Retinoblastoma Deficiency Increases Chemosensitivity in Lung Cancer

William A. Zagorski, Erik S. Knudsen, and Michael F. Reed

Abstract

The retinoblastoma (RB) tumor suppressor is mutated or functionally inactivated in the majority of human malignancies, and p16INK4a-cyclin D1-cyclin-dependent kinase 4-RB pathway aberrations are present in nearly all cases of non-small cell lung cancer (NSCLC). Here, the distinct role of RB loss in tumorigenic proliferation and sensitivity to chemotherapeutics was determined in NSCLC cells. Attenuation of RB deficiency resulted in bypass of the checkpoint response to multiple chemotherapeutic agents. In vitro analysis showed that RB deficiency enhances sensitivity to chemotherapeutic challenge, efficient and sustainable response is highly dependent on the specific therapeutic regimen, in addition to the molecular environment.

Introduction

Lung cancer is the leading cause of cancer mortality worldwide with over one million deaths annually (1). It is estimated that >170,000 new cases will be diagnosed in the United States in 2006 alone (2). Lung cancer accounts for more deaths than the combined fatalities of breast, colon, and prostate cancers, the next three leading causes of cancer mortality. The 5-year survival rate after diagnosis of lung cancer is merely 14%, compared with 86% for breast cancer, 61% for colon cancer, and 96% for prostate cancer (2). First-line therapy for early-stage non–small cell lung cancer (NSCLC), which accounts for >80% of lung cancer, remains surgical resection; however, for patients who receive surgical resection, 5-year survival rates are still below 50%. Small cell lung cancer (SCLC), accounting for <20% of lung cancer, is primarily treated with chemotherapy and radiation therapy. Nonsurgical treatments for NSCLC, including chemotherapy and radiation therapy, constitute the primary therapeutic modalities for advanced disease. Importantly, adjuvant chemotherapy after complete surgical resection has recently been shown to improve survival (3). Thus, chemotherapeutic treatment is used for most stages of NSCLC. Hence, a better understanding of the molecular determinants of chemotherapeutic response will directly affect outcome in the majority of patients with lung cancer and would be useful for better directing of such regimens.

The retinoblastoma (RB) tumor suppressor is a potent inhibitor of cellular proliferation which is functionally inactivated via an assortment of mechanisms, mutated, or genetically lost in the majority of human tumors (4–6). The p16INK4a-cyclin D1-cyclin-dependent kinase 4 (CDK4)-RB pathway is inactivated in the majority of NSCLC (7–13). In NSCLC, RB is inactivated through disparate mechanisms including mutation (7–9), deregulated phosphorylation (14, 15), alternative splicing (16), or homozygous deletion or point mutation (16–19). Additionally, p16INK4a deficiency is particularly frequent in NSCLC (20, 21), resulting in compromised RB function. Conversely, abnormalities of RB are present in >90% of SCLC whereas p16INK4a abnormalities are rare (21). In its active hypophosphorylated state, RB acts as a transcriptional co-repressor at the promoters of E2F-regulated genes, thereby blocking cell cycle progression (22–24). Upon mitogenic signaling, RB is inactivated through hyperphosphorylation by the action of CDK4-cyclin D and CDK2-cyclin E complexes (22, 25, 26). These actions are sufficient to release RB from E2F promoters relieving transcriptional repression, thereby allowing cell cycle progression. In contrast, in the presence of antimitogenic signaling, RB remains in its active hypophosphorylated state, inhibiting cell cycle progression.

Appropriate coordination of the cell cycle maintains genomic integrity by ensuring faithful replication and partitioning of the genome (27, 28). Loss of RB function leads to deregulation of cell cycle control such that cells respond inappropriately to chemotherapeutic challenge (29–31). The mechanisms of this are not completely understood, but discrete targets of RB-mediated transcriptional repression include genes involved in cell cycle control and DNA repair. Some of these genes are, in fact, the targets of certain chemotherapeutic agents commonly used in the clinical arena (32). For example, the known RB targets thymidylate synthase and topoisomerase I are the targets of 5-fluorouracil (5-FU) and etoposide (VP-16), respectively.

With functional inactivation of the p16INK4a-cyclin D1-CDK4-RB pathway occurring in most human cancers and deregulation of RB activity disrupting cell cycle checkpoint control and thereby altering chemosensitivity (33–36), RB status represents a significant modifier of chemosensitivity in lung cancer. As the p16INK4a-cyclin D1-CDK4-RB pathway is inactivated in the majority of NSCLC and chemotherapies are the primary nonsurgical treatment modalities for most stages of disease, NSCLC can serve as an ideal system to evaluate the discrete mechanisms of p16INK4a-cyclin D1-CDK4-RB pathway inactivation on therapeutic response. Here, we use short hairpin RNA (shRNA) to effectively knockdown RB expression in NSCLC cells, resulting in deregulation of E2F target genes and...
increase in proliferative capacity in vitro. Additionally, we show that RB deficiency allows cells to inappropriately progress though the cell cycle in the presence of chemotherapeutic agents, resulting in elevated levels of apoptosis. Moreover, NSCLC xenograft tumors deficient in RB activity exhibit accelerated tumor growth and respond more effectively to chemotherapeutic challenge. Together, these results show that RB deficiency enhances proliferative and tumorigenic capacity, as well as elicits more efficacious responses to chemotherapeutic challenge in lung cancer cells in vitro and in vivo.

Materials and Methods

Cell culture and transfection. NSCLC lines H1299, H520, and H522 (National Cancer Institute) were obtained from American Type Culture Collection. All three lines harbor wild-type RB and all express greatly reduced or undetectable p16INK4a. H1299 cells are p53 null, whereas H520 cells express greatly reduced levels of p53 mRNA relative to normal lung tissue but exhibit no gross structural DNA abnormalities and H522 cells express mutant p53. All cell lines were maintained in RPMI 1640 (Cellgro, Mediatech) containing 10% fetal bovine serum (Biofluids) and supplemented with 100 units/mL penicillin-streptomycin and 2 mmol/L L-glutamine (Mediatech). Cells were cultured at 37°C in a 5% CO2 humidified incubator. H520 cells were transfected via the FuGene6 lipid-based reagent (Roche) with either pMSCVpuro (MSCV)-Rb3c containing a validated Rb shRNA construct (37) and a puromycin resistance gene or MSCV containing an empty vector control and a puromycin resistance gene. Transfectants were selected and maintained in 2.5 μg/mL puromycin (CalBiochem). H1299 and H522 cell lines were infected with a retrovirus encoding a shRB plasmid (MSCV-LMP-Rb88) or the virus encoding the

![Figure 1](https://www.aacrjournals.org) Efficient knockdown of RB expression in lung cancer cells results in deregulation of RB/E2F target genes. A, H1299 and H522 cells were infected with retrovirus encoding empty vector (MSCV) or si-Rb88 (Rb88). H1299 cells were additionally infected with retrovirus encoding a nonspecific shRNA (si-NS). H520 cells were transfected with MSCV vector or MSCV-Rb3c shRNA vector and selected with puromycin for stable incorporation of the plasmids. Whole-cell lysates were collected from both lines and immunoblotted for expression levels of RB, p130, and p107. Cdk4 served as a loading control. B, stable lines were screened for RB expression by immunofluorescence. Images were taken at equal exposures. Bars, 20 μm. C, lysates represented in (A) were immunoblotted for expression levels of topoisomerase IIα, cyclin A, and cyclin E. Cdk4 was used as a loading control.
empty vector control (MSCV-LMP) as previously described (38). In addition, H1299 cells were infected with a retrovirus encoding a nonspecific sh-control. Cells were washed 18 h postinfection and fed with medium containing puromycin for selection and maintenance. Growth curves were established by seeding $1.0 \times 10^4$ cells in 6-cm plates. Cells were trypsinized and counted by trypan blue exclusion every 48 h.

**Immunoblotting.** For protein analyses, exponentially growing cells were harvested and lysed in a radioimmunoprecipitation assay buffer

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**Figure 2.** Knockdown of RB results in an increase in proliferation in vitro and oncogenic growth in vivo. A, exponentially growing cells were pulsed with BrdUrd for 1 h, stained with propidium iodide and subjected to flow cytometric analysis. Histograms represent 20,000 events. B, $1.0 \times 10^4$ cells were initially plated, and viable cells were counted by trypan blue exclusion every 48 h. Samples were counted thrice, and experiments were done in triplicate. C, $2.5 \times 10^5$ cells in 150 μL PBS with 50 μL Matrigel were injected s.c. in contralateral flanks of nude mice. Tumors were measured every 2 d with calipers. Tumor volume was calculated as $v = \pi \times (\text{width}^2 \times \text{length})/6$. 

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supplemented with a protease inhibitor mixture (35). After brief sonication and clarification, protein concentrations were determined with the Bio-Rad DC Protein Assay (Bio-Rad) and equal protein was loaded and subjected to SDS-PAGE. After transfer to Immobilon-P membrane (Millipore Corp.), specific proteins were probed using the following primary antibodies: RB polyclonal antiserum (30), topoisomerase IIα (H-231 polyclonal; Santa Cruz Biotechnology, Inc.), Cyc A (H-432 polyclonal; Santa Cruz), Cyc E (HE-12 monoclonal; Santa Cruz), p107 (C-18 polyclonal; Santa Cruz), p130 (C-20 polyclonal; Santa Cruz), and cdk4 (H-22 polyclonal; Santa Cruz) used as a loading control. Proteins were detected using horseradish peroxidase–conjugated secondary antibody (Pierce Biotechnologies, Inc.) and were visualized using Enhanced Western Lightning chemiluminescence (Perkin-Elmer Life Sciences, Inc.).

Immunofluorescence. Approximately 3 × 10^5 cells were plated on coverslips and allowed to adhere overnight. Cells were fixed in 3.7% formaldehyde and permeabilized in 0.4% Triton X-100 for 20 min at room temperature. After blocking in 5% goat serum, coverslips were incubated with mouse monoclonal anti-human RB antibody (Rb G3-245; BD Biosciences) for 1 h at 37°C. Coverslips were then incubated with goat anti-mouse rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) in addition to 0.1 μg/mL Hoechst 33258 (Sigma-Aldrich) nuclear stain. Coverslips were mounted on glass slides and visualized by indirect immunofluorescence.

Flow cytometry. Exponentially growing cells were pulsed with BrdUrd (Amersham, GE Healthcare Biosciences Corp.) for 1 h. Cells were trypsinized and fixed in 80% ice-cold ethanol. Cells were stained with FITC-conjugated anti-BrdUrd (BD Biosciences) and propidium iodide (0.2 μg/μL), and then subjected to flow cytometry. Samples were analyzed and quantitated on a Beckman Coulter Cell Lab Quanta SC flow cytometer. Histograms represent 20,000 events.

BrdUrd and TUNEL assays. Approximately 1 × 10^5 cells were seeded onto coverslips and allowed to adhere overnight. Cells were then treated with PBS, 16 μmol/L cis-diaminedichloroplatinum II (CDDP, cisplatin; Ben Venue Laboratories, Inc.), 24 μmol/L VP-16 (Sigma), or 50 μmol/L 5-FU (Sigma) for 72 h. For BrdUrd analysis, cells were pulsed with BrdUrd for 4 h before fixing in 3.7% formaldehyde and stained for BrdUrd as previously described (38). For TUNEL assays, cells were fixed in 3.7% formaldehyde and stained according to the manufacturer's protocol (Roche). Graphs represent three independent experiments, each with at least three fields of at least 200 cells counted per experiment.

Xenografts. Tumors were grown as xenografts in 6-week-old to 8-week-old athymic nude mice (Harlan Sprague-Dawley, Inc.) by s.c. flank injection of 2.5 × 10^5 cells in 150 μL PBS with 50 μL Matrigel (BD Biosciences). Tumors were measured with calipers every 48 h, and volume was calculated as v = π(width^2 × length)/6. When tumors reached a volume of 90 to 110 mm^3, mice were randomized into treatment groups and chemotherapy was initiated. Treatment groups were as follows: 0.9% saline in 100 μL daily for 5 days, 5 mg/kg CDDP in 100 μL 0.9% saline daily for 5 days, 20 mg/kg VP-16 in 100 μL 0.9% saline daily for 3 days, or 30 mg/kg 5-FU in 100 μL 0.9% saline daily for 5 days. Treatments were either given in one course or continued until euthanization with 3 days between courses. All treatments were done through i.p. injection. Two hours before euthanization, mice were injected i.p. with 150 mg/kg BrdUrd in 100 μL 0.9% saline. All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Immunohistochemistry. Tumors were dissected, fixed in 10% neutral buffered formalin for at least 24 h, embedded in paraffin, cut into 5-μm sections, and subjected to BrdUrd staining. Representative micrographs are shown (magnification, 200 ×) for RB-proficient and RB-deficient tumors and quantified (right). The graphs represent at least four independent tumors with three random fields of >500 cells scored per tumor section in a random manner. *** P < 0.001.
sections, and adhered to slides. For immunohistochemical analysis, sections were deparaffinized in xylene and rehydrated through a series of graded ethanol/water solutions. BrdUrd staining was done with a detection kit (Zymed Laboratories, Inc.) as recommended by the manufacturer. Sections were counterstained in Harris hematoxylin (Poly Scientific), dehydrated through a series of ethanol/water solutions, and overlaid with coverslips in permanent mounting medium (Permount, Fisher Scientific). BrdUrd incorporation was scored by counting at least three random fields of at least 500 cells per section. Graphs represent at least three independent tumors per treatment group. TUNEL staining was done with the DeadEnd Colorimetric TUNEL system kit (Promega) as recommended by the manufacturer. Sections were counterstained, dehydrated, covered, and counted as above.

Statistical analysis. All statistical analyses were done with GraphPad Prism (GraphPad Prism Software, Inc.). P values were calculated by performing one-way ANOVA analysis with a Newman-Keuls posttest.

Results

RB deficiency in NSCLC lines results in deregulation of RB/ E2F targets and increased proliferation in vitro and in vivo. The p16INK4a-cyclin D1-CDK4-RB pathway is targeted at high frequency through heterogeneous mechanisms in lung cancer. Here, we determined the specific effect of RB loss on lung cancer cells using the human lung cancer cell lines H1299, H520, and H522, which harbor wild-type RB yet contain other aberrations in p16INK4a-cyclin D1-CDK4-RB pathway function, namely loss of p16INK4a expression (absent in H1299 and H520, greatly reduced in H522), as well as exhibit differing p53 status (null in H1299, greatly reduced at the mRNA level in H520, and mutated in H522). RB knockdown was achieved using validated shRNA vectors (37, 38) that target human Rb. After selection, RB expression was determined via immunoblotting (Fig. 1A) and confirmed by immunofluorescence microscopy (Fig. 1B). The expression of RB was virtually undetectable in all three cell lines, and hence, these cells were considered RB deficient. To show that compensation by other pocket proteins did not occur to a significant degree, levels of p107 and p130 were shown to be minimally altered in cells other pocket proteins did not occur to a significant degree, levels was virtually undetectable in all three cell lines, and hence, these

To determine the influence of RB deficiency on lung cancer proliferation rates, H1299, H520, and H522 cells proficient (Fig. 2A, left and right) and deficient (Fig. 2A, middle) in RB activity were subjected to flow cytometric and BrdUrd incorporation analyses. RB-deficient cells showed an enhanced rate of proliferation shown by an increase in cells in S phase, a nearly 10% increase in BrdUrd incorporation in H1299 and H520 cells, and roughly a 5% increase in H522 cells. This effect was validated in H1299 cells harboring the nonspecific shRNA which showed no significant change in cell cycle distribution, S phase, or BrdUrd incorporation, compared with the empty vector control (Fig. 2A). To determine the long-term effect on proliferation after attenuation of RB expression, cells were subjected to growth assays by counting viable cells via trypan blue exclusion every 48 h for 14 days. RB knockdown resulted in a significant increase in long-term proliferation in all three cell lines compared with controls (Fig. 2B), demonstrating that RB loss greatly accelerates proliferation in NSCLC cells.

To evaluate the biological effect of RB loss on oncogenic growth, RB-proficient and RB-deficient H1299 and H520 cells (2.5 × 10^5) were s.c. injected contralaterally into the flanks of nude mice to be grown as xenografts. Within 5 days postinjection, H1299 and H520 tumors were palpable and were measured by calipers every 48 h throughout the experiment. Xenografts of RB-deficient H1299 and H520 cells grew more rapidly than those of RB-proficient cells. This effect was more pronounced in the H520 cells as a result of the duration of the experiment (Fig. 2C). Mice were injected i.p. with 150 mg/kg BrdUrd 2 h before euthanization, and tumor sections were stained for BrdUrd to confirm that the increase in tumor volume was the cumulative result of increased proliferation. H1299 and H520 RB-deficient tumors showed a higher rate of BrdUrd incorporation (44.3% and 26.3%, respectively) compared with RB proficient tumors (21.2% and 17.8%, respectively; Fig. 2D). Representative micrographs of both RB-deficient and RB-proficient tumor sections are shown in Fig. 2D (middle and right). Thus, loss of RB, even in the context of a highly aggressive NSCLC cell line, accelerates proliferation, as well as tumorigenic progression, in an in vivo NSCLC xenograft model.

RB deficiency causes an increase in sensitivity to chemotherapeutic exposure in vitro. Lung cancer is treated with a variety of chemotherapeutic agents that act through distinct mechanisms. Initially, we determined effective doses of certain clinically relevant agents by performing dose-response analyses (data not shown) for subsequent treatments. To investigate the effect of loss of RB activity on the sensitivity to chemotherapeutic treatment, RB-proficient and RB-deficient H1299, H520, and H522 cells were treated with PBS (control), 16 μmol/L cisplatin (CDDP), 24 μmol/L VP-16, or 50 μmol/L 5-FU for 72 h. These cells were subsequently labeled with BrdUrd, and BrdUrd incorporation was determined by immunofluorescence. RB deficient cells showed a significantly higher incorporation of BrdUrd compared with RB-proficient cells in the presence of therapeutic challenge (Fig. 3A). Thus, RB is required for effective checkpoint control after exposure to chemotherapeutic agents. H1299, H520, and H522 cells exposed to the same drugs as described in Fig. 3A were also investigated to determine the relative level of apoptosis dependent upon RB expression. After chemotherapeutic exposure, cells deficient in RB were found to have significantly higher levels of apoptosis, as determined by TUNEL staining, compared with cells proficient in RB (Fig. 3B). Therefore, RB loss permits progression through the cell cycle despite genotoxic exposure. However, a concomitant greater degree of apoptosis is observed. Thus, the net effect on sensitivity represents the interplay between cellular proliferation and apoptosis.

Lung cancer xenografts deficient in RB exhibit an increase in sensitivity to chemotherapeutic agents. To test the overall influence of RB loss on therapeutic response, we used the H520 and H1299 xenograft models described in Fig. 2C and D. Tumors were grown to a volume of 90 to 110 mm^3, and treatment was then initiated. To determine the role of RB on the effect of short-term exposure to chemotherapeutic agents, mice received either 5 mg/kg CDDP daily for 5 days (H520 and H1299), 20 mg/kg VP-16 daily for 3 days (H520 only), or 30 mg/kg 5-FU daily for 5 days (H520 only). Black bars in graphs (Fig. 4A) represent administration of treatment. After exposure to each of these agents, RB-deficient tumors either regressed slightly in the presence of 5-FU (Fig. 4A, bottom) or ceased growing during treatment in the presence of CDDP and VP-16 (Fig. 4A, middle).
and top). However, with discontinuation of treatment, RB-deficient tumors showed a rapid recovery, and both RB-proficient and RB-deficient tumors resumed growth at similar rates in all therapeutic groups (Fig. 4B).

To investigate the role of RB on long-term chemotherapeutic treatment, mice received the same doses of agents as previously described, but the regimen was maintained with 3 days between repeated administrations until euthanization (Fig. 5A-D: H1299, CDDP; H520, CDDP; H520, VP-16; and H520, 5-FU, respectively). Here, tumors lacking RB expression either ceased proliferating or regressed slightly compared with tumors proficient in RB (Fig. 5). With continual exposure to any of the three chemotherapeutic agents, RB-deficient tumors showed a significantly reduced incorporation of BrdUrd. Therefore, tumors with attenuated RB were significantly less proliferative than RB-proficient tumors in the presence of maintained therapeutic challenge. Not unexpectedly, apoptosis was also found to be significantly higher in RB-deficient tumors in all treatment groups, as determined by TUNEL staining. Representative micrographs of RB-proficient and RB-deficient H1299 tumors from the CDDP treatment group are shown (Fig. 5A). Together, these results show that RB-deficient tumors respond transiently to a single exposure of chemotherapy. Yet, with prolonged exposure, RB deficiency results in decreased proliferation concomitant with increased apoptosis yielding greater chemosensitivity.

Discussion

The RB tumor suppressor plays a critical role in control of cell cycle progression, including arrest mechanisms invoked by genotoxic and cytotoxic insults, such as chemotherapy. The p16<sup>INK4a</sup>-cyclin D1-CDK4-RB pathway is disrupted in majority human malignancies (4–6, 39). In NSCLC, p16<sup>INK4a</sup>-cyclin D1-CDK4-RB pathway activity is disrupted through disparate mechanisms in majority of the cases. Chemotherapy remains the primary modality for treatment in late-stage NSCLC and is also indicated as adjuvant therapy in early-stage, surgically resected disease. However, little is known about the role of RB in regulation and modification of response to chemotherapeutic challenge in NSCLC.

Loss of RB has been implicated in promoting aberrant proliferation in the context of cancer etiology. However, much less is known regarding the effect of RB loss on the progression of tumorigenesis. Furthermore, it remains unclear how the disparate mechanisms, through which the p16<sup>INK4a</sup>-cyclin D1-CDK4-RB pathway is compromised, affect discrete tumor types. In primary fibroblast cell culture models, loss of RB provides a slight proliferative advantage (40).

Figure 3. RB deficiency results in an increase in chemosensitivity via loss of checkpoint control and increase in apoptosis. A, 1.0 × 10<sup>5</sup> cells were plated on coverslips, allowed to adhere overnight, and treated with PBS(−), 16 μmol/L circular CDDP, 24 μmol/L VP-16, or 50 μmol/L 5-FU for 72 h and pulse-labeled with BrdUrd the last 4 h. BrdUrd immunofluorescence was done. B, cells were plated and treated as in (A). TUNEL immunofluorescence was done. All samples in (A) and (B) were scored by counting three random fields of at least 200 cells, and graphs represent three independent experiments. ***, P < 0.001.
However, the extent to which RB participates in proliferative control is highly context-dependent, and in specific settings, loss of RB function is paradoxically associated with inhibition of tumorigenic proliferation (41). Here, we sought to delineate the effect of RB deficiency on the control of proliferation in the more genetically complex, but clinically significant, setting of NSCLC. We have previously shown that a Rb shRNA construct can efficiently attenuate RB expression serving as a model for RB loss in NSCLC, resulting in deregulation of several E2F target genes, including thymidylate synthase and topoisomerase IIα, the molecular targets of 5-FU and VP-16, respectively (37). Here, we showed that, in three lung cancer cell lines, attenuation of RB caused no alteration in expression of other pocket proteins (p107 and p130), but the RB/E2F target genes cyclin A and cyclin E, as well as topoisomerase IIα, are consistently deregulated (Fig. 1). Subsequent studies of proliferation and cell cycle analysis showed that, similar to primary cells (42), loss of RB in NSCLC cells resulted in a modest proliferative advantage in vitro (Fig. 2). This phenomenon is somewhat surprising because these cells harbor deficiency of p16INK4a expression and thus, a priori, would have been expected to already harbor loss of p16INK4a-cyclin D1-CDK4-RB pathway activity. Thus, although p16INK4a-cyclin D1-CDK4-RB activity is compromised in the majority of human cancers, cells must retain sufficient RB function to enforce some control over proliferation. Consistent with these in vitro proliferative characteristics, RB deficiency accelerated tumorigenic growth in NSCLC xenografts. Interestingly, the influence of RB loss on oncogenic growth was very pronounced, particularly in the H520 line, suggesting that under the stress of tumorigenic growth, the effect of RB deficiency may be more significant than in the context of growth in culture. This might suggest that the more common p16INK4a-deficient type of NSCLC is likely to show slower growth than the more rare RB-deficient tumors. Importantly, these effects of RB on proliferation and tumorigenic growth were apparent although these three cell lines exhibit variable p53 status, indicating that perturbations in these

Figure 4. Lung cancer xenografts deficient in RB show a transient increase in sensitivity to chemotherapeutic exposure. A, 2.5 × 10⁶ cells in 150 μL PBS with 50 μL Matrigel were injected s.c. in contralateral flanks of nude mice. Tumors were measured every 2 d. Tumor volume was calculated as $V = \pi(\text{width}^2 \times \text{length})/6$. When tumors reached 100 mm³, a single course of treatment was initiated. Treatment groups were 5 mg/kg CDDP daily for 5 d (top), 20 mg/kg VP-16 daily for 3 d, or 30 mg/kg 5-FU daily for 5 d. Black bars on the X axis, treatment administration. B, the mice whose tumors are represented in (A) were allowed to recover after treatment ended and tumor measurements continued every 2 d until euthanization. All injections were done via i.p. delivery.
Figure 5. RB-deficient lung cancer xenografts exhibit increased chemosensitivity via decreased proliferation and increased apoptosis in the presence of long-term chemotherapeutic challenge. Cells (2.5 x 10^6) in 150 μL PBS with 50 μL Matrigel were injected s.c. into contralateral flanks of nude mice. Tumors were measured every 2 d. Tumor volume was calculated as V = π(width^2 x length)/6. When tumors reached ~100 mm^3, treatment was initiated. H1299 (A) and H520 (B), 5 mg/kg CDDP was injected daily for 5 d; H520 (C), 20 mg/kg VP-16 was injected daily for 3 d; H520 (D), 30 mg/kg 5-FU was injected daily for 5 d. All treatment courses were maintained with 3 d between repeated courses until euthanization (top). Two hours before euthanization, mice were injected with 150 mg/kg BrdUrd. All injections were done via i.p. delivery. Tumors were removed, fixed, embedded in paraffin, and cut into 5-μm sections. Tumor sections were subjected to BrdUrd staining. BrdUrd incorporation was quantified, and graphs represent at least three independent tumors with >500 cells scored in three random fields per tumor section in a blind manner. TUNEL staining was done to determine rates of apoptosis. Apoptosis was quantified, and graphs represent at least three independent tumors with >500 cells scored in three random fields per tumor section in a blind manner. Representative micrographs are shown in (A) at 200× magnification for BrdUrd staining (middle) and 400× magnification for TUNEL staining (bottom). **, P < 0.01; ***, P < 0.001.
two tumor suppressive pathways function independently in the context of NSCLC.

Similar to the majority of other cancers, lung cancer is a widely heterogeneous disease, in terms of genetic anomalies, clinical presentation, natural history, and response to therapy. In NSCLC, surgical resection remains the principal treatment modality for early-stage disease, whereas chemotherapy and radiation therapy remain the therapeutic modalities for unresectable late-stage disease. It has been found that in the genetically homogeneous setting of primary cells, the RB/E2F pathway plays an important role in mediating cell cycle inhibition, and acute loss of RB leads to diminished checkpoint response, genomic abnormalities, and modified sensitivity (29, 35). Here, in the case of NSCLC lines, we have shown that loss of RB activity allowed the bypass of the damage checkpoint induced by the chemotherapeutic challenge of three distinct agents (Fig. 3). Thus, the cell cycle arrest mediated by such agents seems to commonly function through the presence of RB. Again, these specific effects on cell cycle are p53-independent, indicating that the cell cycle responses to 5-FU, VP-16, and CDDP are intrinsically dependent on RB activity. Importantly, these results were recapitulated in additional NSCLC cell lines, indicating that this finding is not unique to a single cell line. Strikingly, in addition to bypass of checkpoint control, levels of apoptosis were significantly higher with RB deficiency. In vitro checkpoint bypass coupled with elevated apoptosis has been observed by others in primary cultures (43, 44) and most recently in breast cancer models (36). There exist a multitude of mechanisms through which p16INK4a-cyclin D1-CDK4-RB deficiency has been suggested to contribute to cell death after genotoxic damage, including as a consequence of cell cycle deregulation and higher damage burden, deregulation of proapoptotic gene products, or via effect on the p53-pathway. At present, we favor the former mechanisms because the cell death is occurring independent of p53 function. Thus, in NSCLC cells, RB deficiency exerted differential effects on therapeutic response, which lead us to directly evaluate the influence on therapeutic response in a tumor model.

The status of RB activity in lung cancer and its role in chemotherapeutic response has not been previously addressed. As the p16INK4a-cyclin D1-CDK4-RB pathway is disrupted in many human cancers (4–6, 39) and the vast majority of lung cancers (10), it is important to address the role of RB in chemosensitivity in a clinically relevant setting. NSCLC xenografts of matched cells, proficient or deficient in RB, provide an appropriate model to investigate this role. Here, we have shown that RB-deficient tumors respond favorably to initial challenge with chemotherapeutic agents, yet shortly thereafter return to an aggressive neoplastic state (Fig. 4). This transient sensitivity mimics the in vitro finding that loss of RB permits an increase in chemosensitivity. However, when tumors were removed from treatment, they rapidly returned to the previously aggressive state. This finding is, indeed, reminiscent of certain clinical responses observed in SCLC, which often harbors the genetic loss of RB (10). This parallels the results of Meuwissen and colleagues (45), who noted that in a murine model that development of the neural phenotype SCLC was dependent on loss of both p53 and RB. In contrast, when a wild-type RB allele was maintained, NSCLC (adenocarcinoma) ensued. In human SCLC, initial response to therapy is frequently quite good; however, recurrent disease commonly arises. Because the RB-deficient NSCLC tumors grow more rapidly, the strong initial response results in little durable effect once therapy is discontinued. These results lead us to question whether multiple treatment regimens would lead to resistance or enable a durable response. Under these conditions of maintenance therapy, we found that RB-deficient tumors either discontinued growth or began to regress, whereas RB-proficient tumors continued to grow, albeit with reduced kinetics under constant treatment with chemotherapeutic agents (Fig. 5). Although the mechanisms of RB-dependent chemosensitivity are not known, it is the subject of much ongoing research. In the case of prolonged exposure to therapy, surprisingly, tumors deficient in RB activity ultimately become less proliferative as shown by diminished BrdUrd incorporation. This could represent a direct cell cycle effect of RB deficiency, resulting in cellular senescence, or could be a result of lack of availability of required growth factors, for example, blood supply or nutrients, which are specifically affecting RB-deficient tumors. Future studies will address the mechanisms of decreased proliferation with long-term exposure to chemotherapeutics in the setting of RB deficiency.

In conclusion, we have shown that attenuation of RB activity in NSCLC cells results in further deregulation of downstream RB/E2F targets and leads to not only accelerated proliferation in vitro but also a heightened tumorigenic capacity in vivo. Furthermore, disruption of p16INK4a-cyclin D1-CDK4-RB pathway activity allows these cells to bypass specific checkpoint responses to chemotherapeutic agents, leading to enhanced apoptosis and ultimately an increase in sensitivity and response to chemotherapeutic challenge in a xenograft model. Further delineation of the mechanisms of altered sensitivity and modes of cytotoxicity with loss of RB function is needed. The improved understanding of this relationship and the knowledge of RB-dependent chemosensitivity will contribute to the individualized targeting of therapy based on the molecular characteristics of human tumors.

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