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1. Introduction

Lung cancer is the leading cause of cancer deaths in the world, which is a cause for more solid tumor-related deaths than all other carcinomas combined. More than 170,000 new cases are diagnosed each year in the United States alone, of whom ~160,000 will eventually die, accounting for nearly 30% of all cancer deaths (Siegel et al., 2012). The annual incidence for lung cancer per 100,000 population is highest among African Americans (76.1), followed by whites (69.7), American Indians/Alaska Natives (48.4), and Asian/Pacific Islanders (38.4). Hispanic people have much lower lung cancer incidence (37.3) than non-Hispanics (71.9) (CDC, 2010). These results identify the racial/ethnic populations and geographic regions that would benefit from enhanced efforts in lung cancer prevention, specifically by reducing cigarette smoking and exposure to environmental carcinogens.

Lung lobectomy provides the best chance for patients with early-stage disease to be cured. African American patients with early-stage lung cancer have lower five-year survival rates than whites, which has been attributed to lower rates of resection in former patients (Wisnivesky et al., 2005). Several potential factors underlying racial differences in receiving surgical therapy include differences in pulmonary function, access to care, beliefs about tumor spread at the time of operation, and the possibility of cure without surgery. Of these, access to care is considered to be the most important factor underlying racial disparities.

The most outstanding modifiable risk factor for lung cancer is cigarette smoking (Swierzewski III, 2011). Other risk factors include asbestos exposure, radon, occupational chemicals, radiation, and alcohol. People who smoke tend to drink more alcohols and consume more non-narcotic pain relievers than non-smokers, thus reducing the intoxicating effects of alcohol, promoting the progression from moderate to heavy drinking. Alcoholism is also associ-
ated with significant immune suppression - therefore, a history of drinking may increase a person's susceptibility to lung cancer.

Lung cancer has a high morbidity because it is difficult to detect early and is frequently resistant to available chemotherapy and radiotherapy. The overall 5-year survival rate for all types of lung cancer is around 15 % at most, and it is even worse in SCLC (~5 %) although SCLC is more sensitive to chemo/radiation therapy than NSCLC (Meuwissen & Berns, 2005; Schiller, 2001; Worden & Kalemkerian, 2000). Non-smokers who develop lung cancer may experience delays in diagnosis due to the fact that many early symptoms of lung cancer mimic those of non-specific respiratory infections (Menon, 2012). Thus, a physician may misdiagnose the malignant disease for asthma or other respiratory illnesses. Another reason for delayed diagnosis of lung cancer is that there is no sensitive and specific biomarker, such as prostate-specific antigen in prostate cancer (Brambilla et al., 2003). Thus several biomarkers will have to be used together for early diagnosis of lung cancer at present, which include mutant Ras, mutant p53, and methylation of a variety of genes using bronchial biopsies or bronchoalveolar lavage (Brambilla et al., 2003).

Certain combinations of clinical signs and symptoms – e.g. endocrine, neurologic, immunologic, and hematologic - are associated with lung cancer as a manifestation of the secretion of cytokines/hormones by tumor cells or as an associated immunologic response (Yeung et al., 2011). These paraneoplastic syndromes occur commonly in patients with SCLC. Since the syndromes can be the first clinical manifestation of malignant disease, increased awareness of these syndromes associated with lung cancer is critical to the earlier diagnosis of malignancies, thereby improving the overall prognosis of patients.

Lung cancer has been categorized into two major histopathological groups: non-small-cell lung cancer (NSCLC) (Moran, 2006) and small-cell lung cancer (SCLC) (Schiller, 2001), the latter of which show neuroendocrine features and thus are different from the former. Approximately 80 % of lung cancers are NSCLC, and they are subcategorized into adenocarcinomas (AdCA), squamous cell (SqCLC), bronchioalveolar, and large-cell carcinomas (LCLC) (Travis, 2002). SCLC and NSCLC show major differences in histopathologic characteristics that can be explained by the distinct patterns of genetic alterations found in both tumor types (Zochbauer-Muller et al., 2002). The K-Ras gene is mutated in 20~30 % of NSCLC while its mutation is rare in SCLC; Rb inactivation is found in ~90 % of SCLC while p16/INK4a is inactivated by gene deletion and/or promoter hypermethylation in ~50 % of NSCLC (Fong et al., 2003; Meuwissen & Berns, 2005). Responsiveness of tumor cells to chemotherapy and/or radiation therapy significantly varies between NSCLC and SCLC, and thus, has a dramatic effect on the prognosis of patients.

Progress in whole genome approaches to detect genetic alterations found in human lung cancer has resulted in the identification of a growing number of genes. Genome-wide association studies, whether they are based on single-nucleotide polymorphism array or in gene copy number assays, have identified mutations in lung cancer-related genes. Identification of these lung cancer-related genes will provide great potential as therapeutic targets for lung cancer intervention. Target validation should be done through intervention studies of specific genetic alterations in human lung cancer cell lines. Since in vitro cell culture studies cannot fully mimic more complex in vivo onset/development of lung carcinogenesis, developing en-
dogenous lung cancer in mice that harbor specific mutations will undoubtedly provide a further insight into the mutation-specific effects on lung tumor initiation/development. Moreover, a high degree of pathophysiological similarity between mouse lung tumors and human lung carcinomas will make it possible to use these mouse models in pre-clinical tests for novel anticancer drug screening. Various intervention strategies against specific mutation can then be tested to evaluate both specificity and efficacy in mouse lung tumors at every developing stage. The number of genetically engineered mouse models for lung cancer is ever expanding. Continuous attempt to manipulate the mouse genome has enabled us to adjust compound mouse models of lung cancer in a way that they start to reproduce the more complex human lung cancer in a higher degree.

While susceptibility and incidence of spontaneous lung tumors vary among well-established mouse strains, endogenous mouse lung tumors share many similarities with human lung cancers. This was clearly demonstrated in early studies where defined chemical carcinogens were used to induce lung tumors in mice (Wakamatsu et al., 2007). The incidence of spontaneous and induced lung tumors were very high (61%) in A/J and SWR strains, but very low (6%) in resistant strains such as C57BL/6 and DBA (Wakamatsu et al., 2007). Contrary to human lung cancer with its complex molecular genetics and four distinct tumor types (adenocarcinoma, squamous cell carcinoma, large-cell carcinoma, and small-cell carcinoma) that easily metastasize, spontaneous and chemically-induced lung lesions in mice often result in pulmonary adenomas and more infrequent adenocarcinomas. Mouse lung adenocarcinomas are usually 5mm or more in diameter; however, they are categorized into carcinomas when nuclear atypia or signs of local invasion/metastasis is found in tumors less than 5mm. Mouse lung tumor development shows initial hyperplastic foci in bronchioles and alveoli, which then become benign adenomas and eventually adenocarcinomas (Shimkin et al., 1975). The tumor latency depends on mouse strain and carcinogen administration protocols. Most potent carcinogens are found in cigarettes, such as polycyclic aromatic hydrocarbons, tobacco-specific nitrosamine, and benzopyrene (BaP) (Pfeifer et al., 2002). It has been especially difficult to reproduce well-characterized pre-malignant lesions found in human airway epithelium in mice (Sato et al., 2007). Nevertheless, major histopathological features remain the same between the two species and molecular characterization of spontaneous and carcinogen-induced murine lung tumors revealed a high degree of similarity as compared to their human counterparts (Malkinson, 2001). A common early event is the occurrence of activating K-ras mutations in hyperplastic lesions. Besides overexpression of c-Myc, inactivation of well-known tumor suppressor genes, such as p53, flit, Apc, Rb, Mcc, p16Ink4a and/or Arf occur in both mice and human lung cancers; only a small percentage of lung adenomas progress into AdCAs (Malkinson, 2001).

2. The first generation mouse models for lung cancer

The first generation transgenic models for lung cancer were created by ectopic transgene expression under control of lung-specific promoters. Thus transgenic expression was constitutive. Transgene expression was mainly found in specific subsets of lung epithelial cells. Lung surfactant protein C (SPC) promoter was used for constitutive gene expression in type II
alveolar cells whereas Clara Cell Secretory Protein (CCSP) promoter was used to target the non-ciliated secretory (Clara) cells that exist on the airways. In early studies, SV40 Tag (Simian virus large T-antigen) that neutralizes the activity of both Rb and p53 was constitutively expressed under the control of CCSP (DeMayo et al., 1991; Sandmoller et al., 1994) or SPC promoters (Wikenheiser et al., 1992). Although each tumor originated from either Clara cells or type II alveolar cells, they both resulted in quite similar aggressive AdCAs without metastases (Wikenheiser et al., 1997). A similar strategy was used to express distinct oncogenes (such as c-Raf and c-Myc [Geick et al., 2001]) in the lung/bronchial epithelium, ending up with a milder phenotype, as both transgenic mice mainly developed adenomas, and a few progressed to AdCAs without any metastases.

Ehrhardt et al. (2001) created transgenic mouse models to study tumorigenesis of bronchio-alveolar AdCAs derived from alveolar type II pneumocytes. Transgenic lines expressing c-Myc under the control of the SPC promoter developed multifocal bronchio-alveolar hyperplasias, adenomas, AdCAs, whereas transgenic lines expressing a secretable form of the epidermal growth factor, TGFα, developed hyperplasias of the alveolar epithelium. Since the oncogenes c-Myc and TGFα are frequently overexpressed in human lung bronchio-alveolar carcinomas, these mouse lines will be useful as those for human lung bronchio-alveolar carcinomas (Ehrhardt et al., 2001).

Sunday et al. created a transgenic model for primary pulmonary neuroendocrine cell hyperplasia/neoplasia using v-Ha-ras driven by the neuroendocrine (NE)-specific calcitonin promoter (named rascal). All rascal transgenic mouse lineages developed hyperplasias of NE and non-NE cells, but mostly non-NE cells developed lung carcinomas (Sunday et al., 1999). Analyses of embryonic lung demonstrated rascal mRNA in undifferentiated epithelium, consistent with expression in a common pluripotent precursor cell. These observations indicate that v-Ha-ras can lead to both NE and non-NE hyperplasia/carcinoma in vivo (Sunday et al., 1999).

A strong correlation exists between p53 mutations and lung malignancies, and LOH for p53 has been reported in 40% of NSCLC with specific primers (Mallakin et al., 2007). Preceding this study, Morris et al. (1998) established a transgenic mouse model with disrupted p53 function in the epithelial cells of the peripheral lung. A dominant-negative mutant form of p53 was expressed from the human SPC promoter. The dominant-negative p53 (dnp53) expressed from the SPC promoter antagonized wild-type p53 functions in alveolar type II pneumocytes and some bronchiolar cells of the transgenic animals, and thereby promoted the development of carcinoma of the lung. This mouse model should prove useful to the study of lung carcinogenesis and to the identification of agents that contribute to neoplastic conversion in the lung. Another group later created CCSP-dnp53 transgenic mice and reported significant increase in the incidence of spontaneous lung cancer in 18-month-old transgenic mice (Tchon-Wong et al., 2002). In addition to the increased incidence of spontaneous lung tumor, these transgenic mice were more susceptible to the development of lung adenocarcinoma after exposure to BaP. The risk of lung tumors was 25.3 times greater in BaP-treated mice adjusted for transgene expression. These results suggest that p53 function is important for protecting mice from both spontaneous and BaP-induced lung cancers.
The receptor tyrosine kinase RON (recepteur d’origine nantais) is a member of the MET proto-oncogene family, which is expressed by a variety of epithelial-derived tumors and cancer cell lines and has been implicated in the pathogenesis of lung adenocarcinomas (Chen et al., 2002). To determine the oncogenic potential of RON, transgenic mice were generated using the lung SPC promoter to express human wild-type RON in type II cell phenotypes (Chen et al., 2002). The mice were born normal without morphological alterations in the lung, however, multiple adenomas appeared as a single mass in the lung around 2 months of age and gradually developed into multiple nodules throughout the lung. Most of the tumors were characterized as cuboidal epithelial cells with type II cell phenotypes which transformed from pre-malignant adenomas to adenocarcinomas. Interestingly, Ras expression was dramatically increased in the majority of tumors without mutation in the ‘hot spots’ of the K-Ras or p53 genes suggesting that SPC-RON is a mouse lung tumor model with unique biological characteristics (Chen et al., 2002).

Many prominent genetic lesions found in human lung cancer clearly link the inactivation of well-known tumor suppressor genes (Sekido et al., 2003) to lung cancer development. Initial attempts to mimic some of these lesions implicated in lung cancer by using conventional knockout mice had limited success with respect to the onset of lung cancer. The main reason for this failure was that germ-line deletion of many essential tumor suppressor genes (such as the retinoblastoma gene (Rb) (Jacks et al., 1992) lead to embryonal lethality. Non-essential tumor suppressor gene (for embryonic survival) knockout mice often had a very broad tumor spectrum of which lung tumors formed only a minor fraction. Thus, p53, p16Ink4a and p19Arf (Meuwissen & Berns, 2005) null allele mice seldom develop lung AdCAs. However, introducing similar mutations into endogenous p53 alleles, such as those prominently found in Li–Fraumeni patients, generated p53R270H+/+ and p53R270H−/− which had a different tumor spectrum compared with p53−/− mice (Olive et al., 2004), although their mean survival times were identical. Interestingly these mice, but especially p53R270H+/− and p53R270H−/− mice, gave rise to more malignant lung AdCAs, and even their metastases, which never occurred in p53−/− mice. These results suggest that “humanized” p53 mutations have a greater impact on lung tumor progression than complete p53 loss (Olive et al., 2004; Lang et al., 2004).

Targeting genes deleted early in human lung tumorigenesis, such as the complete cluster at chromosome 3p21.3, showed that heterozygous deletion for this 370 kb region showed no obvious predisposition for lung cancer development albeit homozygous deletion caused embryonal lethality (Smith et al., 2002). A more specific deletion of candidate tumor suppressor genes on chromosome 3 like RassF1a, FHIT and VHL, showed that 31% of Rassf1a−/− mice produced spontaneous mainly lymphomas but also lung adenomas (Tommasi et al., 2005). Treatment of Rassf1a−/− mice with BP or urethane resulted in an even higher rate of lung tumors. No spontaneous lung tumors were observed in FHIT−/− or VHL−/− mice, but 44% of FHIT−/−;VHL−/− mice developed AdCAs by age 2 years. Again use of mutagens such as dimethylnitrosamine led to 100% adenoma and AdCA induction in FHIT−/−;VHL−/− mice and even adenomas in 40% of FHIT−/− mice by age 20 months (Zanesi et al., 2005). This showed the usefulness of these knockout mice in recapitulating a pattern of early lung cancer development similar to human pattern.
3. K-ras<sup>A</sup> and LSL K-ras models

A different approach to address lung cancer onset was the use of knock-in alleles to activate oncogenes. One example of this is based on the somatic K-ras activation via an oncogenic Kras<sup>G12D</sup> knock-in allele (Kras<sup>A</sup>), which is expressed only after a spontaneous recombination event (Johnson et al., 2001). In this way, sporadic Kras<sup>G12D</sup> expression occurred on an endogenous level, which in turn augments efficient development of lung AdCAs. However, these mice also developed other tumor lesions as K-Ras<sup>G12D</sup> expression was not limited to the lung epithelial tissues.

Dmp1 (Dmtf1) is a Myb-like protein with tumor suppressive activity that had been isolated in a yeast two-hybrid screen with cyclin D2 bait (Hirai and Sherr, 1996; Inoue and Sherr, 1998; for review, Inoue et al., 2007; Sugiyama et al., 2008a). The promoter is activated by oncogenic Ras-Raf signaling and induces cell-cycle arrest in an Arf, p53-dependent fashion (Inoue et al., 1999; Sreeramaneni et al., 2005). Both Dmp1<sup>+/+</sup> and Dmp1<sup>-/-</sup> mice are prone to spontaneous and carcinogen-induced tumor development, indicating that it is haplo-insufficient for tumor suppression, the mechanism of which have not been elucidated yet (Inoue et al., 2000, 2001, 2007). The survival of K-ras<sup>A</sup> mice was shortened by approximately 15 weeks in both Dmp1<sup>+/+</sup> and Dmp1<sup>-/-</sup> backgrounds, the lung tumors of which showed significantly decreased frequency of p53 mutations compared to Dmp1<sup>+/+</sup>. Approximately 40% of K-ras<sup>A</sup> lung tumors from Dmp1 wild-type mice lost one allele of the Dmp1 gene, suggesting the primary involvement of Dmp1 in K-ras-induced tumorigenesis (Mallakin et al., 2007). Tumors from Dmp1-deficient mice showed more invasive and aggressive phenotypes than those from Dmp1 wild-type mice. Loss of heterozygosity (LOH) of the hDMP1 locus was detectable in approximately 35% of human lung carcinomas, which was found in mutually exclusive fashion with LOH of INK4a/ARF or that of p53. Thus, DMP1 is a novel tumor suppressor for both human and murine NSCLC (Mallakin et al., 2007; Sugiyama et al., 2008b).

Integration of gene expression data from a Kras<sup>A</sup> mouse model and KRAS mutated human lung tumors showed a significant overlap but also revealed a gene-expression signature for K-ras mutation in human lung cancer itself (Sweet-Cordero et al., 2005). By using Kras<sup>A</sup> knock-in mouse model and human lung cancer specimen, they compared gene expression patterns between these two species (Sweet-Cordero et al., 2005). They applied this method to the analysis of a model of Kras<sup>A</sup>-mediated lung cancer and found a good relationship to human lung AdCA, thereby validating the usefulness of this transgenic model. Furthermore, integrating mouse and human data uncovered a gene-expression signature of KRAS2 mutation in human lung cancer. They confirmed the importance of this signature by gene-expression analysis of shRNA-mediated inhibition of oncogenic Kras<sup>A</sup> (Sweet-Cordero et al., 2005). However, one problem of Kras<sup>A</sup> mice is that they develop tumors other than lung cancer (Mallakin et al., 2007). To overcome this issue, Jackson et al. (2001) developed a new model of lung AdCA in mice having a conditionally activatable allele of oncogenic K-ras (LSL Kras<sup>G12D</sup>). They show that the use of a recombinant adenovirus expressing Cre recombinase (AdenoCre) to induce Kras<sup>G12D</sup> expression in the lungs of mice allows control of the tim-
ing and multiplicity of tumor initiation. Through the ability to synchronize tumor initiation in these mice, they could characterize the stages of tumor progression. Of particular significance, this system led to the identification of a new cell type contributing to the development of pulmonary AdCA (Jackson et al., 2001). By using this Cre-lox system, the same group later created conditional knock-in mice with mutations in K-ras combined with one of mutant p53 alleles (Jackson et al., 2005). p53-loss strongly promoted the progression of Kras-induced lung AdCAs, yielding a mouse model that precisely recapitulates advanced human lung AdCA. The influence of p53-loss on malignant progression was observed as early as 6 weeks after tumor initiation. They also found that the contact mutant p53R270H behaved in a dominant-negative fashion to promote K-ras-driven lung AdCAs. Of note, a subset of mice also developed sinonasal adenocarcinomas, suggesting specific expression of K-ras in this tissue. In contrast to the lung tumors, expression of the point-mutant p53 alleles strongly promoted the development of sinonasal AdCAs compared with simple loss-of-function, suggesting a tissue-specific gain-of-function of mutant p53 (Jackson et al., 2005).

Since activating K-ras mutation models recapitulate the human lung tumor phenotypes well, closer analyses of early lung tumor initiating events were performed (Ji et al., 2006). A combination of both CCSP-Cre recombinase and LSL KrasG12D alleles (Jackson et al., 2005) resulted in a progressive phenotype of cellular atypia, adenoma and finally AdCA. The activation of K-ras mutant allele in CC10-positive cells resulted in a progressive phenotype characterized by cellular atypia, adenoma and ultimately AdCA. Surprisingly, Kras activation in the bronchiolar epithelium was associated with a robust inflammatory response characterized by an abundant infiltration of alveolar macrophages and neutrophils. These mice displayed early mortality in the setting of this pulmonary inflammatory response. Bronchoalveolar lavage fluid from these mutant mice contained the MIP-2, KC, MCP-1 and LIX chemokines that increased significantly with age. Thus, Kras activation in the lung induces inflammatory chemokines and provides an excellent means to study the complex interactions between inflammatory cells, chemokines, and tumor progression (Ji et al., 2006).

### 3.2. Doxycycline (dox)-inducible/de-inducible lung cancer models

In KrasLA mice, oncogene can be induced, but it cannot be de-induced after lung carcinogenesis. To improve this mouse model, a better method of replicating gene expression patterns of target oncogenes had to be taken into account. Furthermore, a general knock-in or knockout procedure only represents genetic events that occur during sporadic lung cancer since genes are already deleted already in utero (Jonkers & Berns, 2002). Conditional regulation of the temporal-spatial expression of oncogenes or inactivation of tumor suppressor genes in somatic tissues of choice can more accurately mimic the in vivo situation leading to the onset of sporadic cancer (Jonkers & Berns, 2002; Lewandoski, 2001). This is why the second generation of mouse models for lung cancer makes use of a conditional bitransgenic tet-inducible system (Lewandoski, 2001). Most often, the reverse tetracycline (tet)-controlled transactivator (rtTA) inducible system is used. The first transgene with the rtTA element behind a tissue-specific promoter causes the rtTA expression in a specific cell types, e.g. MMTV-rtTA, CCSP-rtTA. This transgene is then combined with a second transgene, consist-
ing of a target gene behind a tet-responsive promoter (tetO) vector, e.g. pTRE-Tight (2nd generation vector from Clontech). The presence of tet/dox ensures stable interaction of the rtTA element with the tetO promoter, which, in turn, expresses the target gene upon exposure to tet or dox.

Therefore, on/off target gene expression is possible depending on administration or withdrawal of tet/dox (Gossen et al., 1992). Both SPC-rtTA and CCSP-rtTA transgenes (Perl et al., 2002) have been used for directing dox-responsive rtTA to either alveolar type II or Clara cells. Although both of these promoters have been used to create lung cancer models of mice, CCSP-rtTA has more widely been used than SPC-rtTA since the CCSP promoter is active in both Clara cells and alveolar type II cells while the SPC promoter is active only in alveolar type II cells (Floyd et al., 2005). Several transgenic mice such as CCSP-rtTA;tetO-FGF-7 and CCSP-rtTA;tetO-KrasG12D have been successfully created to induce lung lesions in response to antibiotics (Tichelaar et al., 2000; Fisher et al., 2001). Induction of FGF-7 caused initial epithelial cell hyperplasia followed by adenomatous hyperplasia after dox application. All hyperplasia disappeared after withdrawal of dox (Tichelaar et al., 2000). However, mouse KrasG12D induction caused epithelial cell hyperplasia, adenomatous hyperplasia and, after 2 months dox application, multiple adenomas and AdCAs. Again, no lesion was detected after 1 month of dox withdrawal (Fisher et al., 2001). When the CCSP-rtTA;tetO-KrasG12D alleles were combined with conventional p53 or Ink4a/Arf-null alleles, AdCAs with a more malignant phenotype appeared after 1 month dox treatment, thus showing a synergy of mutant K-ras and p53 or Ink4a/Arf deficiencies. However, even in these compound tet-inducible mouse models, all lesions disappeared after dox withdrawal. This finding demonstrated the importance of mutant K-ras as a “driving” oncogene not only at tumor onset, but also during maintenance of AdCA in these mice (Fisher et al., 2001).

Other models for early, benign lung tumor lesions have been created by using a bitransgenic tet-inducible human KrasG12C allele that can be expressed in both Clara and/or alveolar type II cells (Tichelaar et al., 2000; Floyd et al., 2005). Expression of human KrasG12C caused multiple, small lung tumors over a 12-month time period. Although tumor multiplicity increased upon continued K-ras expression, most lung lesions were hyperplasias or well-differentiated adenomas (Floyd et al., 2005). This is in good contrast to the more severe phenotypes observed in other transgenic mouse models in which different mutant K-ras alleles were expressed in the lung. Expression of K-rasG12C was associated with a 2-fold increase in the activation of the Ras and Ral signaling pathways and increased phosphorylation of Ras downstream effectors, including Erk, p90 ribosomal S6 kinase, ribosomal S6 protein, p38 and MAPKAPK-2. In contrast, expression of K-rasG12C had no effect on the activation of the JNK and Akt signaling pathways explaining low tumor induction by human KrasG12C. This observation was in strong contrast to the effects of the previously described mouse KrasG12D models (Fisher et al., 2001).

3.3. Cre/loxP or Flp/Frt models

The Cre/loxP or Flp/FRT system (Jonkers & Berns, 2002; Lewandoski, 2001; Dutt et al., 2006) provided excellent tools for reproducing more complicated lung tumor genetics found in
human lung cancers, by introducing somatic mutations in a limited number of differentiated cells of choice whereby other cells of the fully developed lung remained normal. In short, mutations of targeted regions, flanked by loxP (also known as being “floxed”) or flippase recombination target (Frt) sequence sites, were introduced through deletion by their respective site-specific recombinases Cre or Flp. Thus, in the case of tumor suppressor genes, conditional hypomorphic mutations (i.e., lower than normal function of the protein) or null allele, several coding or non-coding exons are floxed and can, therefore, be deleted by its corresponding recombinase. Conversely, floxed transcription stops (Lox-Stop-Lox or LSL) in front of oncogene or knock-in alleles can control their respective conditional activation (Jackson et al., 2001) as in the case of LSL KRas<sup>G12D</sup> mice described in the previous section.

The determining factor of this conditional approach is the control of temporal-spatial Cre or FRT recombinase expression. For that purpose, several Cre transgenic lines have been generated, with or without tet-inducible promoters (Perl et al., 2002). Apart from this, Cre-mediated recombination can also be achieved through the administration of an engineered Adeno-Cre virus via nasal or tracheal inhalation (Meuwissen et al., 2001; Jackson et al., 2001). An advantage of the latter method is that a limited amount of adult lung cells can be targeted in a very concise, localized, and timely fashion. Efficacy of this method was tested with conditional alleles of KRas<sup>G12D</sup> and KRas<sup>G12S</sup> (Jackson et al., 2001; Guerra et al., 2003). Infection of adult lungs with Adeno-Cre virus rapidly resulted in the onset of adenomatous alveolar hyperplasia, followed by the development of adenomas and AdCAs at 3-4 months post-infection. Although a latency of 8 months was also observed (Guerra et al., 2003), no metastases could be found in any of the models. Most probably a single K-ras activation is not enough to allow the AdCAs to progress into a higher state of malignancy as would be required for fully metastasizing lesions. However, these straightforward experiments disclosed the important role of K-ras in human lung cancer onset and progression (Guerra et al., 2003). Another important aspect of this model was that lung tumor multiplicity could be controlled by the dose of Adeno-Cre virus infecting only a subset of lung epithelial cells. This, together with a controlled time-point of Adeno-Cre application, mimics sporadic character of human lung cancer development. However, one has to be careful to note that variability of the Adeno-Cre virus delivery and infection (especially with the intranasal method) might lead to inconsistent experimental results. Nevertheless this versatile method remains powerful in that it resembles human lung cancer events.

4. Specific oncogenes in mouse lung cancer models

4.1. Kras downstream effectors and lung cancer – Roles of Raf

Since Kras mutations are very common (20-25%) in NSCLC, the understanding of the precise signaling cascade of the Kras pathway is very important (Ji et al., 2007). One of the best characterized Ras pathways is Ras/Raf/MEK/ERK. In fact, BRAF gene mutations have been found in a variety of human cancers including NSCLC (Davies et al., 2002; Ji et al., 2007). Oncogenic mutations of BRAF render constitutively phosphorylation of the protein, resulting in
continued ERK activation. Of all the BRAF mutations, BRAF-V600E is the most frequent. (Mercer et al., 2003). Dankort et al. (2007) created Brat(CA) (CA: constitutively active) mice to express normal Brat prior to Cre-mediated recombination after which Brat(V600E) was expressed at physiological levels. Brat(CA) mice infected with an Adenovirus expressing Cre recombinase developed benign lung tumors that only rarely progressed to AdCA. The reason for this is the initial proliferation is halted by increased expression of senescence markers p53 and Ink4a/Arf. Consistent with the tumor suppressor function for Ink4a/Arf and p53, Brat(V600E) expression combined with mutation of either locus led to lung cancer progression. Moreover, Brat(VE)-induced lung tumors were prevented by pharmacological inhibition of MEK1/2.

In another study, Ji et al generated a lung-specific, tet-inducible, mice model in which the CCSP-rtTA;tetO-BRAFV600E induced a development of lung AdCA with bronchioalveolar carcinoma type. The extracellular signal-regulated kinase (ERK)-1/2 (MAPK) pathway was highly activated by the expression of BRAF(V600E) mutant. Upon dox withdrawal, the deinduction of BRAF-mutant expression led to regression of lung tumors together with a marked decrease in phosphorylation of ERK1/2. Furthermore, the in vivo use of a specific MAPK/ERK kinase (MEK) inhibitor also induced lung tumor regression. All these results showed that both activated BRAF and KRAS signaling converge onto the same MAPK pathway, making this pathway a potential target for lung tumor intervention.

The significance of c-Raf was also investigated in K-RasG12V-driven NSCLCs. Ablation of c-Raf in K-RasG12V; c-Raflox/lox mice induced dramatic increase of survival rate and life span due to the decrease of tumor burden. This result suggests the essential role of c-Raf in mediating oncogenic Ras signaling in NSCLCs (Blasco et al., 2011).

Further investigation during KrasG12D-driven lung tumorigenesis showed the MAPK antagonist Sprouty-2 (Spry-2) was upregulated. When Spry-2 was knocked out in Cre/lox dependent Spry-2lox/lox; LSL KrasG12D mice, both tumor number and total tumor area were significantly increased. This clearly suggested a tumor suppressor activity for Sprouty-2 during Kras-dependent lung tumorigenesis by involving in antagonism of Ras/MAPK signaling (Shaw et al., 2007).

By using CCSP-rtTA;TetO-Cre;LSL-Kras(G12D) mice Cho et al. (2011) established a dox-inducible, Kras(G12D)-driven lung AdCA to pursue the cellular origin and molecular processes involved in Kras-induced tumorigenesis. The EpCAM(+)MHCII(-) cells (bronchial origin) were more enriched with tumorigenic cells in generating secondary tumors than EpCAM(+)MHCII(+) cells (alveolar origin). In addition, secondary tumors derived from EpCAM(+)MHCII(+) cells showed diversity of tumor locations compared with those derived from EpCAM(+)MHCII(-) cells. Secondary tumors from EpCAM(+)MHCII(+) cells expressed differentiation marker, pro-SPC, consistent with the notion that cancer-initiating cells display not only the abilities for self-renewal, but also the features of differentiation to generate tumors of heterogeneous phenotypes. High level of ERK1/2 activation and colony-forming ability as well as lack of Sprouty-2 expression were also observed in EpCAM(+)MHCII(-) cells. Their data suggested that bronchiolar Clara cells are the origin of tumorigenic cells for Kras(G12D)-induced lung cancer.
Another important pro-survival pathway that is interlinked with RAS is PI3K/Akt signaling pathway. Phosphoinositide-3-kinase (PI3K) consists of a regulatory (p85) and a catalytic (p110) subunit. The overexpression of both subunits was reported in lung carcinomas (Samuels & Velculescu 2004; Wojtalla et al., 2011). Furthermore, selective PIK3CA amplification was found in lung squamous cell carcinomas (Angulo et al., 2008). To investigate the oncogenic potential of PIK3CA, transgenic mice were generated with a tet-inducible expression of an activated p110α mutant, H1047R, and it was crossed with CCSP-rtTA mice to generate CCSP-rtTA;tetO;PIK3CA(H1047R) compound mice. Upon dox treatment of animals for 14 weeks, double transgenic mice developed AdCAs, which subsequently disappeared after dox withdrawal for 3 weeks (Engelman et al., 2008). To identify the effect of loss of PI3K signaling in Kras-induced lung tumorigenesis, PI3K activity was completely eliminated in p85 knockouts (Pik3r2−/−;Pik3r1−/−), and a dramatic decrease in the number of lung tumors was observed in LSL KrasG12D;Pik3r2−/−;Pik3r1−/− mice (Engelman et al., 2008). The clinical efficacy of NVP-BEZ235, a dual pan-PI3K and mammalian target of rapamycin (mTOR) inhibitor was also evaluated against p110α H1047R-induced mouse lung tumors. Application of this drug led to marked tumor regression. In contrast, NVP-BEZ235 barely had effect on mouse lung cancers driven by mutant Kras. However, a combination of NVP-BEZ235 and a MEK inhibitor ARRY-142886, had marked synergistic effect on tumor regression. These in vivo studies suggest that inhibitors of the PI3K-mTOR pathway when combined with MEK inhibitors, may effectively treat KRAS mutated lung cancers. Of note, Ras proteins directly interact with the p110α subunit of PI3K and introduction of specific mutations (T208D and K227A) in PIK3CA blocks this interaction (Gupta et al., 2007). To study the Ras-p110α interactions in vivo and its effects on tumorigenesis, these point mutations were introduced into the Pik3ca gene in the mice and these mice were crossed with KrasLA2 alleles (Gupta et al., 2007). Interestingly, they were highly resistant to Kras induced lung tumor development, which suggest Ras-p110α interaction is required for Ras-driven tumorigenesis (Gupta et al., 2007). All these results emphasize the importance of PI3K signaling, not only in lung tumor induction, but also maintenance.

Rac is a member of the Rho family of small GTPases, and it mediates the regulation of various important cellular processes including cell migration, proliferation and adhesion, all of which may contribute to tumorigenesis (Mack et al., 2011). The important role of Rac in Ras induced lung tumorigenesis was demonstrated in a mouse model in which an oncogenic allele of Kras was activated by Cre-mediated recombination in the presence or absence of conditional deletion of Rac1. They showed that Rac1 function was required for tumorigenesis in lung carcinogenesis for mice with Rac1 deletion had tumor regression and longer survival. These data showed a specific requirement for Rac1 function in cells expressing oncogenic Kras (Kissil et al., 2007).
4.4. Receptor-type protein tyrosine kinase and lung cancer – Roles of EGFR

4.4.1. EGFR and lung cancer

Epidermal growth factor (EGF) receptor family is one type of RTKs, on which the tyrosine residues phosphorylation lead to activation of downstream TK signaling that contributes to cell proliferation, motility and invasion (Stella et al., 2012). The activation mutations on EGFR gene are found in about 10-20% of advanced NSCLC cases and its protein overexpression is found in more than 60% of all lung cancers (Lynch et al., 2004; Soria, et al., 2012). Lynch et al. reported that EGFR mutation correlated with clinical responsiveness to the tyrosine kinase inhibitor gefitinib (2004). Since these mutations lead to increased growth factor signaling with susceptibility to the inhibitor, screening for such mutations in lung cancers will identify patients who will have a response to gefitinib. To study a specific oncogenic potential of EGFR mutant, the variant III (vIII) deletion, Ji et al. (2006a) produced Tet-op-EGFRvIII; CCSP-rtTA mice, in which the EGFRvIII expression was induced in lung type II pneumocytes upon dox administration. Mice developed atypical adenomatous hyperplasia after 6-8 weeks of dox induction and progressed to lung adenocarcinomas after 16 weeks with high activation of AKT and ERK signaling pathways. De-induction of EGFRvIII resulted in significant tumor regression, supporting the requirement of continuous EGFRvIII expression in lung tumorigenesis. Furthermore, by using an EGFR/ERBB2 inhibitor HKI-272, they found tumor volume in EGFRvIII; CCSP-rtTA; Ink4a/Arf−/− mice was dramatically decreased, suggesting a therapeutic strategy for lung cancers with EGFRvIII mutation by an irreversible EGFR inhibitor (Ji et al., 2006a). Politi et al. (2006) also studied the role of EGFR mutations in the initiation and maintenance of lung cancer, and developed transgenic mice that express an exon 19 deletion mutant (EGFR(ΔL747-S752)) or the L858R mutant (EGFR(L858R)) in type II pneumocytes under the control of dox, and reported that expression of either EGFR mutant lead to the development of lung AdCa. Ji et al. (2006b) later created bitransgenic mice with inducible expression in type II pneumocytes of two common hEGFR mutants (hEGFRDEL and hEGFRL858R) seen in human lung cancer. Both bitransgenic lines developed lung AdCa with hEGFR mutant expression, confirming their oncogenic potential. Maintenance of transformed phenotypes of these lung cancers was dependent on sustained expression of the EGFR mutants. Treatment with small molecule inhibitors (erlotinib or HKI-272) as well as a humanized anti-hEGFR antibody (cetuximab) led to dramatic tumor regression (Ji et al., 2006b). Thus persistent EGFR signaling is required for tumor maintenance in human lung AdCas expressing EGFR mutants. Li et al. (2007) generated another dox-inducible lung cancer mice model harboring both erlotinib sensitizing and resistance mutations L858R and T790M (EGFR TL). They found that specific expression of EGFR TL in lung compartments led to the development of typical bronchioalveolar carcinoma after 4-5 weeks and peripheral adenocarcinoma after 7-9 weeks. Treatment of EGFR TL-driven tumors is most effective when using combined regimen of HKI-272 and rapamycin, suggesting that this combination therapy may benefit patients harboring erlotinib resistance EGFR mutation (Li et al., 2007).
4.5. HER2 and lung cancer

The c-ERBB2 gene is located on chromosome 17q11.2-12 and encodes Human Epidermal Growth Factor Receptor 2 (HER2) (Hu et al., 2011). This is a transmembrane glycoprotein receptor p185HER2, which has been targeted by the humanized monoclonal antibody trastuzumab (Herceptin). HER2 is amplified and overexpressed in approximately 25% of breast cancer patients and is associated with an aggressive clinical course and poor prognosis. HER2 protein overexpression without gene amplification happens in some cases, possibly due to promoter activation and/or protein stabilization. HER2 overexpression stimulates cell growth in p53-mutated cells while it inhibits cell proliferation in those with wild-type p53. The molecular mechanisms for these differential responses have recently been clarified: the Dmp1 promoter was activated by HER2/neu through the PI3K-Akt-NF-κB pathway, which in turn stimulated Arf transcription and p53 activation to prevent tumorigenesis. Conversely HER2 simply stimulate cell proliferation in cells that lack Dmp1, Arf, or p53 (Taneja et al., 2010).

HER2 receptor overexpression has been reported in 11% to 32% of NSCLC tumors, with gene amplification found in 2%-23% of cases (Hirsch et al., 2009; Swanton et al., 2006). High-level ERBB2 amplification occurs in a small fraction of lung cancers with a strong propensity to high-grade adenocarcinomas (Grob et al., 2012). The frequency of HER2 amplification in NSCLC and the widespread availability of HER2 fluorescence in situ hybridization analysis may justify a study of trastuzumab monotherapy in NSCLC cases. However, sensitivity to HER2-directed therapies is complex and involves expression not only of HER2, but also of other EGFR family members (HER1, HER2, and HER4), their ligands, and molecules that influence pathway activity (Swanton et al., 2006). The role played by HER2 as a heterodimerization partner for other EGFR family members makes HER2 an attractive target regardless of receptor overexpression in lung cancer. However, targeted therapies in patients overexpressing HER2 have proven less successful in clinical trials for NSCLC. One reason to explain the failure is intratumoral heterogeneity of ERBB2 amplification, which was found in 4 of 10 cases (Grob et al., 2012). Of note, this heterogeneity is rare in breast cancer that responds relatively well to anti-HER2 therapy. Laboratory data indicate that forced expression of HER2 in a NSCLC line increases sensitivity to gefitinib. They speculated that this may result from the gefitinib-mediated inhibition of HER2/HER3 heterodimerization and HER3 phosphorylation. It might thus be expected that combinatorial approaches, such as EGFR inhibition (by gefitinib) together with HER2 dimerization blockade (by pertuzumab) may be even more effective. Preclinical data indicate this may be the case, with the combination of erlotinib and pertuzumab promoting more than additive antitumor activity in the NSCLC (Swanton et al., 2006).

While HER2 is overexpressed in about 20% of lung cancers, mutations in HER2 also occur in about 2-3% of cases. HER2 mutations typically occur in adenocarcinomas and are more frequent in women and never-smokers (Pinder, 2011). Mutations in HER2 lead to constitutive activation of the HER2 receptor, similar to the situation with EGFR. In good contrast to what we experienced in breast cancer, early clinical trials of Herceptin combined with chemotherapy in lung cancer patients with HER2 overexpression did not show a benefit for patients. However, there are case reports of lung cancer with HER2 mutations who have responded...
well to treatment with Herceptin plus chemotherapy. For instance, BIBW2992 (a small molecule inhibitor of EGFR and HER2) has shown evidence of activity in lung cancer patients with HER2 mutations. Most of the patients described had cancers that had shown resistance to chemotherapy and/or EGFR inhibitors. More patients with SCLC should be screened for HER2 mutations since the number of patients described to date is too small to draw any definitive conclusions (Pinder, 2011).

4.6. Cyclin D1 and lung cancer

The development of human lung carcinogenesis is very complex. Several oncogenes involved in this process have been identified, one of which is cyclin D1 (Meuwissen & Berns, 2005). Cyclin D1 is a crucial regulator in mammalian cell cycle, which drives cells to enter S phase by binding and activating CDK4/6. The cyclin D1/CDK4 complex phosphorylates the retinoblastoma protein (pRb), which releases E2F transcriptional factors from pRb constraint. The E2Fs can then activate genes that are required for the cell to enter S phase (Sherr, 1996, 2004). Cyclin D1 overexpression results in deregulation of phosphorylation of pRB, which can cause loss of growth control. In fact, Cyclin D1 gene and protein products are frequently overexpressed in a wide range of cancers. In NSCLC, the CCND1 locus at 11q13 is amplified in up to 32% of cases, and its protein is expressed at high level in average of 45% of all cases (Gautschi et al., 2007).

The ability of cyclin D1 to cause malignant transformation has been demonstrated in breast cancer transgenic mice model, in which MMTV-Cyclin D1 transgenic mice developed mammary AdCA (Wang et al., 1994). Just like in breast cancer, CCND1 is often found amplified and overexpressed in NSCLC patients. It has been shown that cyclin D1 overexpression is a marker for an increased risk of upper aerodigestive tract premalignant lesions for progressing to cancer (Kim et al., 2011). A polymorphism, G/A870, has been identified in the CCND1 gene and it results in an aberrantly spliced protein (Cyclin D1b) lacking the Thr-286 phosphorylation site necessary for nuclear export (Diehl et al., 1997). It has been shown that the MMTV-D1T286A (analogous to Cyclin D1b in humans) mice developed mammary AdCAs at an increased rate relative to MMTV-D1 mice. Even though cyclin D1b was detected in all NSCLC samples, and the G/A870 polymorphism in CCND1 gene is predictive of the risk of lung malignancy (Gautschi et al., 2007), its impact on lung carcinogenesis has never been investigated. Thus creation of mouse models for aberrant cyclin D1 expression in lung epithelial tissue is needed to test whether it is a key factor in the development of lung carcinogenesis.

Cancer chemoprevention uses dietary or pharmaceutical agents to suppress or prevent carcinogenic progression to invasive cancer. In a recent study, it was shown that a combination of retinoid bexarotene and EGFR inhibitor erlotinib can suppress lung carcinogenesis in transgenic lung cancer cells as well as NSCLC patients in both early and advanced stages. Bexarotene can induce the proteasomal degradation of cyclin D1 and erlotinib can act as an inhibitor of EGFR which represses transcription of cyclin D1 (Kim et al., 2011). This finding implicates cyclin D1 as a chemopreventive target and the combination of bexarotene and erlotinib is an attractive candidate for lung cancer chemoprevention (Dragnev et al., 2011).
fore using this regimen in clinical lung cancer chemoprevention, its activity should first be tested in clinically predictive cyclin D1 mouse lung cancer models.

4.7. PTEN and lung cancer

Since expression of phosphatase and tensin homologue deleted from chromosome 10 (PTEN; reviewed in Inoue et al., 2012) is often down-regulated in NSCLC, several mice models have been generated in which Pten was inactivated in the bronchial epithelium (Yanagi et al., 2007; Iwanaga et al., 2008). PTEN is a tumor suppressor gene that acts by blocking the PI3K dependent activation of serine-threonine kinase Akt (Inoue et al., 2012). Since Pten-/- mice are embryonic lethal, one had to make use of floxed Pten alleles (Ptenfl/fl), combined with CCSP-Cre transgene, targeting Pten deletion into bronchial epithelial cells. However, these Ptenfl/fl,CCSP-Cre mice did not show any aberrant pulmonary development or phenotypic abnormalities even when mice were followed for more than 12 months (Iwanaga et al., 2008). This changed dramatically when the Ptenfl/fl,CCSP-Cre alleles were crossed with LSLKrasG12D. Lung tumor development was markedly accelerated compared in Pten-/-;KrasG12D mice to that of single LSLKrasG12D mice. Pten-deficient, Kras mutant tumors were often of the more advanced AdCA with higher vascularity (Iwanaga et al., 2008), suggesting that Pten-loss cooperates with Kras mutations in NSCLC. Contrary to these results were the findings of another study in which Pten-inactivation was targeted in bronchioalveolar epithelium with SPC-rtTA;tetO-/-Cre (Yanagi et al., 2007). When dox was applied in utero at E10-16 during embryogenesis, most mice died post-natally from hypoxia. Their lungs showed an impaired alveolar epithelial cell differentiation with an overall lung epithelial cell hyperplasia. The few surviving mice developed spontaneous lung AdCAs. Post-natal dox application during P21-27 resulted in a mild bronchiolar and alveolar cell hyperplasia and increased cell size but no lethality. A majority of these animals developed AdCAs in comparison to WT controls. Prior addition of urethane induced an even higher amount of AdCAs. Interestingly, most Pten-/- AdCAs (33%), with or without urethane addition, showed spontaneous Kras mutations. The latter observation again indicates the importance of Kras activity in cooperating with Pten-loss during NSCLC development.

4.8. LKB1 and lung cancer – A novel player

Mutations in liver kinase B1 (LKB1) are found in Peutz–Jeghers syndrome (PJS) patients and are characterized by intestinal polyps (hamartoma) and increased incidence of epithelial tumors, such as hamartomatous polyps in the gastrointestinal tract, as well as breast, colorectal, and thyroid cancers (Giardiello et al., 2000). It is a serine threonine kinase also known as STK11 (Sanchez-Cespedes et al., 2002). LKB1 is a primary upstream kinase of adenine monophosphate-activated protein kinase (AMPK), a necessary element in cell metabolism that is required for maintaining energy homeostasis. It is now clear that LKB1 exerts its growth suppressing effects by activating a group of other ~14 kinases, creating a group of AMPK and AMPK-related kinases. Activation of AMPK by LKB1 suppresses cell growth and proliferation when energy and nutrient levels are low. The LKB1 gene has been implicated in the regulation of multiple biological processes, signaling pathways (Wei et al., 2005), and tu-
morigenesis. It has been reported that LKB1 directly activates AMP-activated kinase and regulates apoptosis in response to energy stress (Shaw et al., 2004).

A large fraction of NSCLC cells have germ-line mutations and impaired expression of LKB1. LOH for LKB1 has been reported in more than 50% in lung cancer (Makowski & Hayes, 2008) and thus LKB1 inactivation is a common event for NSCLC (Sanchez-Cespedes et al., 2002, Sanchez-Cespedes, 2007). The highest numbers of mutations were found in AdCAs, especially in those with KRAS mutations (Matsumoto et al., 2007; Sanchez-Cespedes, 2007). LKB1 inactivation cooperates with KRAS activation, suggesting a role for LKB1 as an active repressor of the KRAS downstream pathway (Ji et al., 2007).

$Lkb1^{lox/lox}; LSLKras^{G12D}$ mice showed a broad spectrum of NSCLCs: the majority of lung tumors were AdCAs, but SqCLCs and large cell carcinoma (LCLC) also occurred. Conversely, no SqCLC or LCLC was detected in $p53^{lox/lox}; LSLKras^{G12D}$ and $(Ink4a/Arf)^{lox/lox}; LSLKras^{G12D}$ mice. Furthermore, 61% of AdCA in $Lkb1^{lox/lox}; LSLKras^{G12D}$ mice developed metastases, but none found for SqCLC and LCLC. These results show that LKB1-loss permits squamous differentiation and facilitates metastases, but these two are independent events. AdCA from $Lkb1^{lox/lox}; LSLKras^{G12D}$ mice had reduced pAMPK (phosphorylated, adenosyl monophosphate-activated protein kinase) and pACCA (phosphorylated, acetyl-CoA carboxylase α-subunit) levels and activated mTOR pathway. It is probable that LKB1-loss influences differentiation of NSCLC into subtypes by affecting discrete pathways (Shah et al., 2008). A large panel of human NSCLC showed LKB1 mutations in AdCA (34%), SqCLC (19%), and LCC (16%) (Ji et al., 2007). Simultaneous mutations in $p53$ or LKB1 suggest non-overlapping roles in NSCLC. Moreover, reconstitution of LKB1 in human NSCLC cell lines showed anti-tumor effects independent of their $p53$ or $INK4A/ARF$ status (Ji et al., 2007). Finally, loss of LKB1 expression in alveolar adenomatous hyperplasia, precursor lesion for AdCA, suggests an early role of LKB1-inactivation during AdCA development (Ghaffar et al., 2003).

The same group conducted a mouse trial that mirrors a human clinical trial in patients with KRAS-mutant lung cancers (Chen et al., 2012). They demonstrated that simultaneous loss of either $p53$ or $Lkb1$, strikingly weakened the response of Kras-mutant cancers to single therapy by docetaxel. Addition of selumetinib provided substantial benefit for mice with lung cancer caused by Kras and Kras and $p53$ mutations, but not in mice with Kras and Lkb1 mutations (Chen et al., 2012). Thus synchronous ‘clinical’ trials performed in mice, not only will be useful to anticipate the results of ongoing human clinical trials, but also to generate clinically-relevant hypotheses that will affect the analysis and design of human studies.

4.9. miRNAs and lung cancer

Not only might genetic mutations in oncogenes and tumor suppressor genes affect their target gene expression during lung tumorigenesis, but also microRNAs (miRNAs) can also perform similar roles. microRNAs are evolutionarily conserved, endogenous, non-protein coding, 20–23 nucleotide, single-stranded RNAs that negatively regulate gene expression in a sequence-specific manner. In order to become active, small interfering RNA (siRNA) must undergo catalytic cleavage by the RNase DICER1. In human lung cancer, increased activities of DICER1 and variant regulations of miRNA clusters have been observed. For the latter, a
frequent down regulation of the let-7 miRNA family as well as an upregulation of miR-17-92 have been reported (Hayashita et al., 2005). miR-17-92 encodes a cluster of seven miRNAs transcribed as single primary transcript. To date, functional analyses of Dicer1 and let-7 have been performed in the background Kras-induced NSCLC models. A conditional deletion of Dicer1 in the background of LSLKrasG12D;Dicer1fl/fl mice led to a marked increase of tumor development (Kumar et al., 2007). However, since the 3′ UTR region of Kras transcripts has been shown to be a direct target of let-7 (Johnson et al., 2005), it has become very tempting to increase let-7 expression in KrasG12D lung tumors. Let-7 inhibits the growth of multiple human lung cancer cell lines in culture, as well as the growth of lung cancer cell xenografts in vivo. Intranasal application of both adenoviral (Esquela-Kerscher et al., 2008) and lentiviral (Kumar et al., 2008) let-7 miRNA caused a significant decrease of KrasG12D;p53−/− lung tumors. These findings provide direct evidence that let-7 acts as a tumor suppressor gene in the lung and indicate that this miRNA might be useful as a novel therapeutic agent in lung cancer.

A large scale survey conducted by a different group to determine the miRNA signature of >500 lung, breast, stomach, prostate, colon, and pancreatic cancers and their normal adjacent tissue revealed that miR-21 was the only miRNA up-regulated in all these tumors (Volinia et al., 2006). Functional studies in cancer cell lines suggest that miR-21 has oncogenic activity. Knockdown of miR-21 in cultured glioblastoma cells activated caspasps leading to apoptotic cell death, suggesting miR-21 is an anti-apoptotic factor (Chan et al., 2005). In MCF-7 cells, miR-21 knock-down resulted in suppression of cell growth both in vitro and in vivo (Si et al., 2007). Knock-down of miR-21 in the breast cancer cells reduced invasion and metastasis (Zhu et al., 2008). Targeted deletion of miR-21 colon cancer cells resulted in tumorigenesis through compromising cell cycle progression and DNA damage-induced checkpoint function by targeting Cdc25a (Wang et al., 2009). miR-21 expression is increased and predicts poor survival in NSCLC. Hatley et al. used transgenic mice with loss-of-function and gain-of-function miR-21 alleles combined with a model of NSCLC (K-rasLA2) to determine the role of miR-21 in lung cancer (Hatley et al., 2010). They showed that overexpression of miR-21 enhances lung tumorigenesis and that genetic deletion of miR-21 protects against tumor formation. miR-21 drives tumorigenesis through inhibition of negative regulators of the Ras/MEK/ERK pathway and inhibition of apoptosis (Hatley et al., 2010). These studies indicate that knocking-down of miR-21 expression in cancer cells results in phenotypes important for tumor biology.

Hennesssey et al. (2012) conducted Phase I/II biomarker study to examine the feasibility of using serum miRNA as biomarkers for NSCLC. Examination of miRNA expression levels in serum from a multi-institutional cohort of 50 subjects (30 NSCLC patients and 20 healthy controls) identified differentially expressed miRNAs. They found that 140 candidate miRNA pairs distinguished NSCLC from healthy controls with a sensitivity and specificity of at least 80% each. Several miRNA pairs involving miRNAs-106a, miR-15b, miR-27b, miR-142-3p, miR-26b, miR-182, 126#, let7g, let-7i (described above) and miR-30e-5p exhibited a negative predictive value and a positive predictive value of 100%. Notably, a combination of two differentially expressed miRNAs miR-15b and miR-27b, was able to discriminate NSCLC from...
healthy volunteers with high sensitivity, specificity (Hennessey et al., 2012). Upon further testing on additional 130 subjects, this miRNA pair predicted NSCLC with a specificity of 84%, sensitivity of 100%. These data provide evidence that serum miRNAs have the potential to be sensitive, cost-effective biomarkers for the early detection of NSCLC.

5. Mouse models for squamous cell lung cancer (SqCLC)

So far genomic alterations in SqCLC have not been comprehensively characterized. The Cancer Genome Atlas group recently profiled 178 lung squamous cell carcinomas to provide a comprehensive view of genomic and epigenomic alterations (Hammerman et al., 2012). They showed that the SqCLC is characterized by hundreds of exonic mutations, genomic rearrangements, and gene copy number alterations. In addition to TP53 mutations found in nearly all specimens, loss-of-function mutations were found in the HLA-A class I gene. In addition, Nuclear factor (erythroid-derived 2)-like 2, Kelch-like ECH-associated protein 1, Squamous differentiation, and Phosphatidylinositol-3-OH kinase pathway genes were frequently altered. CDKN2A and RB1 genes were inactivated in as many as 72% of SqCLC cases. This comprehensive study identified a potential therapeutic target in most tumors, offering new avenues of investigation for the treatment of human SqCLC (Hammerman et al., 2012).

Although squamous cell carcinoma is a common type of lung cancer causing nearly 400,000 deaths per year worldwide, there is no established gene-engineered mouse model for squamous cell carcinoma of the lung. Human lung SqCLC is closely linked with smoking and shows a distinct order of pre-malignant changes in the bronchial epithelium from hyperplasia, metaplasia, dysplasia and carcinoma in situ to invasive and metastatic SqCLC (Brambilla et al., 2000). A better understanding of the cell of origin that give rise to SqCLC and identification of unique genetic alterations that are specific to lung squamous cell carcinoma as reported by the comprehensive study might help to create SqCLC mouse models. One important issue that should be taken into account is that normal human or mouse lungs do not contain squamous epithelium. Mice do not smoke, so only under pathological conditions does squamous metaplasia accompanied by high expression levels of keratins occur in the airway epithelium (Wistuba et al., 2002, 2003). Only a few mouse models reported the onset of SqCLC, mostly after carcinogen application. For instance, intratracheal intubation of methyl carbamate (Jetten et al., 1992) or extensive topical application of N-nitroso-compounds (Nettesheim et al., 1971; Rehm et al., 1991) caused SqCLC in mice. Wang et al. (2004) treated eight different inbred strains of mice with N-nitroso-tris-chloroethylurea by skin painting and found that this chemical induced SqCLCs in five strains (SWR, Swiss, A/J, BALB/c, and FVB), but not in the others (AKR, 129/svJ, and C57BL/6). Besides, specific loci for SqCLC susceptibility have been identified through linkage analyses in several mouse strains (Wang et al., 2004), using 6,128 markers in publically available databases. Three markers (D1Mit169, D3Mit178, and D18Mit91) were found significantly associated with susceptibility to SqCLC. Interestingly, none of these sites overlapped with the major susceptibility loci associated with lung adenoma/adenocarcinomas in mice indicating that different
sets of genes are responsible for SqCLC and AdCA. Their model can be used in determining genetic modifiers that contribute to susceptibility or resistance to SqCLC development.

The other group tried to induce SqCLC through constitutive expression of human K14 by creating CC10-hK14 mice (Dakir et al., 2008). Although hK14 is highly expressed in bronchial epithelium, only precursor lesions varying from hyperplasia to squamous metaplasia were observed (Dakir et al., 2008). Clearly, the increased K14 expression and onset of squamous cell metaplasia alone was not sufficient to generate fully developed SqCLC. As far as transgenic/knockout mice models are concerned, only the LSLKras<sup>G12D;</sup>Lkb1<sup>flox/flox</sup> somatic mouse model has been able to generate advanced SqCLC. By using a somatically activatable mutant Kras-driven model of mouse lung cancer (K-ras<sup>L</sup>), Ji et al. (2007) compared the role of Lkb1 to other tumor suppressors in lung cancer. Although Kras mutation cooperated with loss of p53 or Ink4a/Arf in this system, the strongest cooperation was seen with homozygous inactivation of Lkb1. Lkb1-deficient tumors demonstrated shorter latency, an expanded histological spectrum (adeno-, squamous, and large-cell carcinoma) and more frequent metastasis as compared to tumors lacking p53 or Ink4a/Arf. Interestingly up to 60% of Lkb1 deficient lung tumors had squamous or mixed squamous histology (Ji et al., 2007), which has not been reported in other mouse lung cancer models. Pulmonary tumorigenesis was also accelerated by hemizygous inactivation of Lkb1, confirming its haplo-insufficiency. Consistent with these findings, inactivation of LKB1 was found in 34% and 19% of 144 human lung adenocarcinomas and squamous cell carcinomas, respectively. They also identified a variety of metastasis-promoting genes, such as NEDD9, VEGFC and CD24, as targets of LKB1 repression in lung cancer. These studies established LKB1 as a critical barrier to prevent lung carcinogenesis, controlling initiation, differentiation and metastasis (Ji et al., 2007).

6. Clinical implications and future directions for mouse lung cancer models

Xenograft models where manipulated human lung cancer cell lines are subcutaneously injected into nude mice have been extensively used for pre-clinical testing of novel drugs for lung cancer. The major issue for this approach is that lung cancer cell lines have already been adapted for long-term culture in a plastic dish with artificial medium and acquired stem-cell like phenotypes, and thus are not suitable for models of primary human lung cancer obtained by surgical resection. The more preferred method, however, have been orthotopic transplantation of human lung tumor cells in their lung cavity. To date, the results have shown that xenograft models do not accurately predict the clinical efficacy of anti-tumor drugs. Therefore, a question arises as to whether spontaneous and/or genetically-engineered mouse models for lung cancer would be more useful as tools for pre-clinical drug tests. It is obvious that there are differences in the lung anatomy and physiology between mice and humans, but some of the mouse models that we have described have a striking histological similarity, with an analogous genetic signature to that of human NSCLC. Importantly, genetically-engineered mouse model-derived tumors develop in an innate immune
environment and, therefore, have all the tumor-stromal interactions, such as angiogenesis and degradation of the tissue matrix.

We have described two models for NSCLC in which either the continuous oncogenic activity of Kras (Fisher et al., 2001) or EGFR (Politi et al., 2006) are prerequisites of tumor maintenance since lung tumors underwent spontaneous regression with disappearance of the oncogene by dox withdrawal. This not only shows that tumor growth critically depends on the initiating active oncogenic pathways, but it also stresses the usefulness of these oncogenic pathways as therapeutic targets. Direct tumor intervention studies with tyrosine kinase inhibitors against EGFR mutations proved to be highly effective in several hEGFR-transgenic mouse models. TKIs such as gefitinib, erlotinib, and HKI-272 led to complete tumor regression (Politi et al., 2006; Ji et al., 2006a,b). In addition, treatment of lung cancer with humanized anti-hEGFR antibody (cetuximab) caused a significant tumor regression (Ji et al., 2006a). Further studies will be needed to investigate the signaling cascades that determine the sensitivity and resistance to EGFR-related tyrosine kinase interventions.

Other mouse models for NSCLC have also been used for targeted therapies. First, dox-induced overexpression of the PI3K p110α catalytic subunit PIK3CA, mutated in its kinase domain (H1047R) in CCSP-rtTA;tetO-PIK3CA(H1047R) mice, induces adenocarcinomas (Engelman et al., 2008). Treatment of these lung tumors with NVP-BEZ235, a dual pan-PI3K and mammalian target of rapamycin (mTOR) inhibitor, caused a marked lung tumor regression. Interestingly, when this single agent NVP6-BEZ235 was tested on lung tumors in CCSP-rtTA;tetO-KrasG12D mice, no regression was observed. However, when NVP-BEZ235 was combined with MEK inhibitor ARRY-142886, significant regression of KrasG12D tumors occurred (Engelman et al., 2008). Thus, two major RAS downstream effector pathways needed to be inactivated to get an irreversible regression in Ras mutated NSCLC.

Although K-RAS is mutated in ~30% of human NSCLC, direct targeting of RAS has been unsuccessful for lung cancer therapy. Many small molecules against Ras functions have been tested and farnesyl transferase inhibitors are the most marked examples of these failed attempts (Mahgoub et al., 1999; Omer et al., 2000). Recent results with lung cancer mouse models strongly suggest that KRAS4A, and not KRAS4B is driving the onset of NSCLC. An explanation for this failure can thus be attributed to the fact that only KRAS4B is farnesylated, but not its isoform KRAS4A. Although we still have to study if KRAS4A is important in the pathogenesis of human NSCLC, we can imagine the importance of Kras mouse models in testing functional inhibitors for KRAS4A (To et al., 2008).

The use of optimized, genetically-modified mouse models for lung cancer for therapy research necessitates sophisticated non-invasive tools to follow tumor development and response to therapy in vivo. Measurement of tumor size as a function of time is the most obvious way of doing this and existing techniques such as computed-tomography imaging or magnetic resonance imaging for small animals are now in use (Engelman et al., 2008; Politi et al., 2006). However, these techniques are time-consuming and expensive, making them less suitable for large number of animals. Other techniques, such as fluorescence imaging and bioluminescence, can be used for measuring gene expression or tumor growth in vivo.
(Contag et al., 2000; Hadjantonakis et al., 2003). In case of latter studies, transgenic expression of luciferase allows accurate longitudinal monitoring and good quantification of tumor burden as has been shown in the LSL Kras lung tumor model (Jackson et al., 2001). These novel imaging techniques will greatly enhance the accuracy and reproducibility of mouse models.

Transgenic lung cancer models created by Chen et al. (2002) can be applied to clinics by raising Ron-specific antibodies. O’Toole et al. (2006) conducted an antibody phage display library to generate a human IgG1 antibody IMC-41A10 that binds with high affinity to RON and effectively blocks interaction with its ligand, macrophage-stimulating protein. They found IMC-41A10 to be a potent inhibitor of receptor and downstream signaling, cell migration, and tumorigenesis. It antagonized MSP-induced phosphorylation of RON, MAPK, and AKT in several cancer cell lines. In NCI-H292 lung cancer xenograft tumor models, IMC-41A10 inhibited tumor growth by 50% to 60% as a single agent. This antibody should be tested in vivo using the SPC-RON mice with developing lung AdCAs.

Recent strategies showed the importance of aberrant promoter methylation in lung cancer development, such as a p16\(^{INK4a}\), Death-associated protein kinase 1, and, RAS association domain family 1A (Shames et al., 2006). Since chronic inflammations have been implicated in cancer pathogenesis (Shacter & Weitzman, 2002), altered methylation for lung surfactant proteins are good topics for future lung cancer studies; their signatures may serve as valuable markers in lung cancer detection. The lung surfactant protein (SP) genes, SP-A and SP-D have been identified with high throughput approach that showed an altered methylation pattern in lung cancer compared to normal lung tissue (Vaid & Floros, 2009). However, SP-A-deficient mice were able to survive with no apparent pathology in a sterile environment (Korfhagen et al., 1996), although their pulmonary immune responses were insufficient during immune challenge. SP-D-deficient mice, on the other hand, showed phenotypic abnormalities in alveolar macrophages and type II pneumocytes with increased lipid pools, indicating that SP-D has an important role in surfactant homeostasis (Botas et al., 1998). Paradoxically overexpression of SP-A and/or SP-D as a result of promoter hypomethylation has also been reported in lung cancer suggesting that it is critical to keep these protein levels within physiological ranges to prevent neoplastic transformation. Since the role of these lung surfactant proteins in lung carcinogenesis has never been studied in vivo, it will be worthwhile to cross lung surfactant-deficient mice with available transgenic/knockout strains to elucidate the roles of surfactant proteins in lung cancer initiation and development.

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