Retinoblastoma Function Is Essential for Establishing Lung Epithelial Quiescence after Injury

Nicole A. Mason-Richie, Meenakshi J. Mistry, Caitlin A. Gettler, Asmaa Elayyadi, and Kathryn A. Wikenheiser-Brokamp

Pathology and Laboratory Medicine and Neonatology, Perinatal and Pulmonary Biology, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, Ohio

Abstract

The retinoblastoma gene product (RB) regulates cell cycle, quiescence, and survival in a cell type–dependent and environment-dependent manner. RB function is critical in the pulmonary epithelium, as evidenced by nearly universal RB inactivation in lung cancer and increased lung cancer risk in persons with germline RB gene mutations. Lung carcinomas occur in the context of epithelial remodeling induced by cytotoxic damage. Whereas the role of RB in development and normal organ homeostasis has been extensively studied, RB function in the context of cellular injury and repair has remained largely unexplored. In the current studies, the RB gene was selectively deleted in the respiratory epithelium of the mouse. Although RB was not required for establishing or maintaining quiescence during lung homeostasis, RB was essential for establishing quiescence during epithelial repair after injury. Notably, aberrant cell cycle progression was sustained for 9 months after injury in RB-deficient lungs. Prenatal and postnatal RB ablation had similar effects, providing evidence that timing of RB loss was not critical to the outcome and that the injury-induced phenotype was not secondary to compensatory alterations occurring during development. These data show that RB is essential for repair of the respiratory epithelium after cytotoxic damage and support a critical unique role for RB in the context of epithelial remodeling after injury. Because human cancers are associated with chronic cellular damage, these findings have important new implications for RB-mediated tumor suppression. [Cancer Res 2008;68(11):4068–76]

Introduction

The retinoblastoma gene product (RB) is a critical cell cycle regulator and influences numerous cellular processes, including cellular differentiation, survival, terminal cell cycle exit, and maintenance of the post mitotic state (for review, see refs. 1, 2). The current model of RB function suggests a critical regulatory role in many, if not all, cell types. Consistent with this notion, RB is widely expressed during development and in adult tissues. However, germline RB gene ablation in the mouse leads to relatively mild phenotypes (9–15). This is due in part to functional redundancy and/or compensation by the other RB family proteins, p107 and p130. However, cellular response to loss of RB function is highly context specific. RB ablation in multiple epithelial cell types in the mouse leads to increased proliferation and apoptosis, whereas apoptosis does not accompany the aberrant proliferation observed in RB-deficient skin (12, 14, 15). Hepatocyte-specific RB ablation leads to aberrant S-phase entry that is associated with neither hyperplasia nor apoptosis (9). Finally, RB ablation targeted to the intestinal epithelium using the villin promoter causes no intestinal abnormalities (10). Thus, although RB is inactivated in carcinomas arising in multiple organs, epithelial cell response to RB loss is highly context specific.

Timing of RB ablation seems to play a critical role in determining phenotypic outcomes in vitro. Acute loss of RB in mouse embryo fibroblasts and keratinocytes in culture causes more severe cell cycle abnormalities than RB loss during development in vivo (14, 16). The physiologic relevance of temporally dependent effects of RB loss in culture remains unclear. Nonetheless, the data suggest that RB-related phenotypes may be influenced by the precise timing of RB loss.

RB is almost universally inactivated in lung cancers providing strong evidence that RB is a critical regulator in the pulmonary epithelium (for review, see ref. 17). Moreover, humans with RB germline mutations are at increased risk for developing lung cancer (18, 19). Lung carcinomas are associated with cigarette smoking in 80% to 85% of sporadic cases, as well as in patients with germline RB mutations (18, 19). Additionally, chronic lung diseases characterized by continual epithelial remodeling are associated with an increased risk of lung cancer in the absence of smoking (for review, see refs. 20, 21). Thus, like many other malignancies, lung cancer occurs in the context of chronic epithelial damage.

The present studies were designed to directly test whether RB function is critical in the context of lung epithelial remodeling after injury. Although RB was not required for establishing and
maintaining epithelial cell quiescence during lung homeostasis, RB was essential for establishing cellular quiescence in the context of epithelial regeneration after injury. Aberrant cell cycle progression in RB-deficient lungs was sustained for at least 9 months after injury, whereas the epithelium in RB proficient lungs was quiescent 2 weeks after injury. The phenotype was similar regardless of whether RB loss occurred during development or in the postnatal lung. These studies show that RB is essential in the remodeling lung epithelium and support a more critical role for RB in the setting of epithelial repair after injury compared with lung homeostasis. Because human malignancies are commonly associated with chronic injury, these findings have important implications for RB-mediated tumor suppression.

Materials and Methods

Animal generation and treatment. Mice with RB-deficient lung epithelium were generated by mating CC10-rtTA and tetO-Cre double transgenic mice to RB<sup>Flox/Flx</sup> mice and genotyped using tail and lung DNA, as previously described (15). Primers RB-18 and RB-19 were used to differentiate the floxed (746 bp), wild-type (678 bp), and recombined (321 bp) RB alleles. Thyroid-stimulating hormone β subunit (TSHβ) was amplified as an endogenous internal control to verify template DNA quality and quantity using primers 5′TCCTCAAGATGCTCATTAG3′ and 5′GATACTCAGTCAAGTCGAGT3′ at an annealing temperature of 55°C for 35 cycles resulting in a 386-bp band. Gestational age was determined by detection of a vaginal plug (designated embryonic day E0.5). Prenatal RB ablation was induced by doxycycline (Sigma) administration to pregnant dams with a single i.p. injection (125 mg/0.5 ml saline) on E0.5-E1.5 followed by maintenance on doxycycline in the drinking water (1.0 mg/ml) until birth. Postnatal RB ablation was induced in adult mice at 2 to 3 months of age by a single i.p. injection (125 μg/0.5 ml saline) followed by maintenance on doxycycline in the drinking water (1.0 mg/ml) until death. RB ablation was targeted to the lung epithelium by mating CC10-rtTA and tetO-Cre double transgenic mice bearing the reverse tetracycline responsive transactivator under control of the rat Clara cell 10 kDa protein (CC10)/Scegb1a1 gene promoter and Cre recombinase (Cre) under control of the tet operator and a minimal cytomegalovirus promoter were used to target RB ablation to the lung epithelium (15).

Results

Quiescence is established and maintained in adult RB-deficient lung epithelium. A conditional RB knockout model was used to target RB ablation to the lung epithelium (15). Double transgenic mice bearing the reverse tetracycline responsive trans-activator under control of the rat Clara cell 10 kDa protein (CC10)/Scegb1a1 gene promoter and Cre recombinase (Cre) under control of the tet operator and a minimal cytomegalovirus promoter were assessed on H&E-stained sections. Immunohistochemistry and TUNEL analysis was performed on deparaffinized 5μm sections after antigen retrieval in 10 mmol/L citrate solution microwaved for 7 min. Primary antibodies were diluted in 0.1% bovine serum albumin in PBS, applied to tissue sections, and incubated overnight at 4°C using the following dilutions: Ki67 1:50 (clone B56, BD Pharmingen), phosphorylated (Ser10) histone H3 1:1,000 (U.S. Biological), and Clara cell secretory protein (CCSP) 1:20,000 (kind gift from Steven Brody, Washington University). Antibody staining was detected with Vectastain Elite ABC, MOM Immunodetection, and DAB Substrate kits (Vector Laboratories, Inc.). For dual CCSP/BrdUrd immunolabeling, tissue sections were incubated with anti-CCSP antibody overnight followed by application of secondary antibody and ABC Elite Vectastain ABC-AP reagent (Vector Laboratories, Inc.). Positive staining was detected with Vector Alkaline Phosphatase Blue Substrate kit III (Vector Laboratories, Inc.). Subsequent BrdUrd analysis was performed using Zyomed BrdUrd Staining kit (Zyomed Laboratories, Inc.). Tissues were counterstained with hematoxylin or nuclear fast red. Counts represent evaluation of an average of 350 epithelial cells representing both proximal and distal conducting airways and at least two lung lobes per mouse. TUNEL analysis was performed using ApoTag Peroxidase In situ Apoptosis Detection kit (Chemicon International). Percentage of positive cells were determined on samples blinded to genotype by locating a TUNEL-positive cell and counting 100 cells surrounding the initially identified positive cell. Counts represent 200 to 400 epithelial cells per animal, including both proximal and distal conducting airways and at least two lung lobes per mouse. Only cells that were TUNEL positive, showed morphologic features of apoptosis and remained attached to the basement membrane were counted. Statistical significance was determined by unpaired Student’s t tests.

β-Galactosidase staining. In situ staining for β-galactosidase activity was performed on frozen tissue sections as previously described (15).

Figure 1. RB ablation results in sustained epithelial proliferation after injury. A, inducible RB ablation was targeted to the lung epithelium by mating CC10-rtTA and tetO-Cre double transgenic mice with RB<sup>Flox/Flx</sup> mice. Pregnant dams were treated with doxycycline (circles), which activates rtTA (arches) expressed under control of the lung epithelial specific promoter. Activated rtTA induces Cre expression leading to recombination at LoxP sites flanking exon 19 in the RB gene locus. B, H&E-stained sections of RB ablated and control adult lungs show similar overall morphology. C, immunohistochemical analysis for Ki67 in RB ablated and control adult lungs from mice treated with doxycycline throughout gestation. The percentage of Ki67-positive cells (arrows) was comparable in RB ablated and control lungs before naphthalene treatment (day 0) and at day 4 after injury (day 4). A statistically significant increase in Ki67-positive cells was noted on day 4 compared with day 0 in RB ablated (P = 0.009) and control (P = 0.011) lungs. In contrast, the percentage of Ki67-positive cells was significantly increased in RB ablated lungs versus controls on day 14 after injury (day 14). D, quantitative analysis is presented as average ± SE (*, P = 0.01). Representative of five to seven animals per time point. br, bronchicle. Original magnification, 1,000×.
bred into a \( Rb^{LoxP/LoxP} \) background (Fig. 1). Previous studies showed that Cre-mediated recombination is epithelial specific and occurs in the vast majority of epithelial cells throughout the conducting airway in a doxycycline-dependent manner (15). The rat CC10 promoter differs slightly from the endogenous mouse promoter, and therefore, Cre recombination expression and thus RB ablation is not restricted to Clara cells.

We previously reported that RB ablation in the lung epithelium causes epithelial hypercellularity with increased proliferation and apoptosis at birth (15). Adult lungs showed increased neuroendocrine cells but lacked the morphologic features of hyperplasia and apoptosis present at birth, suggesting that the majority of RB-deficient epithelial cells were capable of compensating for loss of RB function postnatally (Fig. 1; ref. 15). To directly determine whether RB-deficient epithelial cells in the adult lung entered quiescence, lungs from double transgenic mice were analyzed for the proliferation marker Ki67. Ki67 is expressed in all phases of the cell cycle, except \( G_0 \), and thus marks nonquiescent cells (22). Controls for this and subsequent experiments consisted of littermates lacking one or both transgenes required for RB ablation. Ki67 expression was similar in RB ablated and control lungs (Fig. 1), providing evidence that RB function is not essential for establishing and maintaining quiescence in the mature respiratory epithelium despite the marked cell cycle abnormalities present at birth.

RB is critical for establishing quiescence after injury. Mice with RB-deficient lungs were exposed to naphthalene to directly test whether RB function is critical during epithelial remodeling after injury. Cytotoxic damage induced by naphthalene is targeted to the lung epithelium because pulmonary epithelial cells contain high concentrations of the specific P-450 isoenzyme, CYP2F2, required for metabolizing naphthalene to its toxic metabolite (23). Temporal and morphologic characteristics of naphthalene-induced injury and subsequent repair are well characterized (24, 25). Briefly, diffuse epithelial damage occurs within the first 24 hours after naphthalene administration. Thereafter, the denuded airways are repopulated by naphthalene resistant cells. Cellular proliferation peaks on days 2 to 4, and the repair process is largely complete 2 weeks after injury.

Adult mice were treated with a single naphthalene injection at a dose known to induce epithelial injury throughout the conducting airways (26). Epithelial damage occurred in both proximal and distal conducting airways as confirmed morphologically (data not shown). Some death was observed within the first 2 weeks after treatment; however, RB ablated and control mice were similarly affected (21% (21 of 102) versus 26% (15 of 58), respectively). RB ablated and control lung epithelium was largely quiescent before treatment (Fig. 1). On day 4 after injury, epithelial proliferation was significantly increased over baseline levels in RB ablated (26 ± 5% versus 3 ± 1%, \( P = 0.002 \)) and control lungs (21 ± 4% versus 2 ± 0.5%, \( P = 0.002 \); Fig. 1). Increased proliferation was noted in both the proximal (30 ± 7% and 20 ± 5%, RB ablated and controls respectively, \( P = 0.30 \)) and distal (25 ± 4% and 23 ± 4%, respectively, \( P = 0.65 \)) conducting airways providing evidence that epithelial remodeling occurred throughout the airway. Remarkably, Ki67 expression was sustained in RB ablated lungs on day 14 after injury, whereas proliferation in control lungs returned to baseline levels (Fig. 1). Thus, RB is essential for establishing quiescence after naphthalene-induced injury.

Loss of RB function results in sustained cell cycle progression after injury. RB blocks cells in the \( G_1-S \) phase of the cell cycle, except \( G_0 \), and thus marks nonquiescent cells (22). Controls for this and subsequent experiments consisted of littermates lacking one or both transgenes required for RB ablation. Ki67 expression was similar in RB ablated and control lungs (Fig. 1), providing evidence that RB function is not essential for establishing and maintaining quiescence in the mature respiratory epithelium despite the marked cell cycle abnormalities present at birth.

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transition of the cell cycle (for review, see refs. 1, 8). Therefore, an expected consequence of RB loss is aberrant entry into S phase. As expected, the proportion of epithelial cells in S phase was <1% in both control and RB ablated lungs on day 0 and significantly increased by day 4 after injury (Fig. 2 and data not shown). A modest increase in BrdUrd-positive epithelial cells was noted in RB ablated lungs on day 0 compared with controls (Fig. 2). The physiologic significance of this increase is unknown because BrdUrd-positive cells represent <1% of the epithelial cells on day 0, and no concomitant increase in Ki67 expression (Fig. 1) or mitotic cells (Fig. 2) was detected at this time point. Given that RB null fibroblasts have shortened G1 and extended S phases, this finding could reflect a slightly different cell cycle profile in the RB ablated versus control lungs (27, 28). On day 14 after injury, however, BrdUrd incorporation was markedly increased in RB ablated lungs compared with controls (Fig. 2), demonstrating that RB deficiency results in aberrant progression into S phase.

Aberrant cell cycle progression induced by RB loss can result in cell cycle arrest. For example, RB-deficient myocytes are unable to maintain G0 arrest upon restimulation with serum and eventually arrest in the S and G2 phases of the cell cycle (29). RB null cells in the brains of chimeric embryos show increased G2 fractions without progression into mitosis suggesting that RB null neuronal cells arrest in late S or G2 phase of the cell cycle (30). Finally, RB is essential for G1-S phase arrest after DNA damage in mouse embryonic fibroblasts (MEF) in culture; however, RB null MEFs accumulate in G2-M phase, providing evidence that the G2-M phase checkpoint remains intact in the absence of RB (31). RB-deficient lung epithelial cells progressed into mitosis, as evidenced by increased expression of phosphorylated histone H3 (PH3) compared with day 0 (Fig. 2). Whereas these data cannot exclude an arrest in mitosis, they indicate that RB-deficient lung epithelial cells progress through S and G2 phases of the cell cycle. Thus, RB is essential for cell cycle exit during epithelial repair after injury and aberrantly proliferating RB-deficient epithelial cells progress into mitosis.

Nonciliated Clara cells constitute the vast majority of aberrantly proliferating cells in the remodeling RB-deficient lung. The airways are lined by diverse and specialized cell types required for normal lung function. Nonciliated Clara cells function as progenitor cells in the conducting airways (25). Because studies in the hematopoietic system indicate that RB is essential for regulating progenitor cells (32, 33), double immunolabeling for the Clara cell marker, CCSP, and BrdUrd was done to determine whether Clara cells constitute the aberrantly proliferating population. Clara cells accounted for 81 ± 7% of the S-phase population in RB-deficient lungs on day 14 after injury (Fig. 2). This was comparable with the proportion of S-phase Clara cells in RB ablated and control lungs on day 4 after injury (80 ± 16% and 71 ± 15%, respectively; Fig. 2). Thus, Clara cells represent the vast majority of aberrantly cycling cells in repairing the RB-deficient lung epithelium.

Aberrant cell cycle progression in RB-deficient lungs is sustained for at least 9 months after a single episode of injury. Epithelial proliferation was significantly increased in RB-deficient lungs 9 months after injury (Fig. 3). Moreover, the percentage of epithelial cells in S and M phases remained elevated, providing evidence that aberrant cell cycle progression was still occurring 9 months after the initiating event (Fig. 3). A trend toward decreased overall proliferation was noted in RB ablated lungs at 9 months compared with day 14 after injury; however, this decrease reached statistical significance only for BrdUrd-labeled cells.

Figure 3. Aberrant cell cycle progression is sustained in RB ablated lungs 9 mo after a single episode of injury and is associated with increased apoptosis. Quantitative data for cell cycle markers and TUNEL analysis on RB ablated and control adult lungs from mice treated with doxycycline throughout gestation. BrdUrd incorporation and PH3 and Ki67 expression were assessed by immunohistochemistry 9 mo after injury. A, quantified data are presented as average percentage of positive cells ± SE. RB ablated lungs showed a statistically significant increase in S-phase cells (BrdUrd), mitotic cells (PH3), and overall proliferation (Ki67) compared with controls (*P = 0.04, **P = 0.01, and ***P = 0.02, respectively). Apoptosis was assessed by TUNEL assay on lung sections before injury (day 0) and on day 4, day 14, and 9 mo after injury. Representative results from the 9-mo time point are shown for RB ablated (B) and control (C) lungs. Percentages of TUNEL-positive cells (arrow) were quantified, and data were represented as average percentage of positive cells ± SE (D). Apoptosis was increased in RB ablated lungs compared with controls at all time points analyzed with the increase reaching statistical significance on day 14 (*, P = 2.9 × 10⁻⁵) and 9 mo (**, P = 0.006) after injury. Representative of 5 to 11 animals per time point. Original magnification, 1,000×.
Epithelial cell cycle abnormalities were separately assessed in proximal and distal conducting airways at 9 months and day 14 after injury to determine if a regional difference existed. Although, there was a trend toward increased proliferation in the distal airways, this difference was not consistently statistically significant among the assessed cell cycle markers. Whereas it is possible that distal airway epithelial cells are slightly more sensitive to RB loss, the trend toward increased cell cycle abnormalities in the distal airway epithelium may simply reflect the higher proportion of Clara cells in distal versus proximal conducting airways.

Tumor incidence was not increased in RB-ablated lungs at 9 months after injury despite the prolonged period of sustained proliferation. Gross and microscopic examination of RB-deficient lungs from mice at 8 to 16 months of age showed no increase in tumor incidence irrespective of naphthalene treatment (Table 1). A possible explanation for lack of tumor formation despite sustained proliferation is that RB ablated cells are selectively lost after injury resulting in an RB-proficient epithelium. Whereas this explanation is highly unlikely given the relatively uniform RB ablation throughout the conducting airway (15), Cre-mediated recombination was directly assessed at the cellular level by performing in situ β-galactosidase assays on RB-deficient lungs from mice harboring the ROSA26 reporter locus. LacZ is expressed in this reporter strain only in cells expressing functional Cre recombinase and their descendants (34). The vast majority of epithelial cells in RB-deficient lungs at 9 months after injury were β-galactosidase positive (data not shown). Therefore, RB ablated cells are not selectively eliminated during epithelial regeneration after injury.

Increased cell death could account for lack of tumor formation in RB-deficient lungs. Indeed, apoptosis was detected in RB ablated lungs at baseline before injury and on day 14 and 9 months after injury. The increase in TUNEL-positive epithelial cells in RB ablated lungs was not statistically significant over controls before injury, but did reach statistical significance on day 14 and 9 months after injury (Fig. 3). Additionally, apoptosis in RB ablated lungs at day 14 and 9 months after injury was significantly elevated over RB ablated lungs before injury ($P = 0.003$ and $P = 0.008$, respectively; Fig. 3). Finally, RB ablated lungs could be blindly identified based upon morphologic features of apoptosis when assessed by a pathologist (K.A.W.-B.). Thus, sustained cell cycle progression in RB-deficient lungs was accompanied by apoptotic cell death. The 6-fold to 7-fold increase in apoptosis in RB ablated injured lungs compared with controls corresponds to a 4-fold to 8-fold increase in proliferation (Fig. 1). Taken together, these data provide evidence that increased cell death accounts, at least in part, for the absence of tumor formation in RB ablated lungs despite long-term aberrant cell cycle progression.

Postnatal and prenatal RB ablation result in similar phenotypic outcomes. To determine whether timing of RB ablation significantly affects the phenotypic outcomes in the lung epithelium in vivo, RB recombination was induced postnatally rather than during development by treating adult mice with doxycycline. RB recombination was consistently detected in the lungs of double transgenic mice treated with doxycycline but not in controls lacking Cre recombinase (Fig. 4 and data not shown). Surprisingly, RB recombination was also detected in double transgenic adult lungs in the absence of doxycycline treatment (Fig. 4). Despite the finding that postnatal RB ablation occurs independently of doxycycline treatment, this mouse model remains valuable for assessing the effects of developmental versus postnatal RB ablation because RB ablation during development is strictly dependent upon doxycycline induction (15).

The conducting airways in the mature mouse lung are lined predominantly by ciliated cells and nonciliated Clara cells. To determine the extent of postnatal RB ablation in these distinct cell types, in situ β-galactosidase analysis was performed on lungs from double transgenic mice containing the ROSA26 locus. As expected, β-galactosidase–positive cells were restricted to the epithelium (Fig. 4). The majority of epithelial cells in the conducting airways were β-galactosidase positive and included both ciliated and Clara cells. Doxycycline administration during gestation induces Cre recombinase expression in early progenitor cells within the lung epithelium (35). Accordingly, the overall β-galactosidase staining was more uniform after doxycycline treatment during development compared with postnatal treatment (Fig. 4; and ref. 15). A similar pattern of β-galactosidase staining was seen in the conducting airways of double transgenic adult mice not treated with doxycycline (Fig. 4). Scattered β-galactosidase–positive alveolar cells, consistent with the location of type II cells, were detected and seemed to be more frequent in the presence of doxycycline treatment. Thus, both prenatal and postnatal RB ablation is epithelial specific, occurs in the majority of conducting airway epithelial cells, and is present in both Clara and ciliated cells.

Postnatal RB ablation resulted in similar phenotypic outcomes as that seen after RB ablation during development. Epithelial quiescence was maintained in RB-deficient lungs after doxycycline administration to adult mice at 2 to 3 months of age (Fig. 5). Similar to RB ablation during development, postnatal RB loss resulted in aberrant cell cycle progression during epithelial repair after injury (Fig. 5). Nonquiescent Ki67-positive cells were increased in RB ablated lungs on day 14 after injury compared with controls. Moreover, cells progressed into S-phase and mitosis. Despite the aberrant cell cycle progression, lung tumors were not detected in mice analyzed at 8 to 16 months of age, either in the presence or absence of naphthalene-induced injury (Table 1). Taken together, the data further show that RB function is critical during epithelial repair after injury and provide evidence that prenatal versus postnatal timing of RB ablation does not significantly affect phenotypic outcome.

### Discussion

Quiescence is established and maintained in adult RB-deficient lung epithelium, providing evidence that RB is not required for these processes during lung homeostasis. In contrast, RB function
is essential for establishing cellular quiescence in the context of epithelial remodeling after injury. Furthermore, the aberrant epithelial cell cycle progression in RB ablated lungs was noted 9 months after a single episode of injury providing evidence that epithelial cells do not compensate for RB loss during epithelial remodeling after injury. This is in stark contrast to the compensation that occurs in the postnatal lung after RB ablation in the absence of injury. These studies provide evidence that RB plays a critical and unique role in epithelial remodeling after injury.

The essential role for RB in lung regeneration after injury differs from skeletal muscle wherein RB is essential for muscle development but is not required for muscle regeneration after cardiotaxin-induced injury (36, 37). Interestingly, there is a unique requirement for RB in stress erythropoiesis (33). Although RB is not critical for steady-state hematopoiesis, RB loss is associated with increased erythroblasts that fail to undergo terminal maturation under stress conditions. These studies suggest that RB loss confers a growth advantage on progenitor cells. Consistent with this concept, Walkley et al. showed that RB is dispensable in hematopoietic stem cells and raised the hypothesis that the requirement for RB in self-renewal is lineage dependent, with progenitor cells having a greater dependence on RB for their division than stem cells (32). The current studies identify RB as a critical regulator of progenitor cells in the repairing lung epithelium. Thus, our data support the notion that RB has unique and essential functions during cellular regeneration after injury.

### Figure 4.

Prenatal and postnatal RB ablation occurs throughout the lung epithelium and is present in both Clara and ciliated cells. PCR analysis on lung DNA from postnatal (PN) day 1 or adult double transgenic mice treated with doxycycline (Dox) during gestation (Prenatal Dox) or not treated with doxycycline (No Dox, A). Control lanes show migration of the recombinant (RBRec), wild-type (RBWT), and floxed (RBLoxP) RB alleles. Adult mice were homozygous for RBLoxP, and day 1 pups were homozygous or heterozygous for RBLoxP. RBRec was detected in the postnatal day 1 lungs only after doxycycline treatment. In contrast, RBRec was detected in adult lungs in the absence and presence of doxycycline treatment. TSHβ was amplified in each sample as an endogenous internal control to verify equivalent template DNA quality and quantity. Representative of 5 No Dox and 36 Prenatal Dox (15 homozygous and 21 heterozygous for RBLox) PN day 1 lungs and 3 adult lungs for each treatment group. Varied levels of recombination among samples are due, at least in part, to differences in the relative proportion of conducting airway epithelium represented in the lung tissue used to isolate DNA. Enzymatic staining for β-galactosidase performed on lung sections from adult double transgenic mice harboring the ROSA26 reporter locus after prenatal doxycycline treatment (B), postnatal doxycycline treatment (C), or no doxycycline treatment (D). Representative low (top) and high (bottom) power images. β-Galactosidase staining (blue) was epithelial specific and present throughout the conducting airways in all groups indicating active Cre recombinase and thus RB ablation. Epithelial staining in the conducting airways was less uniform after postnatal treatment and no treatment compared with prenatal treatment (compare B to C and D). Ciliated (arrows) and Clara cells (arrowheads) were stained in all three treatment groups. Representative of at least three animals for each treatment group. Original magnification, 200× (top) and 1,000× (bottom).
and that progenitor cells are critically dependent on RB for control of cell division.

**RB is required in specific cellular contexts and time periods.** RB ablation targeted to the lung epithelium resulted in aberrant cellular proliferation and apoptosis in specific cellular contexts, namely in the newborn lung and during regeneration after injury (current data and ref. 15). These findings are consistent with RB being essential during limited time periods, wherein cells are in transition into quiescence. Cells withdraw from the cell cycle and enter G0 in response to mitogen deprivation (for review, see ref. 38). This process is dependent upon p27 accumulation and reduction in cyclin D activity. In contrast, cell cycle arrest in response to DNA damage requires p21, and not p27. Interestingly, RB has previously been reported to transcriptionally regulate p21 specifically in epithelial cells, and to increase p27 stability by targeting Skp2, a component of the Skp1-Cullin-F-box protein E3 ubiquitin ligase complex, for degradation (39, 40). Additionally, p27 was shown to be required for RB-mediated senescence in cells in culture (41). Thus, deregulated p21 and/or p27 activity pose a potential link between loss of RB function and inability to enter quiescence.

RB is classically viewed as an essential regulator of cell cycle; however, RB function is also important in regulating cell survival, chromatin remodeling, genomic stability, and cellular ploidy (1, 2, 42). Many of these functions depend upon RB-mediated gene regulation resulting from RB interactions with the E2F family of transcription factors. Transcriptional control of DNA replication genes by the E2F/RB pathway is important for maintaining proper cell cycle control and regulating additional cellular processes, including ploidy (42). Apoptosis in RB-deficient cells also results, at least in part, from deregulation of E2F/RB target genes. Although the response to RB loss is cell-type specific, apoptosis in RB null cells frequently occurs in a p53-dependent manner. Interestingly, p53 and its proapoptotic target gene Bax are induced in the lung epithelium after hyperoxia-induced and bleomycin-induced injury (43, 44). However, no change in p53, Bax, or activated p53 expression was detected in RB ablated lungs compared with controls, and protein expression was not induced after naphthalene injury (Supplementary figure).

The timing of RB-dependent phenotypes in the lung epithelium has striking parallels with RB function in developing retina and skeletal muscle. RB is critical for cell cycle exit in retinal transitional cells during a limited time window during development (for review, see ref. 45). RB ablation before this critical time period results in abnormal retinal development, whereas RB loss at later time points results in no abnormalities. This seems to be true in skeletal muscle as well. RB function is required in myoblasts whereas RB loss has no effect in mature myocytes (36, 37). The critical requirement for RB in limited time periods is likely relevant to RB-mediated tumor suppression because retinoblastoma is almost exclusively a disease of childhood occurring within the temporal window wherein retinoblasts undergo final maturation (46).

**Postnatal and prenatal RB ablation result in similar phenotypic outcomes.** Germline versus acute loss of RB function results in different phenotypic outcomes in cells in culture. Quiescent MEFs undergo cell cycle reentry after acute, but not germline, RB loss (16). In addition, acute RB ablation in senescent MEFs leads to reversal of the senescence-associated phenotype, whereas MEFs with germline RB loss undergo and maintain senescence similar to wild-type cells. Keratinocytes undergoing acute loss of RB in culture are completely refractory to growth arrest when induced to differentiate (14). In contrast, RB null keratinocytes derived after conditional RB ablation in mice in vivo undergo growth arrest similar to wild-type cells, albeit with a 24-hour delay. These findings suggest that precise timing of RB ablation affects phenotypic outcomes and that RB is critical for maintaining cellular quiescence and senescence. Additionally, these

![Figure 5. Postnatal RB ablation results in aberrant cell cycle progression after injury.](image-url)
studies raise the possibility that phenotypes in adult mouse models could reflect secondary effects of developmental compensation after RB loss during embryogenesis.

RB ablation in the postnatal lung did not lead to aberrant cell cycle control under homeostatic conditions providing evidence that RB is not required to maintain lung epithelial quiescence. RB, however, was essential for establishing lung epithelial quiescence after injury regardless of whether RB was ablated during development or in the postnatal lung. These results directly show that prenatal versus postnatal timing of RB ablation does not significantly affect the injury-induced phenotype. Furthermore, the studies provide direct evidence that the injury-induced phenotype is not simply secondary to epithelial alterations occurring during development.

**Relationship to human carcinogenesis.** Carcinogenesis occurs through sequential steps, including tumor initiation and promotion. Initiation depends upon somatic mutations. Promotion mechanisms are less well defined but involve epigenetic factors, such as inflammation and substances that trigger cell death and proliferation. The current studies show that RB and cytotoxic damage cooperate to transform the normally quiescent lung epithelium into an organ with sustained epithelial cell death and proliferation. Cytotoxic damage could thereby function as a tumor promoter by creating a cellular context, wherein RB function is particularly critical.

Loss of RB function by somatic mutation or deregulation of other proteins in the RB pathway is critical, if not essential, for lung carcinogenesis. However, mechanisms underlying tumor promotion are poorly understood. In the liver, many tumor promoters are cytotoxic and therefore indirectly trigger hepatocyte proliferation by causing cell death (for review, see ref. 47). Cytotoxic damage and compensatory proliferation induced by mitogen production are also important components of the tumor promoting microenvironment linking inflammation and carcinogenesis (48). Because the liver is composed of quiescent-differentiated cells, induction of hepatocyte proliferation is a prerequisite for transformation. In corollary, the adult lung epithelium is quiescent and therefore transition of RB null epithelial cells from a quiescent to a constitutively replicating state after cytotoxic damage may set the stage for tumorogenesis.

Lung carcinogenesis is associated with tobacco smoking in 80% to 85% of cases. Increased cancer incidence in tobacco users has traditionally been attributed solely to the mutagenic affects of cigarette smoke. However, reevaluation of the data suggests that cytotoxic damage and chronic epithelial remodeling induced by tobacco smoke act as critical promoters of carcinogenesis by selecting rather than simply inducing tumorigenic mutations (49, 50). Repetitive smoking may therefore promote carcinogenesis by creating a microenvironment that facilitates preferential expansion of cells with mutations that confer a proliferative advantage or resistance to cytotoxic damage.

If smoking promotes carcinogenesis by causing chronic injury, one would predict that pulmonary diseases that arise in response to chronic lung injury would be associated with an increased risk of lung cancer. Indeed, patients with chronic interstitial lung disease are at increased risk for developing lung cancer independent of smoking history (see ref. 47). Although the pathogenetic mechanisms are unknown, an association between chronic injury and lung cancer provides correlative evidence that cytotoxic damage and epithelial remodeling promote lung carcinogenesis. The current studies identify RB as a critical regulator in the context of lung epithelial repair after cytotoxic damage and suggest RB as a potential molecular link between chronic lung injury and carcinogenesis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**


**Grant support:** National Heart Lung and Blood Institute grant R01 HL079193 (K.A. Wilenhues-Brokamp). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank J.A. Whitsett, S.J. Wells, J.C. Rhodes, and D.S. Askew for critical review of the manuscript.

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Nicole A. Mason-Richie, Meenakshi J. Mistry, Caitlin A. Gettler, et al.


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