Alveolar Type II Cells Possess the Capability of Initiating Lung Tumor Development

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Abstract

Identifying cells of tumor origin is a fundamental question in tumor biology. Answers to this central question will not only advance our understanding of tumor initiation and progression but also have important therapeutic implications. In this study, we aimed to uncover the cells of origin of lung adenocarcinoma, a major subtype of non-small cell lung cancer. To this end, we developed new mouse models of lung adenocarcinoma that enabled selective manipulation of gene activity in surfactant associated protein C (SPC)-expressing cells, including alveolar type II cells and bronchioalveolar stem cells (BASCs) that reside at the bronchioalveolar duct junction (BADJ). Our findings showed that activation of oncogenic Kras alone or in combination with the removal of the tumor suppressor p53 in SPC+ cells resulted in development of alveolar tumors. Similarly, sustained EGF signaling in SPC+ cells led to alveolar tumors. By contrast, BASCs failed to proliferate or produce tumors under these conditions. Importantly, in a mouse strain in which Kras/p53 activity was selectively altered in type II cells but not BASCs, alveolar tumors developed while BADJs retained normal architecture. These results confirm and extend previous findings and support a model in which lung adenocarcinoma can initiate in alveolar type II cells. Our results establish the foundation for elucidating the molecular mechanisms by which lung cancer initiates and progresses in a specific lung cell type.

Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% of lung cancers and most patients present with advanced disease [1]. Despite the investment of an enormous amount of effort and resources in the past few decades, the treatment options remain very limited for advanced or recurrent NSCLC [2,3]. For inoperable tumors, the standard treatment consists of various combinations of chemotherapy, radiotherapy and targeted therapies [4]. Improvements in prognosis have been modest, with an overall 5-year survival rate of only 10–15%. This is in sharp contrast to the drastically improved 5-year survival rates in patients afflicted by many other types of solid tumors such as breast or prostate cancer. These achievements are due to early diagnosis and effective treatment. This highlights an urgent need for basic research to identify the cells from which lung cancer originates and uncover the molecular basis of tumor development in order to design new therapies [5–8].

Based on mutation profiles identified in human NSCLC [9], several mouse models of NSCLC have been created [10–15]. A widely used model combines p53 ablation with Kras activation in the mouse lung epithelium [16–18]. These mice developed lung adenocarcinoma (the major subtype of NSCLC) [19] that recapitulates critical aspects of human NSCLC. However, this approach relies on inactivation of p53 and activation of Kras in the lung epithelium globally [20]. It does not allow for identifying the cells of origin of NSCLC or isolating a pure population of tumor cells at distinct stages of tumor development for genome-wide analysis. Addressing these issues requires additional mouse models in which oncogenes and tumor suppressors can be selectively manipulated in a given cell population to test their role in tumor development.

Another important mouse model of NSCLC is based on the finding that a fraction of patients whose tumors harbor mutations in the gene encoding the epidermal growth factor receptor (EGFR) [21,22] are responsive to tyrosine kinase inhibitors (TKIs) that block EGF signaling. Mice carrying various EGFR mutations found in human lung cancer develop lung tumors [23,24], providing an appropriate animal model for studying NSCLC development and drug resistance. Likewise, the cells of origin of lung tumors in these systems have not been rigorously addressed due to the lack of tools that enable selective manipulation of gene activity in a particular lung cell type.

Investigating the cells of origin of lung cancers will provide new insight into the molecular basis of tumor initiation and progression. It is possible that human lung cancers that originate from different cell types possess characteristic mutation profiles and behaviors. Distinct cell types in the airway and alveoli [25] that fulfill requisite physiological functions of the lung are also candidates as the initiating cells of adenocarcinoma [26]. Secretory non-ciliated Clara cells in the bronchioles are interspersed with ciliated cells and neuroendocrine cells, whereas type I and type II cells line the gas-exchange epithelium of the alveoli. Bronchioalveolar stem cells (BASCs) [27] express markers for Clara cells (CC10) and type II cells (SPC) and are located at the bronchioalveolar duct junction (BADJ) where terminal bronchioles...
connect with the alveoli. The physiological function of BASCs remains unclear [28]. Clara cells, BASCs and type II cells possess proliferative potential in various conditions and could potentially accumulate mutations in these processes to initiate tumor development. Nevertheless, it remains uncertain whether tumor development can also begin in non-proliferative, differentiated cell types or transformation is largely confined to cells with progenitor potential. In this study, we utilize mouse lines that allow the manipulation of gene activities in alveolar type II cells and BASCs to test these ideas. Our results support a model in which lung adenocarcinoma can initiate in type II cells. These studies provide the basis for further investigation to uncover the molecular mechanisms of tumor development and progression.

Results

Inducible Expression of CreER Recombinase or Activation of rtTA Under the Control of the SPC Promoter Allows the Manipulation of Gene Activity in SPC⁺ cells Including Alveolar Type II and BASC cells

Proliferation of alveolar type II cells significantly increases following lung injury. Type II cells also have the potential to generate type I cells, which are responsible for gas exchange in the alveoli [25]. These progenitor-like properties render type II cells a prime candidate for initiating lung adenocarcinoma. To regulate gene activity in type II cells, we generated two mouse lines by (1) introducing CreER into the endogenous mouse SPC (Sftpc, sufficient associated protein C) locus via gene targeting [29] and (2) introducing rtTA into an SPC-containing bacterial artificial chromosome (BAC) for producing transgenic mice. This is based on the finding that SPC constitutes the major peptide in type II cells and is not present in other lung cell types [30] except the SPC⁺CC10⁺ BASCs at the BADJ. The resulting mouse lines are designated SPCCreER/Fig. 1A and SPCrtTA/Fig. 1B) respectively in this study and they enable control of gene activity in SPC-expressing type II cells and BASCs. CreER encodes a fusion protein of Cre recombinase and estrogen receptor (ER) and is activated by tamoxifen (TM) [31]. Moreover, tamoxifen injection into adult SPCCreER mice by pronuclear injection [35] (Fig. 1B), eto-O-Cre was introduced into a separate genomic locus to avoid any potential interference with rtTA. When dox food was used continuously for SPCCreER/+; R26R/+ or SPCCreER⁺; RO-S426TmG⁺/+ mice, extensive labeling of type II cells was observed (Fig. 1H, 1I) in contrast to the spotty labeling in SPCrtTA/+; R26R/+ or SPCrtTA⁺; ROSA26TmG⁺/+ mice without dox (Fig. 1G, 1K). This suggests that rtTA expression from SPCCreER/+ mice should enable manipulating gene activity in SPC⁺ cells, including all genes that are placed under TRE (tetO) control.

Activating Kras Alone or in Combination with p53 Removal in Murine SPC⁺ cells Led to Non-small Cell Lung Cancer (NSCLC) Development in Alveoli

Loss of p53 and activated Kras mutations are frequently found in human NSCLC [9]. This prompted several groups to develop a mouse model of NSCLC through global inactivation of p53 and activation of Kras (G12D mutation) in the lung epithelium, which was achieved via intratracheal injection of adenoviral Cre [16–18]. This mouse model of NSCLC recapitulates critical aspects of human NSCLC, validating the use of mouse models for understanding human NSCLC [12]. However, this approach carries several important caveats, including the side effects of anti-inflammatory agents used to suppress adenovirus-induced inflammation, the inconsistent delivery and expression of adenoviral Cre that could result in variable tumor development and perhaps most importantly, the inability to pinpoint the cell types that initiate transformation.

To address these issues, we employed the SPCCreER mouse line to activate Kras and inactivate p53 in SPC⁺ cells. We took advantage of the widely used KrasLSL-G12D and p53f alleles. KrasLSL-G12D is converted into activated KrasG12D by Cre, while the conditional p53f allele is converted into a null allele in the presence of Cre. This offers a precise genetic system to manipulate p53 and Kras exclusively in SPC⁺ cells. We bred mice to produce SPCCreER⁺; KrasLSL-G12D⁺/+ and SPCCreER⁺; p53f/+; KrasLSL-G12D⁺/+ mice and administered tamoxifen to these adult animals. Lungs were collected at different time points after TM injection for analysis.

By 18 weeks after TM injection, SPCCreER⁺; KrasLSL-G12D⁺/+ mice developed numerous tumors in the alveoli, largely adenomas and a small number of cells with features of malignant transformation (Fig. 2B, 2D), while the airways and BADJs retained normal architecture (Fig. 2A, 2C, 2E, 2G). These tumors stained positive for SPC (negative for CC10 and Sox2) (Fig. 2F, 2H, 2I) and were morphologically similar to those produced in a lung cancer model in which adenoviral Cre was used to induce KrasG12D expression [16–18]. Tumor cells exhibited high proliferation (Fig. 2J). This phenotype is completely penetrant, follows a defined time sequence and does not appear to be affected by genetic background. We noted that a significant fraction of tumors was found in close proximity to the airway or BADJ or the surface of the lung. Interestingly, the BADJ itself did not contain hyperplastic lesions or tumors.

Compared to SPCCreER⁺; KrasLSL-G12D⁺/+ mice at the same stage, SPCCreER⁺; p53f/+; KrasLSL-G12D⁺/+ mice exhibited a more severe...
phenotype in terms of tumor size and tumor growth and grade as a result of p53 elimination in addition to Kras activation. At 3–4 weeks after TM injection, several small adenomas of papillary nature could already be found in the alveoli of SPCCreER/\textsuperscript{+}; p53f/f; KrasLSL-G12D/\textsuperscript{+} mice (Fig. 2L). These lesions expressed SPC but not CC10 (Fig. 2P) or Sox2 (not shown). At 10 weeks after TM injection, large tumors were widespread in the alveoli and they exhibited features characteristic of higher grade lung tumors such as the presence of enlarged pleomorphic nuclei, aberrant mitosis and tumor giant cells (Fig. 2N). In fact, many SPCCreER/\textsuperscript{+}; p53f/f; KrasLSL-G12D/\textsuperscript{+} mice did not survive beyond this stage due to the heavy burden of lung tumors. In mice that did survive to 13 weeks after TM injection, their lungs contained adenocarcinoma in the alveoli consisting of cells with features of malignant transformation surrounded by stromal cells. Similarly, all tumors examined...
Figure 2. Induction of alveolar lung tumors in mice with activated \textit{Kras} alone or in combination with loss of \textit{p53} in SPC-expressing cells. (A–D) Histology of lung sections from \textit{SPC\textsuperscript{CreER\textsuperscript{loxP}}, Kras\textsuperscript{LSL-G12D\textsuperscript{loxP}}} adult mice injected with tamoxifen (TM) to activate \textit{Kras}. These mice developed alveolar tumors while no abnormalities were detected in the airway or bronchioalveolar duct junction (BADJ). Increase in average tumor size with time was observed. (E–J) Immunostaining of lung sections from \textit{SPC\textsuperscript{CreER\textsuperscript{loxP}}, Kras\textsuperscript{LSL-G12D\textsuperscript{loxP}}} adult mice injected with TM to activate \textit{Kras}. Alveolar tumors
shown in (F, H, I) expressed SPC but not CC10 or Sox2. Airways and BADJs displayed normal architecture. Tumor cells were highly proliferative as assessed by Ki67 staining in (J), the entire field of which consists of tumor cells. (K–N) Histology of lung sections from SPCCreER+/p53f/f; KrasLSL-G12D/+ adult mice injected with TM to activate Kras and remove p53 simultaneously. Similar to SPCrtTA/+; tetO-EGFR+ mice, SPCCreER+/p53f/f; KrasLSL-G12D/+ mice developed alveolar tumors while the airway or BADJ appeared normal. The inset in (M) shows the airway. Compared to tumors in SPCCreER+/p53f/f; KrasLSL-G12D/+ mice at the same time, tumor growth was in general accelerated and cell morphology indicates higher-grade tumors in SPCCreER+/p53f/f; KrasLSL-G12D/+ mice, for instance, by the presence of prominent nucleoli (blue arrow), enlarged pleomorphic nuclei (arrowhead), aberrant mitosis (yellow arrow) and tumor giant cells (black arrow) in (N). (O–T) Immunostaining of lung sections from SPCCreER+/p53f/f; KrasLSL-G12D/+ adult mice with activated Kras and loss of p53. Similarly, alveolar tumors (P, R, S) expressed SPC but not CC10 or Sox2. The inset in (O) displays the airway and the inset in (S) showed an alveolar tumor. These tumor cells were highly proliferative as assessed by Ki67 staining (T), the entire field of which consists of tumor cells. (U–X) Immunostaining of lung sections from SPCCreER+/p53f/f; KrasLSL-G12D/+ adult mice with activated Kras and loss of p53. BASCs (SPC+; p53+; KrasLSL-G12D+) at the BADJs rarely proliferated whereas extensive type II cell (TII) (SPC+; p53+; KrasLSL-G12D+) showed an alveolar tumor. These tumor cells were highly proliferative as assessed by Ki67 staining (T), the entire field of which consists of tumor cells. (U–X) Immunostaining of lung sections from SPCCreER+/p53f/f; KrasLSL-G12D/+ adult mice with activated Kras and loss of p53. BASCs (SPC+; p53+; KrasLSL-G12D+) at the BADJs rarely proliferated whereas extensive type II cell (TII) (SPC+) proliferation was observed. Similar results were obtained in SPCCreER+/p53f/f; KrasLSL-G12D/+ adult mice (Y–A') even though SPCCreER was expressed in a significant fraction of BASCs in BADJs (Fig. 1I, 1J). We performed a careful analysis of BASC proliferation in both SPCCreER+/p53f/f; KrasLSL-G12D/ and SPCCreER+/p53f/f; KrasLSL-G12D/+ mice injected with tamoxifen. While extensive type II cell proliferation was observed (Fig. 2U–X), proliferating BASCs were rarely found (Fig. 2U–X) when Kras was activated either alone or in conjunction with p53 removal. BASC proliferation was assessed at eight days, three weeks, six weeks, ten weeks and thirteen weeks after TM injection and similar results were obtained. Interestingly, proliferation of SPC+ type II cells that carry p53 and Kras mutations can be detected in just a few days after TM administration (Fig. 2Y–Z and 2A'–B'). Taken together, our findings suggest that alveolar tumors induced by Kras/p53 suppressor removal and initiate tumor development.

Leaky SPCCreER Expression without Tamoxifen Injection was Found in Some Alveolar Type II Cells and Not BASCs in Certain SPCCreER+/p53f/f; KrasLSL-G12D/+ mice, Resulting in Proliferation and Transformation of Alveolar Type II and Not BASC cells

We noticed spotty expression of CreER in some SPCCreER, ROSA26mTmG adult mice without tamoxifen injection (Fig. 1D, E). Interestingly, leaky CreER expression was only detected in alveolar type II cells (Fig. 3A) and not in BASCs or other lung cell types (Fig. 3B). We took advantage of this observation to further test the cells of origin of lung adenocarcinoma developed in SPCCreER+/p53f/f; KrasLSL-G12D/+ mice without tamoxifen injection. This would allow us to directly follow the fate of type II cells carrying Kras/p53 mutations without the complication of BASCs. We performed a careful immunohistochemical analysis of lungs collected from SPCCreER+/p53f/f; KrasLSL-G12D/+ mice without tamoxifen injection. These mice with leaky CreER expression without TM injection survived a few more months than those injected with TM. Similar to SPCCreER+/p53f/f; KrasLSL-G12D/+ mice injected with tamoxifen, invasive lung adenocarcinomas observed in SPCCreER+/p53f/f; KrasLSL-G12D/+ mice with leaky CreER expression localized to the alveoli (Fig. 3D, 3E). Tumor cells expressed type II cell marker (SPC) and were highly proliferative (Fig. 3E). BADJs, where BASCs reside, retained normal architecture (Fig. 3C) in SPCCreER+/p53f/f; KrasLSL-G12D/+ mice with leaky CreER expression and no obvious proliferation was observed at the BADJ (Fig. 3F). This provides additional evidence to support the notion that alveolar type II cells can be the initiating cells for lung adenocarcinoma.

Activating EGFR in Murine SPC+ cells Led to Tumor Development in Alveoli

To further test the notion that alveolar type II cells can be the originating cells for NSCLC, we utilized our SPCrtTA mice to activate EGFR signaling in SPC+ cells. We bred mice to produce SPCrtTA/+; tetO-EGFRL858R animals. The tetO-EGFRL858R mice harbor a transgene in which a mutant form of EGFR (EGFRL858R) is under the control of tetO; thus EGFRL858R expression can be induced upon doxycycline (dox) administration [23]. Importantly, the L858R mutation in EGFR is frequently found in human adenocarcinoma and is known to confer ligand-independent EGF signaling [36,37]. SPCrtTA/+; tetO-EGFRL858R animals provide a means to regulate EGF signaling in cells that express rtTA. In the absence of dox, the lung morphology of SPCrtTA/+; tetO-EGFRL858R mice (Fig. 4A, 4B) was similar to that in wild-type mice. This suggests that even if low levels of EGFRL858R were expressed in some SPC+ cells in the absence of dox, they are insufficient to cause any phenotypes. Consistent with this, only spotty labeling of SPC+ cells by R26R or ROSA26mTmG reporters was observed in the absence of dox as described above (Fig. 1G, 1K), reflecting a low degree of leaky rtTA expression. By contrast, dox administration promoted rtTA binding to tetO and activated EGFRL858R in SPC-expressing cells. We found that after 12 weeks of continuous dox administration, SPCrtTA/+; tetO-EGFRL858R mice developed widespread tumors in the alveoli (Fig. 4C, 4D) but not in other parts of the lung, including the airways (Fig. 4H) and BADJ (Fig. 4I). No hyperplasia was detected in the airways or BADJs (Fig. 4C, 4D) and proliferating BASCs were rarely found. Tumor cells in SPCrtTA/+; tetO-EGFRL858R mice stained positive for SPC and negative for CC10 (Fig. 4G, 4J), and were highly proliferative (Fig. 4L) compared to non-tumor tissues (Fig. 4K). In conjunction with studies using SPCCreER, these results provided additional evidence to support the idea that NSCLC can be initiated in SPC+ type II cells. Interestingly, alveolar tumors regressed after dox withdrawal (Fig. 4E, 4F), suggesting that neoplasia is dependent on active EGFR signaling, consistent with previous reports [23]. This genetic system thus offers the advantage of studying the molecular process of tumor regression and resistance.
Mouse Alveolar Tumors Induced by \textit{Kras/p53} Mutations are Associated with Notch Pathway Activation

As a first step toward understanding the molecular mechanisms that underlie tumor development and confer tumor properties, we investigated whether major signaling pathways were perturbed in lung tumors developed in \textit{SPC^{CreER}; p53f/f; KrasLSL-G12D} mice. We extracted RNA from dissected tumors and adjacent non-tumor tissues and performed qPCR analysis to examine the expression of target genes for major signaling pathways. Interestingly, we found that Notch signaling was consistently upregulated in tumor versus non-tumor tissues (Fig. 5A). By contrast, several other major signaling pathways, including the Platelet-derived growth factor (PDGF), Wnt, Bone morphogenetic protein (Bmp) and Hedgehog (Hh) pathways, showed modest or no apparent changes in target gene expression in tumor tissues compared to non-tumor tissues (Fig. 5A). Interestingly, expression of \textit{Itgb4} (integrin beta 4) was also increased in mouse lung tumor tissues (Fig. 5A) as well as in human non-small cell lung cancer cell lines (Fig. 5B). Notch signaling has been associated with survival of lung

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**Figure 3. Leaky expression of \textit{SPC^{CreER}} in alveolar type II cells and not BASCs is associated with alveolar tumors while BADJs retain normal architecture.** (A–F) Histology and immunostaining of lung sections from adult \textit{SPC^{CreER}; ROSA26mTmG} mice without tamoxifen injection. In \textit{SPC^{CreER}; ROSA26mTmG} mice that displayed leaky Cre activity without tamoxifen injection, leaky Cre expression was detected in alveolar type II cells (A) and not BASCs (B). Only alveolar lung tumors were detected in \textit{SPC^{CreER}; p53f/f; KrasLSL-G12D} mice with leaky Cre activity (D) and BADJs retained normal architecture (C). Tumor cells expressed SPC (type II cell marker) and were highly proliferative (E), suggesting their origin from type II cells. By contrast, no obvious proliferation was observed at BADJs in these mice (F) consistent with their normal structure. Scale bar = 50 \textmu m for panels in each row except (C, D), which is 100 \textmu m.
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adenocarcinoma cells in vitro [38] and tumor metastasis [39]. Our findings suggest that activated Notch signaling in response to \(Kras/ p53\) mutations could be involved in tumor development or confer tumor properties. The genetic system we developed provides an ideal setting to isolate tumor cells at different stages of tumor development and ask how Notch signaling controls tumor initiation, progression and invasion/metastasis. These studies will provide mechanistic insight into the roles of major signaling pathways in lung tumor development.

**Discussion**

Through the development of new genetic tools in mice, we provide strong evidence to support the notion that human NSCLC can originate from alveolar type II cells. Our results confirm and extend the findings in a recent report [40], which primarily analyzed mice carrying \(Kras\) mutations. No evidence was presented in the published article [40] to demonstrate tumor invasion or metastasis. It is worth noting that earlier reports using adenoviral-Cre to infect lung epithelium globally showed that mice carrying the \(Kras\) mutation and one loss-of-function allele of \(p53\) in the lung exhibited phenotypes similar to mice carrying the \(Kras\) mutation alone [41]. This suggests that loss of both copies of \(p53\) is critical for invasive tumors to develop. Our experimental setting enabled us to achieve simultaneous \(Kras\) activation and \(p53\) ablation. As a result, tumor invasiveness was observed in \(Kras/p53\) mutants but not in mice carrying activated \(Kras\) alone under our experimental conditions. Importantly, taking advantage of the leaky expression of our \(SPC\text{-}CreER\) line only in alveolar type II cells but not \(BASC\)s, we were able to attribute lung adenocarcinoma developed in these mice to type II cells and not \(BASC\)s. Taken together, these new findings strongly support the idea that development of lung adenocarcinoma (beyond adenoma or carcinoma \(in situ\)) originates from \(SPC^+\) alveolar type II cells.

A previous report [23] relied on a \(CCSP\text{-}rtTA\) transgene to investigate how EGFR mutations in different lung cell types could influence tumor development. The \(CCSP\text{-}rtTA\) transgene utilizes a short fragment of the rat \(CCSP\) promoter to drive rtTA expression. The short rat \(CCSP\) promoter does not recapitulate mouse \(CC10\) expression and displays significant leaky expression in \(SPC\)-expressing cells, raising the possibility that it is expressed in \(BASC\)s. How EGFR mutations affect \(BASC\) proliferation/transformation was not addressed in previous work [23]. Our \(SPC\text{-}rtTA\) mouse line in which rtTA is under the control of the endogenous \(SPC\) promoter provides the first mouse model to assess the effects of EGFR mutations on transforming \(SPC^+\) type II cells.

While our results suggest that NSCLC could initiate in type II cells, the exact nature of tumor-initiating cells requires further investigation. Without tracing the early events at the molecular level, it is not possible to decipher how differentiated type II cells that carry oncogenic mutations undergo progressive transformation. It is possible that all \(SPC\)-expressing type II cells share a
KrasLSL-G12D/+ and multiple mice were analyzed. An increase in tumor tissues. Multiple lung tumors from a single mouse were assessed for changes in their expression of several major signaling pathways, including the lung (SPC) and NCI-A549 (lung adenocarcinoma). The numbers represent the fold of increase in marker expression relative to housekeeping genes.

**Figure 5.** Analysis of major signaling pathways in mouse Kras/ p53 tumors and marker expression in human lung cancer cell lines. (A) Quantitative PCR (qPCR) analysis of RNA extracted from tumors and adjacent non-tumor tissues derived from SPCtetO-EGFRL858R/+; p53f/f; KrasLSL-G12D/+ adult mice injected with tamoxifen. Target genes of each major signaling pathway were assessed for changes in their expression in tumor versus non-tumor tissues. The numbers represent fold of increase in tumor tissues. Multiple lung tumors from a single mouse and multiple mice were analyzed. Ccd1 (cyclin D1) was upregulated, consistent with increased Ki67 staining on tumor sections. Notch signaling was consistently upregulated in tumor versus non-tumor tissues whereas Platelet-derived growth factor (PDGF), Wnt, Bone morphogenetic protein (Bmp) and Hedgehog (Hh) pathways showed modest or no apparent changes in target gene expression. Expression levels of Nkx2.1 and Myc were also significantly increased, similar to findings in human adenocarcinoma. Interestingly, expression of Itgb4 (integrin beta 4) was also increased in tumor tissues. Future studies will determine whether Itgb4 plays a role in tumor development. (B) qPCR analysis of RNA extracted from representative human lung cancer cell lines, including NCI-H292 (pulmonary mucoepidermoid carcinoma), NCI-H82 (small cell lung cancer), NCI-H441 (papillary adenocarcinoma of the lung) and NCI-A549 (lung adenocarcinoma). The numbers represent fold of increase in marker expression relative to housekeeping genes. SPC expression was upregulated in the A549 cell line. Itgb4 (integrin beta 4) was also highly expressed in non-small cell lung cancer cell lines compared to small-cell lung cancer cell lines.

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Similar potential of being transformed. Alternatively, tumors may only initiate in a small population of uncharacterized type II cells, for instance, those expressing low levels of SPC and presumably possessing proliferative potential in various conditions [42] or those residing at specific anatomical locations. Without markers currently available to define subpopulations of type II cells, it is not possible to assess the transformation potential of distinct subpopulations of type II cells, if they do exist. It is also uncertain how the transformation potential of type II cells is compared to other lung epithelial cell types such as Clara cells, BASCs, ciliated cells, goblet cells and type I cells under various genetic perturbations. Perhaps, distinct cell types are susceptible to specific insults and a systematic investigation is required to delineate the molecular basis of proliferative potential among distinct lung cell types or populations. These studies would shed light on the source of heterogeneity or subtypes of NSCLC, the very properties of which could originate from distinct cell types carrying different mutation profiles. Finally, we cannot rule out the possibility that cells with altered p53 and Kras activities may exert non-cell autonomous effects on neighboring cells and induce tumor development in these cells containing wild-type p53 and Kras.

We found some degree of leakiness present in both SPCtetO- and SPCtetO/+ mouse strains that we used to manipulate gene activity in type II cells. In particular, more sensitive reporter lines (e.g., ROSA26Gt(rosa)26) appear to detect only very low levels of leaky expression. In some cases, variations in background expression among individual mice were also noted. Consistent with these findings, when leaky CreER expression reached a critical threshold in SPCtetO/+; KrasLSL-G12D/+ or SPCtetO/+; p53f/f; KrasLSL-G12D/+ mice, cell hyperplasia and even tumor development was observed without TM administration albeit at a lower frequency than in mice injected with TM. Interestingly, even in mice in which leaky CreER expression led to tumor development, tumors were never detected at the BADJ. By contrast, SPCtetO+; tetO-EGFRL858R mice without dox do not develop hyperplasia or tumors. This probably reflects differential sensitivities of type II cells to diverse perturbations.

Given that SPCtetO confers the ability to manipulate gene activity in BASCs, it is somewhat surprising that hyperplasia at the BADJ was not observed in SPCtetO+/+; KrasLSL-G12D/+ or SPCtetO+/+; p53f/f; KrasLSL-G12D/+ mice when CreER is activated by TM. This raises the possibility that BASCs are not as sensitive to Kras/p53 perturbation as other SPC-expressing cells. Similarly, active EGF signaling in SPC-expressing cells only led to alveolar tumor development and not BASC hyperplasia or neoplasia. Since BASCs express both SPC and CC10, it would be interesting to compare the transformation potential between BASCs and Clara cells. Whereas our results do not provide evidence to support a role for BASCs in tumor development, it is conceivable that additional genetic perturbation not utilized in this study may be required to transform BASCs. It is also possible that a longer time period is needed for tumors to develop in BASCs. This is consistent with the observation that human lung tumors usually develop after many years or even decades of repeated epithelial injury such as from cigarette smoking. Though BASC proliferation is rare in our experimental setting, we cannot formally rule out the possibility that BASCs can be transformed, expanded and differentiated into type II-like cells. Lineage-tracing studies in which Kras/p53 mutant BASCs and type II cells are labeled and followed during early tumor development will provide new insight into this important issue.

Human NSCLC is a heterogeneous group of diseases that share common histology but carry different mutations and perhaps even originate from different cell types [43]. For instance, some NSCLC...
Materials and Methods

Mice

All animal work was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

To produce the SpcCAGR mouse line, the translational start ATG in the mouse SPC genomic locus was replaced with a CreER-FRT-Neo-FRT cassette through gene targeting in E14 embryonic stem (ES) cells. The FRT-Neo-FRT cassette was subsequently removed by crosses with Flp mice [45].

The SpcCAGR mouse line harbors an engineered bacterial artificial chromosome (BAC) in which rTAiPA was placed at the translational start ATG of the SPC genomic locus and tetO-Cre was inserted at the translational start ATG of the Nudt18 genomic locus. To generate the SpcCAGR BAC, an rTAiPA targeting construct containing homologous arms to the SPC locus, the recombined BAC underwent a second recombination to introduce tetO (TRE-Tight)-Cre to the Nudt18 locus. The Nudt18 gene is located ~57 kb upstream of the translational start ATG of SPC. BAC DNA was linearized with PstI and introduced into CD-1 zygotes by pronuclear injection to produce transgenic mice.

Histology, Immunohistochemistry and β-galactosidase Staining

Mouse lungs were inflated with 1 ml of 2% paraformaldehyde (PFA) and fixed for 4 hrs-overnight at 4°C in 2% PFA. After dehydration and processing, lungs were embedded in paraffin for sectioning and immunohistochemistry. All the tissues collected were sectioned at 6 μm thickness for histological analysis. Paraffin sections were stained with the following primary antibodies: chick anti-GFP (1:200, Abcam), goat anti-CC10 (1:200, Santa Cruz), rabbit anti-proSP-C (1:400, Millipore), hamster anti-CC10 (1:200, Developmental Studies Hybridoma Bank), mouse anti-Kit67 (1:200, BD) and goat anti-Sox2 (1:200, Santa Cruz). Secondary antibodies and conjugates used include: donkey anti-chick DyLight 488 (1:1,000; Jackson ImmunoResearch Laboratories), donkey anti-rabbit Alexa Fluor 594 (1:1,000; Life Technologies), donkey anti-goat Alexa Fluor 594 or 647 (1:1,000; Life Technologies), and DAPI (1:10,000; Sigma). For biotinylated secondary antibodies (goat anti-rabbit, 1:1,000, donkey anti-goat, 1:1,000 and horse anti-mouse, 1:1,000, Jackson ImmunoResearch Laboratories), the signal was detected using HRP-conjugated streptavidin (1:1,000, Vector Laboratories) in combination with either the chromogenic substrate DAB (Vector Laboratories) or fluorescent substrate Alexa Fluor 594 tyramide (1:100, TSA kit, Perkin-Elmer). Antibodies against Kit67 may require the biotin-streptavidin amplification step for optimal signal detection. Fluorescent images were acquired using a SPOT 2.3 CCD camera connected to a Nikon E1000 epifluorescence microscope. Adjustment of RGB histograms and channel merges were performed using SPOT Advanced software or ImageJ. Confocal images were captured on a Leica laser-scanning confocal microscope. Multiple optical sections were obtained to distinguish cell boundaries. For X-gal staining, lungs were inflated with 1 ml of 1% PFA, 0.2% gluteraldehyde and fixed 1 hr at 4°C in 1% PFA and 0.2% gluteraldehyde. To detect β-galactosidase activity, whole lungs or frozen sections (10 μm) were stained in X-gal staining solution following standard procedures.

Analysis of Target Gene Expression for Major Signaling Pathways in Mouse Lung Tumors and Lung Marker Expression in Human Lung Cancer Cell Lines

Tumor and adjacent non-tumor tissues were microdissected from the mouse lungs. Human lung cancer cell lines were purchased from ATCC. Total RNA was extracted using Trizol (Invitrogen) and RNeasy Mini Kit (Qiagen) and subsequently reverse-transcribed to cDNA using the SuperScript Kit (Invitrogen). Quantitative PCR (qPCR) was carried out on the ABI Prism 7900HT Sequence Detection System.

The following primers for mouse genes were used: Cond1 F: 5’GCAGAAGAGATTTGTCGATGCC3’; Cond1 R: 5’AGGAACGCCTTCAGGTATCC3’; Notch1 F: 5’GGTGCCTCTTTGATGGCTTCGA 3’; Notch1 R: 5’CATTTCGCCCAGTTCAGGAC3’; Hes1 F: 5’CAGAATGGACTGTTCCGACC3’; Hes1 R: 5’GAAGCGGGTTCCACTGCCTATG3’; Igf4 F: 5’ACACACAGCTCCAGACAGAGG3’; Igf4 R: 5’TCCACCGTCTTTCTCCTGAGC3’; Nkx2.1 F: 5’CAGAGCACCAGTGCCCTATG3’; Nkx2.1 R: 5’GCCAGTGCTTCTGTCGATGCC3’; Myc F: 5’TGGTGGTGCTCTGTCGAGTCC3’; Myc R: 5’GGTTTGGCTTCTGTCGAGTCC3’; PDGFRα F: 5’GGATTGTCCTAGACTCCAG3’; PDGFRα R: 5’GGTTGAGGAATCTTCAGACCC3’; PDGFRβ F: 5’AC-
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