Cancer Cell
Article

Cell of Origin of Small Cell Lung Cancer: Inactivation of *Trp53* and *Rb1* in Distinct Cell Types of Adult Mouse Lung

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DOI 10.1016/j.ccr.2011.04.019

SUMMARY

Small cell lung cancer (SCLC) is one of the most lethal human malignancies. To investigate the cellular origin(s) of this cancer, we assessed the effect of *Trp53* and *Rb1* inactivation in distinct cell types in the adult lung using adenoviral vectors that target Cre recombinase to Clara, neuroendocrine (NE), and alveolar type 2 (SPC-expressing) cells. Using these cell type-restricted Adeno-*Cre* viruses, we show that loss of *Trp53* and *Rb1* can efficiently transform NE and SPC-expressing cells leading to SCLC, albeit SPC-expressing cells at a lesser efficiency. In contrast, Clara cells were largely resistant to transformation. The results indicate that although NE cells serve as the predominant cell of origin of SCLC a subset of SPC-expressing cells are also endowed with this ability.

INTRODUCTION

Small cell lung cancer (SCLC) is a devastating disease and is, along with non-small cell lung cancer (NSCLC), the most common cause of cancer related mortality worldwide (Jemal et al., 2008). Uncovering the identity of the cell type(s) that give rise to both lung cancer subtypes would constitute an important advancement for the field. Tissue stem cells are attractive candidates for the cells of origin of tumors, as their long in vivo life span would allow them to accumulate genetic mutations that drive tumorigenesis (Smalley and Ashworth, 2003). It is possible that committed progenitors and differentiated cells can also serve as the primary target, although this may depend upon their turnover rate and the acquisition of specific and/or combinations of genetic modifications that confer self-renewal capacity. Identifying the cell(s) of origin and elucidating the genetic and molecular changes associated with their transformation are of critical importance and will help to focus intervention strategies on eliminating the cancer propagating cells that most likely retain many of the characteristics of its cell of origin.

Understanding the normal cellular hierarchy within the lung is an important prerequisite to identify the cells of origin of cancer. To date, most of our understanding of the relationship between cell types and the existence of stem/progenitor cells in the lung originates from studies of mouse models. The adult mouse lung can be compartmentalized along the proximal-distal axis into three structurally distinct domains: the first compartment is composed of trachea and extrapulmonary bronchi, the second compartment includes intrapulmonary bronchi, bronchioles, and terminal bronchioles, and the third compartment is the alveolar ducts or gas-exchanging airspaces (for review, see Rock et al., 2010). The epithelium of the mouse trachea contains an abundant population of basal cells as well as ciliated epithelial lining cells, goblet cells, and some Clara cells. Solitary neuroendocrine (NE) cells are also present but are far more rare. In the trachea, Keratin 5 positive ($Krt5^+$) basal cells have been shown to act as stem/progenitor cells, with the ability to self-renew and give

Significance

The identification of the cell type(s) from which SCLC originates is critical in the development of methods for early diagnosis and treatment. In this paper, we validate the use of cell type-restricted Adeno-Cre vectors in directing *Trp53* and *Rb1* loss to distinct cell populations in the adult mouse lung. In doing so, we show that NE cells are the predominant cells of origin of SCLC. Our study provides additional tools to address questions related to the cell of origin of lung cancer, and highlights the importance of specifically targeting NE cells for the treatment of SCLC.

rise to the Clara cells that constitute the upper surface (Rock et al., 2009). In the more distal airways, Clara cells are abundant and line the small bronchi and bronchioles. In this region of the lung Clara cells can both self-renew and generate ciliated cells under both homeostatic conditions and in response to epithelial injury (Rawlins et al., 2009b). In the intrapulmonary airways, a limited number of pulmonary NE cells are present, the majority of which can be found in clusters commonly referred to as neuroepithelial bodies (NEBs). The latter seem to have a higher prevalence near airway junctions in the bronchioles. The most distal region of the lung is organized into a complex system of alveoli, composed of alveolar type 1 (AT1) cells and cuboidal alveolar type 2 (AT2) cells. In the alveoli, AT2 cells are considered to be the major stem/progenitor cell of the alveolar epithelium based upon their ability to self-renew and give rise to AT1 cells (Adamson and Bowden, 1974; Evans et al., 1975). The transition between the terminal bronchioles and the alveoli is known as the bronchioalveolar duct junction (BADJ) (Giangreco et al., 2002).

The specific cell type(s) that upon genetic alteration gives rise to SCLC is not yet known. This is largely due to the advanced stage of disease in most patients at the time of diagnosis (Jackman and Johnson, 2005). Interestingly, lung tumor subtypes generally follow a proximal-to-distal distribution pattern, moving distally from the trachea: squamous cell carcinoma (SCC), SCLC, and adenocarcinoma/bronchioalveolar carcinoma (for review, see Giangreco et al., 2007). It is therefore speculated that different tumor subclasses arise from cells of origin located within a defined regional compartment/microenvironment. For example, human and mouse SCLC predominately localize to the midlevel bronchioles, and express markers of NE cells, while markers defining other cells types are not expressed (Wistuba et al., 2001). However, whether SCLC arises from differentiated NE cells or from stem/progenitor cells has not been established. It is also unclear whether SCLC and NSCLC originate from different cells. The observation that SCLC can exhibit epidermoid carcinoma, adenocarcinoma, and/or large cells carcinoma-like features, would argue for the existence of a ''common'' cell of origin for these lung cancers (Travis, 2002).

Analysis of the underlying genetic alterations of lung cancer has revealed an association between the histopathology of the disease and the observed genetic lesions (Sekido et al., 2003). For example, in the lung loss of *Rb1* function has been shown to negatively regulate NE cell differentiation, and this may explain why *Rb1* loss is predominately associated with SCLC. Conversely, activating mutations in *K-ras* are almost exclusively found in NSCLC, while *Trp53* loss-of-function is observed in both SCLC and NSCLC (for review, see Meuwissen and Berns, 2005). Although distinct lesions are clearly associated with specific tumor cell types, it remains unclear to what extent the cell of origin is a determining factor in the tumor phenotype. Mouse models are well suited to sample distinct cell populations for their propensity to give rise to specific tumors (for review, see Visvader, 2011). This has been nicely demonstrated in pancreatic cancer models using cell type-specific Cre expression (Gidekel Friedlander et al., 2009; Wang et al., 2009). Here, we have addressed this question in lung focusing on the likely cell of origin of SCLC.

RESULTS

Restricting Cre-Recombination to Specific Lung Epithelial Cells using Recombinant Adenoviral Vectors

To target specific cell types, we chose to use the Cre-loxP system, which is an effective method for expressing or deleting a target gene in Cre-expressing cells. We generated a series of recombinant adenoviruses expressing Cre recombinase from specific lung epithelial gene promoters. We chose to target Clara cells, AT2 cells, and NE cells. These cell types, based on previous studies, might prove the most likely candidates either on the basis of markers that are shared between tumors and normal cells, or on the basis of studies that illustrated the regenerative capacity of distinct cell types after lung injury.

In order to target Clara cells, we utilized a DNA fragment that contains 2.1 kb of upstream 5′ flanking sequences of the mouse *CC10* gene (Ray et al., 1993). CC10 is expressed in tracheal Clara cells, bronchiolar Clara cells, including bronchioalveolar stem cells (BASCs) (Kim et al., 2005) and was more recently shown to be expressed, albeit to a low level in a rare cell population in the alveoli, that also expresses SPC (Rawlins et al., 2009b). Alveolar type 2 (AT2) cells were targeted using the Surfactant Protein C (*SPC*) gene promoter. Surfactant protein C is expressed in AT2 cells present in the alveoli and in BASCs. We utilized a murine DNA fragment that extends 4.8 kb upstream of the transcriptional start site of the *SPC* gene (Glasser et al., 2005). This promoter construct shows sequence homology to the 3.7 kb human *SPC* promoter fragment that has been used in the generation of a transgenic mouse (*SPC-rtTA*) (Perl et al., 2002b). The neural/neuroendocrine specific calcitonin/calcitonin-gene related peptide (*CGRP*) promoter (Johnston et al., 1998) was used to target pulmonary NE cells. This promoter has been shown in a number of studies to limit transgene expression to neuroendocrine and neural cells (Baetscher et al., 1991). Using these adenoviral vectors (Ad5-CC10-Cre, Ad5-SPC-Cre, and Ad5-CGRP-Cre), we can delete one or more tumor suppressor genes in a cell type-restricted manner in the adult mouse lung.

To validate the specificity of adenovirus promoter targeting, we made use of the *Rosa26R-LacZ* and *mT/mG* reporter mouse strains (Muzumdar et al., 2007; Soriano, 1999). In order to target pulmonary epithelial cells, high titer recombinant adenoviral vectors were administered intratracheally and targeted cells were identified by immunohistochemical staining, cellular location and morphology. Histochemical staining for β-galactosidase activity in lung tissue sections obtained from *Rosa26R-LacZ* mice 2–3 weeks following Ad5-CC10-Cre infection showed a restricted pattern of $LacZ^+$ cells (Figure 1A). Beta-galactosidase activity was exclusively observed in cells lining the lobar conducting airways, bronchioles and terminal bronchioles (Figure 1A). Immunostaining with an anti-CC10 antibody on consecutive sections revealed that the $LacZ^+$ cells were indeed Clara cells (Figure 1B). No $LacZ^+$ cells were observed in the alveoli, and consistent with this observation, no colocalization was observed upon immunostaining for pro-SPC, a marker for type 2 cells (Figure 1C). An identical recombination pattern was also observed in Ad5-CC10-Cre-infected *mT/mG* animals, where multilabel confocal colocalization studies confirmed the highly predominant infection of CC10-positive Clara cells (data not shown). ''Clara-like'' cells surrounding the NEBs were also found

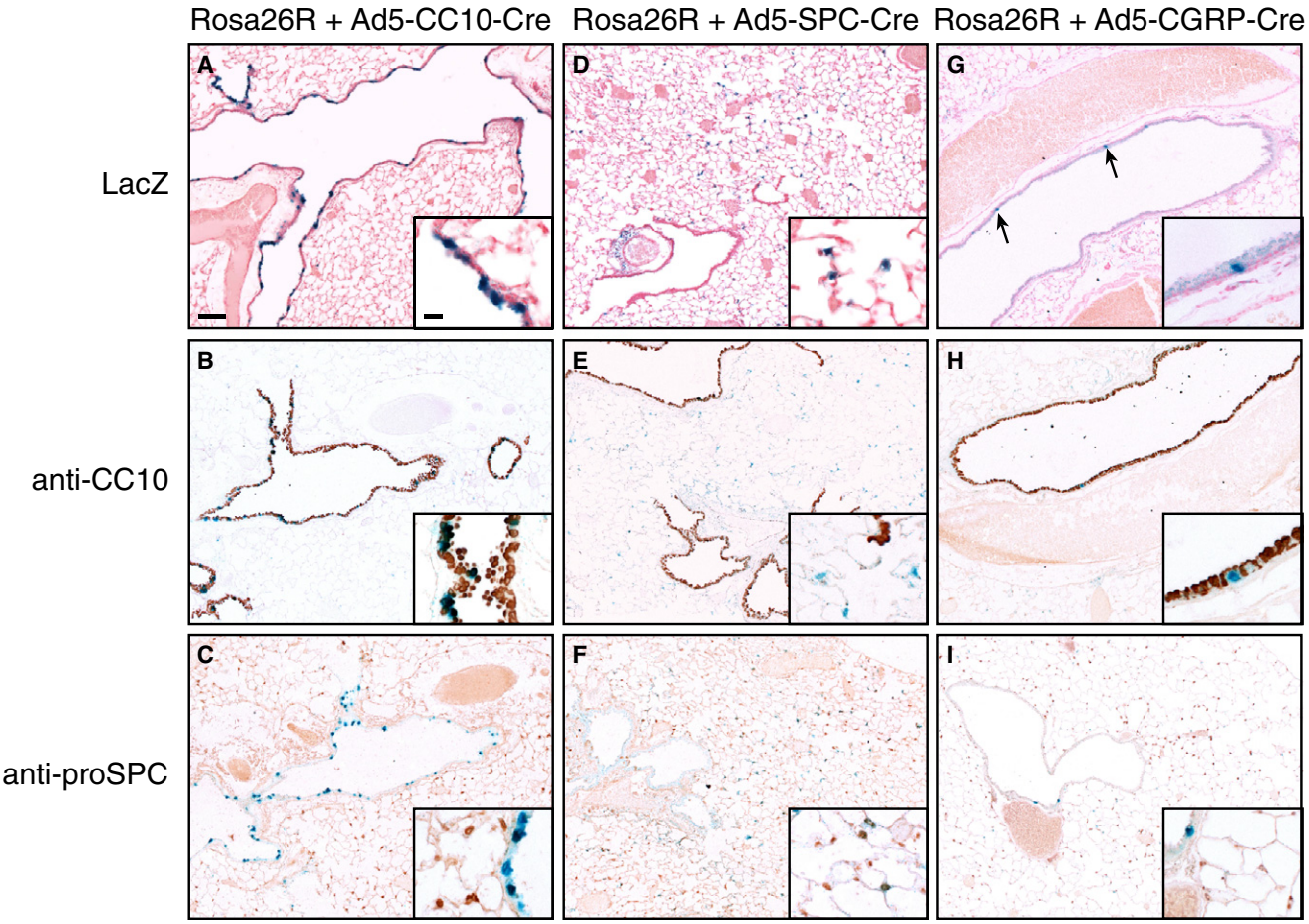

Figure 1. *Rosa26R-LacZ* Mice Show a Distinct Pattern of Reporter Gene Expression following Intratracheal Injection of Cell Type-Restricted Ad5-Cre Viruses

(A–C) Adult lung sections of *Rosa26R-LacZ* mice infected with Ad5-CC10-Cre virus (titer = 1.5 × 10^9). (A) $LacZ^+$ cells observed in the lining of the lobar bronchus, bronchiole, and terminal bronchiole (inset). No $LacZ^+$ cells were observed in the alveoli. (B) Colocalization of $LacZ^+$ cells with anti-CC10, Clara cells. (C) No colocalization of $LacZ^+$ cells with anti-pro-SPC, alveolar type 2 cells.

(D–F) Adult lung sections of *Rosa26R-LacZ* mice infected with Ad5-SPC-Cre virus (titer = 2.5 × 10^9). (D) Switching was observed in type 2 cells resident in the alveoli, whereas no switching was observed in cells lining the bronchioles/terminal bronchioles. (E) Clara cells are strongly stained by anti-CC10; however, no colocalization was observed with $LacZ^+$ cells. (F) A number of $LacZ^+$ AT2 cells are colocalized with anti-pro-SPC.

(G–I) Adult lung sections of *Rosa26R-LacZ* animals infected with Ad5-CGRP-Cre virus (titer = 1.5 × 10^9). (G) Two distinct $LacZ^+$ cells were observed lining the bronchioles. Arrows indicate putative $CGRP^+$ NE cells. (H) No colocalization of $LacZ^+$ cells was observed with Clara cells following anti-CC10 staining. (I) No colocalization of $LacZ^+$ was observed with AT2 cells following anti-pro-SPC staining.

Scale bars in (A)–(I) equal 100 μm; inserts equal 20 μm.

to be switched following Ad5-CC10-Cre infection of *mT/mG* reporter animals, however no colocalization was observed following immunofluorescence staining with an anti-synaptophysin (Syn) antibody, a marker of NE cells (Figure 2A). In scanning a large number of cryosections, we found only a single NE cell that showed Cre-mediated switching after infection with Ad5-CC10-Cre virus.

Conversely, administration of Ad5-SPC-Cre virus yielded β-galactosidase activity in alveolar pneumocytes resident within the parenchyma (Figure 1D). Very infrequently, a $LacZ^+$ cell was observed in close proximity to the BADJ, in a continuum with the bronchiole lining (data not shown). However, as we did not detect colocalization following anti-CC10 staining we cannot confirm whether these switched cells are indeed BASCs. Overall we did not observe colocalization of $LacZ^+$ cells residing within the parenchyma with anti-CC10 (Figure 1E). Ad5-SPC-Cre-switched cells did, however, colocalize with AT2 cells, as determined by costaining with an anti-pro-SPC antibody (Figure 1F). Moreover, examination of around 150–200 NEBs in *mT/mG* sections obtained from Ad5-SPC-Cre-infected mice, revealed no colocalization of GFP^+ switched with Syn^+ NEBs (data not shown) whereas such staining was easily detected when Ad5-CGRP-Cre virus was used.

Pulmonary NE cells represent an extremely rare cell population in the adult mouse lung and are scattered throughout the epithelium of conducting airways, predominantly as small clusters of cells termed neuroepithelial bodies (NEBs). In line with the low abundance of pulmonary NE cells in the adult lung,

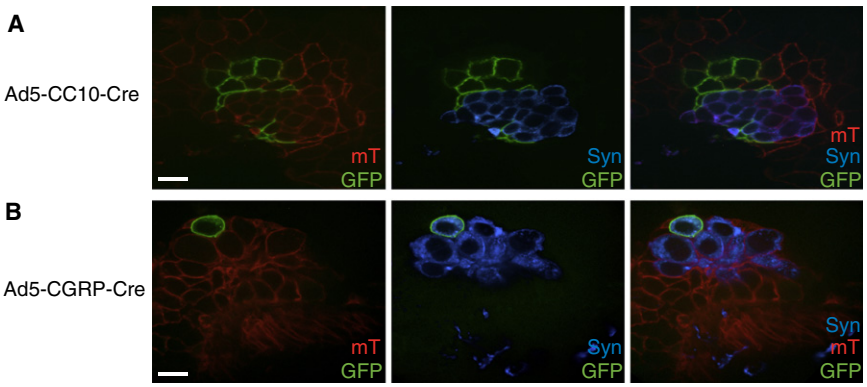

Figure 2. Neuroendocrine Cells Are Selectively Targeted by Cell Type-Restricted Ad5-Cre Viruses

(A) Adult lung cryosections of *mT/mG* reporter mice infected with Ad5-CC10-Cre virus (titer = 1.5×10^9). GFP$^+$, recombined cells (green, FITC), were observed in close proximity to the NEB. No colocalization of GFP$^+$ cells with Syn$^+$ cells present within the NEB.

(B) Adult lung cryosections of *mT/mG* reporter mice infected with Ad5-CGRP-Cre virus (1.5×10^9). One GFP$^+$, recombined cell (green, FITC) was observed in the epithelium of the conducting airway. Colocalization of the GFP$^+$ cell with Syn$^+$ cells present in the NEB.

Scale bars in (A) and (B) equal 10 μm.

very few LacZ$^+$ cells were detected in the epithelium of intrapulmonary conducting airways in *Rosa26R-LacZ* animals following intratracheal injection with Ad5-CGRP-Cre virus (Figure 1G). Moreover, LacZ$^+$ cells were generally found as apparently single cells, tightly surrounded (enclosed) by other airway epithelial cells, likely representing NE cells (Figure 1H). Ad5-CGRP-Cre-switched cells did not colocalize with AT2 cells, as detected by costaining with an anti-pro-SPC antibody (Figure 1I). To confirm that the Ad5-CGRP-Cre-switched cells are in fact NEB cells, we stained 25 μm-thick cryosections obtained from *mT/mG* mice infected with Ad5-CGRP-Cre with anti-Syn and colocalization with GFP$^+$ cells was determined by confocal microscopy. As expected GFP$^+$ cells colocalized with Syn$^+$ cells in the airway epithelium. All of the switched NE cells were found to be present within a limited number of NEBs, which typically harbored just one or two GFP$^+$ cells per NEB (Figure 2B), clearly indicating that the Ad5-CGRP-Cre virus preferentially targets NE cells in NEBs of the adult mouse lung. However, we also observed switching of a small fraction of the Clara cells and ciliated cells indicating that these cells can express Cre after infection with Ad5-CGRP-Cre. Although Ad5-CGRP-Cre does cause a very low level switching in these cell types, the virus is by several orders of magnitude more effective in switching NE cells than either Ad5-CC10-Cre or Ad5-SPC-Cre adenovirus.

Taken together, these results confirm that Cre-mediated recombination is confined to a specific cell population as dictated by the promoter utilized (Table 1), thereby validating a series of cell type-restricted Ad5-Cre viruses, which can be used to address the lineage specificity and cell of origin in murine lung cancer.

Table 1. Relative Frequency of Observed Ad5-Cre-Switched Cells

Adenovirus	Neuroendocrine Cells	Clara/Ciliated Cells	Alveolar Type 2 Cells
Ad5-CMV-Cre	++++	++++	++++
Ad5-CC10-Cre	+/−-[a]	++++	-
Ad5-SPC-Cre	-	+/−-	++++
Ad5-CGRP-Cre[b]	++++	+/−-	-

[a] One NE cell present within an NEB was found to be switched.
[b] Total cells switched with the Ad5-CGRP-Cre virus is >100-fold lower than with other viruses.

Loss of *Trp53* and *Rb1* in Specific Populations of Airway Epithelial Cells Results in Different Tumor Pathologies and Rates of Tumor Onset

The specific cell type(s) that gives rise, upon genetic alteration to SCLC, is unknown. To gain insight into the cell of origin we utilized a sporadic mouse model of lung cancer based on the conditional inactivation of the *Trp53* and *Rb1* tumor suppressor genes (Meuwissen et al., 2003). Loss of *Trp53* and *Rb1* is achieved in a variety of lung epithelial cell types by intratracheal infection of *Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals with Ad5-CMV-Cre virus. Infected *Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals develop lung tumors with striking morphological and immunophenotypical similarities to human SCLC (Calbo et al., 2011; Meuwissen et al., 2003). To gain insight into the target cell for transformation in SCLC, we infected *Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals with our cell type-restricted Adeno-Cre viruses: Ad5-CC10-Cre, Ad5-SPC-Cre and Ad5-CGRP-Cre. *Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals also carried the *Rosa26R-LacZ* reporter allele (Soriano, 1999), allowing us to monitor Cre-mediated switching of *Trp53* and *Rb1* in the various cell populations. Mice were sacrificed when they became moribund. Consistent with prior observations, the tumors that most frequently arose in our cohorts were SCLC (Figure 3A and Table 2).

The time for *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ to succumb from NE tumors following injection with cell type-restricted Ad5-Cre viruses is depicted in Figure 3B. In line with previous findings, the majority of animals injected with Ad5-CMV-Cre virus developed NE lung tumors with a median latency of 273 days (Figure 3B; Meuwissen et al., 2003). A similar trend was observed in animals following Ad5-CGRP-Cre injection, with the majority of animals developing NE tumors with a median latency of 362 days (Figure 3B). Approximately half of *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals injected with Ad5-SPC-Cre virus developed NE lung tumors (Figure 3B). Although the tumor cell characteristics of the NE tumors observed in *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice infected with either Ad5-CGRP-Cre or Ad5-SPC-Cre virus were very similar, the time of occurrence of NE tumors in mice infected with Ad5-SPC-Cre virus was further delayed (454 days). The latency of neuroendocrine tumors of animals injected with Ad5-CC10-Cre virus could not be reliably estimated due to the paucity of tumors that were observed and the predominance of other causes of death after 18 months.

Recently, a relationship between naphthalene-induced lung injury and NSCLC has been demonstrated (Kim et al., 2005).

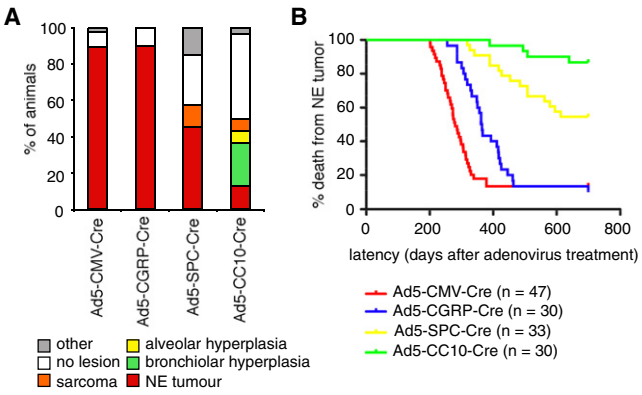

Figure 3. Loss of *Trp53* and *Rb1* in Specific Cellular Compartments Results in Different Tumor Pathologies and Rates of Tumor Onset

(A) Histological spectrum of lesions in *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice infected with cell type-restricted Ad5-Cre viruses. The bar graph represents the histopathology spectrum of *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals infected with cell type-restricted Ad5-Cre viruses.

(B) The incidence of NE lung tumors in mice carrying conditional *Trp53* and *Rb1* alleles following infection with cell type-restricted Ad5-Cre viruses. Curves showing the percentage of *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice that died from an NE tumor following infection with Ad5-CMV-Cre virus (red curve), Ad5-CGRP-Cre virus (blue curve; n = 30; median survival age (T_{50}) = 363.5 days; p = 0.0095**), Ad5-SPC-Cre virus (yellow curve; n = 33; T_{50} = undefined; p < 0.0001***), and Ad5-CC10-Cre virus (green curve; n = 30; T_{50} = undefined; p < 0.0001***).

See Figure S1 for the effect of naphthalene-induced damage on small-cell tumor initiation in *Trp53*$^{F/F}$;*Rb1*$^{F/F}$ compound mice.

Moreover, elevated proliferation of pulmonary NE cells (PNECs) is observed in murine lungs following acute naphthalene injury (Stevens et al., 1997). Given these observations, we explored whether a relationship exists between naphthalene-induced lung injury and *Trp53*- and *Rb1*-induced SCLC. *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice were treated with naphthalene (or vehicle control) and tumorigenesis was initiated (*Trp53* and *Rb1* were inactivated) by administration of the cell type-restricted Ad5-Cre viruses 3–4 days following lung injury (see Figure S1A available online). No difference, however, was observed in the median tumor-free survival of mice infected with either Ad5-CMV-Cre or Ad5-CGRP-Cre viruses depending upon whether or not naphthalene had been administered prior to switching (Figure S1B). Thus, naphthalene-induced injury and the accompanied induction of cell proliferation, does not accelerate SCLC development through loss of *Trp53* and *Rb1*.

Neuroendocrine Cells Are the Predominant Cells of Origin of SCLC

In *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice infected with Ad5-CGRP-Cre virus neoplastic lesions with NE differentiation were identified by H&E staining in lungs of 25/30 mice. All tumors were located in the central region of the lung (Figure S2A). Focal areas of intraepithelial neoplasia were also detected in the epithelial lining of conducting airways (Figures 4A and 4B), strengthening the idea that Ad5-CGRP-Cre-induced tumors originate from NE cells. The tumors were composed of uniform small- to middle-sized round cells that contained a course chromatin structure and thick nuclear membrane (Figure 4C). They showed high mitotic and apoptotic activities, with local fields of necrosis. Immunohistochemical analysis of the lung tumors confirmed their similarity to human SCLC. Many markers that stain positive in human SCLC were also detected in our murine SCLC such as neural cell adhesion molecule (Ncam1) (Figure 4D), synaptophysin (Syn), CGRP and thyroid transcription factor-1 (TTF-1) (data not shown). The tumors arising in these mice were highly invasive, with tumor cells invading the mediastinum and thoracic wall. Moreover, metastasis was observed in extrapulmonary sites, such as liver (Figure 4E) and kidney, and their morphological appearance was identical to that of the primary lung tumors. Moreover, like their primary tumor counterpart, metastases expressed markers of NE differentiation, such as Ncam1 (Figure 4F), Syn, and CGRP (data not shown).

In *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice following Ad5-SPC-Cre administration, lung tumors displaying characteristics of neuroendocrine differentiation were also observed, although at a lower frequency. Interestingly however, three cases (20%) of the Ad5-SPC-Cre cohort showed only a peripheral tumor location (Figure S2B). This peripheral-only subset of NE lung tumors was unique for the Ad5-SPC-Cre virus, as no such cases were observed in the Ad5-CGRP-Cre animal cohort supporting the notion that a peripherally located SPC-positive cell can transform into an NE tumor following loss of *Trp53* and *Rb1*. In contrast, SCLC could not be induced effectively following loss of *Trp53* and *Rb1* in cells expressing CC10 with only 4/30 animals showing NE tumors in the lung following infection with Ad5-CC10-Cre virus after a very long latency. The fact that we observed a rare switched NE cell after Ad5-CC10-Cre infection in one of the cryosections described above might indicate that in these few cases NE cells served as the cell of origin. The histopathology of NE tumor cells after infection with Ad5-SPC-Cre and Ad5-CC10-Cre was identical to those observed following Ad5-CGRP-Cre infection (data not shown). PCR analysis indicated that all primary NE lung tumors from *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice that were examined from each of the Adeno-Cre cohorts had undergone Cre-mediated deletion of *Trp53* and *Rb1* conditional alleles (Figure S2C). This was also the case in liver metastasis isolated from individual animals (Figure S2C). Moreover, quantitative reverse-transcription PCR (RT-PCR) analysis confirmed the decrease in expression of *Trp53* and *Rb1* in both primary lung tumors (Figure S2D) and liver metastasis (Figure S2E).

Large cell sarcomatoid-like lesions were observed in the periphery of the lungs in a small number of animals following either Ad5-SPC-Cre or Ad5-CC10-Cre administration. The tumor cells were large to giant in size and contained single and multiple nuclei (Figure S3A). Metastatic lesions in the liver were also present in these animals. The tumor cells displayed a histiocytic immunophenotype with cells staining positive for F4/80 (Figure S3B), and partially positive for vimentin (data not shown). Tumor cells stained negative for cytokeratin 8 (CK8) (Figure S3C) and were also negative for pro-SPC (data not shown). The precise origin of these tumors is unclear. However, the fact that they arose upon either Ad5-SPC-Cre or Ad5-CC10-Cre administration suggests that they arose from CC10/SPC positive cells and therefore could have arisen from BASCs.

A large fraction (14/30) of *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice infected with Ad5-CC10-Cre virus, showed no neoplastic lesions in the lung (Figure 3A and Table 2). The absence of cellular

Table 2. Occurrence of Lung Tumors in Conditional Mutant Mice

Adenovirus	Total Number of Mice	# Mice Sacrificed with Described Pathology/# of Mice Analyzed	Histopathology of Lung Lesions	Lesion Latency (days)
Ad5-CMV-Cre	47[a]	4/47	No lesions	297–517
		3/47	NE hyperplasia	196–286
		40/47	NE tumor	201–339
Ad5-CGRP-Cre	5[b]	3/5	Simple hyperplasia (NE cells)	182–275
	30[a]	1/5	NE tumor	238
		4/30	No lesions	224–287
		1/30	Spindle cell NE tumor	206
		25/30	NE tumor	255–464
Ad5-SPC-Cre	2[b]	2/2	No lesions	252
	33[a]	9/33	No lesions	386–684
		15/33	NE lung tumor	319–613
		4/33	Sarcoma	529–649
		1/33	Papillary carcinoma	407
		1/33	Adenocarcinoma	642
		3/33	Other	562–573
Ad5-CC10-Cre	2[b]	1/2	No lesion	280
	30[a]	1/2	Simple hyperplasia (Clara cells)	280
		14/30	No lesions	236–684
		3/30	NE tumor	389–640
		1/30	NE tumor and alveolar hyperplasia	601
		7/30	Bronchiolar hyperplasia	333–684
		2/30	Alveolar hyperplasia	333–601
		2/30	Sarcoma	663–679
		1/30	Other	204

The total number of mice analyzed for each genotype and the occurrence of NE tumors and other lung lesions at different time points is presented.
[a] Mice sacrificed when symptoms occurred or died of old age. See also Figure S3 for additional histopathology.
[b] Mice arbitrarily sacrificed.

transformation was not due to a lack of Ad5-CC10-Cre-mediated recombination, as β-galactosidase positive cells, a readout of *Trp53* and *Rb1* recombination, were readily detected in *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals 410–684 days following Ad5-CC10-Cre virus infection (data not shown). Interestingly, atypical hyperplasia of bronchiolar epithelial lining cells was observed in 7/30 (23%) mice 383–684 days following Ad5-CC10-Cre injection. The cells in these lesions were characterized by an increased nuclear to cytoplasm ratio and hyperchromatic nuclei (Figure S3D). Moreover, the lesions showed a high proliferation index, as determined by Ki67 immunostaining (Figure S3E). In contrast to the simple hyperplastic lesions, the atypical hyperplasic cells would appear to be solely of Clara cell origin, as they stained positive for CK8, CC10, but were negative for Syn (Figure S3F, data not shown).

Involvement of SPC-Positive Cells in *Trp53*- and *Rb1*-Induced SCLC

Given the fact that loss of *Trp53* and *Rb1* in SPC-positive cells gives rise to SCLC, although at a much lower efficiency, we were interested in assessing the expression level of pro-SPC in primary lung tumors and liver metastasis isolated from *Rosa26*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals. Consistent with the NE features of SCLC, the NE cell marker CGRP (*Calca*) was highly expressed in all primary NE lung tumor samples isolated from mice infected with different cell type-restricted Ad5-Cre viruses. In contrast, the Clara cell marker, CC10 (*Scgb1a1*) was not detected in the vast majority of NE tumors examined (Figure 5A). Interestingly, the alveolar cell marker, SPC (*Sftpc*) was detectable in a high number of primary NE tumor samples, although at a much reduced level to that observed in normal lung tissue (Figure 5A). As we cannot exclude the possible contamination of normal lung tissues in our tumor preparations, we made use of a series of clonal cell lines derived from mouse SCLC. A subset of cell lines, termed NE cells, grow as suspending cell aggregates and express markers of NE differentiation. The remaining subset of cell lines grow as an attached monolayer composed of larger cells with visible cytoplasms and based on these phenotypic observations were termed non-NE cells (Calbo et al., 2005, 2011). Consistent with previous observations, high expression of *Calca* was detected in NE clonal cell lines, but absent in non-NE cell lines. The expression of *Sftpc* was also detected, although at extremely low levels, in clonal NE cell lines, but absent in non-NE cells (Figure 5B). Expression of *Scgb1a1* was below detectable levels in all cell lines examined (data not shown). Although, this observation would point to the possibility that NE cells can be switched, although relatively inefficient, by Ad5-SPC-Cre viruses, our inability to find even a single switched

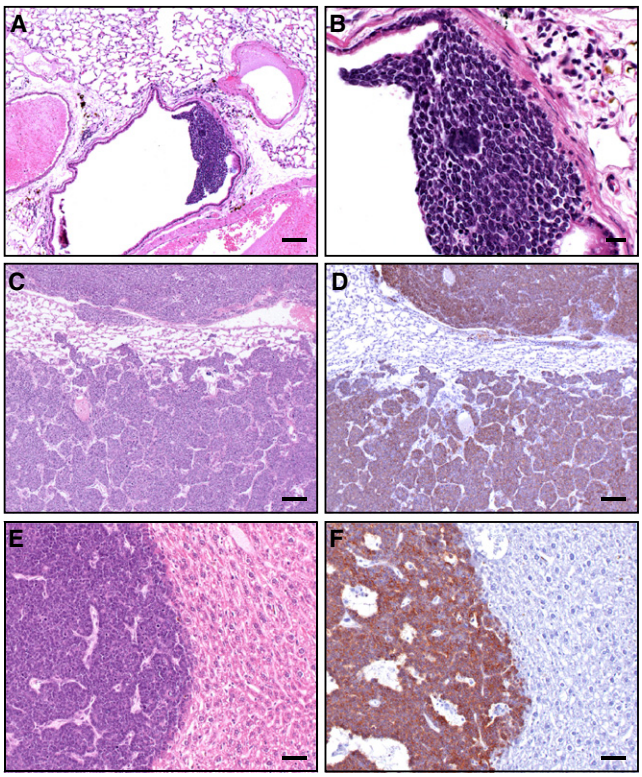

Figure 4. NE Cells Are Target Cells for Transformation in *Trp53*- and *Rb1*-Induced SCLC

Representative lesions are shown from *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ mice 238–464 days following Ad5-CGRP-Cre administration.

(A and B) H&E-stained section of an intraepithelial neoplasia of NE cells.

(C) H&E-stained section of an NE lung tumor showing large mass formation and massive infiltration into the surroundings.

(D) Immunostaining with antisera raised against the NE cell marker Ncam1.

(E) H&E-stained section of metastasis to the liver.

(F) Immunostaining of liver metastasis with antisera raised against Ncam1.

Scale bars: (A), (C), and (D) equal 100 μm; (B) equal 20 μm; (E) and (F) equal 50 μm. See Figure S2 for images related to tumor location, *Trp53* and *Rb1* recombination efficiencies and expression analysis.

NE cells after Ad5-SPC-Cre infection, makes this an unlikely explanation.

DISCUSSION

In this study, we have directly addressed questions related to the cell-of-origin of lung cancer through the generation of cell type-restricted adenoviruses, which have the ability to direct Cre recombinase expression to a defined, yet small number of adult lung cells in vivo. This strategy is advantageous as it allows the induction of sporadic mutations in a small number of cells in their normal tissue microenvironment, and thus more closely mimics the way in which tumors develop in man. Transgenic models directing inducible expression of Cre recombinase to the proximal and distal airways using the 2.3 kb rat *Scgb1a1* promoter and the 3.7 kb human *SFTPC* promoter, respectively, have been described previously (Perl et al., 2002a; Stripp et al., 1992). However, in these systems problems related to misexpression of Cre recombinase in cells other than the cells of interest were noted (for review, see Perl et al., 2009). With our adenoviral vectors we obtained a very high level of cell type specificity as based on Cre reporter assays performed in *Rosa26R-LacZ* and *mT/mG* reporter mice (Figures 1 and 2 and Table 1).

Although our Ad5-CGRP-Cre virus targets an extremely small number of cells, induced loss of *Trp53* and *Rb1* in these rare cells gives rise to a very high incidence of lung tumors that display NE differentiation and SCLC morphology (Figure 4). This tumor incidence is similar to that achieved with Ad5-CMV-Cre virus, which infects over 100-fold more cells in the lung. As seen in humans, Ad5-CGRP-Cre-induced tumors were centrally located and aggressive in nature, exhibiting a marked capacity to metastasize to liver. Moreover, mouse SCLC tumors express numerous cellular markers that are also used to mark human SCLC tumors, such as high levels of TTF-1. Taken together, these tumors show a striking similarity to human SCLC. Thus, our study supports the notion that Ad5-CGRP-Cre infects the predominant cell of origin of SCLC. Our findings therefore provide strong in vivo evidence that NE cells serve as the prevalent target cell of origin of SCLC (Figure 6).

Interestingly, directed expression of *H-ras* to pulmonary NE cells using a *CGRP* promoter-driven transgenic construct, resulted in the formation of only bronchial adenocarcinomas (Sunday et al., 1999). These observations, together with our own, indicate that different genetic lesions can drive the same target cells down a divergent differentiation path. It suggests that an active Ras pathway in these cells does not permit the cells to retain their neuroendocrine features, while loss of both *Rb1* and *Trp53* promotes the neoplastic transformation of NE cells. Our recent studies in which introduction of an activated *Ras* can convert NE SCLC cells into cells that completely change their phenotype and now resemble more closely NSCLC adenocarcinomas supports this and argues that mutant Ras can act dominantly in commanding the lung cancer phenotype (Calbo et al., 2011).

In the adult mouse lung, NEBs are found within niches containing Clara cells and variant Clara cells, some of which cover the luminal surface of the NEB (De Proost et al., 2008). From these observations, a relationship between NE and Clara cells has long thought to exist and thus, Clara cells have also been speculated to serve as cells of origin of SCLC. Our data argue against this possibility. From our *mT/mG* reporter animal analysis, we observed upon screening through a series of thick cryosections colocalization between one Ad5-CC10-Cre-switched cell and a Syn^{+} cell, present within a NEB. This rare aberrant Cre expression in NE cells could account for the few cases of SCLC observed following administration of Ad5-CC10-Cre. More frequently, however we observed switching of Clara cells adjacent to NEBs. Alternatively, the rare occurrence of SCLC after administration of Ad5-CC10-Cre could result from the uptake of Cre protein in NE cells from an adjacent highly Cre-expressing Clara cell.

Although NE cells appear the predominant cancer-initiating population in SCLC, our data also support the presence of an SPC-positive progenitor cell population that can give rise to SCLC following loss of *Trp53* and *Rb1*, albeit with a lower penetrance (Figure 6). We do not believe that our Ad5-SPC-Cre virus

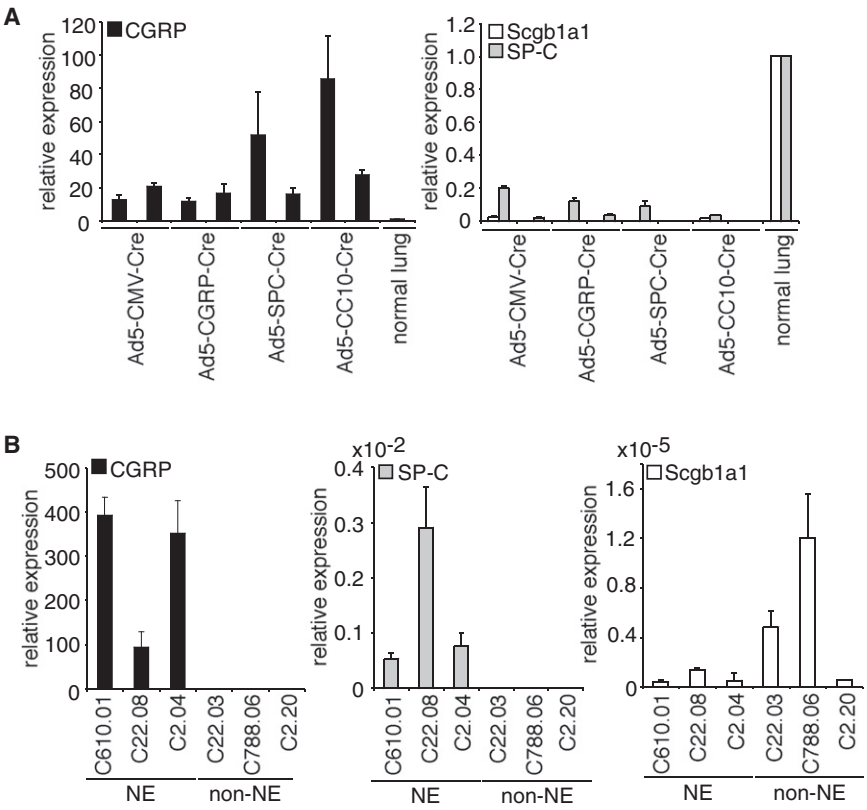

Figure 5. Low Expression of SPC in Primary SCLC Tumors and Mouse SCLC Clonal Cell Lines

(A) The expression of *CGRP* (black bars), *Scgb1a1* (white bars), and *SPC* (gray bars) transcripts relative to *Hprt* was determined by quantative PCR analysis of RNA derived from primary lung tumors from *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals infected with Ad5-CMV-Cre, Ad5-CGRP-Cre, Ad5-SPC-Cre, and Ad5-CC10-Cre viruses. Two tumor samples are shown for each virus. Values are represented relative to levels detected in RNA extracted from normal lung. Data shown represent mean ± SD.

(B) The expression of *CGRP* (black bars), *SPC* (gray bars), and *Scgb1a1* (white bars) relative to *Hprt* was determined by quantative PCR analysis of RNA derived from mouse SCLC clonal cell lines: neuroendocrine (NE) cell lines and non-neuroendocrine (non-NE) cell lines. Three cell lines are shown for each.

Values are represented relative to levels detected in RNA extracted from normal lung. Data shown represent mean ± SD.

exhibits ectopic expression to a cell type not detectable in our reporter assays. In agreement with previous studies our findings in reporter animals indicate that the mouse *SPC* promoter exhibits a high degree of cell type specificity to AT2 cells (Figure 1) (Glasser et al., 1990, 2005). This observation implies that there might be a population of SPC-positive cells that, upon loss of *Trp53* and *Rb1*, acquire the capacity to give rise to SCLC. A cell type that immediately comes to mind is the BASC. BASCs have been proposed to be the cell of origin of K-*ras*-induced NSCLC (Kim et al., 2005). This rare cell type located at the BADJ expresses both CCSP and SPC, and thus would be targeted by both our Ad5-CC10-Cre and Ad5-SPC-Cre viruses. If BASC cells acted as cancer-initiating cells for SCLC, however, one would expect that both Ad5-CC10-Cre and Ad5-SPC-Cre viruses are similarly effective in inducing SCLC. Contrary to this assumption, only a very small number of *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals exhibited SCLC following Ad5-CC10-Cre virus inoculation, while a pronounced fraction of animals exhibited SCLC following Ad5-SPC-Cre virus administration. Indeed, it is feasible, that recombination efficiencies may vary among the adenoviral vectors due the to activity of Cre-driven promoters within a distinct cellular compartment. However, given the robust switching we observe with Ad5-CC10-Cre and to a lesser extent Ad5-SPC-Cre of the *Rosa26R-LacZ* reporter which switches less efficient than either the *Trp53* and *Rb1* conditional alleles (Vooijs et al., 2001), it is very unlikely that BASCs are the cell of origin of SCLC in Ad5-SPC-Cre-infected animals.

It is more likely that other cell types that express SPC have the capacity to give rise to SCLC. In this respect it is noteworthy that a fraction of the SCLC cell lines also express *SPC* (Figure 5), suggesting that *SPC* is expressed in the lineage of the cell of origin of SCLC. This points to the existence of an early progenitor that expresses low levels of *SPC* and has the capacity to differentiate along the neuroendocrine lineage. This could be a common progenitor residing in the lung or a committed progenitor that trans-differentiates under pressure of the concomitant loss of *Trp53* and *Rb1*. In support of the former, it is interesting to note that there is evidence for a multipotent progenitor cell in the immature and developing lung (Rawlins et al., 2009a; Wuenschell et al., 1996). However, the existence of a corresponding multipotent progenitor population in the adult lung remains illusive and is currently the focus of much investigation.

We should also not underestimate the influence specific genetic mutations may have on the target cell. *Rb1* is a critical controller of NE differentiation and its loss could be a mechanism by which a progenitor-like AT2 cell could differentiate toward a more NE cell-like state. The observed peripheral location of SCLC in a distinct subset of the Ad5-SPC-Cre but not the Ad5-CGRP-Cre induced tumors supports this notion. In line with this hypothesis it has been shown that *Rb1* can regulate cell fate choice and lineage commitment both in epithelial (Wikenheiser-Brokamp, 2004) and mesenchymal cells (Calo et al., 2010). It should be noted that it is unlikely that every SPC-positive cell has the capacity to develop a NE tumor following loss of *Trp53* and *Rb1*. Instead, the susceptibility of a subset of SPC-positive cells to tumor development might be a consequence of intrinsic capabilities inherent to progenitor cells. In this regard, it is interesting to note that activation of *K-ras*^{V12} in lung causes only a small fraction of the cells to give rise to hyperplastic growth arguing that specific cell autonomous features or microenvironmental conditions are required to support tumor initiation (Guerra et al., 2003).

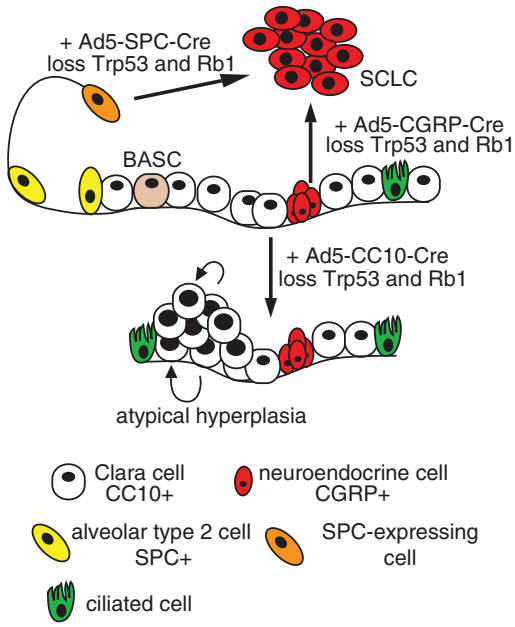

Figure 6. Hypothetical Schematic of *Trp53*- and *Rb1*-Induced SCLC
Loss of *Trp53* and *Rb1* was restricted to specific cell types in the adult lung by cell type-restricted Ad5-Cre viruses. Loss of *Trp53* and *Rb1* in CGRP-positive NE cells (red) efficiently gave rise to NE lung tumors, indicating that NE cells are the predominant cells of origin for SCLC. SCLC was detected in a substantial number of *Rosa26R*;*Trp53*$^{F/F}$;*Rb1*$^{F/F}$ animals following Ad5-SPC-Cre infection. It remains unclear whether this is due to loss in a differentiated AT2 cell (yellow), or whether loss occurs in a more rare SPC-expressing progenitor cell type (orange) that has the ability to differentiate along the NE cell lineage. SCLC could not be effectively induced following directed loss of *Trp53* and *Rb1* to CC10-positive Clara cells (white). Ciliated cells, (green), were not targeted by the cell type-restricted Ad5-Cre viruses.

Finally, our study identifies the rarely occurring NE cells as the predominant cell of origin of SCLC. Targeting *Trp53* and *Rb1* loss to any other cell results in a much lower or even negligible incidence. These findings are also relevant for the treatment of SCLC that is notoriously difficult to control as it invariably relapses after an initial response to chemotherapy (Jackman and Johnson, 2005). The notion that NE cells are the predominant cells of origin of SCLC implies that we should concentrate on this specific cell type in the design of therapies and be less concerned about an elusive cancer-initiating cell with different characteristics. Very little is still known about the lineage relationship of NE cells to other cell types in the lung. Although NE cells are also found in microenvironments that have been shown to maintain stem cell populations, it would appear that NE cells do not behave as lung stem cells. Rather they exhibit properties of uni-potent progenitor cells (Reynolds et al., 2000a, 2000b). If this is indeed the case, then designing strategies to specifically eradicate rapidly dividing NE cells may have acceptable toxic side effects. At the same time, we should keep an eye on the propensity of these neuroendocrine tumors to undergo rather dramatic changes upon the activation of other signaling pathways as is evident from activation of the Ras pathway in these cells. This might be one of the routes of SCLC to become refractory to treatment by Cisplatin.

In conclusion, our strategy to manipulate specific adult lung cell populations in a controlled manner by cell type-restricted somatic gene transfer vectors can help to answer the question of whether distinct lung pathologies have a unique cell of origin and whether this cell of origin is a determining factor in the drug resistance profile of the various tumor subtypes.

EXPERIMENTAL PROCEDURES

A detailed description of the materials and methods utilized in this work can be found in the available online Supplemental Experimental Procedures.

Mouse Strains

All experiments involving animals comply with local and international regulations and ethical guidelines, and have been authorized by our local experimental animal committee at The Netherlands Cancer Institute (DEC-NKI). The mouse small cell lung cancer model based on conditional *Trp53*$^{F2\text{-}10/F2\text{-}10}$ and *Rb1*$^{F19/F19}$ animals was previously described (Meuwissen et al., 2003). These animals were crossed with *Rosa26R-LacZ* reporter animals (Soriano, 1999). The *mT/mG* double fluorescent reporter animal (Muzumdar et al., 2007) was genotyped using the following primers: 5′- CTCTGCTGCCTCCTGGCTTCT-3′, 5′- CGAGGCG GATCACAAGCAATA-3′, and 5′- TCAATGGGCGGGGGTCGTT-3′.

Generation of Recombinant Cell-Specific Adeno-Cre Viruses

An *attB*-flanked PCR linker, containing multiple restriction enzyme cloning sites (linker #7 5′-AAA AAG CAG GCT CAG ATC TAG CGA AGC TTG CGC CAT CCC TGG AATTCGCCCCGCTCGAGCATAACCCAGCTTTCT-3′, linker #9 5′-AAAAAGCAGGCTCGGTACCAGCAGATCTAGCGAAGCTTGCGCCATC CCTGGAATTCGCCC CGCTCGAGCATAACCCAGCTTTCT-3′), was amplified and subsequently recombined into the pDONR™221 vector (Invitrogen). For cell type-specific expression of *Cre* recombinase, a fragment containing the *Cre* open reading frame with an N-terminal synthetic intron and C-terminal polyadenylation signal was isolated from pOG231 (O'Gorman et al., 1997). This fragment was then inserted either behind the 2.1 kb mouse *CC10* promoter (Linnoila et al., 2000) in pDONR™221 or behind the 4.8 kb mouse surfactant protein C promoter (Glasser et al., 2005) in pDONR™221. Cloned pDONR™221 constructs were then recombined into promoter-less pAd-PL DEST vectors (Invitrogen) by Gateway LR recombination, to generate Ad5-CC10-Cre and Ad5-SPC-Cre adenoviral constructs. The 2.0 kb rat calcitonin/CGRP promoter (Johnston et al., 1998) was cloned upstream of the *Cre* open reading frame as described. High titer adenoviruses were amplified and purified for use in vivo by the University of Iowa Gene Transfer Vector Core, supported in part by the NIH and the Roy J. Carver Foundation, for viral vector preparation.

Intratracheal Adeno-Cre Virus Administration

Seven-week-old mice were treated with cyclosporine A (Novartis) orally in the drinking water, 1 week prior to adenovirus administration and 2–3 weeks following adenovirus infection. Mice were intratracheally intubated with 20 μl of purified Adeno-Cre viruses.

Immunohistochemistry and X-Gal Staining

For histological analysis, lungs were inflated with ethanol-acetic acid-formalin (EAF) and fixed for 24 hr in EAF. Fixed tissues were subsequently dehydrated, embedded in paraffin and sections (2 μm) prepared and stained by hematoxylin and eosin (H&E). We carried out immunohistochemistry for: anti-Ncam1 (rabbit polyclonal, Millipore), anti-synaptophysin (rabbit polyclonal, DAKO), anti-pro-SPC (rabbit polyclonal, Chemicon), anti-CCSP (goat polyclonal), Ki67 (rat monoclonal, DAKO), Cytokeratin 8 (rat monoclonal, Developmental Studies Hybridoma Bank). Streptavidin-peroxidase (DAKO) or PowerVision Poly-HRP (Leica Micosystems), were used for visualization and diaminobenzidine as a chromagen (DAKO). To detect β-galactosidase activity, whole lungs were stained in X-gal staining solution as previously described (Sum et al., 2005), and then processed for paraffin embedding. Three sections were prepared, 50 μm apart, stained with nuclear fast red and examined by light microscopy.

Immunofluorescence Microscopy

For immunofluorescence microscopy, lungs were inflated with 4% paraformaldehyde (PFA) and fixed 4 hr in 4% PFA. Lungs were washed in PBS, sucrose-protected and mounted in cryomatrix. Immunocytochemical staining was performed using: anti-CCSP (goat polyclonal), anti-pro-SPC (rabbit polyclonal, Chemicon), anti-synaptophysin (Syn, Guinea-pig polyclonal, Synaptic Systems), and Calcitonin gene-related peptide (CGRP, rabbit polyclonal, Sigma). Detailed images were obtained using a microlens-enhanced dual spinning disk confocal microscope (UltraVIEW, PerkinElmer).

Gene Expression Analysis

Total RNA was isolated from primary mouse lung tumors using TRIzol reagent (Life Technologies) according to the manufacturer's instructions, and then treated with DNA-free™ Kit (Ambion). cDNA was prepared using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche), according to the manufacturer's specifications. For real-time PCR, cDNA was subjected to 40 cycles of amplification using either the Applied Biosystems TaqMan gene expression assays or the Applied Biosystems SYBR Green expression assay, as per the manufacturer's instructions.

Mouse SCLC Cell Lines

Clonal cell lines derived from SCLC tumors excised from compound *Trp53*$^{F/F}$; *Rb1*$^{F/F}$ animals has been previously described (Calbo et al., 2005, 2011). Cells are maintained in modified HITES medium DMEM/F12 (1:1) (GIBCO) supplemented with Glutamax, 4 μg/ml Hydrocortisone (Sigma), 5 ng/ml murine EGF (Invitrogen), Insulin-Transferrin-Selenium mix/solution (GIBCO) and 10% Fetal Bovine Serum (Greiner Bio one). Cells are cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.ccr.2011.04.019.

ACKNOWLEDGMENTS

We thank J. Zevenhoven, E. Riem, and J. van Ooij for their expert technical assistance; R. Kerkhoven for his expert help with performing array CGH analysis, and E. van Montfort for his assistance in interpreting the data; the personnel of the animal facility for their excellent animal husbandary; M. C. Kwon and P. Krimpenfort for critically reading the manuscript. We acknowledge J. Calbo for providing us with the mSCLC clonal cell lines; B. Stripp for providing us with the mouse *SP-C* promoter, and R. I. Linnoila for kindly providing us with the mouse *CC10* promoter and the *rascal* construct. K.D.S was a recipient of a National Health and Medical Research Council of Australia Overseas based Biomedical Training Fellowship (No. 516781). I.B. and D.A. are supported by the Fund for Scientific Research-Flanders (No. G.0081.08). This work was also supported by a grant of the Dutch Cancer Society.

Received: September 28, 2010
Revised: February 23, 2011
Accepted: April 18, 2011
Published: June 13, 2011

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