Retinoblastoma Tumor Suppressor Status Is a Critical Determinant of Therapeutic Response in Prostate Cancer Cells

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Abstract

The retinoblastoma tumor suppressor protein (RB), a critical mediator of cell cycle progression, is functionally inactivated in the majority of human cancers, including prostatic adenocarcinoma. The importance of RB tumor suppressor function in this disease is evident because 25% to 50% of prostatic adenocarcinomas harbor aberrations in RB pathway. However, no previous studies challenged the consequence of RB inactivation on tumor cell proliferation or therapeutic response. Here, we show that RB depletion facilitates deregulation of specific E2F target genes, but does not confer a significant proliferative advantage in the presence of androgen. However, RB-deficient cells failed to elicit a cytostatic response (compared with RB proficient isogenic controls) when challenged with androgen ablation, AR antagonist, or combined androgen blockade. These data indicate that RB deficiency can facilitate bypass of first-line hormonal therapies used to treat prostate cancer. Given the established effect of RB on DNA damage checkpoints, these studies were then extended to determine the impact of RB depletion on the response to cytotoxic agents used to treat advanced disease. In this context, RB-deficient prostate cancer cells showed enhanced susceptibility to cell death induced by only a selected subset of cytotoxic agents (antimicrotubule agents and a topoisomerase inhibitor). Combined, these data indicate that RB depletion dramatically alters the cellular response to therapeutic intervention in prostate cancer cells and suggest that RB status could potentially be developed as a marker for effectively directing therapy. [Cancer Res 2007;67(13):6192–203]

Introduction

Prostatic adenocarcinoma is the most commonly diagnosed malignancy and the second leading cause of cancer related death in men (1). The majority of prostate cancers are androgen dependent and respond to androgen deprivation therapies, which include bilateral orchiectomy, administration of luteinizing hormone–releasing hormone agonists to suppress testicular androgen production, and/or administration of antiandrogens (e.g., bicalutamide; refs. 2, 3). Unfortunately, median time to the formation of recurrent tumors is only 24 to 36 months with relapse occurring in a great majority of treated patients (4). Cells of the recurrent tumors proliferate in the absence of androgen, and few treatments exist for this stage of disease (5). Given the failure rate of first-line therapy, several cytotoxic agents are currently being tested in clinical trials as putative second-line therapeutics for advanced prostate cancer, including DNA-damaging agents, antimicrotubule agents, and histone deacetylase (HDAC) inhibitors (6, 7). Importantly, biomarkers to use as determinants for therapeutic response to either hormone-based or cytotoxic therapies remain elusive.

We have shown that the retinoblastoma tumor suppressor protein (RB) plays a critical role in the proliferative response to androgens in prostate cancer cells (8, 9). RB belongs to a family of pocket proteins (RB, p107, and p130) and is present throughout the cell cycle, but phosphorylation of the protein is regulated in a cell cycle-dependent manner (10). In quiescent cells, RB is hypophosphorylated and assembled transcriptional repressor complexes on promoters of E2F-regulated genes to inhibit cell cycle progression. In response to mitogenic signals, RB becomes phosphorylated by sequential activity of cyclin–cyclin-dependent kinase (cyclin-cdk) complexes. These modifications are sufficient to disrupt the interaction of RB with E2F family members, thereby relieving transcriptional repression and facilitating cell cycle progression (10–12). We and others have shown that androgens exert their effect on the cell cycle by triggering accumulation of cyclin D1 (thereby activating cdk4) and through post-translational activation of cdk2 (8, 9, 13). The culmination of these events results in RB hyperphosphorylation and S-phase progression. Thus, RB is hypothesized to play a critical role in androgen-dependent proliferation.

RB function is disrupted in a multitude of tumor types, including prostate cancer (14). RB inactivation can occur through disparate mechanisms, and these events are often tissue specific. For example, RB is inactivated through excessive cdk activation (e.g., non–small cell lung carcinoma), loss of the cdk inhibitor, p16INK4a (e.g., melanomas) or through direct mutation or loss of the RB locus (e.g., retinoblastoma; ref. 10). The latter mechanism is most common in prostate cancer, wherein loss of RB function has been attributed to allelic loss [loss of heterozygosity (LOH)] that has been reported to occur in 25% to 50% of cases (15, 16). Despite the high frequency of RB inactivation in prostate cancer, few studies have addressed the impact of this event on cellular response to therapeutic outcome.

Here, we challenged the molecular and proliferative response of androgen-dependent prostate cancer cells to RB depletion using models of both acute and stable RB disruption. We show that RB depletion triggers deregulation of only a select group of E2F target genes, and some compensation by RB-related proteins was noted. These events failed to induce a proliferative advantage in cells.
cultured in the presence of androgen; however, RB deficiency induced a marked increase in cell proliferation upon androgen ablation. This disparity in proliferation was most apparent under conditions of combined androgen blockade, wherein RB-depleted cells continued to proliferate with robust kinetics. These collective data indicate that RB ablation is sufficient to render prostate cancer cells refractory to hormone-dependent strategies and suggest that RB status may be a critical determinant in predicting the efficacy of first-line therapies. However, it has been shown in fibroblasts that RB deficiency can sensitize cells to DNA damage, as attributed to a loss of checkpoint control (17). Remarkably, our data show no perceptible advantage to the cytotoxic effects of an HDAC inhibitor and conferred modest resistance to a platinating agent. However, RB-depleted cells were significantly sensitized to cell death induced by antimitotubule agents or a topoisomerase inhibitor. Together, these data reveal a critical role for RB disruption in bypassing the response to hormonal based therapies, but indicate that RB-deficient tumors may be sensitized to specific cytotoxic agents.

**Materials and Methods**

**Reagents.** Paclitaxel, docetaxel, and etoposide were purchased from Sigma Chemical Co. Casodex (bicalutamide) was a generous gift from AstraZeneca Pharmaceuticals (London, United Kingdom). Clinical grade 1,2-diaminocyclohexaneplatinum II (CDDP) was purchased from Bedford Laboratories. Suberoylanilide hydroxamic acid (SAHA) was a kind gift from Dr. Ching-Shih Chen (Ohio State University, Columbus, OH). Paclitaxel, docetaxel, and SAHA were dissolved in DMSO to 10−2 mol/L. Antibodies used were RB (554136, BD PharMingen), RB phospho-Ser780 (3039-1, Santa Cruz Biotechnology). Antibodies used were RB (554136, BD PharMingen), RB phospho-Ser780 (3039-1, Santa Cruz Biotechnology), RB knockdown or control LAPC-4 stable clones. Following selection with MSCV-LMP Rb88; targeted sequence: 5'-GAAAGGCATGT-C2 immuno-preparation assay (RIPA) buffer [150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8.0)] supplemented with protease inhibitors and phenylmethylsulfonyl fluoride. After brief sonication and clarification, protein concentrations were determined using Bio-Rad DC Protein Assay, and an equal amount of protein was subjected to SDS-PAGE and transferred to Immobilon-P membrane (Millipore Corp.). The membrane was immunoblotted for RB, RB phospho-Ser780, cyclin A, cyclin C, cyclin D1, cyclin D3, cdk-2, cdk-4, p16INK4a, and β-tubulin with indicated antibodies by standard techniques and visualized using enhanced Western lightening chemiluminescence (Perkin-Elmer Life Sciences). Development of membrane and quantification of band intensities for cyclin-A, cyclin-E, mcm-7, PCNA, p107, p130, and β-tubulin was done using Odyssey IR Imaging System (LI-COR, Biosciences).

**Immunofluorescence.** Immunofluorescence staining for RB was done by seeding cells with or without stable and transient RB knockdown at a density of 3 × 105 cells/well on poly-L-lysine–coated coverslips resting in six-well dishes. The coverslips were then transferred into a humidified chamber, and cells were fixed with 3.7% formaldehyde for 5 min. After washing with PBS (Mediatech), cells were treated with 0.3% Triton X-100 (Ameresco) for 15 min. Following additional wash with PBS, cells were blocked with 5% goat serum (Invitrogen) for 1 h at room temperature. Cells were then incubated with primary antibody solution containing RB antibody (mouse anti-human, 554136, BD PharMingen) in 5% goat serum solution for 1 h. After incubation, cells were washed with PBS (thrice, 5 min each) followed by incubation with secondary antibody solution containing goat anti-mouse rhodamine (Jackson Immunoresearch) and Hoechst 33258 (Sigma-Aldrich). After 1 h incubation, cells were washed with PBS (thrice, 5 min each), followed by mounting on a glass slide using gelvatol (Air Products and Chemicals). Cells were then visualized for RB as previously described (18).

**Bromodeoxyuridine incorporation assay.** To monitor bromodeoxyuridine (BrdUrd) incorporation for transient RB knockdown, LNCaP cells were seeded (3 × 105 cells/well) on poly-L-lysine–coated coverslips in six-well plates in 5% FBS-IMEM. After 24 h in culture, cells were transfected with 1.5 μg MSCV-Rb3C or 1.5 μg MSCV donor and 0.5 μg H2B–green fluorescent protein (GFP) plasmid in IMEM (serum free). Following transfection, cells were supplemented with 5% CDT-IMEM or 5% AB8s-IMEM and treated with 1 μmol/L Casodex or 0.1% DMSO. Cells were resuspended with 1 μmol/L Casodex or 0.1% DMSO after 48 h. BrdUrd labeling reagent (Amersham Biosciences) was added after 10 h for a 14-h labeling period, after which the cells were fixed and stained with anti-BrdUrd antibody, and the percentage of GFP-positive cells exhibiting BrdUrd incorporation were counted using indirect immunofluorescence as previously described (8). A minimum of 200 H2B-GFP–transfected cells per experimental condition were counted for BrdUrd incorporation in at least three independent experiments and results plotted as BrdUrd incorporation for RB knockdown cells relative to RB-proficient control cells set to 1. To monitor BrdUrd incorporation for stable RB knockdown, shRB1 and shCon1 LNCaP cells were seeded as described above and treated with 1 μmol/L Casodex and 0.1% DMSO. Cells were resuspended with 1 μmol/L Casodex or 0.1% DMSO after 48 h. BrdUrd was added 24 h later, and cells were labeled for 14 h, after which they were fixed, stained, and counted for BrdUrd using indirect immunofluorescence. A minimum of 200 Hoechst-stained cells per sample were counted for BrdUrd incorporation in at least three independent experiments and results plotted as relative BrdUrd incorporation. For the asynchronous BrdUrd pulse experiment, 4.7 × 105 cells (shRB1 and shCon1 for LNCaP; L4-shRB and L4-shCon for LAPC-4) were cultured in 6-cm dishes. Following 48 h in culture, the cells were pulsed with BrdUrd for 1 h before harvesting. Cells were later fixed in 95% ethanol, pelleted, resuspended in 2N HCl + 0.5 mg/mL pepsin, and incubated at room temperature for 30 min. Sodium tetraborate (0.1 mol/L) was then added to neutralize HCl. The cell pellet was then washed with IFA buffer, centrifuged, and then washed with IFA + 0.5% Tween-20 solution. The pellet was then suspended in IFA solution containing FITC conjugated anti-BrdUrd antibody (BD Bioscience) and incubated in the dark at room temperature for 45 min. Cell solution was then washed with IFA + 0.5% Tween-20 and pelleted. The pellet was then suspended in PBS containing propidium iodide (0.2 μg/mL) and analyzed for BrdUrd incorporation using Beckman Coulter Cell Lab Quanta SC flow cytometer.

**Flow cytometry.** shCon1 LNCaP cells and L4-shCon LAPC-4 cells (7.8 × 105 cells per 10-cm dish) were infected with p16INK4a adenovirus for 12 h as previously described (19). Following wash PBS, cells were cultured for an additional 36 to 48 h and then fixed in 95% ethanol, stained with propidium iodide (0.2 μg/mL), and subjected to flow cytometry as described previously (20). Histograms represent ~10,000 cells. Analysis of flow
cytometric data was done using ModFit LT flow cytometry modeling software.

**Cell growth and survival assays.** shCon1 and shRB1 LNCaP cells, and L4-shCon and L4-shRB LAPC-4 cells were seeded at \(-1.5 \times 10^5\) cells per well in poly-L-lysine-coated six-well dishes into appropriate media with the indicated concentration of drugs. For cell growth assays analyzing the effect of Casodex, cells were supplemented with 1 \(\mu\)M/L Casodex or 0.1% DMSO after 24 h in culture (0 h time point). The cells were resupplemented with 1 \(\mu\)M/L Casodex or 0.1% DMSO at 72 h in culture (48 h time point), and viable cells were counted using trypan blue exclusion at the indicated time points. Total cell number was determined in triplicate biological samples, and each experiment was repeated at least thrice. For all the other growth curves, cells were seeded in 5% FBS-IMEM and indicated doses of paclitaxel, docetaxel, CDDP, etoposide, SAHA, or 0.1% DMSO were added to each well after 24 h in culture and challenged for 48 h. Following treatment, cells were resupplemented with fresh media without drugs and cultured for an additional 48 h. Viable cells were then counted using hemocytometer and trypan blue exclusion. For the growth assays using Casodex, relative cell number was reported as compared with untreated control at time 0 h. Doubling time was determined using the following equation: \(t = \frac{\ln(c_t)}{\ln(c_0)}\), where \(t\) is the time interval in hours, \(C_t\) is the final cell count, and \(C_0\) is the initial cell count (21). Cell survival in the presence of cytotoxic agents was determined by quantifying cell number after each drug treatment as compared with untreated control by trypan blue exclusion. Total cell number was determined for each condition in duplicate biological samples, and each experiment was replicated at least thrice.

**Statistical assessment.** Quantitative results are expressed as mean ± SD. Statistical analyses were done using one-way ANOVA followed by Newman-Keuls Multiple Comparison post-test. The criterion for statistical significance was \(P < 0.05\).

**Results**

RB depletion triggers selected RB target gene deregulation and compensatory induction of RB family members. RB inactivation is observed during prostate cancer progression and has been potentially correlated with poor outcome (22). To challenge the consequence of RB loss in prostate cancer cells, LNCaP cells were used. These cells reflect the salient features of early disease in that they are AR positive and androgen dependent for cellular proliferation. Androgen induction triggers a discrete cascade of biochemical changes that result in RB phosphorylation and G1 to S phase progression (8, 9). Consistent with previous reports, integrity of the RB pathway was evident in that ectopic expression of p16INK4a (which causes activation of endogenous RB), resulted in RB dephosphorylation and repression of target genes (e.g., cyclin A; Fig. 1A, left and middle). As expected, these collective events culminated in enforcing G1 arrest (right). Therefore, LNCaP model system was used for subsequent analyses of RB depletion.

To determine the effect of RB depletion on response to therapeutic regimens, shRNA technology was employed to generate

**Figure 1.** RB knockout in prostate cancer cells causes deregulation of RB/E2F target genes and compensation by RB family members. A, LNCaP cells were infected with p16INK4a adenovirus. Following culture for 36 to 48 h, cells were harvested, lysed, and immunoblotted as indicated. \(\beta\)-Tubulin is included as the loading control (left and middle). p16INK4a-infected cells were analyzed in parallel for cell cycle position (right). B, cells were transfected with pMSCV-puroRb3C or pMSCV-puro and H2B-GFP as a marker for transfection. Transfected cells were visualized for RB staining by indirect immunofluorescence (40× magnification; left), and corresponding lysates were monitored for RB expression levels by immunoblot (right). C, stable clones of RB knockout (shRB1) or control (shCon1) were generated and tested for RB expression levels using immunofluorescence (40× magnification; left) and immunoblot analysis (right). D, immunoblot analysis of shRB1 and shCon1 cells to monitor alteration of E2F targets (cyclin A, cyclin E, Mcm-7, PCNA). \(\beta\)-Tubulin is included as a loading control (left). Relative expression in shRB1 (normalized to \(\beta\)-tubulin and compared with shCon1) is indicated as determined by LICOR fluorescence. Lysates from shRB1 and shCon1 cells were subjected to immunoblot analyses to examine p107 and p130. \(\beta\)-Tubulin served as a loading control, and relative expression is indicated (right).
model systems of RB depletion. Knockdown was initially done transiently, wherein wherein cells were cotransfected with a plasmid encoding shRNA against Rb (MSCV-Rb3C) or a control plasmid (MSCV-donor) and H2B-GFP as a marker for transfection efficiency. Following transfection, cells were visualized for RB staining using indirect immunofluorescence (Fig. 1B, left). As can be seen, transfected (GFP+) cells exhibited little RB immunoreactivity, whereas neighboring untransfected (GFP−) cells retained RB expression (Fig. 1B, arrows). RB status was also verified using immunoblot analysis (Fig. 1B, right), wherein RB expression was reduced by at least 50%, consistent with the observed transient transfection efficiency (45–50% GFP positive, data not shown). These data indicate that RB expression can be efficiently ablated in this model system using transient assays. Parallel experiments were done in which a puromycin resistance marker was included in the cotransfections to generate stable RB knockdown. Multiple clones were isolated, expanded, and validated for RB depletion and showed similar characteristics in subsequent functional assays (data not shown). Representative clones for RB knockdown (shRB1) and control stable (shCon1) are shown in Fig. 1C. As expected, no RB expression was detected by either immunofluorescence (left) or immunoblot (right). Through these dual model systems, the impact of RB depletion was subsequently challenged.

RB exerts its antiproliferative activity through its function as a transcriptional repressor and thus represses expression of target genes that control cell cycle transitions (23). The cohort of RB-regulated target genes could potentially be cell context specific, but repression of E2F target genes that govern S-phase entry and progression is commonly observed (24). To determine the molecular consequence of RB depletion in prostate cancer cells, immunoblot analysis was done in the stable depletion model for expression patterns of this central cohort (Fig. 1D, left). RB depletion resulted in a marked increase in cyclin-E expression, indicating that derepression of cyclin-E is a downstream consequence of RB depletion in prostate cancer cells. Expression of minichromosome maintenance-7 (mcm-7), a component of the pre-replication complex, was modestly induced by RB knockdown. These observations are consistent with previous reports in other model systems, wherein RB depletion triggers apparent induction of only a subset of target genes (25). Mechanisms underlying this have been partially attributed to compensation by other RB family members, p107 and p130 (26). Therefore, relative p107 and p130 expression was examined in the stable cell lines, and representative blots are shown (Fig. 1D, right). In RB-depleted cells, p107 was modestly elevated (1.3-fold increase), whereas a robust increase in expression levels of p130 (6-fold increase) was observed. Together, these data indicate that RB depletion is sufficient to initiate deregulation of selected target genes, and compensatory mechanisms are invoked after stable RB depletion to induce RB family member expression.

Acute or chronic RB ablation is insufficient to enhance cellular proliferation in prostate cancer cells. Given the importance of RB in regulating cellular proliferation, the impact of RB deficiency on proliferation and cell cycle progression was determined. For these studies, strategies to assess the impact of both transient and chronic RB depletion were employed so as to reveal any potentially confounding effects of p107 or p130 induction. The proliferative capacity of RB-depleted cells was analyzed by monitoring BrdUrd incorporation in asynchronous cultures. For transient RB depletion, asynchronous cultures were cotransfected with plasmids encoding shRNA or control shRNA vector and H2B-GFP. Post-transfection, cells were pulse labeled with BrdUrd and fixed, and BrdUrd incorporation in the GFP-positive cells was monitored by indirect immunofluorescence. For the stable cell lines, asynchronous cultures were removed from puromycin selection, seeded at identical densities, and similarly pulse labeled to monitor BrdUrd incorporation. For comparative purposes, the BrdUrd incorporation rate of RB-proficient cells in each experiment was set at “1.” As shown, there was no significant difference in the proliferative index in either cell system, regardless of RB status (Fig. 2A). To validate these observations, cell number was monitored in parallel cultures (Fig. 2B). Subconfluent, exponentially proliferating cultures of shRB1 and shCon1 cells were seeded at equal density and counted at indicated time points using trypan blue exclusion. As shown, expansion of the cultures occurred with indistinguishable kinetics. However, it has been previously shown in specific cellular systems that RB loss can shorten the duration of $G_1$ phase without necessarily altering the doubling time of cells (27). To determine if this held true for androgen-dependent prostate cancer cells, asynchronously growing population of shRB1 and shCon1 cells were cultured in androgen-proficient medium (ΔFBS), pulsed with BrdUrd for 1 h, harvested, and analyzed for propidium iodide intensity and % BrdUrd incorporation using bivariate flow-cytometric analysis. As shown in Fig. 2C, there was no significant difference in the $G_1$ population (56.6 ± 1.7% for shCon1 versus 58.7 ± 2% for shRB1) and amount of BrdUrd incorporation (20.9 ± 2.3% for shCon1 versus 23.5 ± 1% for shRB1), indicating that RB loss does not significantly shorten the timing of $G_1$ phase in prostate cancer cells. These findings are consistent with previous reports (8, 9), wherein androgen induces RB phosphorylation and inactivation; thus, RB depletion is unlikely to elicit an effect under these conditions. Together, these data show that RB depletion is insufficient to alter cellular proliferation rates in the presence of androgen.

RB depletion triggers bypass of hormone ablative therapies. Despite the failure of RB ablation to enhance the proliferative response in the presence of androgen, in at least one study of prostate cancer, it has been suggested that RB inactivation correlates with poor response to therapeutic intervention because a significant number of patients undergoing hormone ablation therapy harbored tumors with low RB mRNA levels (22). Indeed, it has been shown that RB is phosphorylated and inactivated as a consequence of androgen signaling in prostate cancer cells, thus indicating that the antiproliferative effects of RB may be masked by the presence of the hormone (8). These observations suggest that the hormonal milieu or status of the androgen signaling pathway may influence the impact of RB depletion. Given the importance of hormone therapy for first-line treatment of prostate cancer (3), this concept was directly challenged using the cell systems of acute or chronic RB depletion. First, the impact of RB status on response to the most commonly used androgen receptor (AR) antagonist, Casodex (CSDX, bicalutamide), was assessed. Casodex triggers the AR to form transcriptional repressor complexes and thereby acts to prevent activation of AR target genes (28); however, the influence of RB on cellular response to Casodex has yet to be ascertained. Here, transient RB depletion was induced as described in Fig. 2A, but subsequent to transfection, serum was supplemented with 1 μmol/L Casodex (Fig. 3A). As expected, RB-proficient cells showed a decreased proliferative capacity in response to Casodex treatment (e.g., reduction of ∼65.5% BrdUrd incorporation for
LNCaP cells transfected with control vector and 56.4% for shCon1 cells relative to the same cells in the absence of an antagonist; raw data not shown). This is also visualized as a significant reduction of doubling time for the shCon1 cells in the presence of Casodex (~49 h without antagonist and 72 h in the presence of Casodex; compare growth curves from Figs. 2B and 3A, right), consistent with previous reports and with the established dependence of this cell type on androgen receptor signaling for cell cycle progression (8). By contrast, acute and chronic RB depletion yielded a significant proliferative advantage to cells treated with Casodex, as indicated by a 2-fold increase in BrdUrd incorporation in RB-deficient cells relative to RB-proficient cells (Fig. 3A, left). Similar effects were observed through the analyses of cell number (Fig. 3A, right). These data indicate that RB depletion may facilitate the bypass of response to AR antagonists, and that the deleterious effects of RB depletion are not reversed through compensation by other pocket protein family members.

Given the impact of RB status on the response to therapeutic agents that block androgen signaling, the influence of RB depletion on response to androgen deprivation was assessed. To implement androgen ablation, cells (shRB1, shCon1, or LNCaP cells transiently transfected as in Fig. 2A) were cultured in serum devoid of steroid hormones (5% CDT, charcoal dextran–treated serum). As expected, RB-proficient cells were markedly reduced in the rate of cell cycle progression (72% reduction in BrdUrd incorporation in cells transfected with control vector and 85% in shCon1 cells as compared with parallel samples cultured in the presence of hormone; raw data not shown) and cellular proliferation compared with cells cultured in androgen-proficient media, compare growth curves from Figs. 2B and 3B (right). However, the RB-deficient cells showed a significant increase in both indicators of cellular proliferation (Fig. 3B, left and right). Combined, these data indicate that RB depletion enables LNCaP cells to bypass various single-agent hormonal therapies used in prostate cancer treatment.

Although these results indicate that RB status may be a significant modifier of the response to single-agent hormonal therapies, frequently, these modes of therapeutic intervention are used in combination. Coadministration of agents to deplete androgen production and antagonize residual AR activity through...
the use of direct AR antagonists is referred to as "combined androgen blockade" (29). To assess the impact of RB on this maximal hormone-manipulative regimen, the experimental strategy used in Fig. 3B was modified to include 1 μmol/L Casodex or 0.1% DMSO (vehicle control). After 48 h of treatment, cells were then monitored for relative BrdUrd incorporation (left), or cell number was monitored at indicated time points (right). B, experiments were repeated as in (A), except that cells were cultured in steroid-depleted serum (CDT) for 48 h before BrdUrd labeling (left) or monitored for cell viability as indicated (right). Data represent at least three independent experiments wherein data for each time point were collected with triplicate biological replicates. *, P < 0.05, ANOVA.

RB status renders prostate cancer cells more resistant to selected chemotherapeutic agents. Although the above data indicate that RB status renders prostate cancer cells resistant to hormone ablative therapies, it has been suggested in other cell types that RB ablation can sensitize cells to specific modes of genotoxic stress used in cancer therapy (17, 18). For example, it has been previously shown that RB-deficient murine embryo fibroblasts (MEFs) fail to induce cell cycle checkpoints in response to cisplatin, etoposide, and mitomycin C, resulting in deleterious DNA replication and concomitant cell death (17). In light of these observations, it could be hypothesized that RB depletion in prostate cancer cells may sensitize cells to specific chemotherapeutic agents. To test this hypothesis, response of shRB1 and shCon1 cells to cytotoxic intervention was monitored by following cell viability after exposure to representative classes of agents that are currently in clinical trials to treat prostate cancer. First, SAHA was analyzed because this HDAC inhibitor has been shown to inhibit growth of prostate cancer cells both in vitro and in vivo through induction of apoptosis (30, 31). To determine the effect of chronic RB depletion on response to SAHA, shRB1 and shCon1 cells were cultured at equal density in hormone-complete media (5% ΔFBS) and, after 24 h, were treated with increasing concentrations (10^{-9}, 10^{-7}, 10^{-6}, and 10^{-5} mol/L) of SAHA or vehicle (0.1% DMSO) control for 48 h. Following cytotoxic challenge, cells were washed free of drug, media was replaced, and cells were allowed to propagate for an additional 48 h recovery period; subsequently, cell number was determined using hemocytometer and trypan blue exclusion (Fig. 5A). As expected, SAHA was cytotoxic in cells as cell survival steadily decreased as a function of increasing drug concentration. However, survival of shRB1 cells was indistinguishable from that observed with shCon1, thus indicating that SAHA offers no therapeutic advantage for RB-deficient androgen-dependent prostate cancer cells. By contrast, in cells treated using an identical protocol with a DNA-damaging agent, cisplatin (CDDP), modest but statistically distinct alterations were observed as a function of RB status. Surprisingly, at all clinically relevant doses tested (4, 8, and 16 μmol/L), the shRB1
cells proved less sensitive to the effects of cisplatin as compared with shCon1 cells (Fig. 5B; *, P < 0.05, ANOVA). These unexpected results indicate that analyses of MEFs may not accurately predict the response of prostate cancer cells to cytotoxic intervention, and that RB depletion may actually confer modest resistance to platinum-based cytotoxic agents.

**RB-deficient prostate cancer cells are sensitized to cell death by antimicrotubule agents and topoisomerase inhibitor.** Recent studies have indicated that treatment of hormone refractory prostate cancers with antimicrotubule agents (i.e., taxanes) can extend survival in prostate cancer patients, thus putting forward new possibilities for second-line therapies (7). Because our data indicated that RB deficiency may contribute to the development of hormone refractory disease, it was imperative to assess the impact of RB status on the response to taxanes. For these studies, two distinct agents were used (paclitaxel and docetaxel), each of which is administered clinically. Using the experimental strategy analogous to that in Fig. 5A, shRB1 and shCon1 cells were treated with increasing doses of paclitaxel (Fig. 5C, top) or docetaxel (Fig. 5C, bottom) and 0.1% DMSO (vehicle control). As shown, the RB-deficient cells showed 10%, 28.5%, and 57% reduction in cell survival relative to RB-proficient isogenic controls at 10^-9, 10^-8, 10^-7, and 10^-6 mol/L doses of paclitaxel, respectively (*, P < 0.05, ANOVA). Similarly, RB-deficient cells showed enhanced sensitivity to docetaxel at all doses tested (14.5%, 58%, and 54.5% reduction in cell survival relative to RB-proficient isogenic controls at doses 10^-10, 10^-9, and 10^-8 mol/L, respectively; *, P < 0.05, ANOVA), indicating that RB-deficient prostate cancer cells are more sensitive to cytotoxic effects of antimicrotubule agents. Lastly, the impact of RB status on etoposide was considered because this topoisomerase II inhibitor is currently in phase II clinical trials for hormone refractory prostate cancer (32). As shown in Fig. 5D, at each dose tested, RB depletion resulted in significantly reduced cell survival; ~32%, 22%, and 24% reduction in cell survival relative to isogenic control at doses 10^-9, 10^-7, and 10^-6 mol/L, respectively (*, P < 0.05, ANOVA). These results indicate that whereas RB deficiency confers resistance to hormone therapies, RB depletion can sensitize prostate cancer cells to antimicrotubule agents and topoisomerase inhibitors.

**Impact of RB depletion is conserved in AR-dependent prostate cancer cell line.** Given the dramatic impact of RB depletion in LNCaP cells on chemotherapeutic response and to confirm that the aforementioned results were not a cell line–specific response, the specificity of these findings were subsequently challenged in an alternative AR- and RB-proficient cell line, LAPC-4. These cells express requisite G1 machinery (p16INK4a, cyclin A, cyclin E, cyclin D1, cyclin D3, cdk-2, and cdk-4) and retain a functional cyclin D1-RB pathway, as shown by the ability of ectopic p16INK4a to induce activation of endogenous RB, repression of RB target genes (e.g., cyclin A), and cell cycle arrest (Supplementary Figs. S1 and S2). To determine the effect of RB depletion in this cell line, multiple RB knockdown clones were isolated, expanded, and validated for RB depletion (data not shown). Representative clones for RB knockdown (L4-shRB) and control stable (L4-shCon) are shown in Fig. 6A. Subconfluent, exponentially proliferating cultures of L4-shRB and L4-shCon were then seeded at equal density and counted at indicated time points using trypan blue exclusion. As shown in Fig. 6B (top left), the RB depletion yielded no significant effect on growth kinetics of these cells in the presence of the androgen (AFBS). Furthermore, RB depletion did not alter the percentage of cells in G1 or S phase as compared with RB-proficient counterparts (Supplementary Fig. S3). Consistent with data from LNCaP cells, these data again show that RB depletion is insufficient to alter cellular proliferation in prostate cancer cells. However, when media was supplemented with 1 μmol/L Casodex (Fig. 6B, top right), RB-depleted cells bypassed this mode of therapeutic intervention, as L4-shRB cells proliferated more profitably than L4-shCon cells (evident from doubling times of 34.8 h for L4-shRB and 40.4 h for L4-shCon cells). Next, to implement androgen-ablative and combined androgen-ablative therapeutic regimens, these cells were cultured in serum devoid of steroid hormones (CDT) supplemented without or with 1 μmol/L Casodex. As shown in Fig. 6B (bottom), RB-deficient cells showed an increased proliferation profile compared with RB-proficient cells under both therapeutic regimens. These data collectively indicate that although RB depletion confers little effect on cellular proliferation in the presence of the androgen, RB status does modify response to conventional hormonal therapies in multiple prostate cancer model systems. To assess the impact of RB depletion on cytotoxic intervention, these cells were then subjected to cell survival assays as previously described for LNCaP cells (Fig. 5). First, the effect of HDAC inhibitor, SAHA, was analyzed. As shown in Fig. 6C (top),
although SAHA was cytotoxic in these cells as cell survival steadily decreased as a function of drug concentration, no significant difference was observed in the survival of L4-shRB cells relative to L4-shCon cells, thus indicating that HDAC inhibitor like SAHA may offer no therapeutic advantage for RB-deficient prostate cancer cells. When treated with cisplatin (Fig. 6B, bottom), L4-shRB cells proved to be less sensitive than L4-shCon cells at all doses tested, indicating that resistance of RB-deficient prostate cancer cells to cisplatin is conserved among AR-positive prostate cancer cells, and that RB depletion may actually confer modest resistance to platinating agents. To determine the impact of RB status on the response to taxanes and topoisomerase inhibitor in this model system, L4-shRB and L4-shCon cells were treated with increasing concentrations of paclitaxel (Fig. 6D, left), docetaxel (Fig. 6D, right) or etoposide (ETOP, Fig. 6D, bottom). As shown, the RB-deficient cells showed 10.62%, 29.3%, and 36.4% reduction in cell survival relative to RB-proficient isogenic controls at doses 10^{-10}, 10^{-9}, and 10^{-8} mol/L of docetaxel, respectively (*, P < 0.05, ANOVA). Similarly, RB-deficient cells showed enhanced sensitivity to etoposide at all doses tested (17%, 21.1%, and 25.7% reduction in cell survival relative to RB-proficient isogenic controls at doses 10^{-6}, 10^{-7}, and 10^{-8} mol/L, respectively *, P < 0.05, ANOVA) indicating that RB-deficient prostate cancer cells are more sensitive to cytotoxic effects of antimicrotubule agents and topoisomerase inhibitor. Combined, the data herein identify RB as a key modulator of the response to therapeutic intervention in prostate cancer cells.

**Discussion**

Given the frequency of RB inactivation in prostate cancer, the present study assessed the consequence of acute and chronic RB deficiency in androgen-dependent prostate cancer cells. These cells retain an active RB pathway and express the required components important for G_{1} to S phase control (Fig. 4 and Supplementary Figs. S1 and S2). Effective RB depletion was

![Figure 5. RB depletion sensitizes prostate cancer cells to selected chemotherapeutics. Stable clones were cultured in androgen-containing media (ΔFBS), and following 24 h in culture, cells were supplemented with indicated concentrations of SAHA (A), cisplatin (CDDP; B), antimicrotubule agents paclitaxel (PTX) and docetaxel (DCTX; C), and etoposide (ETOP; D) or 0.1% DMSO (vehicle control) and challenged for 48 h. Cells were resupplemented with fresh media without drugs and allowed to propagate for another 48 h and then counted using trypan blue exclusion method. The number of cells remaining after drug treatment was set relative to condition without cytotoxic challenge (100% survival). Data represent at least three independent experiments, wherein data were collected with duplicate biological replicates. *, P < 0.05, ANOVA.](image-url)
achieved through either acute or chronic mechanisms and molecular analyses of stable RB depletion revealed selected derepression of E2F target genes and compensatory induction of RB family members, p107 and p130 (Fig. 1). Although these events were insufficient to confer growth advantage in the presence of androgen (Figs. 2 and 6B and Supplementary Fig. S3), RB-deficient cells showed resistance to cell cycle arrest as induced by androgen ablation or AR antagonists (Figs. 3 and 6B), and were highly refractory to combined androgen blockade (Figs. 4 and 6B). These data indicate that RB ablation is sufficient to bypass the most frequently used therapeutic modality for disseminated prostate cancer. Further dissection of the consequence of RB status revealed that RB depletion sensitized cells to selected chemotherapeutics (antimicrotubule agents and topoisomerase inhibitor; Figs. 5C and D and 6D) and conferred modest resistance to platinating agent (Figs. 5B and 6C). Combined,

Figure 6. Impact of RB depletion is conserved in multiple prostate cancer cell systems. A, stable clones of RB knockdown (L4-shRB) or control (L4-shCon) were generated from parental LAPC-4 cells and tested for RB expression levels using immunoblot analysis. B, L4-shRB and L4-shCon cells were cultured in 10% FBS or CDT media supplemented with or without Casodex, and cell number was monitored at indicated time points using trypan blue exclusion. Data represent at least three independent experiments wherein data for each timepoint were collected with triplicate biological replicates. C and D, stable clones were cultured in 10% FBS, treated with SAHA, cisplatin, paclitaxel, docetaxel, and etoposide as described in Fig. 5, and cell number was measured using trypan blue exclusion. The number of cells remaining after drug treatment was set relative to condition without cytotoxic challenge (100% survival). Data represent at least three independent experiments wherein data were collected with duplicate biological replicates. * P < 0.05, ANOVA.
these data strongly implicate RB status as a determinant of therapeutic response in prostate cancer cells.

RB functional inactivation occurs with relatively high frequency in prostate cancer and can occur through multiple mechanisms. Point mutations in the coding region of the RB gene, deletion of the RB promoter, LOH, decreased RB protein expression levels, and loss of p16INK4a (an upstream regulator of RB pathway) have all been reported in prostate adenocarcinoma (14–16). It is believed that RB inactivation occurs in late stages of the disease, as one study has shown that the loss of RB expression was more profound in metastatic tissue specimens compared with primary tissue samples. Furthermore, these alterations of RB expression showed a correlation with increasing tumor stage and grade (16). These results suggest that the loss of RB expression occurs more frequently in higher stages and grades of prostate cancer and, thus, may be contributing to the malignant progression of human prostate cancer. Based on these data, it has been proposed that RB plays a significant role in prostate cancer development and/or progression. Tissue recombination studies and mouse models have also implicated the role for RB in these processes. Prostate epithelium from RB−/− embryonic pelvic viscera, when recombined with wild-type rat urogenital mesenchyme under the kidney capsule of male nude mice, became hyperplastic in 40% of grafted samples (33). Similarly, conditional RB deletion in murine prostate resulted in focal hyperplasia that is potentially reminiscent of early-stage disease (34). A more specific challenge of RB action in the prostate was reported by Hill et al. (35), who expressed a mutant of SV40 large T antigen that specifically targets RB and related pocket proteins; this event resulted in prostatic intraepithelial neoplasia lesions followed by focally invasive, well-differentiated adenocarcinomas. These effects are exacerbated by combinatorial p53 deletion, which results in fast progressing metastatic carcinomas of the prostate (36). Moreover, mouse models like TRAMP based on prostate-specific expression of the SV40 large T antigen in prostate epithelial cells have been reported to encompass the full spectrum of neoplastic progression from prostatic intraepithelial neoplasia to invasive cancer (37). Despite these reports implicating RB inactivation in prostate cancer, few studies have dissected the ramifications of this event on cellular or clinical outcomes relevant to tumor management.

Here, we have used two different model systems, LNCaP and LAPC-4 androgen-dependent prostate cancer cells that reflect early-stage prostate cancer. These cells are dependent on androgen for growth and proliferation, express p16INK4a, retain functional cyclin D1-RB pathway, and express various components important for cell cycle (refs. 8, 9, 38 and Supplementary Figs. S1 and S2). It is important to note that adenocarcinoma cells exhibit a cell autonomous proliferative response to androgen whereas in the normal epithelia, the proliferative effects of androgen are indirect as mediated through stromal-derived paracrine factors (39). Thus, the program by which androgen induces the cell cycle machinery is unique to adenocarcinoma cells.

Our data show that the ability of RB depletion to confer a proliferative advantage is dependent on androgen status. Specifically, no significant proliferative advantage was conferred by RB depletion in the presence of androgen, whereas RB-deficient cells were resistant to cell cycle arrest induced by androgen depletion and/or AR antagonists. These data are consistent with the hypothesis that androgen inactivates RB as a part of its mitogenic program. Zhou et al. have recently shown that RB- and p53-deficient murine prostate epithelial cells had similar proliferative indices in the presence or absence of androgen, indicating that RB deficiency may enable bypass of androgen deprivation therapy in vivo (36). Conversely, previous studies have shown that the introduction of SV40 large T antigen or viral oncoprotein E1A, which act in part to sequester RB function, can bypass the response to androgen ablation in prostate cancer cells (8). Combined, these collective data reveal a major role for RB in the response to hormone or AR manipulation. Although this concept has yet to be rigorously challenged in the clinic, at least one study showed that 12 out of 33 patients that received hormonal therapy harbored tumors with RB abnormalities (22). Furthermore, fluorescence in situ hybridization (FISH) analysis of RB gene aberrations in advanced prostate tumor specimens before and after hormonal therapy have reported that loss of the RB gene was almost four times more frequent after androgen deprivation therapy than before therapy, thus indicating that RB loss may enhance survival of prostate cancer cells (40). These collective observations highlight the importance of assessing the impact of RB status on the efficacy of hormone-based therapies in vivo.

The mechanisms by which RB depletion facilitates the bypass of hormonal therapies remain an active area of investigation. Although it would be suspected that RB depletion would initiate unscheduled cell cycle progression, derepression of only a subset of E2F target genes was observed. Compensation by p130 may account for this phenomenon, and future studies will be directed at dissecting the precise cohort of genes deregulated by RB depletion that confer androgen-independent proliferation. A recent study has shown that the elevation of E2F1 may contribute to the progression of hormone-independent prostate cancer through its ability to repress AR transcription (41). This indicates that RB depletion may lead to bypass of hormonal therapies through deregulation of the AR transcriptional pathway; however, this area still needs further investigation. The present report is the first to link RB status to the efficacy of AR antagonists used in therapy (e.g., Casodex). This agent functions by promoting the recruitment of transcriptional repressors to the AR complex (28). Disruption of this process (as occurs through inflammation-induced displacement of NCoR; ref. 42) nullifies the effect of AR antagonists. Given this precedent, it will be of interest to determine the impact of RB (and downstream targets) on corepressor recruitment.

Despite the negative impact of RB depletion on the response to first-line intervention, there is strong precedent in the literature to indicate that such a genetic disruption can be capitalized on in cancer management. Specifically, RB is a critical component of DNA damage checkpoint signaling as elicited by the exposure to specific chemotherapeutics (17). Although these effects are often cell type specific, failure to arrest in response to DNA damage can result in enhanced cell death. Our data clearly show that RB status has little bearing on the response to cytotoxic intervention with SAHA, an HDAC inhibitor shown to inhibit prostate cancer growth (30, 31) and currently in clinical trials. A recent study has shown that the combination of low, subeffective doses of SAHA and Casodex resulted in synergistic reduction in cellular proliferation and increase in caspase-dependent cell death (43). This indicates that although SAHA may not be very effective as a single agent therapy, it may prove beneficial when used in combinatorial treatments. Unexpectedly, RB-depleted prostate cancer cells were less sensitive to cisplatin (CDDP) than RB-proficient isogenic controls. This result was unexpected because RB ablation has been shown to sensitize breast cancer cells and murine embryonic fibroblasts to the cytotoxic effects of cisplatin through the deregulation of the cell cycle (17, 18). Thus, future
studies will be directed at determining the mechanism behind the resistance to platinating agents. Finally, whereas RB deficiency did not sensitize prostate cancer cells to SAHA or CDDP, RB depletion enhanced the cytotoxic response to antimicrotubule agents (paclitaxel and docetaxel) and a topoisomerase inhibitor (etoposide). These are consistent with a previous study that showed that DU-145 cells (which are RB deficient) are more sensitive to the apoptotic effects of paclitaxel than PC-3 cells (which are RB proficient; ref. 44). Similar results were observed in ovarian cancer cells (CAOV-3), which were sensitized to camptothecin, a topoisomerase I inhibitor through RB phosphorylation (45). Although not a direct measure, induction of E2F activity in different tumor cell lines has been shown to correlate with increased sensitivity to etoposide (46). Together, these data suggest that deregulation of RB/E2F signaling pathway may be one of the mechanisms for increased sensitivity to antimicrotubule agents and topoisomerase inhibitors.

One remaining question is whether the consequence of RB depletion on therapeutic response is functionally equivalent to the loss of RB function as achieved by other mechanisms, e.g., p16INK4a loss which renders RB inactive and is known to induce cellular and transcriptional outcomes that are distinct from RB loss (18). Alternatively, RB can be inactivated through the loss of SWI/SNF activity (47, 48), although whether this holds true in prostate cancer cells has not been addressed. As such, future studies will be directed at dissecting differential mechanisms of RB loss as a function of therapeutic outcome.

Together, these studies show that RB deficiency confers no significant growth advantage under steady-state conditions, but triggers bypass of hormone therapies. However, RB depletion significantly sensitized cells to cytotoxic effects of chemotherapeutic agents that function by inhibiting topoisomerase II or microtubule organization. These collective data establish RB as a potentially critical determinant of therapeutic efficacy for first-line and putative second-line therapies in prostate cancer and provide the foundation for future studies directed at using RB status as a potential metric for optimizing therapeutic regimens in effective prostate cancer management.

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References

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