk)	2.89	1.57	−1.84			
AC002397	12034	B-cell receptor–associated protein 37	2.91	1.62	−1.80			
Fatty acid metabolism (three unique transcripts)								
M21285	20249	Stearoyl-coenzyme A desaturase 1 (Scd1)	−3.86	−1.66	*2.33*			
M21285	20249	Stearoyl-coenzyme A desaturase 1 (Scd1)	−2.63	−1.46	1.80			
AJ223066	16592	Fatty acid binding protein 5, epidermal (Fabp5)	−2.13	−2.49	−1.17			
AF017175	12894	Carnitine palmitoyltransferase 1, liver (Cpt1a)	1.97	2.70	1.37			i
Miscellaneous (19 unique transcripts)								
Z29532	14313	Follistatin (Fst)‡	−5.16	−2.09	*2.47*	b	e	
AI047076	60530	Fidgetin-like 1 (Fignl1)	−4.20	−3.00	1.40			
AI852645	69716	Thyroid hormone-R interactor 13 (Trip13)	−3.76	−5.03	−1.34			
U42385	14211	Fibroblast growth factor–inducible 16 (Fin16)	−3.38	−3.65	−1.08		c,d	
D55720	16647	Karyopherin (importin) alpha 2 (Kpna2)	−3.37	−2.94	1.15			c,i,j
AA032310	22695	Zinc finger protein 36 (Zfp36, tristetraprolin)	−3.22	−3.03	1.06			
AW227345	57806	U2 small nuclear ribonucleoprotein polypeptide A′	−2.93	−2.03	1.44			
AI838853	56207	Ubiquitin carboxyl-terminal esterase L5 (Uchl5)	−2.63	−2.34	1.12			
AA913994	20425	Serine hydroxymethyl transferase 1 (Shmt1)	−2.52	−1.89	1.33			
AF058055	20501	Solute carrier family 16, member 1 (Slc16a1)	−2.46	−1.95	1.26			
AF103875	26357	ATP-binding cassette, subfamily G, member 2	−2.19	−2.69	−1.23			
AJ002390	11752	Annexin A8 (Anxa8)	1.34	3.46	*2.58*		c,d	
AI840339	58809	Ribonuclease, RNase A family 4 (Rnase4)	1.42	2.39	1.68			
U60593	17988	N-myc downstream–regulated 1 (Ndr1)	1.62	2.53	1.56			i,j
AI839138	56338	Thioredoxin-interacting protein (Txnip)	1.64	3.20	1.95			
M32017	16784	Lysosomal membrane glycoprotein 2 (Lamp2)	1.89	2.39	1.26			
U52073	17990	N-myc downstream–regulated–like (Ndrl, Tdd5)	1.97	2.70	1.37		d	
AI853960	67183	Ribosomal protein L22 (Rpl22)	1.98	2.43	1.23			
AW124337	56615	Microsomal glutathione *S*-transferase 1 (Mgst1)	2.34	6.29	*2.69*			
Genes of undefined function (34 unique transcripts)								
AA184423	73644	2210039B01Rik: unknown	−5.84	−2.26	*2.58*			
AA590345	106108	EST AA408511: unknown	−5.45	−6.30	−1.16			
AW259499	101449	EST AA410048: unknown	−4.83	−3.46	1.40			
AW123269	97521	EST C78101: similar to anillin, actin-binding prtn	−4.58	−4.58	−1.00	a	g	
AI122538	68026	2810417H13Rik: 93% similar to pyrimidine binding prtn 2	−4.56	−6.48	−1.42			
AI848479	73644	2210039B01Rik: unknown	−4.45	−2.01	*2.21*			
AA822539	73644	2210039B01Rik: unknown	−3.88	1.17	*4.54*			
AI595322		EST: 94% similar to retinoblastoma-associated Hec1	−3.74	−4.45	−1.19		c,g	
AA681998	66197	1110038L14Rik: 94% CDC28 prtn kinase 2 (CKS2)	−3.66	−3.66	1.00	a		
AI131895	101761	EST AU021460: similar to kinesin-like 4 (KID)	−3.45	−4.32	−1.25	a	g	i
AA989957	67196	2700084L22Rik: similar to ubiquitin-conjugating enzyme	−3.36	−2.58	1.30		g	c,i
AA856349	52439	D7Ertd348e: similar to prtn regulator of cytokinesis 1	−3.29	−4.65	−1.41	a	g	
AI852985	69071	1810014L12Rik: unknown	−3.17	−2.69	1.18			
AV228594		EST: similar to D.m. barren (mitosis)	−3.17	−2.68	1.18			
AW060793	98772	EST: 87% R.n. ADP-ribosylation factor–like prtn 5	−3.01	−1.35	*2.23*			

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Table 1. (*continued*)

Accession	Locus Link	Gene	wtT/wtU*	KOT/KOU	Fold Change	Repressed by p16/pRb†	Bound or Regulated by E2Fs	Cell Cycle–Regulated
AI510131	30866	EST: 98% M.m. hepatoma upregulated protein	−2.92	−2.83	1.03			
AI851230	66949	2310035M22Rik: unknown	−2.89	−2.38	1.21			
AA275196	99457	EST: 100% similar to M.m. nucleolar protein ANKT	−2.84	−4.54	−1.60			
AW122331	68105	4831429J16Rik: unknown	−2.75	−3.22	−1.17			
AI197337	52089	D4Ertd27e: unknown	−2.71	−2.39	1.13			
AI462312		EST: unknown	−2.70	−2.88	−1.07			
AI843655	102464	EST AI256758: similar to H.s. topoisomerase (DNA) II bp	−2.65	−1.64	1.62			
AW213883		EST: 98% similar to similar to barr-P1 (D.m. barren)	−2.57	−2.62	−1.02			
AI848382		EST: similar to M.m. ubiquitin-specific protease	−2.56	−1.84	1.39	a		
AW213865	66929	1700003K02Rik: unknown	−2.53	−3.15	−1.25			
AA007891	73804	4930402F02Rik: similar to mitotic centromere–associated kinesin	−2.50	−3.05	−1.22			
AW214617	70385	2600001J17Rik: unknown	−2.44	−3.05	−1.25			
AW229141	69482	2310006I24Rik: 97% similar to mitotic phosphoprotein 44	−2.40	−2.65	−1.10			
AA409629	102848	EST: 93% similar to R.n. polypyrimidine tract binding prtn	−2.38	−3.19	−1.34			
AA716963		EST: similar to isopentenyl-diphosphate delta isomerase	−2.37	−3.22	−1.36			
AI850288	71747	1300002E07Rik: unknown	−2.34	−2.93	−1.25			
AI591702	97568	EST C87313: unknown	−2.27	−3.27	−1.44			
AI846439	66578	2610039C10Rik: unknown	−2.21	−2.31	−1.05			
AA914734	27985	D4Wsu24e: unknown	1.69	2.62	1.55			
AW124049	52331	D5Ertd593e: unknown	2.04	2.76	1.35			
AI043040	98264	EST AI118496: 99% similar to M.m. polyductin (Pkhd1)	2.25	1.12	*−2.01*			
AA755260		EST: 100% similar to deleted in polyposis 1-like 1	2.64	3.16	1.20			

*Responses of wild-type (wt) and *Rb* knockout (KO) prostate epithelial cells to 48-hour culture in serum-free conditions are represented by the ratio of average signal from treated (T) to untreated (U) samples. Fold changes for each transcript between wtPrE and $Rb^{-/-}$PrE were calculated by dividing wt and KO ratios.
†Genes that have been previously described as being repressed by $p16^{ink4a}$ or pRb [(a) Ref. [44]; (b) Ref. [46]], bound or regulated by E2Fs [(c) Ref. [38]; (d) Ref. [43]; (e) Ref. [45]; (f) Ref. [16]; (g) Ref. [17]; (h) Ref. [47]], or cell cycle–regulated [(i) Ref. [37]; (j) Ref. [38]; (j) Ref. [39]].
‡Represent genes confirmed by semiquantitative RT-PCR.

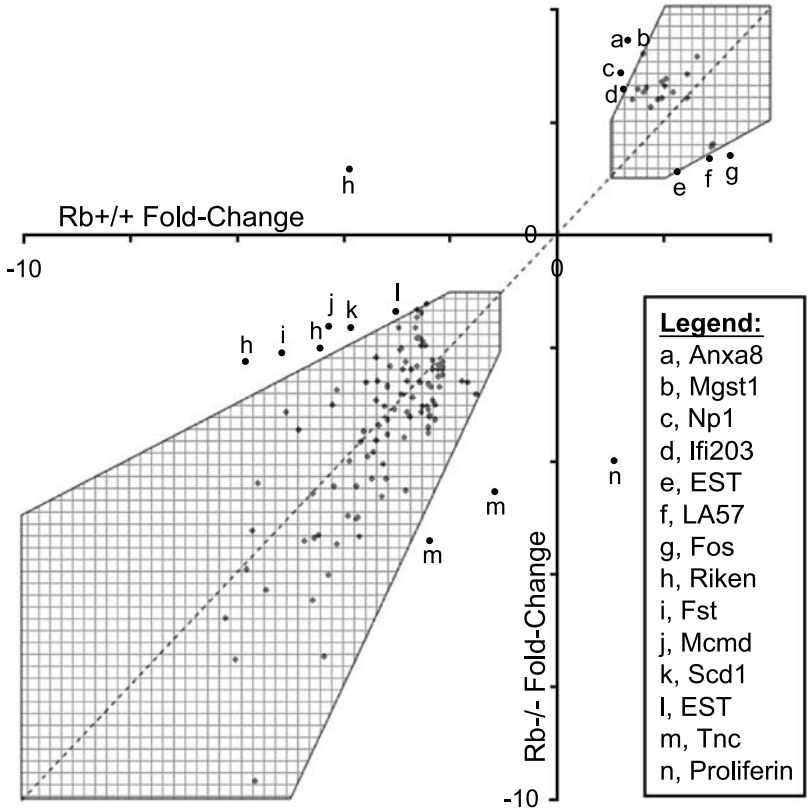

Figure 2. *Comparison of gene expression profiles of growth-arrested $Rb^{+/+}$ and $Rb^{-/-}$ cell lines. Gene expression alterations in response to serum withdrawal in $Rb^{+/+}$ cell lines were plotted along the x-axis, and gene expression alteration in response to serum withdrawal in $Rb^{-/-}$ cell lines was plotted along the y-axis. The dashed line represents a 1:1 correlation between wild-type cell line fold change and $Rb^{-/-}$PrE cell line fold change. Shaded areas represent the limits of the two-fold change between the two cell lines. Each filled circle represents the combined wild-type and $Rb^{-/-}$PrE cell line data for an individual gene transcript.*

difference (annexin A8 [2.58]; Mcmd [2.64]; Mgst1 [2.69]; tenascin C [−3.86]; proliferin [−4.33]; and 2210039B01Rik [4.54]; Table 1). These highly conserved gene expression profiles between wild-type and *Rb*$^{-/-}$ cells suggested that pRb was not required for the vast majority of gene expression alterations associated with growth arrest in murine prostate epithelial cells.

Validation of Gene Expression in Both wtPrE and Rb$^{-/-}$ *PrE Cells*

To confirm gene expression alterations observed with the Affymetrix GeneChips, we performed semiquantitative RT-PCR for several randomly selected transcripts from each of the main functional clusters of genes. Total RNA was isolated in triplicate from three independently derived wtPrE or Rb$^{-/-}$PrE cell lines under log-phase or growth-arrested conditions, and RT-PCR was performed with primers specific to each transcript. To assure that amplification was within a linear range when analyzed, samples were removed from each reaction following cycles 25, 28, 31, and 34, consistent with other previously reported methods [40]. Results of the semiquantitative RT-PCR experiments (Figures 3, 4, and 5) correlated closely with those obtained from the microarray experiments (Table 1). When several genes from the G_1/S cluster were analyzed, all transcripts exhibited downregulation following growth arrest similar to that identified in the microarray analysis. Ribonucleotide reductase M2 (Rrm2), myeloblastosis oncogene (*c-Myb*), minichromosome maintenance–deficient (Mcmd/Mcm3), flap structure endonuclease 1 (Fen1), DNA primase p49 (Prim1), and thymidylate synthase (Tyms) were all considerably downregulated in

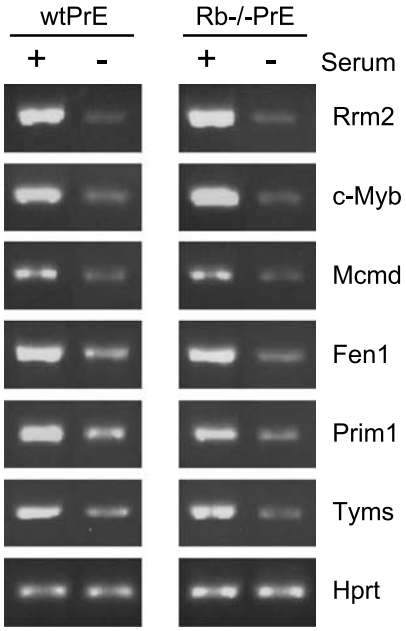

Figure 3. *Semiquantitative RT-PCR confirmation of G_1/S phase genes repressed following growth arrest. wtPrE and Rb$^{-/-}$ PrE cells were cultured in the presence or absence of serum for 48 hours. Equal cDNA generated from total RNA was utilized in RT-PCR reactions with primers specific to Rrm2, c-Myb, Mcmd, Fen1, Prim1, or Tyms. The HPRT transcript is a housekeeping gene that serves as a loading control. Only the reactions that best represented the linear range of amplification are depicted.*

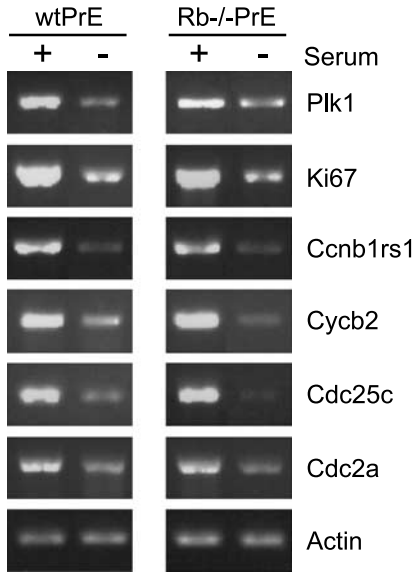

Figure 4. *Semiquantitative RT-PCR confirmation of G_2/M phase genes repressed following growth arrest. wtPrE and Rb$^{-/-}$PrE cells were cultured in the presence or absence of serum for 48 hours. Equal cDNA generated from total RNA was utilized in RT-PCR reactions with primers specific to Plk1, Ki67, Ccnb1rs1, Cycb2, Cdc25c, or Cdc2a. The actin transcript serves as a loading control.*

both wtPrE and Rb$^{-/-}$PrE compared to the hypoxanthine guanine phosphoribosyl transferase (HPRT) control transcript (Figure 3). Interestingly, Mcmd, which appeared from the microarray results to be differentially regulated between the wild-type and Rb$^{-/-}$PrE cell lines, was not observed to be more strongly downregulated in the wtPrE by semiquantitative RT-PCR in four replicate studies (Figure 3; data not shown). These findings suggest that if differences exist between wild-type and *Rb*$^{-/-}$ prostate epithelial cells, they may be even more conservative than suggested by microarray analysis.

Validation of genes in the G_2/M functional cluster was performed in an identical fashion as described above. As indicated in Figure 4, total RNA was isolated from log-phase or growth-arrested wtPrE and Rb$^{-/-}$PrE and semiquantitative RT-PCR was performed for polo-like kinase homolog (Plk1), Ki67, cyclin B1-related sequence 1 (Ccnb1-rs1), cyclin B2 (Cycb2), cell division cycle 25 homolog c (Cdc25c), and

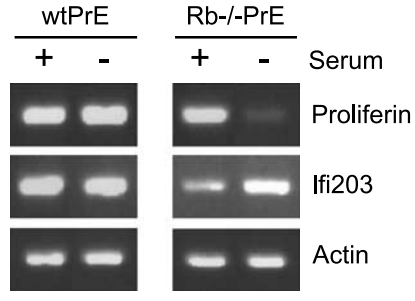

Figure 5. *Semiquantitative RT-PCR confirmation of genes identified by microarray analysis as being differentially regulated between wtPrE and Rb$^{-/-}$ PrE during growth arrest. wtPrE and Rb$^{-/-}$ PrE cells were cultured in the presence or absence of serum for 48 hours. Equal cDNA generated from total RNA was utilized in RT-PCR reactions with primers specific to proliferin or Ifi203. The actin transcript serves as a loading control.*

Cdc2a. Semiquantitative RT-PCR results correlated precisely with microarray results, suggesting that little difference exists between the ability of $Rb^{+/+}$ and $Rb^{-/-}$ cells to repress transcription of genes involved in G_2/M phase functions following growth arrest. These data demonstrate that overall gene expression changes following growth arrest are nearly identical in prostate epithelial cells independent of pRb status.

Finally, due to the potential for random variation and nonspecific hybridization intrinsic to such large-scale microarray studies, six transcripts were selected from the 14 genes identified as being differentially regulated to determine whether these genes were also differentially regulated when assessed by semiquantitative RT-PCR. For this analysis, semiquantitative RT-PCR was performed for tenascin C (Tnc), follistatin (Fst), interferon-activated gene 203 (Ifi203), FBJ osteosarcoma oncogene (c-Fos), proliferin (Plf), and nuclear protein 1 (Nupr1; Figure 5). Triplicate analysis of mRNA levels for these genes in three $Rb^{+/+}$ and $Rb^{-/-}$ prostate epithelial cell lines indicated that differences observed in the microarray experiments for Tnc, Fst, c-Fos, and Nupr1 did not exist when analyzed by RT-PCR. Tnc and Fst were both strongly downregulated and Nupr1 was upregulated following growth arrest of prostate epithelial cell lines independent of pRb status (data not shown). On the other hand, semiquantitative RT-PCR confirmed gene expression differences between $Rb^{+/+}$ and $Rb^{-/-}$ cell lines for Ifi203 and proliferin (Figure 5). Ifi203 was repeatedly upregulated several-fold in growth-arrested compared to log-phase $Rb^{-/-}$PrE cells, but this was not observed in the wtPrE cells. Additionally, proliferin was downregulated in growth-arrested $Rb^{-/-}$PrE cells, but not in wtPrE cells. Therefore, the Ifi203 and proliferin transcripts were confirmed to exhibit altered regulation in $Rb^{-/-}$PrE following growth arrest. Additional research will be required to determine the role of these alterations in the susceptibility of $Rb^{-/-}$ prostate tissue to tumorigenesis.

Discussion

In an effort to identify any unique functions contributed by pRb in the regulation of cell cycle and growth arrest, we have analyzed the gene expression profiles of three independently established $Rb^{+/+}$ and $Rb^{-/-}$ prostate epithelial cell lines under log-phase or growth-arrested conditions. Considering the role of pRb as a tumor-suppressor gene and a purported major regulator of the G_1/S phase transition, it was surprising that both wild-type and $Rb^{-/-}$ prostate epithelium proliferated with identical kinetics and growth-arrested similarly in response to short-term serum withdrawal. Additionally, when Affymetrix gene expression analysis was performed on these two cell lines, it was unanticipated that among all the genes identified as being altered in response to growth arrest conditions, only 14 of these exhibited a $\geq$2.0-fold difference in response between the $Rb^{+/+}$ and $Rb^{-/-}$ cell lines. These findings demonstrate that in the absence of pRb, prostate epithelial cells retain the ability to perform functions previously associated with pRb, including growth arresting under serum-free culture conditions and inhibiting transcription of genes involved in several cellular processes including DNA replication and G_2/M phase regulation.

pRb Is Not Strictly Required for G_1 Growth Arrest

Studies from our group and others have now demonstrated that MEFs and prostate epithelial cells lacking pRb as a result of homologous recombination retain the ability to growth-arrest in the G_1 phase of the cell cycle *in vitro* [31,32,34]. These findings suggest that mechanisms other than those involving pRb exist to induce growth arrest when pRb is genetically inactivated. Current data support an underlying role for the remaining pocket protein family members, p107 and p130, in this compensatory growth arrest mechanism [31,32]. Studies in which the individual pocket proteins were inactivated revealed that loss of pRb, p107, or p130 alone had no major impact on cell cycle profile, rate of immortalization, growth in soft agar, or tumorigenicity in immune-compromised mice [31,32]. Yet, when the effects of various combinations of pocket protein deletions were studied, it became clear that a triple knockout of the entire pocket protein family ($Rb^{-/-}p107^{-/-}p130^{-/-}$) was required to obtain convincing loss of the G_1 growth arrest function and increased susceptibility to immortalization [31,32]. The results of these cellular studies are also supported by biochemical data. For example, in normal $Rb^{+/+}$ neural precursor cells, pRb is found complexed primarily with E2F3, whereas p107 and p130 interact primarily with E2F4 and E2F5 [41]. However, in neural precursor cells isolated from $Rb^{-/-}$ mice, p107 was upregulated and was uncharacteristically found interacting with E2F3 [41]. These data reveal a compensatory mechanism whereby the loss of one pocket protein is balanced by the upregulation of the remaining pocket proteins and interactions of these remaining proteins with additional E2Fs normally regulated by the lost pocket protein. Therefore, the increased levels of p107 observed in our $Rb^{-/-}$ prostate epithelial cell lines and the potential interaction of p107 with the activating E2Fs may result in normal growth kinetics and growth arrest following serum depletion despite the lack of pRb.

Perhaps complicating these initial findings are recent results from Sage et al. [42], who utilized a conditional allele of the *Rb* gene (*cRb*), which allowed for acute deletion of *Rb* when $cRb^{lox/lox}$ MEFs were infected with an adenovirus vector expressing the Cre recombinase gene to generate $cRb^{-/-}$ cells. In these experiments, acute loss of pRb was sufficient to induce cell cycle reentry in quiescent or senescent MEFs, suggesting that pRb is normally required for maintenance of the growth-arrested phenotype [42]. However, through the use of siRNA-based knockdown experiments, this group also demonstrated that if the elevated p107 levels in growth-arrested $Rb^{-/-}$ MEFs were decreased, these cells reentered the cell cycle as measured by BrdU incorporation [42]. Therefore, these data are in agreement with our findings that prostate epithelial cells that constitutively lack pRb due to homologous recombination maintain the ability to growth-arrest due to pocket protein compensatory mechanisms, yet they also raise useful questions as to which model will be more informative for cancer research. Perhaps

in the end, it will be determined that both models will be of use as even the acutely deleted *cRb*$^{-/-}$ MEFs compensated for pRb loss by upregulating p107 within 2 days of *Rb* deletion and some of these cultures eventually reentered senescence approximately 10 passages following acute *Rb* deletion [42].

Regulation of E2F Target Genes in the Absence of pRb

Microarray-based and chromatin immunoprecipitation-based analyses have recently been used by several groups to identify large numbers of genes targeted by specific transcription factors including pRb and the E2F family [16,17,38,43–47]. The majority of these studies has helped to identify genes that were regulated by ectopic expression of various E2Fs or a constitutively active (phosphorylation sites mutated; PSM-Rb) pRb. As denoted in Table 1, our study demonstrated that *Rb*$^{-/-}$ prostate epithelial cells retained the ability to transcriptionally regulate 97% (30/31) and 93% (37/40) of the PSM-Rb and E2F target genes, respectively, that were altered in the growth-arrested wild-type cells. These findings demonstrate that although a constitutively active pRb has the ability to repress these genes in an inducible Rat-16 fibroblast model system [44], pRb is not the only protein capable of regulating these genes under growth-arrested conditions. In agreement with our findings, Hurford et al. [48] demonstrated that *Rb*$^{-/-}$ MEFs exhibited derepressed expression in G_0/G_1 of only 2 of 21 E2F-regulated genes tested by Northern blot. Taking into account the aforementioned findings of several groups, the most likely explanation for our observation is functional compensation for pRb loss by p107 and possibly p130.

Recently, Black et al. [49] utilized microarray to analyze the gene expression phenotypes of wild-type, *Rb*$^{-/-}$, and *p107*$^{-/-}$*p130*$^{-/-}$ MEFs grown under serum-starved (0.25% FBS) conditions. The goal of their experiments was similar to ours in that they aimed to obtain a global evaluation of the gene expression alterations coinciding with the loss of the pocket protein family members. Intriguingly, the results of their studies in a MEF system differed greatly from those of differentiated prostate epithelial cells. In their study, *Rb*$^{-/-}$ MEFs exhibited greatly elevated levels (in many cases 10- to 20-fold increases) of many E2F target genes [49]. However, 6 and 29 of the 100 genes identified in their study were also investigated either by Hurford et al. [48] or our group, respectively, and were not found to be responding differently based on *Rb* genotype. The differing results may be due to the use of different model systems (MEFs *versus* prostate epithelial cells), or due to an earlier analysis of gene expression in the MEF study compared to ours (passage 4 MEFs *versus* passage 15 prostate epithelial cells); however, these differences cannot explain the differences between the Hurford and Nevins studies. Further investigation of these differences may provide information on the tissue-specific role of pRb in regulating E2F-mediated gene expression.

Although the results of our study demonstrate a nonessential role for pRb in the regulation of growth arrest in an epithelial cell line constitutively lacking *Rb*, we do not suggest that pRb only plays redundant roles simultaneously regulated by the other pocket proteins. The goal of this study was to understand the molecular response of *Rb*$^{+/+}$ and *Rb*$^{-/-}$ cells to serum depletion–induced growth arrest, and does not measure the ability of pRb to perform other functions including regulation of differentiation or cooperative activation of transcription by certain non-E2F transcription factors. This is an important clarification as the ability of pRb to induce growth arrest and repress E2F-mediated transcription is separable from the ability of pRb to induce flat cell morphologies, differentiation, and cooperative transcriptional activation with tissue-specific transcription factors involved in differentiation [50]. Clearly, an important role for pRb regulation of E2F1 in development has already been established genetically as homozygous mutation of E2F1 in *Rb*$^{-/-}$ and *Rb*$^{+/-}$ mice decreased the severity of their respective phenotypes [51,52]. Additionally, pRb may be involved in posttranslational regulation of proteins involved in cell cycle arrest and other cellular processes not identified by microarray studies that focus solely on transcriptional regulation.

In summary, we have generated a gene expression profile of *Rb*$^{+/+}$ and *Rb*$^{-/-}$ prostate epithelial cells as they respond to serum removal and growth arrest. Surprisingly, very minor differences were observed in the ability of these two cell lines to repress the same set of growth-regulated genes. Among the genes that are equivalently regulated between the two cell lines are many E2F targets. Current data would suggest that in the *Rb*$^{-/-}$ cells, the regulation of E2F target genes is occurring through direct functional compensation by p107. Further studies to assess novel interactions between p107 and/or p130 and E2Fs 1–3 in the *Rb*$^{-/-}$ prostate epithelial cells are required to provide further clarity to this proposal.

Acknowledgements

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