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The comparative pathology of genetically engineered mouse models for neuroendocrine carcinomas of the lung

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Abstract

Introduction—Because small cell lung carcinomas (SCLC) are seldom resected, human materials for study are limited. Thus, genetically engineered mouse models (GEMMs) for SCLC and other high-grade lung neuroendocrine (NE) carcinomas are crucial for translational research.

Methods—The pathologies of five GEMMs were studied in detail and consensus diagnoses reached by four lung cancer pathology experts. Hematoxylin and Eosin and immunostained slides of over 100 mice were obtained from the originating and other laboratories and digitalized. The GEMMs included the original Rb/p53 double knockout (Berns laboratory) and triple knockouts from the Sage, MacPherson and Jacks laboratories (double knockout model plus loss of *p130* (Sage laboratory) or loss of *Pten* (MacPherson and Jacks laboratories). In addition, a GEMM with constitutive co-expression of SV40 large T antigen (Tag) and Ascl1 under the Scgb1a1 promoter from the Linnoila laboratory was included.

Results—The lung tumors in all of the models had common as well as distinct pathological features. All three conditional knockout models resulted in multiple pulmonary tumors arising mainly from the central compartment (large bronchi) with foci of in situ carcinoma and NE cell hyperplasia. They consisted of inter- and intra-tumor mixtures of SCLC and large cell NE cell carcinoma (LCNEC) in varying proportions. Occasional adeno- or large cell carcinomas were also

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seen. Extensive vascular and lymphatic invasion and metastases to the mediastinum and liver were noted, mainly of SCLC histology. In the Rb/p53/Pten triple knockout model from the MacPherson and Jacks laboratories and in the constitutive SV40/Tag model many peripherally arising NSCLC tumors having varying degrees of NE marker expression were present (NSCLC-NE tumors). The resultant histological phenotypes were influenced by the introduction of specific genetic alterations, by inactivation of one or both alleles of specific genes, by time from Cre activation and by targeting of lung cells or NE cell subpopulations.

Conclusion—The five GEMM models studied are representative for the entire spectrum of human high-grade NE carcinomas and are also useful for the study of multistage pathogenesis and the metastatic properties of these tumors. They represent one of the most advanced forms of currently available GEMM models for the study of human cancer.

Introduction

For a number of clinical, therapeutic, pathological and biological reasons, small cell carcinoma of the lung (SCLC) is regarded as an entity distinct from the more common non-small cell carcinomas (NSCLC).[1, 2] SCLC is neuroendocrine (NE) tumor and it is the most common and aggressive subtype within the spectrum of NE lung tumors. NE tumors of the lung are a distinct subset of tumors, which share morphologic, ultrastructural, immunohistochemical and molecular characteristics although these tumors are classified into different morphologic categories within the WHO classification.[3, 4] Pulmonary NE tumors may be divided into two categories: a) high-grade NE carcinomas consisting of SCLC and large cell NE carcinomas (LCNEC) and b) low grade NE tumors consisting of the carcinoid tumors, typical and atypical.[5] High-grade NE lung carcinomas are characterized by strong association with tobacco usage, high mitotic and proliferative indices, initial response to chemotherapy, widespread metastases, almost universal inactivation of the *TP53* and *RB1* genes, and other characteristic molecular alterations. Whether all NE tumors arise from respiratory tract NE cells, from less differentiated multipotent cells, or cells committed to other lineages is disputed.[6, 7] While all pulmonary NE tumors may originate from the same pulmonary precursor cells, precursor lesions have not been convincingly identified for high-grade NE carcinomas.[8] Pulmonary neuroendocrine cell hyperplasia, has been observed in association with carcinoids, but no clear association is recognized with other lung cancers including SCLC.[9, 10] Multiple potential targets for individualized therapy have been identified in SCLC cells.[11, 12] However, despite several clinical trials, effective targeted therapies for SCLC are not currently available.[13] Because curative intent resections are seldom performed for SCLC, there is a paucity of tumor materials for the performance of translational research. Biological and preclinical studies of SCLC largely depend on the availability of modest sized banks of human cell lines.[14] Thus, the introduction of a genetically engineered mouse model[15] resulting from the somatic inactivation of the *Tp53* and *Rb1* genes represented an important step.[2] These mice developed aggressive NE lung cancers, termed SCLC, which gave rise to extrapulmonary metastases and required bi-allelic inactivation of both genes. A reported preinvasive feature was the presence of hyperplastic and dysplastic foci and nodules, particularly in the larger airways. However, the latent period for tumor formation was relatively long (7-12 months). Later, Schaffer et al. reported that the additional conditional loss of *p130*, a cell cycle inhibitor in the *Rb1* gene family,

shortened the latent time in the *Rb/p53/p130* triple-knockout mouse model.[16] The histopathology of these metastatic mouse tumors was also reported to closely resemble human SCLC. More recently, another triple knockout model (with the additional conditional inactivation of the *Pten* gene in the *Rb/Tp53* floxed model) has been described.[17] Heterogeneous inactivation of the *Pten* gene resulted in SCLC like tumors after a shorter latent period, but also in adenocarcinomas with varying degrees of NE cell differentiation (NSCLC-NE tumors). Homozygous inactivation of *Pten* resulted in NSCLC carcinomas with varying degrees of NE cell differentiation. Another variation of the *Rb/p53/Pten* triple knockout model has been described by McFadden et al.[18] A further complicating factor is that the classification of NE carcinomas is that some otherwise typical appearing human NSCLC tumors, usually adenocarcinomas, express much or all of the neuroendocrine cell program - so called NSCLC with NE features (NSCLC-NE). These tumors remain largely unstudied with differing views on incidence and therapeutic options.[19-24] However, microarray expression profiling identifies a subgroup of human lung adenocarcinomas that express NE cell features, confirming the presence of NSCLC-NE as a subset of NSCLC.[25, 26] Congress passed into law the Recalcitrant Cancer Research Act in 2013, calling on the National Cancer Institute (NCI) “to develop scientific frameworks that will help provide the strategic direction and guidance needed to make true progress against recalcitrant cancers”, defined as those with a five-year relative survival rate below 50 percent. Following a workshop held in Bethesda, MD in 2013, a report on “The Scientific Framework for Small Cell Lung Cancer” was issued (http://www.lungcanceralliance.org/News/SCLC%20Congressional%20Response%206-30-14%20FINAL%20with%20appendices.pdf). One of the priorities identified in the report was the development of better models for SCLC including GEMMs. As described below, five GEMM models for NE lung carcinomas have been described, and more are under development. However, descriptions of the detailed pathology of most of these models are lacking. Recently we (AFG and EB) had the opportunity to review the pathology of the GEMMs propagated at our respective institutions (UT Southwestern Medical Center and Institut Albert Bonniot). We found similarities and differences between the histological appearances of the mouse models and human SCLC, and also between the different mouse models. We undertook detailed analyses of the pathology of the currently described NE mouse models and their preinvasive changes, and invited the senior initiators of the models (AB, JS, DM, IL and TJ) to collaborate with us and submit pathological materials of the GEMMs from their respective laboratories for pathological examination. The primary purpose of the study was to determine the suitability of the GEMMs as models for the study of human SCLC and other neuroendocrine carcinomas.

Materials and Methods

GEMMs

Five GEMMs for NE lung tumors were obtained from seven independent laboratories -the originating laboratory, as well as from multiple sources for some models (Table 1). These models have been described previously, and details are available from the cited references. For the conditional models, tumors were initiated by adenoviral delivery of Cre.[27]

Pathology examination

Tissues from over 120 mice were examined, over 80 from the *Rb/p53* double knockout model, and five to 15 each from the other four models. Mice were sacrificed either when symptomatic or at defined intervals after Cre activation. Lungs and other tissues (liver, mediastinum, regional lymph nodes) were fixed in neutral buffered formalin, paraffin embedded and 5μ H. & E. stained sections prepared. For representative cases immunostains for NE cell markers (Ascl1, ChgA, Cgrp and Syn) were performed on corresponding sections. NKX2-1 staining, a marker for both adenocarcinoma and NE lung cancers, was available for some tumors. Entire slides were digitally scanned at high (40X) resolution using the NanoZoomer 2.0 HT Digital Pathology System (Hamamatsu Photonics, Hamamatsu City, JP) and examined using the manufacturer's software. One pathologist (AFG) examined all of the scanned images in detail and captured multiple representative images. These were distributed to the other three pathologists (EB, WDT and IL) and consensus diagnoses were reached about each model.

Pathologic criteria for diagnosis

For diagnosis, we used standard definitions as stated by the World Health Organization (WHO) classification of tumors of the lung:[3, 4] 1) Small cell carcinoma (SCLC): A NE carcinoma having cells of a small size, with scant cytoplasm, nuclei with finely granular nuclear chromatin, inconspicuous nucleoli, high mitotic rate, frequent necrosis often covering large zones (“geographic necrosis”). Another criterion we used was the presence of the Azzopardi effect in ischemic areas, a feature present in about 30% of human SCLC tumors.[28] This feature, highly characteristic of SCLC, represents deposition of basophilic DNA-containing material on blood vessel walls resulting from release of nucleic acids in large amounts from degenerating cellular neoplastic tissues. 2) Large cell neuroendocrine carcinoma (LCNEC): A tumor with a neuroendocrine morphology (organoid nesting, palisading, rosettes, trabeculae), high mitotic rate, frequent area of necrosis, often geographic, cytologic features of a NSCLC (large cell size, low nuclear to cytoplasmic ratio, vesicular, coarse or fine chromatin, and/or frequent nucleoli. Some tumors have fine nuclear chromatin and lack nucleoli, but qualify as NSCLC because of large cell size and abundant cytoplasm.[10] 3) Non small cell carcinoma with neuroendocrine features (NSCLC-NE). These are defined as otherwise typical NSCLC tumors (often adenocarcinomas or large cell carcinomas) expressing one or more NE cell properties, but lacking the typical morphological features of NE carcinomas (see above). Demonstration of NE cell properties by positive immunostaining for one or more NE markers (other than neuron specific enolase) and/or presence of cytoplasmic NE granules by electron microscopy. These tumors remain largely unstudied with differing views on incidence and therapeutic options.[22-24, 29]

Results

A brief overview of the major pathological changes observed in the various GEMMs is presented in Table 2.

Pathology of *Rb/p53* Double knockout and *Rb/p53/p130* Triple knockout GEMMs

These represent the original Rb/p53 double knockout established in the Berns laboratory in 2003. In an effort to shorten the lengthy latency time, the Sage laboratory created the first of the triple knockouts in 2010. Both of these models gave rise to central tumors arising from non-invasive lesions originating in the large bronchi, although occasional tumors appear to have arisen in the peripheral lung from respiratory bronchioles or alveolar ducts. Most of the preinvasive lesions consisted of cells having similar morphology to the accompanying invasive component (see below), and were regarded as carcinoma in situ lesions. The distinction between SCLC (Fig.1-2) and LCNEC (Fig. 3) was more difficult in the in situ lesions, especially small ones. Neuroepithelial bodies and foci of NE cell hyperplasia protruding into the lumen were occasionally present although most appeared independent of the in situ and invasive components. The *Rb/p53* double knockout was the most abundant source of materials for the present study, with over 80 affected mice available from all five participating laboratories. Irrespective of the laboratory source, the pathology of the resultant mouse tumors was uniform and virtually identical. Mice were sacrificed 10-15 months after Cre activation. Mice sacrificed early had mainly in situ lesions, while mice sacrificed later had multiple tumors occupying up to 60% of the lung volume, with vascular and peribronchial invasion and perivascular and peribronchial intralymphatic metastases (Fig. 2). They were accompanied by extensive mediastinal spread and lymph node and hepatic metastases. In some cases extensive mediastinal involvement was noted with only modest intrapulmonary tumor burdens. The histology of most of the metastases consisted largely or solely of the SCLC cell component (see below). The majority of the tumor cells in the *Rb/p53* double knockout model were very similar to those of human SCLC. The cells formed sheets of small cells having high mitotic rates, scant poorly defined cytoplasm, nuclei with the presence of small (but distinct) nucleoli, areas of geographic necrosis and foci of Azzorpardi effect. Minor differences compared to human SCLC were the lack of small “salt and pepper” like chromatin granules and small but distinct nucleoli in most of the tumor cells. The granules in this GEMM were somewhat larger and more distinct, with some surrounding perinucleolar clearing. A feature occupying about 10% of the tumors was the presence of foci compatible with LCNEC – the cells were larger, more clearly outlined, with larger nuclei and sometimes with prominent nucleoli. Features indicative of NE tumors included organoid nests, palisading, trabeculation and rosette formation. The LCNEC foci occurred both as distinct tumor nodules, as well as being interspersed with the more typical SCLC component. In the mixed foci, transition zones between the two histological types were observed. Of interest, the regions with Azzopardi effect were limited to the SCLC areas. About 10% of the tumors resembled NSCLC, especially adenocarcinoma or large cell carcinoma and they lacked expression of NE cell markers. Metastases of the NSCLC tumors were not observed.

The Triple *Rb/p53/p130* knockout tumors from the Sage and Johnson laboratories had a shorter latent time and the mice were sacrificed electively 5 months post Cre activation or at later times when showing signs of distress. Most mice had centrally arising NE tumors, although occasional NSCLC tumors were noted. Mice sacrificed early had mainly in situ lesions, while mice sacrificed later had multiple tumors occupying up to 60% of the lung volume, with vascular and lymphatic invasion and perivascular and peribronchial

intralymphatic metastases. In contrast to the Rb/p53 double knockout model, the *Rb/p53/p130* triple knockout tumors had a predominantly LCNEC component when the mice were sacrificed early, but the SCLC component became more prominent when sacrificed at later time points. In mixed histology tumors, the Azzorpardi effects were limited to the SCLC component. In some of these mixed tumors there was not a clear distinction between the two NE cell components, but a gradual transition from one to the other. However, lymphatic, vascular and hepatic metastases had a predominantly SCLC histology, suggesting that this component had a longer latent time but also had a greater metastatic potential. As with the Rb/p53 double knockout model, occasional foci of NE cell hyperplasia or neuroepithelial bodies (NEBs) were noted in bronchi, although there was no obvious relationship to the in situ or invasive tumors.

Pathology of *Rb/p53/Pten* Triple knockout^{Pten} GEMMs

The pathology of the tumors, induced by the addition of *Pten* knockout (either *Pten*$^{lox/lox}$ or *Pten*$^{lox/+}$)[17] to the original *Rb/p53* double knockout model developed in the Berns lab, was more complex than any of the other models included in this study. In the MacPherson lab, all lung cells were targeted using intratracheal infection with Ad-CMV-Cre and two genotypes were studied – with either one or both *Pten* alleles inactivated by Cre. Mice were sacrificed when symptomatic. Tumors developed much faster after adenoviral Cre delivery with rapid mortality, especially for the Rb/p53/*Pten*$^{lox/lox}$ mice usually 4-5 months post Cre and gross metastases were not noted. In the Rb/p53/*Pten*$^{lox/+}$ model, dominant tumors emerged mostly from mice that got sick 7-9 months post Cre, and about two thirds had liver metastases. Both of the Rb/p53/*Pten* subtype mice had multiple tumors apparently arising both from the central and peripheral airways. The tumors consisted of two major subtypes – those resembling SCLC and those with NSCLC features, particularly adenocarcinoma. However the SCLC-like component was more prominent in the *Pten*$^{lox/+}$ mice. The cytological resemblance to human SCLC was not as striking as the double Rb/p53 knockout (Berns) model with many cells having distinct small nucleoli and defined outer cell borders. We refer to these cells as SCLC-like. The NSCLC component consisted of adenocarcinomas with acinar and palisading features and occasional mucin-like secretory material, both intra- and extra-cellular. Multiple large linear, multilayered regions of in situ carcinoma were noted. These consisted largely of the SCLC cells, although occasionally of the NSCLC component or admixtures of the two. By contrast, the in situ lesions in the *Rb/p53* double and *Rb/p53/p130* triple knockout models consisted almost entirely of the NE cell component, and were smaller and more globular in shape. Foci of hyperplastic basal cells or NEBs were rarely identified. Metastases to the mediastinal nodes and liver were frequent in the *Rb/p53/Pten*$^{lox/+}$ model, and the SCLC-like cell component dominated in the metastases. Immunostaining of both morphologic phenotypes showed considerable heterogeneity, with some foci of both SCLC-like and NSCLC staining uniformly, while others were negative or were variable in intensity and distribution. This heterogeneity extended to in situ and metastatic lesions.

A similar Rb/p53/*Pten* triple knockout model was developed in the Jacks laboratory, but using adenoviral Cre vectors driven by the CGRP promoter and targeting NE lung cells specifically. Both *Pten* alleles were inactivated in this model. These mice developed tumors

rapidly with frequent liver metastases but the tumor histologies showed somewhat different features than the model from the MacPherson laboratory. Mild-modest NE cell hyperplasia and NEBs were present in the large bronchi. While most of the in situ lesions were LCNEC, and occasionally SCLC, three types of invasive cancers were noted: About 60% were LCNEC, 20% SCLC and 20% NSCLC. Heterogeneous expression of CGRP expression was present in all three forms of invasive cancers, as well as in the in situ lesions. However the metastases to peribronchial and perivascular lymphatics and to the liver were almost all SCLC.

Pathology of CC10-SV40Tag-Ascl1 model GEMM

In this model there is constitutive co-expression of SV40 large T antigen (Tag) and *Ascl1* under the *Scgb1a1* (also known as *CC10*) promoter.[30-32] At a relatively young age (2-4 months) mice develop extensive acinar adenocarcinomas, mainly peripheral, but with some arising in larger bronchi (Fig. 5). In addition to the adenocarcinomas, foci of NE cell hyperplasia that appeared linear along the epithelium were present in the large bronchi. While the foci of NE cell hyperplasia expressed the NE cell markers (Ascl1, Cgrp and Syn) strongly and uniformly, expression in the adenocarcinomas was focal, weaker and heterogeneous. Nkx2.1 was also expressed in both the NE cell hyperplasias and in the adenocarcinomas.

Discussion

As appropriate GEMMs are a key component for the understanding of SCLC and other high-grade NE lung carcinomas, we undertook a detailed pathological review of the multiple mouse models currently available to us. We obtained these models both from the originating laboratories, as well as from other laboratories that had replicated the models. We are aware that several other GEMMs for NE lung cancers are currently under development or study. However, as these have not been described in the literature, we chose not to include them in this study even though, in some instances, the originators were willing to share them with us.

Early GEMM models were created by ectopic transgene expression under the control of lung-specific promoters.[33, 34] More advanced GEMMs allow for inducible, tissue-specific expression of oncogenes as well as conditional, tissue-specific deletion of tumor suppressors. We included in our study one early model, described more than a decade ago from the Linnoila laboratory, as it represented a model for the poorly understood and studied NSCLC-NE. In this model, lung tumors are generated by constitutive expression of Ascl1 in combination with SV40 Tag under the secretoglobin1a1 gene promoter. The other four models were more advanced models that utilized or modified the original double knockout concept from the Berns laboratory. As Berns postulated, because biallelic inactivation of *TP53* and *RB1* genes are near universal in human SCLC, knocking out these two genes in mouse lung epithelial cells would result in SCLC like tumors. However, in this GEMM, the latent period for tumor development was long (about 12 months). Human SCLC tumors almost always occur in patients having lengthy and extensive smoke exposure histories, and are accompanied by numerous molecular changes.[11, 12, 35] By contrast, GEMMs for NE

lung cancers are not exposed to tobacco carcinogens, and require spontaneous development of further genetic changes for tumor development including frequent amplification of the *Nfib* and *L-Myc* genes.[36, 37] The secondary changes in the GEMM model for SCLC from the Jacks lab included alterations in DNA copy number and complex genomic rearrangements but a relatively low somatic point mutation frequency in the absence of tobacco mutagens.[17,18] Alterations targeting the tumor suppressor *Pten* occurred in the majority of murine SCLC studied. The relatively lengthy time required for these secondary changes to occur results in long latent periods for tumor development. The Sage, MacPherson and Jacks laboratories, in efforts to shorten the latent time, utilized triple knockout GEMMs, modifying the original *Rb/p53* double knockout model with the additional inactivation of a third tumor suppressor gene. Further refinements include Cre activation in all exposed lung cells or promoter activation in specific lineage subpopulations such as pulmonary NE cells. A further confounding factor is the inactivation of one or both alleles of more of the utilized genes. As described herein, these additional alterations affected the pathological features of the resulting tumors.

The five models studied shared some pathological features, although there were also individual features characterizing each GEMM. Most tumors arising in the *Rb/p53* Berns laboratory double knockout model closely resembled human SCLC, although some minor cytological differences were noted. A minor subpopulation of LCNEC was present in most mice, either as individual foci or admixed with the SCLC foci, with transition areas. About 10% of the tumors appeared to be NSCLC, especially adenocarcinoma, and lacked NE cell differentiation. The original report from the Berns lab indicated that biallelic inactivation of the *Rb1* gene was essential for SCLC tumors in the double knockout model, and that NSCLC may arise in the absence of biallelic inactivation.[15, 38] These NSCLC tumors lacked NE cell differentiation. Another interesting feature was the presence of multiple, often large, nodular and protruding foci of in situ NE cell carcinoma. Occasional foci of basally located NE cell hyperplasia or increased numbers of NEBs were noted, usually distinct from the carcinoma in situ foci. NEBs represent basally located focal collections of NE cells in the respiratory epithelium.[39] As premalignant or preinvasive lesions are very seldom recognized in human SCLC tumors, the GEMMs provide unique models to study the multistage pathogenesis of these tumors. We, and others, have suggested that lung carcinomas may arise from the central or peripheral compartments of the lung, with most squamous cell and SCLC carcinomas arising from the former, and most adenocarcinomas arising from the latter.[40] The in situ findings from the GEMMs confirm the central origin of most SCLC and LCNEC tumors. This is consistent with the findings of Sutherland et al that most SCLC tumors arise from centrally located NE cells while occasional tumors may also arise from peripherally located SPC positive cells.[41]

The *Rb/p53/p130* triple knockout model from the Sage laboratory had LCNEC as the most prominent of the in situ and early invasive lesions, with a SCLC component becoming more prominent when mice were sacrificed at a later time point. However SCLC formed the majority of the metastatic lesions. There appeared to be plasticity between the two components, with individual tumors expressing both phenotypes with transitional zones where the demarcation was not clear. Thus addition of *p130* knockout to the original Berns

double knockout model resulted in shorter latent periods, but was accompanied by alterations of the major tumor cell phenotype that altered with time to sacrifice. At all time points, SCLC was the predominant component of metastases to lymph node, mediastinum or liver. These observations suggest the close relationship and inter-relationship of SCLC and LCNEC. While the SCLC component was slower to develop, perhaps because more secondary genetic changes were needed for its development, it was the predominant phenotype present in metastases of all the GEMM models studied.

The *Rb/p53/Pten* triple from the MacPherson laboratory (with mono- or biallelic inactivation of *Pten* added to the original double knockout model)[17] had the most complex and varied pathology of the models studied. The resultant tumors had two major phenotypes: Centrally arising SCLC tumors and multiple peripherally arising NSCLC, usually adenocarcinomas, with intra- and inter-tumor heterogeneity of NE marker expression. While the pathological description in the original report of this model suggested major differences between the mono- and biallelic *Pten* inactivated tumors, we interpret them as being part of a spectrum, with the SCLC-like component being dominant in the monoallelic (heterozygous) tumors and the NSCLC-NE tumors dominant in the biallelic model. One possible explanation is that the short latent period for the development of the extensive NSCLC component in the biallelic model resulted in death of the mice before the SCLC-like component had time to fully develop.

The triple *Rb/p53/Pten* triple knockout model from the MacPherson laboratory targeted all available lung cells using Ad-CMV-Cre while the similar model from the Jacks lab targeted NE cells using Ad-CGRP-Cre. Perhaps as a result, the tumors from the Jacks laboratory demonstrated a mixture of LCNEC, SCLC and NSCLC, while the equivalent model from the MacPherson laboratory had NSCLC with varying expression of NE features as a prominent component.

While the Rb/p53/*Pten* triple knockout model resulted in NSCLC-NE tumors, the constitutive SV40/Ascl1 model from the Linnoila laboratory also induced NSCLC-NE tumors, but without the prominent SCLC-like component seen in the *Rb/p53/Pten*$^{lox/+}$ model. In both SV40/Ascl1 and *Rb/p53/Pten*$^{lox/lox}$ models the NSCLC-NE tumors demonstrated considerable intra and inter-tumor heterogeneity of NE cell markers. NE marker expression was less intense than in SCLC or LCNEC components of the *Rb/p53* double knockout or *Rb/p53/p130* triple knockout models. It is of interest to point out the contrasting features of the GEMMs for the NE carcinoma models as summarized in this report, and those of the many GEMM models for NSCLC (Table 3). Most NSCLC GEMMs arise peripherally and are characterized by intense hyperplastic lesions and adenoma formation, with foci of true invasive carcinoma and metastases occurring occasionally and relatively late in the disease process.[42, 43] By contrast, the GEMMs for NE carcinomas have relatively long latent periods, with the exception of the SV40 driven constitutive model from Linnoila laboratory, arise from the central compartment, hyperplastic foci are rare, adenomas are not seen, and invasive carcinomas and metastatic lesions are frequent.[42-44]

Metastatic lesions were present in all the models for which metastatic lesions were available for examination. These were most frequent and extensive in the Berns laboratory model,

where mice were sacrificed late, often when symptomatic. The extent and pattern of metastatic spread, sometimes in the presence of modest intrapulmonary tumor load, were highly reminiscent of human SCLC – perivascular and peribronchial spread, large mediastinal node involvement, frequent and multiple liver metastases. As previously mentioned, most metastases in all the models had SCLC cells as the principal or sole component, whether or not this was the dominant tumor cell component in the intrapulmonary tumors.

GEMMs for NE carcinomas of the lung present a unique set of models for the study of an important human disease for which human tissues are seldom available. The pathological features of the four GEMMs that form the basis of this report all share some features, but also have individual characteristics. They represent the entire spectrum of high-grade NE carcinomas of the lung including LCNEC. While the original double knockout model from the Berns laboratory showed the greatest resemblance to human SCLC, many tumors also demonstrated features of LCNEC. These two NE cell components often showed mixed patterns in individual tumors, with transitional features from one to the other. These findings indicate that the distinction between the two main forms of high-grade NE lung carcinomas is not absolute but relative, and that transitions between them may occur. NSCLC tumors, with or without expression of NE cell markers were a prominent feature of the *Rb/p53/Pten* triple knockout from the MacPherson laboratory and the constitutive SV40/Ascl1 model from the Linnoila laboratory.

These models offer a spectrum of pathological phenotypes ranging from SCLC, LCNEC, NSCLC and the poorly understood NSCLC-NE tumors. In most cases, the widely metastatic pattern of the conditional models closely resembles the pattern of spread of human SCLC. It appears that multiple factors can influence the resultant tumor phenotypes including introduced genetic changes, targeting of niche subpopulations such as NE cells, mono- or biallelic inactivation of genes, and time period to sacrifice after Cre activation.

Bock et al have recently described the development and advancement of mouse models for human cancer.[45] They hierarchically cluster mouse models of cancer into five stages of development and sophistication. The fifth stage, largely futuristic, includes earlier stage models mimicking metastatic progression, with metastasis becoming rate limiting for tumor growth. In our opinion, the conditional NE carcinoma models fulfill these criteria, and thus represent one of the most advanced of the currently available mouse models for cancer.

Conclusions

In conclusion, GEMMs offer appropriate and potentially useful models for the study of the multistage development, invasion, metastases and therapy of the entire spectrum of human high-grade NE lung carcinomas. A detailed understanding of the pathology and biology of the individual GEMM models for NE carcinomas is essential for the selection of the most appropriate model for future studies.

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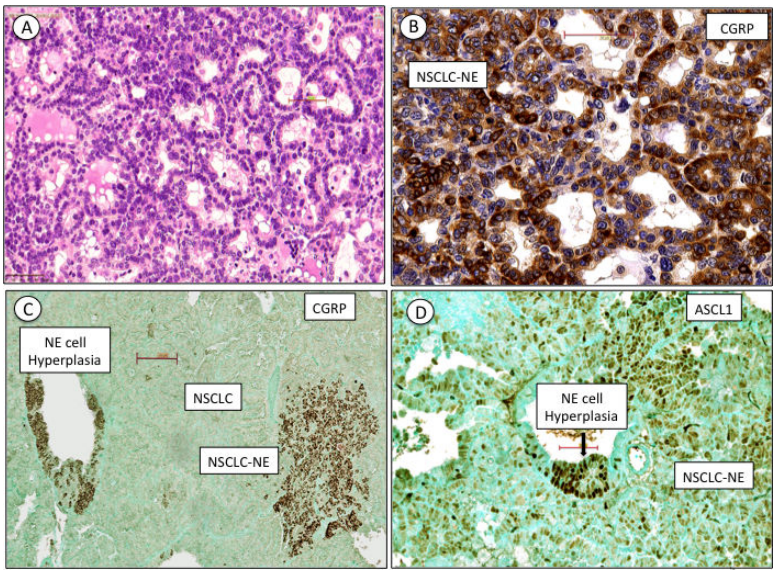

Fig. 1.

SCLC tumors. A and C: Berns laboratory, (p53;Rb1 double CKO); B and D: Sage laboratory, (p53;Rb1;p130 triple CKO). A, whole lung section demonstrating multiple in situ lesions arising in large airways and a few small invasive carcinomas. Bar indicates 10 microns. B, SCLC with area of necrosis and Azzopardi effect. C, high power view of SCLC morphology. D, combined SCLC carcinoma, with focal areas of poorly differentiated NSCLC.

The pathologies of various GEMMs are illustrated. Except where indicated. Horizontal bars = distance in microns as indicated.

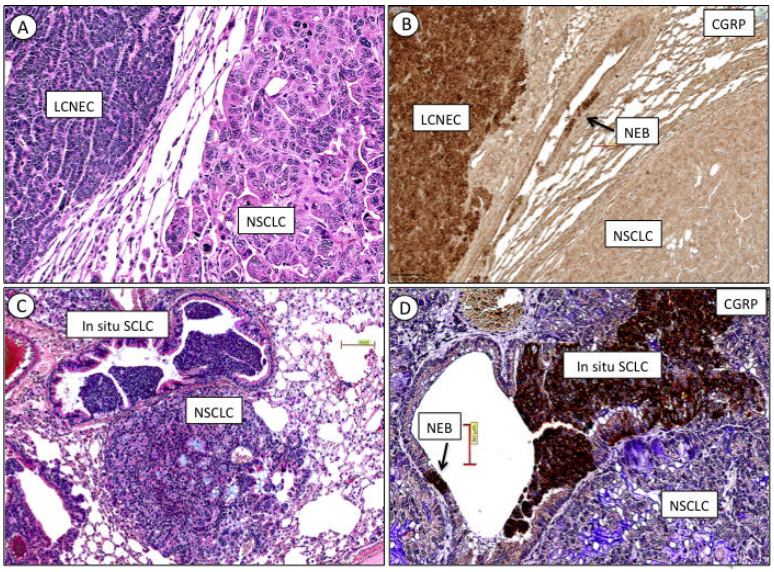

Fig 2.

Metastases from SCLC. In all models examined, the metastatic tumors usually had SCLC morphology irrespective of the GEMM or the dominant pulmonary tumor phenotype. A and C: Berns laboratory, B: Sage laboratory, D: Jacks laboratory (p53; Rb1; Pten triple CKO). A. Extensive mediastinal spread, modest intrapulmonary tumor. B. Intrapulmonary perivascular lymphatic spread with focal invasion of blood vessel (BV) wall. C. Metastasis to mediastinal node. D. Metastases to liver.

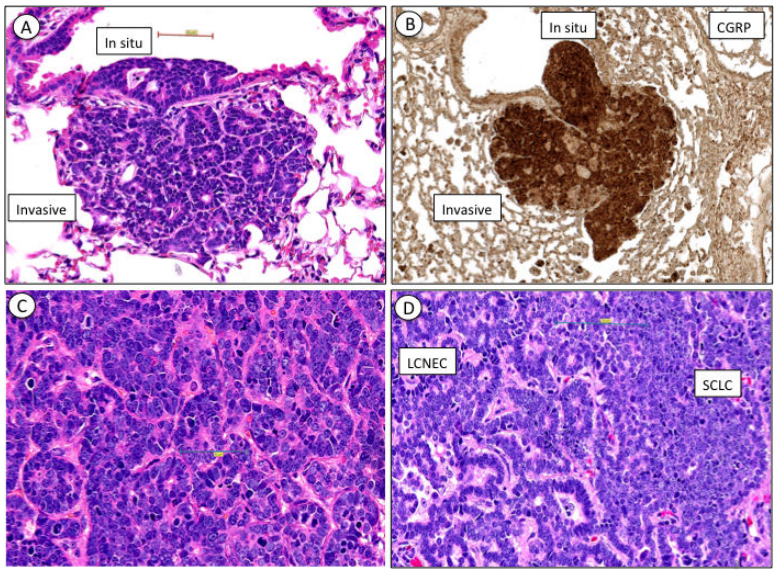

Fig 3.

LCNECs. A and B: Johnson laboratory; C and D: Sage laboratory (both laboratories used the same p53;Rb1;p130 triple CKO). A. Intrabronchial in situ lesion with underlying invasive component. B. A lesion similar to the one illustrated in A, CGRP immunostain. Strong cytoplasmic expression of the NE cell product CGRP in both the in situ and invasive tumor components. C. High power view of LCNEC morphology. D. Both LCNEC (left field) to SCLC (right field) morphologies are present within a single lesion.

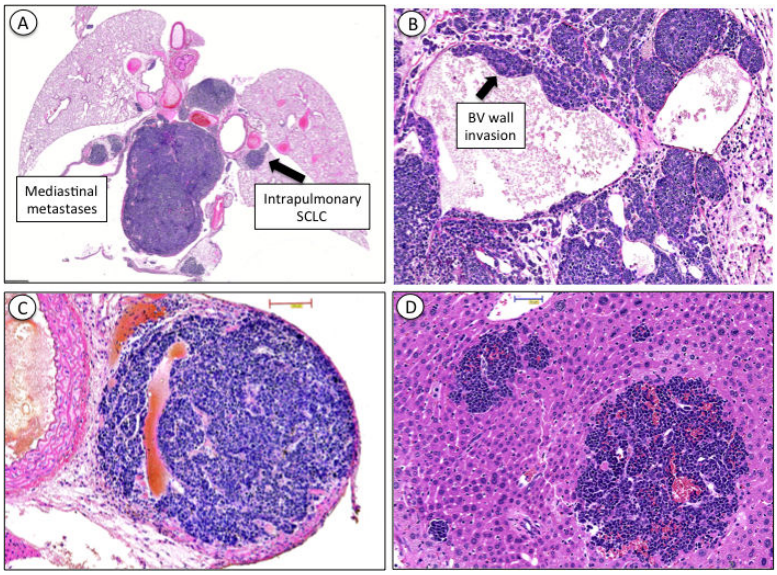

Fig. 4.

NSCLC tumors. A and B: Johnson laboratory; C and D MacPherson laboratory (p53;Rb1;PTEN triple CKO). A and B, Adjacent LCNEC (left field) and NSCLC (right field) tumors in the *p130* triple knockout model. CGRP immunostaing (B) is limited to the LCNEC tumor and an adjacent neuroepithelial body in (NEB) within a bronchus separating the two tumors. C and D. In situ SCLC and adjacent invasive NSCLC-NE arising in a *Pten*$^{lox/+}$ triple knockout mouse. CGRP immunostaining (D) demonstrates that the NE cell marker expression is limited to the in situ SCLC component and to a neuroepithelial body (NEB) in the same bronchus.

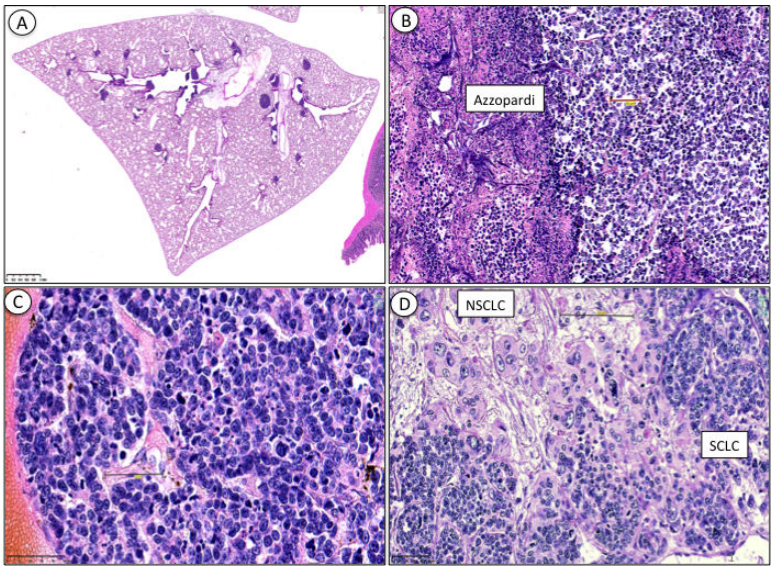

Fig. 5.

Non small cell lung carcinomas with expression of NE cell markers (NSCLC-NE). A and B: MacPherson Laboratory; C and D Linnoila laboratory (SV40/ASCL1 driven by CC10 promoter). A, Adenocarcinoma. B, same tumor as A immunostained for CGRP expression. C and D Poorly differentiated NSCLC immunostained for Cgrp (C) or Ascl1 (D). There is focal and variable NE marker expression in the tumor. Immunostaining of the foci of NE cell hyperplasia in adjacent bronchi show more intense and uniform expression of Ascl1.

Table 1

GEMMs used in the study

Identification	Laboratory Source	Brief description of induced genetic alteration	Target cells	Tissues examined	Reference
Rb/p53 double knockout	*Berns, Sage, Brambilla, Linnoila, Jacks	Conditional inactivation of *Rb1* and *Tp53* in lung cells under CGRP or CMV promoter	Lung (NE cells)	Lung, liver, mediastinum	15
Rb/p53/p130 Triple knockout	*Sage, Johnson	Conditional inactivation of *Rb1* and *Tp53* and *p130* in lung cells under CMV promoter	Lung (Non specific)	Lung, liver mediastinum	16
Rb/p53/Pten triple knockout$^{PtenLox/+}$	*MacPherson	Conditional inactivation of *Rb1*, *Tp53* and *Pten*$^{lox/+}$ in lung cells under CMV promoter	Lung (Non specific)	Lung, liver, mediastinum	17
Rb/p53/Pten triple knockout$^{PtenLox/lox}$	*MacPherson	Conditional inactivation of *Rb1*, *Tp53* and *Pten*$^{lox/lox}$ in lung cells under CMV promoter	Lung (Non specific)	Lung, liver, mediastinum	17
Rb/p53/Pten triple knockout$^{PtenLox/lox}$	*Jacks	Conditional inactivation of *Rb1*, *Tp53* and *Pten*$^{lox/lox}$ in lung cells under CGRP promoter	Lung (NE cells)	Lung, liver	18
CC10-SV40Tag-ASCL1	*Linnoila	Constitutive expression of human ASCL1 in combination with SV40 Tag under Scgb1a1 (CC10) promoter in lung cells	Lung (Peripheral epithelium)	Lung	32

*Laboratory responsible for developing the original model

Table 2

Major pathological findings in the GEMMs studied

Identification	Laboratory Source	Time to sacrifice or symptom development	Major tumor type	Minor tumor type	Comments
Rb/p53 double knockout	*Berns, Sage, Brambilla, Linnoila, Jacks	Elective and symptomatic, 4-15 months	SCLC	LCNEC, NSCLC	Mostly late, centrally arising tumors; widespread metastases, especially after one year
Rb/p53/p130 Triple knockout	*Sage, Johnson	Electively, 3-5 months or when symptomatic, 6-8 months	LCNEC early, SCLC later	NSCLC	SCLC component predominates in metastases
Rb/p53/Pten triple knockout$^{PtenLox/+}$	*MacPherson	Symptomatic, 6-10 months	SCLC	NSCLC-NE	Widespread liver metastases. Heterogeneous expression of NE cell markers in both phenotypes
Rb/p53/Pten triple knockout$^{PtenLox/lox}$	*MacPherson	Symptomatic, 3-5 months	NSCLC-NE	NSCLC-NE	Liver metastases absent. Heterogeneous expression of NE cell markers in all phenotypes
Rb/p53/Pten triple knockout$^{PtenLox/lox}$	*Jacks	Symptomatic, 5-8 months	LCNEC	SCLC, NSCLC-NE	Heterogeneous expression of NE cell markers in all phenotypes. SCLC component predominates in metastases
CC10-SV40Tag-ASCL1	*Linnoila	Electively, 2-9 months	NSCLC-NE	NSCLC	Peripherally arising tumors, linear NE cell hyperplasia in large airways

Nb. Mice were infected with Adeno-Cre 2-10 weeks after birth. They were sacrificed electively or when symptomatic at the times as indicated. Except for the CC10-SV40Tag-ASCL1 model, preinvasive lesions and invasive tumors were predominantly central in origin from large airways, although occasional peripherally arising tumors were also noted. In all models in which lymph node, mediastinal or liver metastases were observed, the predominant histology of the metastases was SCLC, irrespective of the predominant histology of the primary tumors arising in the lung.

Table 3

Short summary of the main pathological features of the main GEMM models for NE and NSCLC lung carcinomas.[&]

Feature	GEMM models for NE lung carcinomas	GEMM models for NSCLC
Major driver mutation(s)	Inactivation of *TTp53* and *Rb1* (+/− others)	Activation of *KRAS*, *EGFR*, or other genes
Invasive tumors	Frequent	Occasional[*]
Latent time to tumor induction	Relatively long (months)	Relatively long (months)[*]
Site of origin	Predominantly from central compartment	Predominantly from peripheral compartment
Predominant tumor type(s)	SCLC, LCNEC, occasional NSCLC	NSCLC, usually adenocarcinoma
Hyperplasia	Nodular and linear NE cell foci in bronchi	Extensive hyperplasia of peripheral airways
Adenoma formation	Rare/absent	Frequent
Carcinoma in situ	Frequent	Occasional
Metastatic lesions	Frequent, usually of SCLC component	Rare

Nb: As multiple GEMMs exist for both NE and NSCLC carcinomas, the above table reflects composite features that may not apply fully to all individual models.[33] The NSCLC GEMMs include mainly models for adenocarcinomas, as the recently developed GEMMs for squamous cell carcinomas have not been fully characterized to date.[33, 46]

[*]While extensive preneoplastic lesions appear relatively early in most GEMMs for NSCLC, true invasive cancers appear late during multistage pathogenesis and they rarely metastasize