Rb family proteins differentially regulate distinct cell lineages during epithelial development

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Summary

pRb, p107 and p130 are important regulators of cell cycle and have extensive overlapping functions; however, only Rb has been shown to be a bone fide tumor suppressor. Defining the overlapping versus distinct pocket protein functions is therefore an important step to understanding the unique role of Rb. Using lung as a model, the present studies demonstrate that pocket proteins are important not only in regulating cell cycle and survival but also in cell lineage specification. An inducible lung-specific Rb knockout strategy was used to demonstrate that Rb is specifically required for restricting neuroendocrine cell fate despite functional compensation for Rb deficiency in other cell types. Ablation of total Rb family function resulted in opposing effects in specification along distinct cell lineages, providing evidence that pocket proteins inhibit neuroendocrine cell fate while being required for differentiation in other cell types. These findings identify a novel role for pocket proteins in cell fate determination, and establish a unique cell lineage-specific function for Rb that explains, at least in part, why Rb and p16 are inactivated in phenotypically distinct carcinomas.

Key words: Rb, p107, p130, Cre-LoxP system, Lung development, CC10

Introduction

The retinoblastoma protein (Rb) is a prototype tumor suppressor and is required for embryonic development. The current model of Rb function suggests a crucial role in cell cycle regulation, operative in all cell types. A universal role for Rb in tumor suppression has also been implicated by the frequent inactivation of the cell cycle regulatory pathway centered on Rb in most human cancers (Nevins, 2001; Sherr, 1996; Weinberg, 1995). Challenging the ubiquitous role for Rb in development, however, is the fact that embryos nullizygous for Rb alone, or in combination with other family members, show cell lineage-specific defects (Classon and Dyson, 2001; Lin et al., 1996; Lipinski and Jacks, 1999). Additionally, although proteins in the Rb pathway are commonly altered in human tumors, Rb gene mutations are frequently observed in only a very small subset of human malignancies, including retinoblastoma and small cell lung cancer (SCLC) (Sherr, 1996; Weinberg, 1995). These data suggest that Rb has cell-specific functions important in development as well as in tumor suppression.

Cellular sensitivity to Rb deficiency may reflect differing degrees of functional redundancy among family members in distinct cell types. Rb is a member of a protein family that also includes p107 and p130. These three family members share extensive structural homology, especially in a bipartite pocket domain that functions as the binding site for viral oncoproteins and acts as a transcriptional repressor motif (Classon and Dyson, 2001). All three pocket proteins inhibit E2F responsive promoters, recruit chromatin remodeling enzymes, actively repress transcription, and growth arrest cells when overexpressed (Classon and Dyson, 2001; Harbour and Dean, 2000). These structural and biochemical similarities probably explain the functional overlap among pocket proteins during development, and provide rationale for the observation that p107 and p130 can at least partially compensate for loss of Rb function in vivo (Lipinski and Jacks, 1999). An important distinction among the pocket proteins, however, is that Rb, but not p107 and p130, has been shown to be a tumor suppressor in humans (Classon and Dyson, 2001). Defining the overlapping versus distinct functions of Rb family proteins is therefore an important step to understanding the unique role of Rb.

The lung provides a manipulatable model system in which to explore Rb family function in vivo, both in cell cycle control and in cellular differentiation. The respiratory epithelium comprises markedly diverse and specialized cell types that are derived from a common progenitor population. Cellular proliferation and differentiation are coordinately regulated to create this diversity, which is required for normal lung function (Kauffman, 1980). Rb, p130 and p107 are all expressed in the developing and adult lung. p107 expression is fairly uniform in the developing lung, with levels peaking during mid-gestation (embryonic day 12.5-13.5) and then declining thereafter (Jiang et al., 1997; Kim et al., 1995). By contrast, p130 and Rb expression increase during late embryonic development and are maintained at relatively high levels in the adult lung (Bernards et al., 1989; Chen et al., 1996a; Garriga et al., 1998; Jiang et al., 1997; Levine et al., 1998; Pertile et al., 1995). Furthermore, accumulation of the active, hypophosphorylated form of Rb is coincident with epithelial

cell differentiation and growth arrest (Levine et al., 1998). Taken together, these data suggest that pocket protein function is important for lung development.

Rb has also been implicated as a crucial tumor suppressor in the lung epithelium. Lung carcinomas are divided into small cell (SCLC) and non-small cell lung cancers (NSCLC), based upon distinct clinical and pathologic features. Rb gene mutations are found in nearly all SCLC, an aggressive neoplasm that shares a neural phenotype with retinoblastoma (Kaye, 2001; Minna et al., 2002). The nearly universal and selective occurrence of Rb gene mutations in SCLC provides evidence that Rb loss is essential in the genesis of this malignancy, and suggests that specific cell types within the lung epithelium are particularly sensitive to loss of Rb function.

The current studies were designed to determine putative cell-type-specific functions of Rb that are not functionally redundant with other family proteins. The results provide convincing evidence that pocket protein function is essential for lung epithelial development. Unexpectedly, pocket proteins also have opposing roles in specification along distinct cell lineages; they inhibit neuroendocrine cell fate but are required for differentiation in other cell types. Remarkably, Rb is specifically required for restricting neuroendocrine cell fate despite functional compensation for Rb deficiency in other cell types. The selective occurrence of Rb gene mutations in human SCLC correlates well with the cell lineage-specific functions for Rb seen in the currently generated mouse model. Thus, these studies demonstrate that Rb acts as a cell-specific regulator in epithelial development and provide a mouse model that mimics human disease associated with Rb deficiency. The results also identify and define a novel role for pocket proteins in cell lineage specification.

Materials and methods

Generation of mice

The human 3.7 kb SP-C gene promoter (Glasser et al., 1991) and the rat 2.4 kb CC10 gene promoter (Stripp et al., 1992) were cloned upstream of T121 or TK1 coding sequences derived from pLST1137 or pLST1137K1 (Symonds et al., 1994), respectively. T121 encodes the first 121 amino acids of the SV40 large T antigen, and is capable of binding Rb family proteins but not p53. TK1 encodes the same truncated T antigen with a single amino acid change (E107 to L) that disrupts binding of T antigen to Rb family proteins. Transgenic mice were generated and maintained on a C57Bl6 background. Mice were screened using a PCR-based assay on tail DNA (Symonds et al., 1994). Transgene expression was confirmed by western blot analysis on lung extracts using anti-SV40 large T/small t PAb 108 antibody (PharMingen). T121 lines could not be established due to neonatal lethality. TK1 control lines were generated; however, these lines were not maintained after completion of the studies and are thus no longer available. CC10-rtTA (Perl et al., 2002a; Tichelaar et al., 2000) and tetCre transgenic mice (Perl et al., 2002b) were mated to $Rb^{LoxP/LoxP}$ (Vooijs and Berns, 1999; Vooijs et al., 2002) and $Rb^{+/-}$ (B6.129S2-$Rb1^{tm1Tyj}$, Jackson Laboratory) mice. Mice were genotyped using tail or lung DNA in PCR based assays using primers 5′-TGCC-ACGACCAAGTGACAGCAATG-3′ and 5′-AGAGACGGAAATC-CATCGCTCG-3′ for tetCre, and previously established primers given in references above.

Doxycycline administration

Gestation was dated by detection of vaginal plug (E0.5). Dams were treated with 125 μg doxycycline (Sigma) in 0.5 ml PBS by intraperitoneal injection on E0.5-E1.5 and administered doxycycline in the drinking water at a final concentration of 1.0 mg/ml. A 50× doxycycline stock solution (50 mg/ml in 50% ethanol) was freshly prepared prior to each administration of drug and diluted in water or in PBS for injection. Doxycycline water was replaced three times per week because of the light sensitivity of doxycycline.

Histology, immunohistochemistry and TUNEL analysis

Lung tissue was fixed in 10% neutral buffered formalin and paraffin embedded. Sections were prepared and stained with Hematoxylin and Eosin for histological analysis. Immunohistochemistry was performed using Vectastain Elite ABC, M.O.M. Immunodetection, and DAB Substrate Kits (Vector Laboratories). Methanol/hydrogen peroxide pre-treatment and microwave/10 mM citrate antigen retrieval were performed. Antibodies were diluted in 0.1% bovine serum albumin in PBS, applied to sections and incubated overnight at 4°C. Antibodies and dilutions used were as follows: SV40 T Ag Ab-2, 1:80 (Oncogene); PCNA PC10, 1:200 (PharMingen); Ki67, 1:50 (BD PharMingen); CGRP, 1:10,000; (Sigma); and CCSP and Foxj1, 1:20,000 and 1:500, respectively (kindly provided by Steve Brody, Washington University, St Louis, MO, USA). TUNEL analysis was performed on sections using ApoTag Peroxidase Detection Kit (Intergen). Slides were counterstained with Hematoxylin. Quantitation of apoptosis and proliferation was carried out by counting the number of TUNEL- or Ki67-positive cells, respectively. Percentages were determined by evaluating 400 cells representing proximal and distal conducting airways and at least two lung lobes per mouse. Quantitation of neuroendocrine cell foci was performed by counting CGRP-positive foci per mm of airway. Counts represent analysis of 8.5-10 mm of airway and at least two lung lobes per mouse. Littermates were used as controls for all studies when possible. Statistical significance was determined by Student's *t*-test.

Laser capture microdissection (LCM)

Five micron sections of formalin-fixed, paraffin-embedded tissue were placed on uncharged, non-coated glass slides, dried and put into a dessicator. Slides were deparaffinized, rinsed in distilled water and stained in Hematoxylin for 30 seconds and Eosin for 11 seconds. LCM was performed with the Pixcell II Laser Capture Microdissection and Image Archiving Workstation systems (Arcturus Engineering). Epithelial cells were captured on thermoplastic caps and digested in proteinase K digestion buffer at 55°C overnight. Samples were heated at 95°C for 15 minutes to inactivate the protease and used directly for PCR analysis. The Rb alleles were analyzed using two separate primer sets: (1) primers Rb-18 and Rb-19 that yield products of 678 bp for the wild-type and Rb knockout alleles, 746 bp for the floxed Rb allele and 321 bp for the recombined Rb allele (Vooijs et al., 2002); and (2) primers Rb-212, Rb-18 and Rb-19E that yield products of 235 bp for the wild-type and Rb knockout alleles, 283 bp for the floxed Rb allele and 260 bp for the recombined Rb allele (Meuwissen, 2003).

β-Galactosidase staining

Whole-mount and frozen section staining for β-galactosidase activity was performed as described (Nagy, 2003). Briefly, whole lung was fixed in 0.4% paraformaldehyde, rinsed in detergent buffer and incubated at 37°C overnight in stain solution. For section staining, tissue was fixed in 0.2% paraformaldehyde, cryoprotected in 30% sucrose and embedded in OCT compound. Three or five micron sections were postfixed in 0.2% paraformaldehyde, rinsed in saline and detergent buffers and incubated at 37°C overnight in stain solution. Slides were subsequently counterstained with Nuclear Fast Red or used in a modified immunoassay for CGRP, wherein methanol/hydrogen peroxide pre-treatment and antigen retrieval were eliminated, and antibody was incubated at room temperature for 1 hour.

Results

Mouse model showing inducible lung epithelial-specific Rb ablation

To delineate the role of Rb in the lung epithelium, a conditional lung-specific Rb-knockout model was developed. Double-transgenic mice bearing (1) the reverse tetracycline responsive transactivator (rtTA) under control of the rat Clara cell 10 kDa protein (CC10) promoter (CC10-rtTA) (Tichelaar et al., 2000), and (2) Cre recombinase under control of the tet operator (tetCre) (Perl et al., 2002b) were bred into $Rb^{LoxP/LoxP}$ (Vooijs et al., 2002) or $Rb^{LoxP/-}$ backgrounds. The rat CC10 promoter directs transgene expression to lung epithelial progenitor cells early in development (embryonic day 12.5) before differentiation into specialized cell types (Hackett and Gitlin, 1994) and to the adult lung epithelium. Thus, rtTA is specifically expressed in lung epithelial cells throughout development and in the adult. Rb gene ablation is accomplished when rtTA in combination with doxycycline activates expression of tetCre leading to recombination at floxed Rb alleles (Fig. 1A). To assess Cre function double-transgenic mice were also mated to the ROSA26 reporter strain in which *lacZ* expression is only present in Cre-expressing cells and their descendants (Soriano, 1999). Doxycycline was continuously administered to pregnant dams throughout development. Pups were initially analyzed on the day of birth to assess the role of Rb in prenatal lung development. PCR analysis on lung and tail DNA demonstrated that recombination at the Rb allele was lung specific and strictly dependent upon the presence of both transgenes and doxycycline treatment (Fig. 1B).

The respiratory epithelium comprises specialized cell types, including ciliated, non-ciliated columnar (Clara) and neuroendocrine cells in the conducting airway, and Type I and Type II cells in the distal respiratory airway. In situ enzymatic staining for β-galactosidase (β-gal) revealed epithelial-specific staining throughout the conducting airway (Fig. 1C-F). Although scattered epithelial cells within the conducting airways were not stained, the vast majority of ciliated and non-ciliated cells were positive for *lacZ* expression. By contrast, the distal respiratory epithelium showed only scattered β-gal-positive cells, morphologically consistent with Type II cells. Functional Cre was also expressed in a subset of pulmonary neuroendocrine cells, as demonstrated by co-expression of *lacZ* and the neuroendocrine cell marker calcitonin gene-related peptide (CGRP) (Fig. 1E). β-Gal staining was strictly dependent upon the presence of both transgenes, and identical results were seen in lungs from day 1 and adult mice treated with doxycycline in utero (Fig. 1C-F). These data confirm that Cre-mediated recombination occurs in the vast majority of epithelial cells throughout the conducting airway. Moreover, the data show that Rb gene recombination, and thus loss of Rb function, is confined to the lung epithelium and strictly dependent upon doxycycline induction during lung development.

Rb regulates epithelial cell proliferation and survival during development

Lungs from double-transgenic mice treated with doxycycline (hereafter referred to as CC10-rtTA) were grossly normal and showed normal branching morphogenesis. Microscopic analysis, however, showed marked epithelial cell abnormalities throughout the conducting airways. The epithelium was hypercellular and composed of dysplastic cells with increased nuclear to cytoplasmic ratios and cells containing pyknotic nuclei (Fig. 2A). These epithelial changes were not present in the absence of doxycycline treatment (Fig. 2B), or in control littermates lacking one or both transgenes required for Rb gene ablation (Fig. 2C). The phenotype was similar in double transgenic mice with $Rb^{LoxP/LoxP}$ or $Rb^{LoxP/-}$ alleles, and therefore results shown for all analyses are representative of both genotypes.

To determine whether increased epithelial cell proliferation contributed to the observed hypercellularity, immunohistochemical analysis was performed for the proliferation marker Ki67 (Scholzen and Gerdes, 2000). Rb ablation resulted in a marked increase in Ki67 expression in CC10-rtTA lungs compared with untreated and control mice lacking one or both transgenes (Fig. 2G-I). In addition, the morphologic impression of apoptosis was confirmed by a marked increase in TUNEL-positive cells in CC10-rtTA lungs when compared with controls (Fig. 2D-F). Quantitation showed highly statistically significant increases in both apoptosis and proliferation throughout the conducting airways (Fig. 2M). A more marked increase in proliferation was seen in distal when compared with proximal conducting airways. Interestingly, apoptotic rates were similar throughout the conducting airway and therefore did not directly correlate with ectopic proliferation. Thus, Rb ablation promotes epithelial cell proliferation resulting in epithelial hypercellularity despite increased apoptotic cell death.

Rb ablation leads to increased proliferating cell nuclear antigen (PCNA) expression

To confirm that Rb function is indeed lost in the lung epithelium of double-transgenic mice treated with doxycycline, immunohistochemical analysis was performed for the E2F-regulated gene product PCNA. Rb/E2F complexes repress gene transcription (Harbour and Dean, 2000). Therefore, loss of Rb function would be expected to lead to derepression of E2F-regulated genes and thus increased PCNA expression. Immunohistochemical analysis demonstrated uniform PCNA expression throughout the conducting airways in CC10-rtTA lungs (Fig. 2J). Derepression of PCNA was dependent upon Rb ablation, as increased PCNA expression was not detected in controls lacking one or both transgenes, or in the absence of doxycycline (Fig. 2K,L). These data provide evidence that Rb function is lost in the vast majority of epithelial cells upon doxycycline treatment.

Rb loss selectively effects neuroendocrine cell fate during development

Rb gene mutations are preferentially found in neuroendocrine versus non-neuroendocrine lung carcinomas (Kaye, 2001; Minna et al., 2002), implying that Rb has cell lineage-specific functions. To explore this possibility, epithelial differentiation was assessed in CC10-rtTA lungs by morphology, along with immunohistochemical analysis for markers of Clara cell [Clara cell specific protein (CCSP)] and neuroendocrine cell (CGRP) differentiation. Clara and ciliated cell differentiation was similar in Rb-deficient and control lungs (Fig. 3A-C, and data not shown). Interestingly, however, Rb-deficient lungs showed an increase in neuroendocrine cells within the airway (Fig. 3D-

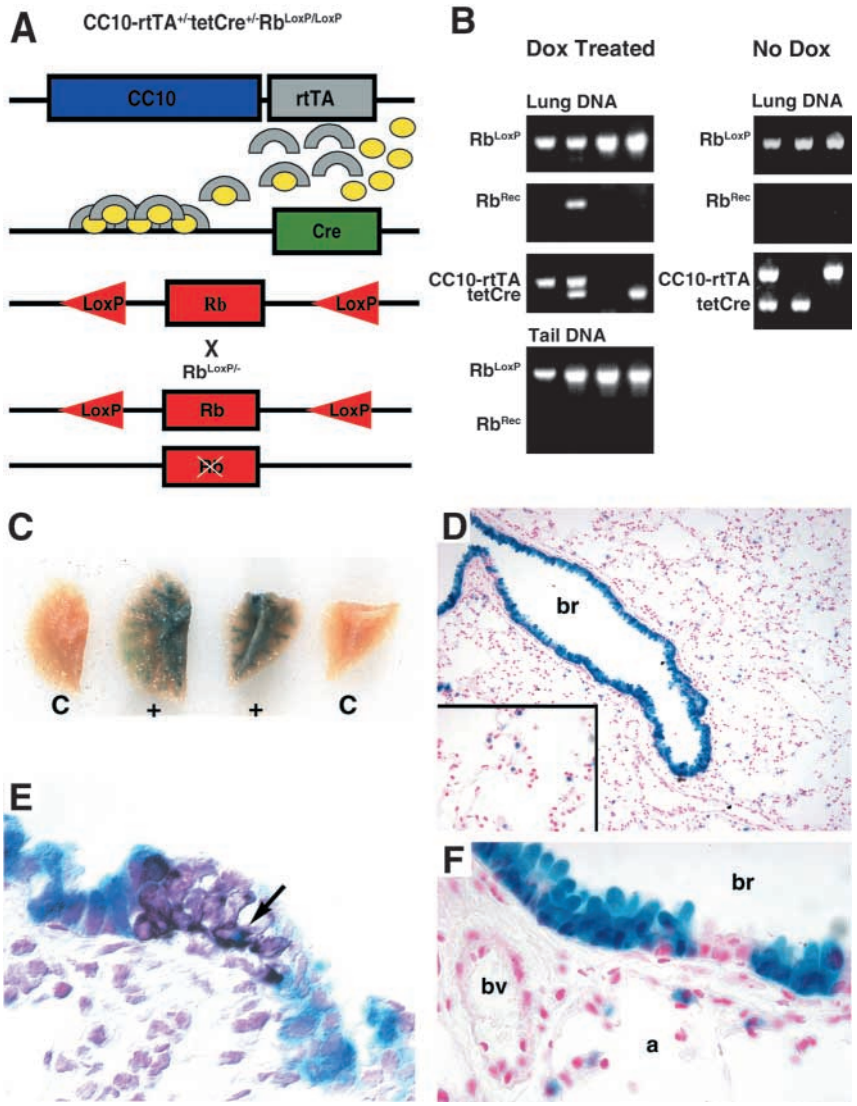

Fig. 1. Inducible lung epithelial-specific ablation of Rb. (A) Male mice heterozygous for both CC10-rtTA and tetCre transgenes, and homozygous for the floxed Rb gene allele (Rb^{LoxP}) were bred to female mice bearing a floxed Rb allele and a mutated null Rb allele (Rb^{-}). Dams were treated with doxycycline (ovals), which activates the rtTA (arches) expressed specifically in lung epithelium under control of the CC10 promoter. Activated rtTA induces Cre expression leading to excision and functional loss of Rb. (B) PCR analysis on lung or tail DNA obtained from day 1 pups taken from doxycycline-treated dams, or control dams not treated with doxycycline. Representative results are shown. Recombination at the floxed Rb allele (Rb^{Rec}) is dependent upon the presence of both CC10-rtTA and tetCre transgenes, and is detected in lung but not tail DNA. No recombination is detected in lung DNA obtained from control pups not treated with doxycycline. (C-F) Enzymatic staining for β-gal (blue) in lungs from ROSA26 reporter mice harboring the CC10-rtTA and tetCre transgenes, and treated with doxycycline in utero. (C) Whole-mount staining of lungs from double transgenic day 1 pups (+) and control littermates (C) lacking one or both transgenes. (D,F) Staining of adult lung sections. Note that majority of ciliated and non-ciliated epithelial cells show staining. Inset in D highlights the scattered punctuate staining observed in the alveolar region, consistent with the localization of Type II cells. (E) Enzymatic staining for β-gal (blue) followed by immunohistochemistry for CGRP (brown/black) in day 1 lung. Arrow marks a cell positive for both β-gal activity and CGRP. br, bronchi/bronchioles; bv, blood vessel; a, alveoli.

F). Immunohistochemical analysis for CGRP showed scattered single cells and small cell aggregates predominantly at airway branchpoints in control lungs, consistent with the location of pulmonary neuroendocrine cells. By contrast, Rb-deficient lungs showed an increase in CGRP immunoreactive epithelial aggregates. Quantification of CGRP immunoreactive foci per mm of airway showed a statistically significant increase in CC10-rtTA mice, when compared with controls lacking one or both transgenes required for Rb ablation, and lungs from mice not treated with doxycycline [2.3±0.7 versus 1.4±0.5 (P<0.02) for control and 1.6±0.3 (P<0.05) for No Dox lungs]. By contrast, there was no statistically significant difference between untreated mice and controls lacking one or both transgenes. Thus, Rb is not essential for non-neuroendocrine cell differentiation. Instead, Rb specifically restricts the neuroendocrine cell lineage.

Compensation for Rb loss during development occurs postnatally

Rb deficiency during lung epithelial development leads to ectopic proliferation, apoptosis and increased neuroendocrine cells. To determine the physiological consequences of these alterations postnatally, Rb-deficient pups were allowed to develop and lungs were analyzed from mice at 9-15 weeks of age. Unexpectedly, CC10-rtTA adult lungs lacked the epithelial hypercellularity and apoptosis noted in day 1 lungs. In fact, the majority of epithelial cells in adult lungs were morphologically normal (Fig. 4A-D). This finding could not be explained by the possibility that severely affected mice died prior to analysis because genotyping revealed the expected percentage of double transgenic mice (Table 1). These results confirm that Rb deficiency does not result in postnatal lethality despite the marked epithelial alterations seen at birth.

A plausible explanation for the lack of overall epithelial hypercellularity and apoptosis in adult lungs is that Rb is not uniformly ablated throughout the epithelium in CC10-rtTA lungs. In this case, cells lacking Rb function could be selectively eliminated and the epithelium subsequently restored by cells with functional Rb. To test this possibility directly, epithelium was microdissected from adult CC10-rtTA lung sections using laser capture and tested for recombination at the Rb locus (Fig. 4E-H). PCR analysis demonstrated loss

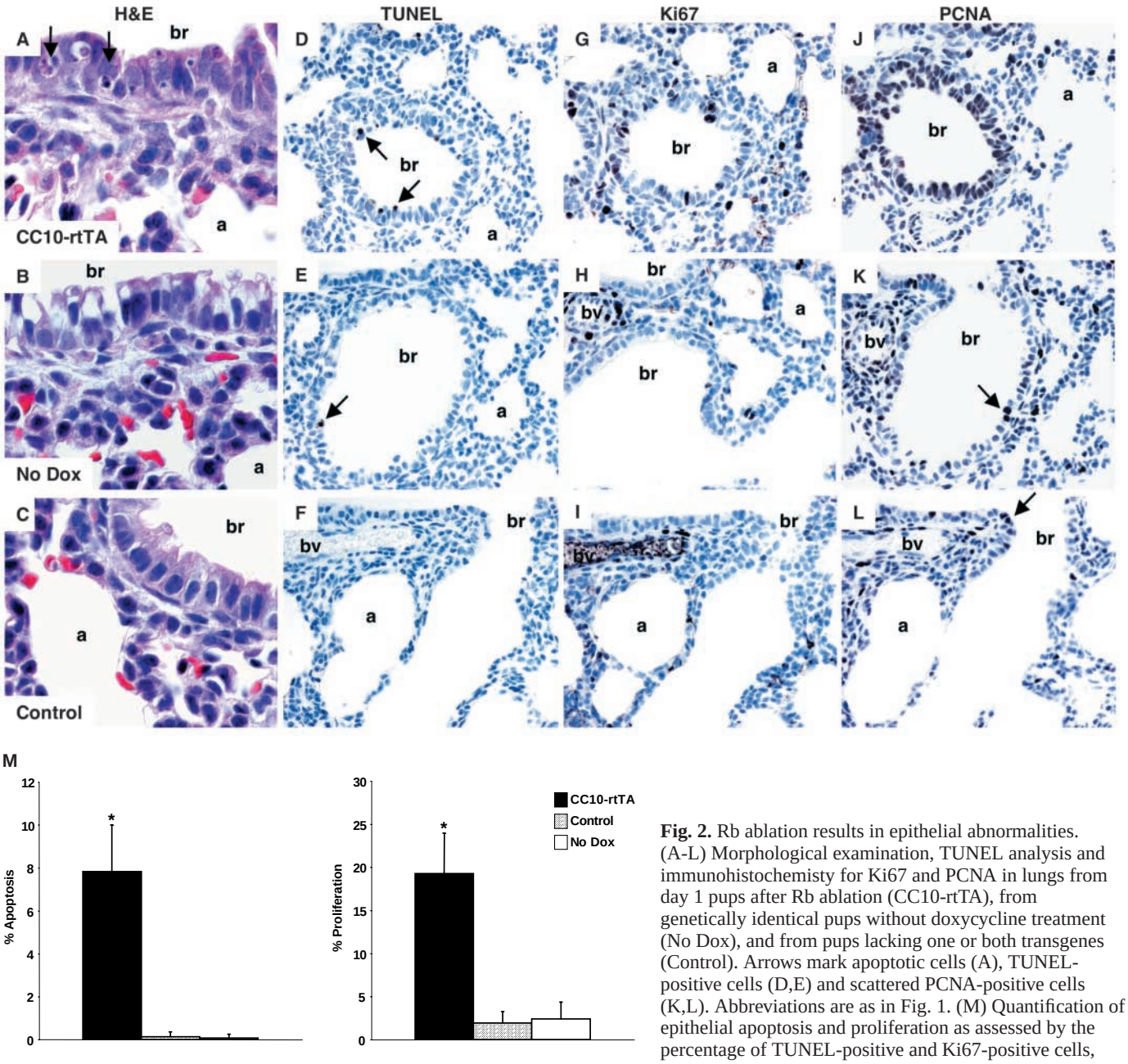

Fig. 2. Rb ablation results in epithelial abnormalities. (A-L) Morphological examination, TUNEL analysis and immunohistochemisty for Ki67 and PCNA in lungs from day 1 pups after Rb ablation (CC10-rtTA), from genetically identical pups without doxycycline treatment (No Dox), and from pups lacking one or both transgenes (Control). Arrows mark apoptotic cells (A), TUNEL-positive cells (D,E) and scattered PCNA-positive cells (K,L). Abbreviations are as in Fig. 1. (M) Quantification of epithelial apoptosis and proliferation as assessed by the percentage of TUNEL-positive and Ki67-positive cells, respectively. Rb ablation results in statistically significant increases in epithelial apoptosis and proliferation (*P<0.0002). Data represents the analysis of seven CC10-rtTA, eight control and four No Dox mice.

of the floxed Rb allele along with emergence of the recombined Rb allele in epithelial DNA. A faint band indicative of the floxed allele was present in some samples, which is likely to represent the few scattered epithelial cells lacking Cre function and/or minimal contamination by surrounding mesenchymal cells. Nevertheless, these data provide direct evidence that the vast majority of epithelial cells in the adult lungs are nullizygous for Rb and thus capable of compensating for loss of Rb function.

Rb deficiency leads to hypercellular neuroendocrine lesions

Although the epithelium in adult CC10-rtTA mice was remarkably restored in comparison with lungs from day 1 pups, neuroendocrine cell abnormalities were present. Lungs from adult mice showed multifocal hypercellular epithelial lesions located predominantly at airway branchpoints and bronchiolo-alveolar duct junctions (Fig. 4A-C). These lesions were composed of epithelial cells showing high nuclear to cytoplasmic ratios that protruded into the airway lumens. Sloughed cellular aggregates were also noted within airway lumens. The cells exhibited a neuroendocrine phenotype, as evidenced morphologically and confirmed by CGRP expression (Fig. 4C). Thus, Rb has a cell lineage-specific role that is essential for regulation of neuroendocrine cell fate.

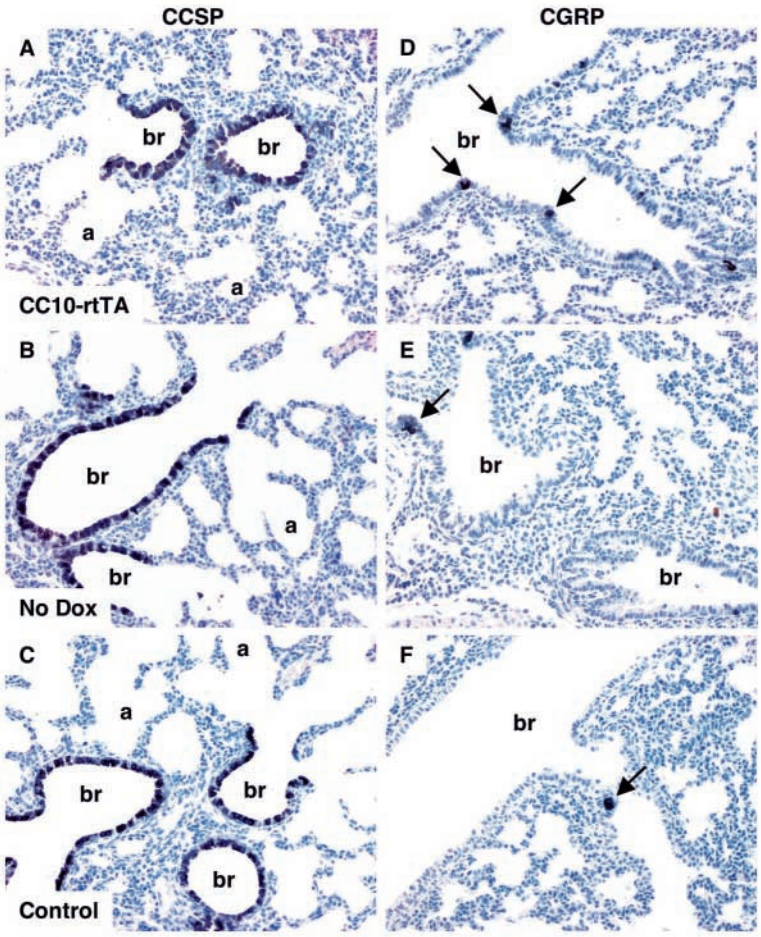

Fig. 3. Rb ablation leads to cell lineage-specific effects in differentiation. (A-F) Immunohistochemical analysis for CCSP and CGRP in lungs from day 1 pups after Rb ablation (CC10-rtTA), from genetically identical pups without doxycycline treatment (No dox), and from pups lacking one or both transgenes (Control). Arrows mark neuroendocrine cells. Abbreviations are as in Fig. 1. Data represent analysis of seven CC10-rtTA, eight Control and four No Dox lungs.

Rb family function is essential for lung epithelial development and neonatal survival

Compensation for loss of Rb function in non-neuroendocrine cells could be due to functional redundancy with other Rb family proteins, namely p107 and p130. To test this possibility, transgenic mice were created wherein total Rb family function was ablated in a lung epithelial specific manner. A truncated SV40 large T antigen oncoprotein (T121) that binds and specifically perturbs pocket protein function (Symonds et al., 1994) was targeted to the developing lung epithelium using promoters from the rat CC10 (Stripp et al., 1992) and the human surfactant protein C (SPC) (Glasser et al., 1991) genes. Although endogenous SPC is expressed in distal Type II cells and CC10 is confined to Clara cells in the adult mouse lung (Zhou et al., 1996), both promoters direct transgene expression to progenitor cells in the lung epithelium early in development (embryonic day 10-12.5), before differentiation into specialized cell types (Hackett and Gitlin, 1994; Wert et al., 1993). The CC10 promoter directs expression throughout the developing epithelium, whereas transgene expression directed by the SPC promoter is confined to progenitor cells that give rise to the distal respiratory airway. To ensure the phenotype is dependent upon loss of pocket protein function, transgenic mice were also generated with the same promoters driving expression of the truncated SV40 large T antigen containing a single base pair mutation (TK1) that is known to disrupt Rb family binding (Symonds et al., 1994).

Potential transgenic founder animals screened at the time of weaning showed a significantly lower percentage of positive animals with T121 versus the control TK1 transgene (Table 2). Additionally, surviving CC10-T121 and SPC-T121 founders did not express the transgene (data not shown). These results suggest that Rb family ablation resulted in lethality prior to weaning. To address this possibility, potential CC10-T121 founder animals were delivered by Caesarean section one day prior to expected delivery as lung function is first required at birth. Seventy-five percent (9/12) of transgene-positive pups died within the first 15 minutes after Caesarean delivery because of respiratory failure characterized by irregular gasping and failure to establish a regular breathing pattern and pink color. Surviving transgenic founders maintained using foster mothers did not express the transgene. These results indicate that Rb family function in the lung epithelium is essential for neonatal survival.

Rb family proteins regulate epithelial cell proliferation, differentiation and survival

CC10-T121 transgenic lungs were grossly indistinguishable from wild-type controls and showed normal growth and branching morphogenesis (Fig. 5A,B). Microscopic analysis, however, showed marked abnormalities throughout the epithelium. Conducting and distal respiratory airways were

Table 1. Genotype frequencies of mouse progeny from a CC10-rtTA$^{+/-}$tetCre$^{+/-}$Rb$^{LoxP/LoxP}$× CC10-rtTA$^{-/-}$tetCre$^{-/-}$Rb$^{LoxP/-}$ cross

CC10-rtTA	+/–	–/–	+/–	–/–
tetCre	–/–	+/–	+/–	–/–
Number of progeny	13	22	19	24
Percentage	17%	28%	24%	31%
*Expected percentage	25%	25%	25%	25%

*Expected percentage assumes Mendelian inheritance.

Table 2. Transgene founder frequencies

Transgene	Founders/total* (percentage)
CC10-T121	4/111 (4)
CC10-TK1	6/39 (15)
SPC-T121	3/85 (4)
SPC-TK1	8/22 (36)

*Transgene-positive founders/total mice weaned.

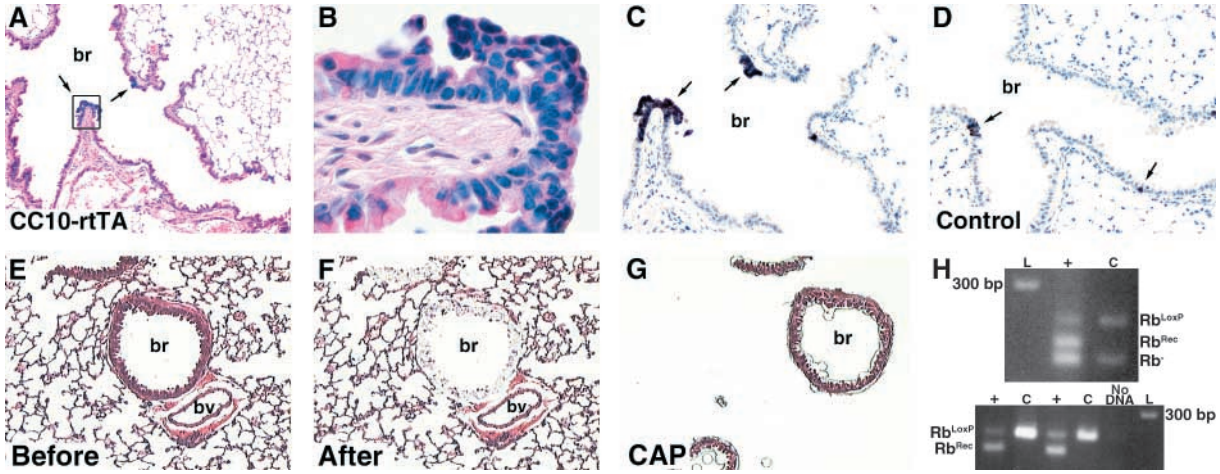

Fig. 4. Rb ablation leads to hypercellular neuroendocrine lesions. Mice were mated as detailed in Fig. 1A, and pregnant dams were treated with doxycycline throughout gestation. Doxycyline was discontinued on the day of birth. (A-D) Morphological examination and immunohistochemical analysis for CGRP in adult lungs after Rb ablation (CC10-rtTA), and in control littermates lacking one or both transgenes (Control). B represents a higher magnification of the boxed area in A. Arrows mark neuroendocrine cells. Note the remainder of the airway epithelium is essentially normal. Data is representative of three CC10-rtTA mice. (E,F) Adult lung sections before (E) and after (F) microdissection of the epithelium. (G) Microdissected epithelium on thermoplastic cap used for PCR analysis. Abbreviations are as in Fig. 1. (H) PCR analysis of microdissected epithelium from double transgenic adult mice (+) with $Rb^{LoxP/-}$ (top) or $Rb^{LoxP/LoxP}$ (bottom) alleles, and control littermates lacking one or both transgenes (C). Representative results are shown. Note loss of the Rb^{LoxP} allele and the emergence of the recombined allele (Rb^{Rec}). L, ladder.

lined by a hyperplastic epithelium with focal epithelial cell aggregates protruding into airway lumens (Fig. 5C). Epithelial cell proliferation was markedly increased as demonstrated by Ki67 expression throughout the epithelium (Fig. 5E-F). The epithelial cells were also uniformly dysplastic, showing increased nuclear to cytoplasmic ratios and abnormal chromatin aggregation. Cells with pyknotic nuclei characteristic of apoptosis were also present (Fig. 5C). Accordingly, there was a marked increase in TUNEL-positive cells in transgenic lungs when compared with wild-type controls (Fig. 5G,H). In addition to hyperplasia and dysplasia, the epithelium lacked morphologic characteristics of specialized cell types including cilia and apical cytoplasmic protrusions indicative of Clara cells (Fig. 5C,D). Thus, the data suggest that Rb family proteins are not only important in regulating cell cycle and survival but are also essential for differentiation of epithelial cells into specialized cell types.

Western blot analysis of whole lung lysates confirmed expression of the transgene and, as expected, showed no expression of full-length SV40 large T antigen or small t antigen (an alternate splice product encoded within the wild-type T antigen sequence; Fig. 6A). Transgene expression was confined to the epithelium (Fig. 6B), consistent with the previously described epithelial cell-specific activity of the CC10 promoter (Stripp et al., 1992). Control CC10-TK1 mice expressing the transgene with a mutation known to disrupt Rb family binding survived and lacked epithelial abnormalities (Fig. 6C). Although these studies do not definitively rule out the possibility that higher T121 versus control TK1 transgene expression during development may play a role in the phenotypic differences, the data strongly suggest that the lung abnormalities result from the loss of Rb family function.

Epithelial cell alterations were restricted to epithelial cells expressing the T121 transgene, providing further evidence that the lung phenotype resulted from inactivation of Rb family function. Transgene expression directed by the human SPC promoter is confined to epithelial cells lining the distal respiratory airway (Wert et al., 1993). This expression pattern differs from the uniform expression throughout the lung epithelium seen with the CC10 promoter (Hackett and Gitlin, 1994). SPC-T121 pups were generated by mating a mosaic SPC-T121 founder female with a wild-type male. Transgene-positive offspring died at birth (6/6), and showed epithelial cell hyperplasia, dysplasia and increased apoptosis in the distal respiratory epithelium despite normal epithelial cell development in conducting airways (Fig. 6F-H). Consistent with the characteristics of the SPC promoter, transgene expression was confined to the distal epithelium (Fig. 6D), correlating with the location of epithelial cell defects. Control SPC-TK1 mice survived and showed normal lung morphology, despite transgene expression (Fig. 6E). Thus, taken together the data provide strong evidence that Rb family proteins are important in regulating lung epithelial cell proliferation, survival and differentiation into specialized cell types.

Loss of Rb family function leads to increased expression of PCNA

To confirm that pocket protein function is lost in the epithelium of CC10-T121 lungs, immunohistochemical analysis was performed for the E2F-regulated gene product PCNA. Indeed, PCNA was detected throughout the epithelium in CC10-T121 lungs, which is in marked contrast to the scattered immunopositive cells observed in wild-type lungs (Fig. 5I,J). These data provide evidence that Rb family function is lost in CC10-T121 lungs.

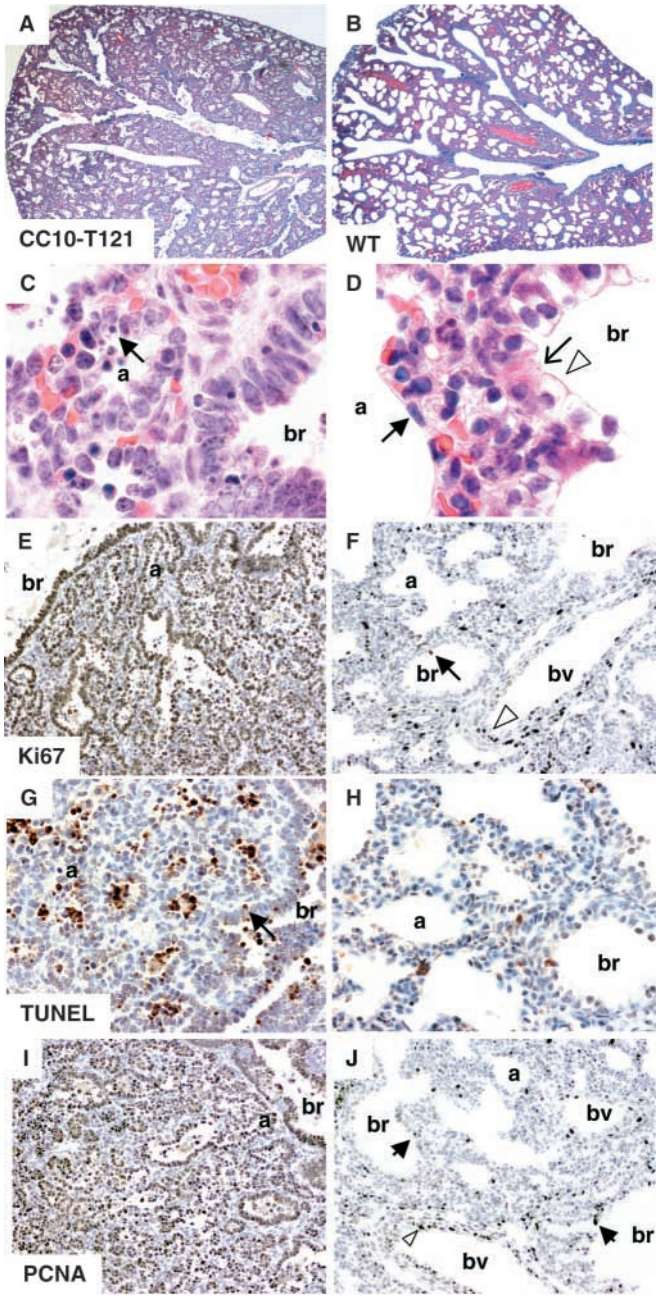

Fig. 5. Rb family deficiency results in lung epithelial abnormalities. (A-D) Morphological examination of transgenic (CC10-T121; A,C) and control wild-type (WT; B,D) lungs. A pyknotic nucleus in transgenic lungs is marked with an arrow (C). Note lack of morphologic indicators of differentiation in transgenic lungs, including flattening of Type I cells (D, closed arrow), ciliated cells (D, open arrow) and apical cytoplasmic protrusions indicative of Clara cells (D, white arrowhead). Images are representative of seven CC10-T121 founder mice. (E-H) Immunohistochemistry for Ki67 (E,F) and TUNEL (G,H) analysis. Rare focal epithelial cells positive for Ki67 are marked with an arrow in wild-type lungs (F). Note the majority of Ki67 immunoreactivity is localized to mesenchymal cells in wild-type lungs (F, white arrowhead). Arrow indicates TUNEL-positive epithelial cells in transgenic lungs (G). No specific TUNEL staining is present in wild-type lungs (H).
(I,J) Immunohistochemistry for PCNA. Scattered focal PCNA immunoreactive epithelial (arrows) and mesenchymal (white arrowhead) cells are indicated in wild-type lungs (J). Ki67, TUNEL and PCNA data is representative of four CC10-T121 founder mice. Abbreviations are as in Fig. 1.

Ablation of Rb family function blocks cellular differentiation along both Clara and ciliated cell lineages

Morphological characteristics typical of Clara and ciliated cell lineages were lacking in CC10-T121 transgenic lungs indicating impaired cellular differentiation. To further investigate cell lineage specification, immunohistochemistry was performed for markers of Clara cell (CCSP) (Zhou et al., 1996) and ciliated cell [hepatocyte nuclear factor-3/forkhead homolog 4 (HFH-4/Foxj1)] (Blatt et al., 1999; Tichelaar et al., 1999) differentiation. CCSP and Foxj1 are expressed in the respective cell types prior to the appearance of morphologic indicators of differentiation. CCSP and Foxj1 expression were lacking or markedly reduced in CC10-T121 transgenic lungs (Fig. 7A and data not shown).

As with the abnormalities in epithelial proliferation and survival, lack of cellular differentiation correlated directly with transgene expression. Although the majority of CC10-T121 transgenic mice showed epithelial cell abnormalities and transgene expression uniformly throughout the conducting and respiratory airways, several founders showed a less severe phenotype. The lung epithelium in these founders showed focal areas of normal-appearing epithelium interspersed among hyperplastic and dysplastic cells. Interestingly, focal CCSP and Foxj1 expression was detected in these lungs (Fig. 7G and data not shown). As expected, these cells showed morphologic characteristics of Clara or ciliated cells, respectively, and did not express the transgene. By contrast, adjacent transgene-expressing cells were dysplastic and lacked indicators of differentiation. In addition, distal respiratory airways do not contain Clara and ciliated cells and therefore ablation of Rb family function in the distal epithelium using the SPC promoter would not be expected to alter differentiation along these cell lineages. Consistent with this prediction, SPC-T121 lungs showed normal Clara and ciliated cell differentiation (Fig. 7C and data not shown). Taken together, these results demonstrate that pocket protein function is required for Clara and ciliated cell differentiation.

Ablation of Rb family function leads to an increase in neuroendocrine cells

Neuroendocrine cell differentiation was assessed by immunohistochemical analysis for CGRP expression. CC10-T121 transgenic lungs showed an increase in the number of neuroendocrine cell aggregates when compared with wild-type controls (Fig. 7D,E,H). Additionally, larger neuroendocrine cell aggregates were found in transgenic lungs when compared with wild-type control lungs (18% of aggregates were composed of >10 cells in CC10-T121 lungs versus 4% in wild-type lungs; P=0.026). Thus, the data support cell lineage-specific functions for Rb family proteins in the airway epithelium; acting as essential promoters of non-neuroendocrine cell differentiation while suppressing neuroendocrine cell fate. Interestingly, lungs with only Rb deficiency show neuroendocrine-specific abnormalities. Taken together, these results provide strong evidence that Rb has a

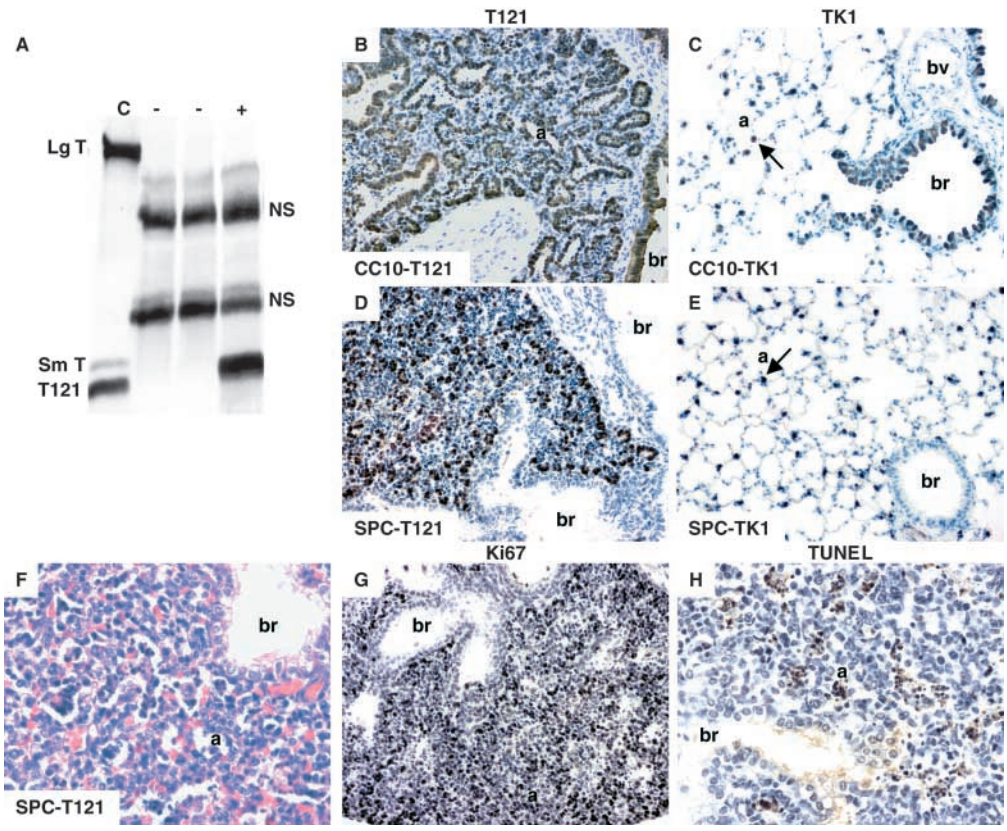

Fig. 6. Transgene expression correlates with epithelial abnormalities. (A) Western blot analysis of whole lung lysates, showing specific expression of truncated large T antigen (T121) in CC10-T121 transgenic (+) but not wild-type (–) lungs. Full-length large T antigen (Lg T) or small t antigen (Sm T) is not detected in transgenic lungs but is seen in control cell lines (C). NS, nonspecific bands. (B-E) Immunohistochemical analysis for T121 (B,D) or TK1 (C,E) expression in transgenic lungs. Arrows indicate transgene expression in alveolar Type II cells. (F-H) Morphological examination, Ki67 expression and TUNEL analysis in transgenic SPC-T121 lungs. Data is representative of seven founder CC10-T121 mice and two SPC-T121 mice. Transgene expression was detected in two of six CC10-TK1, and seven of eight SPC-TK1 transgenic lines; adult lungs are shown. Abbreviations are as in Fig. 1.

cell lineage-specific role that is essential for the regulation of neuroendocrine cell fate despite family member compensation for Rb deficiency in non-neuroendocrine cell lineages.

Discussion

The current studies show that Rb is an essential regulator of lung epithelial development. Furthermore, the data provide strong evidence that Rb family proteins show functional redundancy in regulation of epithelial cell proliferation, survival and differentiation along non-neuroendocrine cell lineages. Interestingly, selective loss of Rb function leads to neuroendocrine hypercellularity, supporting a unique cell lineage-specific role for Rb. The dependency of neuroendocrine cells on Rb function, coupled with the requirement for Rb loss in the genesis of human neuroendocrine SCLC, points toward common regulatory mechanisms operative in the mouse epithelium and in neoplastic processes associated with Rb deficiency in humans. Accordingly, the model generated for these studies provides a valuable system with which to explore how temporal variations in Rb expression (either alone or in combination with other oncogenes and tumor suppressors) alter development and promote carcinogenesis.

Rb provides a unique cell lineage-specific function in epithelial development

The phenotypes of Rb and total pocket protein-deficient lungs show that pocket proteins are essential in lung epithelial development for the regulation of epithelial cell proliferation, survival and differentiation. Specifically, pocket proteins augment cell survival and non-neuroendocrine differentiation, while suppressing proliferation and neuroendocrine cell fate. It remains possible that the T121 transgene used in the present studies may alter cellular functions in addition to Rb family function. Nevertheless, the lack of phenotypic abnormalities in control mice expressing the same transgene with a single base pair mutation known to eliminate Rb family binding provides evidence that pocket protein function is essential during epithelial development.

The current studies provide direct evidence that Rb has a unique and essential role in negatively regulating neuroendocrine cell fate in vivo. The epithelial-specific Rb knockout model generated in the current work demonstrates that Rb itself is capable of suppressing proliferation and enhancing cell survival during epithelial development. Interestingly, compensation for loss of Rb function occurred with regard to these functions, enabling restoration of the airway epithelium. By contrast, Rb function was absolutely required for regulating the neuroendocrine cell lineage. This unique cell lineage-specific function of Rb is not only essential in development but also in tumor suppression, as Rb ablation led to hypercellular neuroendocrine lesions in the current study, and somatic inactivation of both Rb and p53 in the mouse lung induces small cell lung cancers (Meuwissen et al., 2003). Furthermore, the observation that Rb is specifically, and nearly universally, mutated in human SCLC suggests that this unique cell lineage-specific Rb function is also important in the suppression of human malignancies.

Rb family proteins have opposing roles in differentiation along distinct cell lineages

The present work provides evidence that pocket protein

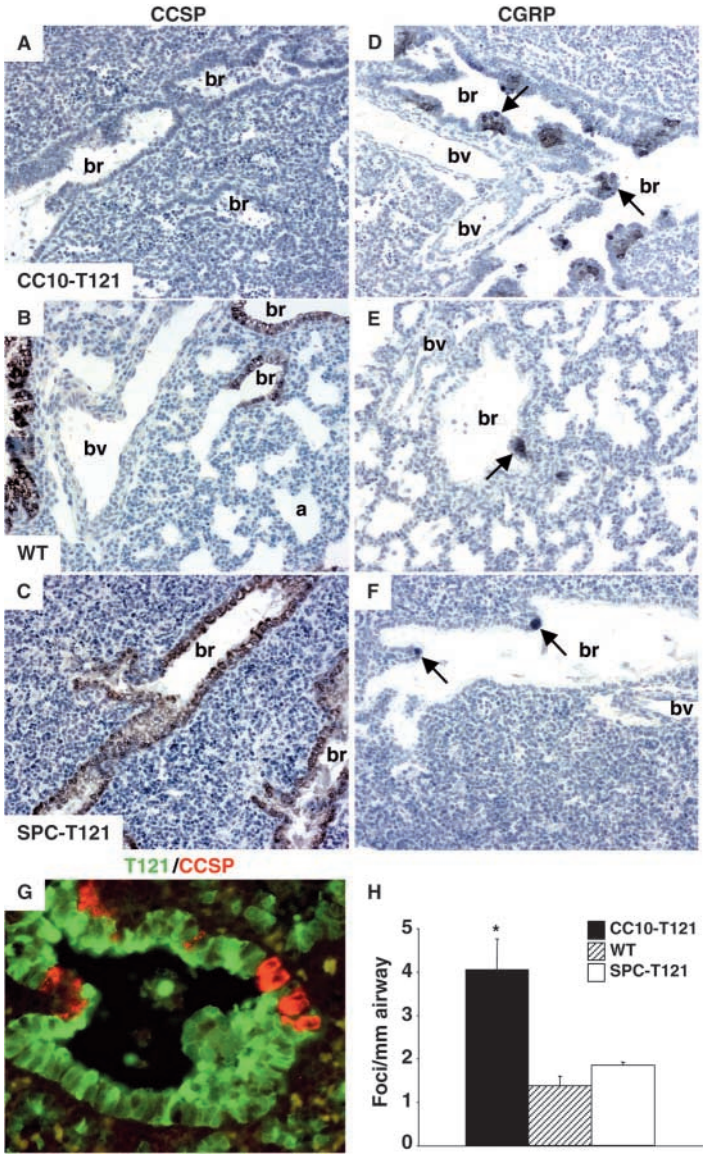

Fig. 7. Rb family deficiency results in cell lineage-specific effects in differentiation. (A-F) Immunohistochemical analysis for CCSP and CGRP in transgenic and wild-type (WT) control lungs. Arrows mark neuroendocrine cells. Data is representative of three to six CC10-T121 founder mice and two SPC-T121 pups. (G) Immunohistochemistry for the transgene (T121) and CCSP in a CC10-T121 founder with a less severe phenotype. Note that transgene expression (green) and CCSP expression (red) do not overlap. Abbreviations are as in Fig. 1. (H) Quantification of CGRP-immunoreactive foci. CC10-T121 mice show increased CGRP-reactive foci when compared with wild-type (WT) (**P*=0.0002) and SPC-T121 (**P*=0.0008) mice. Data represent six CC10-T121 founder mice, four wild-type mice and two SPC-T121 mice.

function is crucial for restricting neuroendocrine cell fate while promoting differentiation in other cell types. There are three mechanisms by which pocket protein inactivation could lead to an increase in neuroendocrine cells: (1) decreased apoptosis, (2) increased proliferation, or (3) increased differentiation. Decreased apoptosis is unlikely as there is no evidence that neuroendocrine cell number is regulated by apoptosis in normal lung development, and apoptosis was not detected in wild-type lungs in the present studies. Furthermore, loss of pocket protein function has been shown to induce rather than inhibit apoptosis (Dannenberg et al., 2000; Lipinski and Jacks, 1999; Sage et al., 2000). Enhanced neuroendocrine cell proliferation is the most obvious mechanism. Although increased proliferation would explain an overall increase in neuroendocrine cell number, this mechanism does not account for the increase in neuroendocrine foci seen in pocket protein-deficient lungs. Moreover, the majority of neuroendocrine cells in CC10-T121 transgenic lungs are not immunoreactive for the proliferation marker Ki67 in co-localization studies (data not shown). The prominence of neuroendocrine cells in pocket protein-deficient lungs is therefore not likely to occur simply as a result of increased cellular proliferation. Thus, the data suggest that loss of Rb family function leads to increased neuroendocrine differentiation.

Although pocket proteins have previously been shown to augment differentiation (Chen et al., 1996b; Gu et al., 1993; Novitch et al., 1996; Thomas et al., 2001), Rb has not previously been implicated in suppressing a differentiation pathway. Interestingly, SCLC and retinoblastomas share a neural phenotype and are the only human malignancies that exhibit Rb gene mutations in nearly all cases (Sherr, 1996). $Rb^{+/-}$ mice and chimeric animals made from $Rb^{-/-}$ ES cells also develop neuroendocrine malignancies (Hu et al., 1994; Jacks et al., 1992; Maandag et al., 1994), albeit not the same tumors associated with Rb loss in humans. In addition, ES cells lacking pocket protein function show exclusive neural differentiation, whereas normal totipotent ES cells differentiate along endodermal, ectodermal and mesodermal cell lineages (Dannenberg et al., 2000). Taken together, these data suggest a novel role for Rb in suppression of differentiation. Moreover, the mechanisms underlying Rb function in cellular differentiation and tumor suppression are likely to be linked, given the strong association between Rb loss and neuroendocrine differentiation in tumors.

Functional redundancy among pocket proteins provides an explanation for why Rb and p16 are differentially targeted in phenotypically distinct carcinomas

Lung carcinomas are divided into SCLC and NSCLC, based upon distinct clinical and pathologic features. Rb gene mutations occur in nearly all SCLC, whereas p16 is the preferential target for inactivation in NSCLC (Kaye, 2001; Minna et al., 2002). The p16 protein inhibits cyclin D/cdk4,6 kinase activity thus maintaining Rb in its active, hypophosphorylated state. Inactivation of p16 occurs in many human cancers and results in constitutive hyperphosphorylation and thus inactivation of Rb (Sherr and McCormick, 2002). The remarkably tight inverse correlation between mutational inactivation of Rb and loss of p16 expression suggest that these proteins function in a common regulatory pathway (Otterson et al., 1994; Shapiro et al., 1995). Why then are different components of the Rb pathway selectively mutated in distinct carcinomas? One hypothesis is that Rb gene mutations are seen in SCLC because neuroendocrine cells are exquisitely sensitive to Rb loss because of a lack of functional compensation by p107 and/or

p130 in this cell lineage. By contrast, Rb mutations are not detected in NSCLC because these tumors arise from non-neuroendocrine cell lineages (Minna et al., 2002) that show functional compensation for Rb deficiency. In support of this hypothesis, Rb function was demonstrated to be specifically required for regulation of neuroendocrine but not other epithelial cell lineages in the current studies. Total pocket protein inactivation resulted in marked epithelial abnormalities throughout the epithelium, implying that p107 and/or p130 provide a redundant or compensatory function in other cell lineages. Inactivation of p16 alters total pocket protein function, thereby eliminating family member compensation that occurs with Rb loss alone (Classon and Dyson, 2001; Sherr and McCormick, 2002; Tedesco et al., 2002). Moreover, p107 or p130 is required along with Rb for p16-mediated growth arrest in mouse embryo fibroblasts (Bruce et al., 2000). Thus, the data support the hypothesis that Rb gene mutation is sufficient to generate SCLC but that p16 inactivation is required to generate NSCLC because of differing degrees of functional redundancy among pocket proteins in distinct cell types. In human cancers, p16 inactivation occurs with much greater frequency than Rb gene mutations (Sherr, 1996) suggesting that, in contrast to lung neuroendocrine cells and retinoblasts, most cells require loss of total pocket protein function rather than simply Rb to progress to cancer.

Rb deficiency in the mouse lung epithelium mimics human disease

$Rb^{+/-}$ mice develop pituitary and thyroid tumors but unexpectedly do not develop retinoblastoma (Hu et al., 1994; Jacks et al., 1992; Maandag et al., 1994; Williams et al., 1994). Furthermore, genetically altered mice with Rb-deficient photoreceptor cells show no retinal abnormalities, even in a p53 null background (Vooijs et al., 2002). Importantly, the mouse retina is not intrinsically resistant to the development of retinoblastoma as this tumor occurs in transgenic mice expressing viral oncoproteins in photoreceptor cells (Vooijs and Berns, 1999). The striking discordance between the development of retinoblastomas in humans with Rb germline mutations versus mice has raised the general question as to whether engineered mice can be used to model human disease resulting from Rb deficiency.

The lung phenotype seen upon conditional Rb gene activation in the current studies shows similarities with lung disease resulting from loss of Rb function in humans. First, the nearly universal and selective occurrence of Rb gene mutations in neuroendocrine as opposed to non-neuroendocrine human lung carcinomas correlates well with the hypercellular neuroendocrine lesions observed after Rb ablation in the mouse. Second, germline Rb mutations in humans would not be predicted to lead to global lung abnormalities, based on the remarkable compensation for loss of Rb function seen in the mouse lung. However, germline Rb mutations would be predicted to predispose individuals to neuroendocrine tumors, specifically to SCLC. Indeed, multiple studies have now established that germ line Rb mutations in humans confer an increased risk to lung cancer (Kleinerman et al., 2000; Leonard et al., 1988; Sanders et al., 1989; Strong et al., 1984). Epidemiological studies show that carriers of a mutant Rb allele are 15 times more likely to die from lung cancer than the general population (Sanders et al., 1989). Moreover, the tumors that arise in these patients are predominantly SCLC and develop at a younger age than in the general population (Leonard et al., 1988; Sanders et al., 1989; Strong et al., 1984). The mouse model generated for these studies therefore provides evidence that genetically engineered mice can be used to model human lung disease resulting from Rb deficiency.

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