Eur Respir J 2010; 35: 426–443
DOI: 10.1183/09031936.00124709


**SERIES "LUNG CANCER"**
**Edited by C. Brambilla**
**Number 11 in this Series**

# Progress and applications of mouse models for human lung cancer

**S. de Seranno and R. Meuwissen**

**ABSTRACT: The continued progress of modelling lung cancer in mice has led not only to new means of understanding the molecular pathways governing human lung cancer, but it has also created a vast reservoir of alternative tools to test treatments against this malignancy. More sophisticated somatic mouse models for nonsmall cell lung cancer, small cell lung cancer and pulmonary squamous cell carcinoma have been generated that closely mimic human lung cancer. These models enable us to identify the cells of origin and the role of stem cells in the maintenance of the various types of lung cancer. Moreover, results of lung cancer intervention studies are now starting to reveal the full potential of these somatic mouse models as powerful pre-clinical models.**

**KEYWORDS: Lung cancer, mouse models, nonsmall cell lung cancer, small cell lung cancer**

Progress in whole genome approaches to detect genetic alterations in human lung cancer has resulted in the identification of a growing number of lung cancer-related genes. Genome-wide association studies, whether they are based on single-nucleotide polymorphism array studies or detecting changes in gene copy numbers *via* comparative genomic hybridisation arrays, link the occurrence and frequency of mutations in lung cancer-related genes to the well-defined phenotype of high numbers of human lung cancer. These lung cancer-related genes provide great potential as therapeutic targets for lung cancer intervention. Target validation then occurs through *in vitro* intervention studies of these specific genetic mutations and their respective molecular pathways in human lung cancer cell lines. However, *in vitro* cell culture studies are limited and cannot fully mimic the more complex *in vivo* onset of tumorigenesis and response to tumour therapy. Developing lung cancer in mouse models that harbour specific mutations will undoubtedly provide a further and better insight into the mutation-specific effects on lung tumour biology. Moreover, a high degree of pathophysiological similarity between lung tumours from mouse models and their human counterparts will make it possible to use these mouse models for pre-clinical tests. Various intervention strategies against specific mutations can then be tested based on better evaluation of both specificity and efficacy in mouse lung tumours of every developing stage. Continuous innovation of techniques to manipulate the mouse genome has enabled us to adjust compound mouse models of lung cancer in such a way that they start to reproduce the more complex human lung cancer in a higher degree. Complementary to this, the number of genetically engineered mouse models for lung cancer is ever expanding.

## FIRST MOUSE MODELS FOR LUNG CANCER

The use of mouse models for spontaneous or chemically induced lung tumours has a long

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Received:
Aug 04 2009
Accepted after revision:
Aug 10 2009

**Previous articles in this series: No. 1:** De Wever W, Stroobants S, Coden J, *et al.* Integrated PET/CT in the staging of nonsmall cell lung cancer: technical aspects and resection for lung cancer. *Eur Respir J* 2009; 33: 201–212. **No. 2:** Rami-Porta R, Tsuboi M. Sublobar resection for lung cancer. *Eur Respir J* 2009; 33: 426–435. **No. 3:** McWilliams A, Lam B, Sutedja T. Early proximal lung cancer diagnosis and treatment. *Eur Respir J* 2009; 33: 656–665. **No. 4:** Sculier J-?P, Moro-Sibilot D. First- and second-line therapy for advanced nonsmall cell lung cancer. *Eur Respir J* 2009; 33: 916–930. **No. 5:** van Tilburg PMB, Stam H, Hoogsteden HC, *et al.* Pre-operative pulmonary evaluation of lung cancer patients: a review of the literature. *Eur Respir J* 2009; 33: 1206–1215. **No. 6:** Brambilla E, Gazdar A. Pathogenesis of lung cancer signalling pathways: roadmap for therapies. *Eur Respir J* 2009; 33: 1482–1494. **No. 7:** Horváth I, Lázár Z, Gyulai N, *et al.* Exhaled biomarkers in lung cancer. *Eur Respir J* 2009; 34: 261–275. **No. 8:** Ocak S, Sos ML, Thomas RK, *et al.* High-throughput molecular analysis in lung cancer: insights into biology and potential clinical applications. *Eur Respir J* 2009; 34: 489–506. **No. 9:** Field JK, Liloglou T, Niaz A. *et al.* EUELC project: a multi-centre, multipurpose study to investigate early stage NSCLC, and to establish a biobank for ongoing collaboration. *Eur Respir J* 2009; 34: 1477–1486. **No. 10:** Demedts IK, Vermaelen KY, van Meerbeeck JP. Treatment of extensive-stage small cell lung carcinoma: current status and future prospects. *Eur Respir J* 2010; 35: 202–215.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

history. While susceptibility and incidence of spontaneous lung tumours varies between well-established mouse-inbred strains, all their molecular pathologies share many similarities with human lung cancer [1]. This was clearly established in early studies in which defined chemical carcinogens were used to induce lung tumours [1]. Incidence of spontaneous and induced lung tumours were both at the highest (61%) in sensitive strains such as A/J and SWR, but very low (6%) in highly resistant strains C57BL6 and DBA for males at 2 yrs [1]. Contrary to human lung cancer with its complex molecular genetics and four distinct tumour types that readily metastasise, spontaneous and chemically induced lung lesions in mice often result in pulmonary adenomas [2] and more infrequent adenocarcinomas, which almost never metastasise. Mouse lung tumour development shows initial hyperplastic foci in bronchioles and alveoli, which then become benign adenomas and eventually adenocarcinomas [2]. The very reproducible tumour latency obviously depends on strain susceptibility and/or application of carcinogen-induction protocols. Most potent carcinogens are the cigarette smoke carcinogens, such as polycyclic aromatic hydrocarbons, tobacco-specific nitrosamine and benzo[a]pyrene (B[a]P) [3]. However, cigarette smoke itself is sufficient to induce reproducibly lung tumours in A/J mice after a 5-month exposure period followed by a crucial 4-month recovery period [4]. Exposure of B6C3F1 female mice to life-time (30 months) cigarette smoke resulted in 48% benign and malignant lung tumours through distinct (epi)genetic pathways [5]. Therefore, there are clear differences between (spontaneous) murine and human lung tumours. It has been especially hard to replicate the well-characterised pre-malignant lesions in human airway epithelium [6]. Nevertheless, major histopathological similarities remain and molecular characterisation of spontaneous and carcinogen-induced murine lung tumours revealed a high degree of genetic lesions compared to their human counterparts [7]. A prominent early event is the occurrence of activating Kras mutations [7] in hyperplastic lesions. Besides over expression of cMYC, inactivation of well-known tumour suppressor genes, such as Tumour suppressor protein 53 (Trp53), Fragile histidine triad protein (fhit), adenomatous polyposis coli protein (APC), retinoblastoma protein (RB), mutated in colorectal carcinoma protein (MCC) and $p16^{INK4A}$ (CDKN2A) [7], readily occur; most frequently adenomas develop which only sporadically progress into adenocarcinomas. Differences in susceptibility to lung cancer development between various mice strain remain, however, very intriguing. Most susceptible strains, such as A/J and BALB/C, do have a polymorphism in intron 2 of Kras [8, 9] and a CDKN2a polymorphism was found in BALB/C, influencing their sensitivity to lung cancer. Due to the genome wide sequence analysis, efforts in more interesting polymorphism information of various mice strains is likely to follow and can then be compared with human syngeneic counterparts found in large panels of human lung cancer genome data. Moreover, as we will see in later in this review, more direct transgenic models will be very suitable to cross into resistant strains in order to facilitate the direct search for modifying genes of lung tumour susceptibility.

## TRANSGENIC MICE

### *First generation*

The first generation of transgenic models was based on ectopic transgene expression under control of heterologous promoters. Expression is mainly targeted to specific subsets of lung epithelial cells: surfactant protein-C promoter directs expression primarily to type II alveolar cells whereas Clara cell secreatory protein (CCSP)/CC10 promoters mainly target the nonciliated secretory (Clara) cells along the airways. *SV40 Tag* (Simian virus large T-antigen) was constitutively expressed behind CCSP [10, 11] or surfactant protein (SP)-C promoters [12]. Although each tumour originated from either type II alveolar or Clara cells, they both resulted in quite similar aggressive adenocarcinomas without metastases [13]. A similar strategy was used for different oncogenes (such as *cRaf* and *cMyc* [14]), however, with a milder phenotype, as both transgenic mice mainly ended up in having adenomas and a few progressed adenocarcinomas, again without any metastases.

Many prominent genetic lesions found in human lung cancer clearly link the inactivation of well-known tumour suppressor genes [15] to lung cancer development. Initial attempts to mimic some of these lesions implicated in lung cancer by way of conventional knockout mice studies had rather limited success with respect to the onset of lung cancer. The main reason for this was that germ-line deletion of many essential tumour suppressor genes (such as the retinoblastoma gene (*Rb*) and wilms tumour-1 homolog gene (*Wt-1*) [16]) lead to embryonal or perinatal lethality. Nonessential tumour suppressor gene knockout mice with a longer life-span often had a very broad tumour spectrum of which lung tumours formed only a minor fraction. Thus, *Trp53*, $p16^{INK4A}$ and $p19^{ARF}$ [17] homozygous null allele mice seldom develop lung adenocarcinomas. However, introducing similar mutations into endogenous *Trp53* alleles, such as those prominently found in Li–Fraumeni patients, generated $Trp53^{R270H/+}$ and $Trp53^{R172H/+}$ which had a different tumour spectrum compared with $Trp53^{+/-}$ [18], although their mean survival times were identical. Interestingly these mice, but especially $Trp53^{R270H/+}$ and $Trp53^{R270H/-}$, gave rise to more malignant lung adenocarcinomas, desmoplasia and even metastases which never occur in $Trp53^{-/-}$ mice. These results suggest that ''humanised'' *Trp53* mutations have a greater impact on lung tumour progression than complete *Trp53* loss [18, 19]. 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 [20]. A more specific deletion of candidate tumour suppressor genes on chromosome 3 like *RassF1a*, *FHIT* and *VHL*, showed that 31% of $RassF1a^{-/-}$ mice produced spontaneous mainly lymphomas but also lung adenomas [21]. Treatment of $RassF1a^{-/-}$ mice with B[a]P or urethane resulted in an even higher rate of lung tumours. No spontaneous lung tumours were observed in $Fhit^{-/-}$ or $Vhl^{+/-}$ mice, but 44% of $Fhit^{-/-}/Vhl^{+/-}$ mice developed adenocarcinomas by age 2 yrs. Again use of mutagens such as dimethylnitrosamine led to 100% adenoma and adenocarcinoma induction in $Fhit^{-/-}/Vhl^{+/-}$ mice and even adenomas in 40% of $Fhit^{-/-}$ mice by age 20 months [22]. This showed the usefulness of these knockout mice in recapitulating a pattern of early lung cancer development similar to human pattern.

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 Kras activation *via* an oncogenic $Kras^{G12D}$

knock-in allele ($Kras^{LA2}$), which is expressed only after a spontaneous recombination event (fig. 1) [23]. In this way, sporadic $Kras^{G12D}$ expression occurred on an endogenous level, which in turn augments efficient development of lung adenocarcinomas. However, these mice also developed other tumour lesions as $Kras^{G12D}$ expression was not limited to the lung epithelial tissues.

### *Second generation*

In order to refine the current mouse models, a better method of replicating true expression patterns of oncogenes during lung tumorigenesis had to be taken into account. Furthermore, a general knock-in or knockout procedure only poorly represents genetic events that occur during sporadic lung cancer. Too great an extended expression of dominant oncogenes and/or inactivation of tumour suppressor genes creates a microenvironment that simply does not correspond to sporadic cancer development in which just a limited amount of tumour cells are surrounded and interact with normal cells [24]. Conditional regulation of the temporal-spatial expression of oncogenes or deletion of tumour suppressor genes in somatic tissues of choice can more accurately mimic the *in vivo* situation leading to the onset of sporadic cancer (fig. 1). This is why the second generation of mouse models for lung cancer makes use of a conditional bitransgenic tetracycline inducible system [25]. Most often, the reverse tetracycline-controlled transactivator (rtTA) inducible system is used. The first transgene with the rtTA element behind a tissue-specific promoter guaranties the rtTA expression in a cell or tissue type of choice. This transgene is then combined with a second transgene, consisting of a target gene behind a tetracycline-responsive promoter ($TetO_7$). The presence of tetracycline/doxycycline ensures stable interaction of the rtTA element with the $TetO_7$ promoter, which in turn expresses the target gene.

Therefore, on/off target gene expression is possible depending on administration or withdrawal of tetracycline/doxycycline [26]. Both *SP-C-rtTA* and *CCSP-rtTA* transgenes [27] have been used for directing doxycycline responsive rtTA to either alveolar type II or Clara cells. Several bitransgenic mice such as *CCSP-rtTA;*$TetO_7$ *FGF-7* [28] and *CCSP-rtTA;*$TetO_7$ $Kras^{G12D}$ [29] have been successfully used to induce lung lesions. Induction of FGF-7 caused initial epithelial cell hyperplasia followed by adenomatous hyperplasia after doxycycline application. All hyperplasia disappeared after withdrawal of doxycycline [28]. However, $Kras^{G12D}$induction caused epithelial cell hyperplasia, adenomatous hyperplasia and, after 2 months doxycycline application, multiple adenomas and adenocarcinomas. Again, no lesions could be detected after 1 month of doxycycline withdrawal [29]. When the *CCSP-rtTA;*$TetO_7$ $Kras^{G12D}$ alleles were combined with conventional *Trp53* or *Ink4a/ARF* null alleles, adenocarcinomas with a more malignant phenotype had already appeared after 1 month doxycycline application, thus showing a synergy of mutant-Kras and both tumour suppressor deficiencies. However, even in these compound Tet-inducible mouse models, all lesions disappeared after doxycycline withdrawal. This showed the importance of mutant-Kras as a ''driving'' oncogene not only at tumour onset but also during maintenance of adenocarcinoma in these mouse models. Although the histopathological similarities with human adenocarcinomas were quite obvious, no metastases were found to arise from these murine adenocarcinomas [29].

Simulating more complex lung tumour genetics in human lung cancer required an expanded approach with other conditional genetic systems. The Cre/loxP or Flp/FRT system [24, 30] provided excellent tools for this by introducing somatic mutations in a limited number of differentiated cells of choice whereby other cells of the fully developed lung remained normal [23, 31]. In short, mutations of targeted regions, flanked by loxP (also known as being ''floxed'') or FRT sequence sites, are introduced through deletion by their respective site-specific recombinases Cre or Flp. Thus, in the case of tumour suppressor genes, conditional hypomorphic mutations or null allele, several (non)coding exons are floxed and can, therefore, be deleted by its corresponding recombinase (fig. 1). Conversely, floxed transcription stops (Lox-Stop-Lox or LSL) in front of oncogene or knock-in alleles can control their respective conditional activation [32]. 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 [27]. Apart from this, Cre-mediated recombination can also be achieved through the administration of an engineered Adeno-Cre virus *via* nasal or tracheal instillation [31, 33]. An advantage of the latter method is that a limited amount of adult lung cells can be targeted in a very concise, localised and timely fashion. Efficacy of this method was tested with conditional alleles of $Kras^{G12D}$ and $Kras^{G12V}$ [31, 33, 34]. Infection of adult lungs with Adeno-Cre virus rapidly resulted in the onset of adenomatous alveolar hyperplasia, followed by the development of adenomas and finally adenocarcinomas at 3–4 months post-infection. Although a latency of 8 months was also observed [34], no metastases could be found in any of the models. Most probably a single Kras activation is not enough to allow the adenocarcinomas to progress into a higher state of malignancy as would be required for fully metastasising lesions. However, these straightforward experiments disclosed the important role of Kras in human lung cancer onset and progression [17, 34]. Another important aspect of this model was that lung tumour 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, indeed ensures a precise mimicking of the 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.

In the next part of our review we want to give an update of most of the recent advances in mouse models for human lung cancer. For this we will focus on the proceedings on the three main types of lung cancer, namely nonsmall cell lung carcinoma (NSCLC), small cell lung carcinoma (SCLC) and squamous cell carcinoma (SCC).

The types of available genetically modified mouse models, causal genotype-phenotypic correlations and implications for

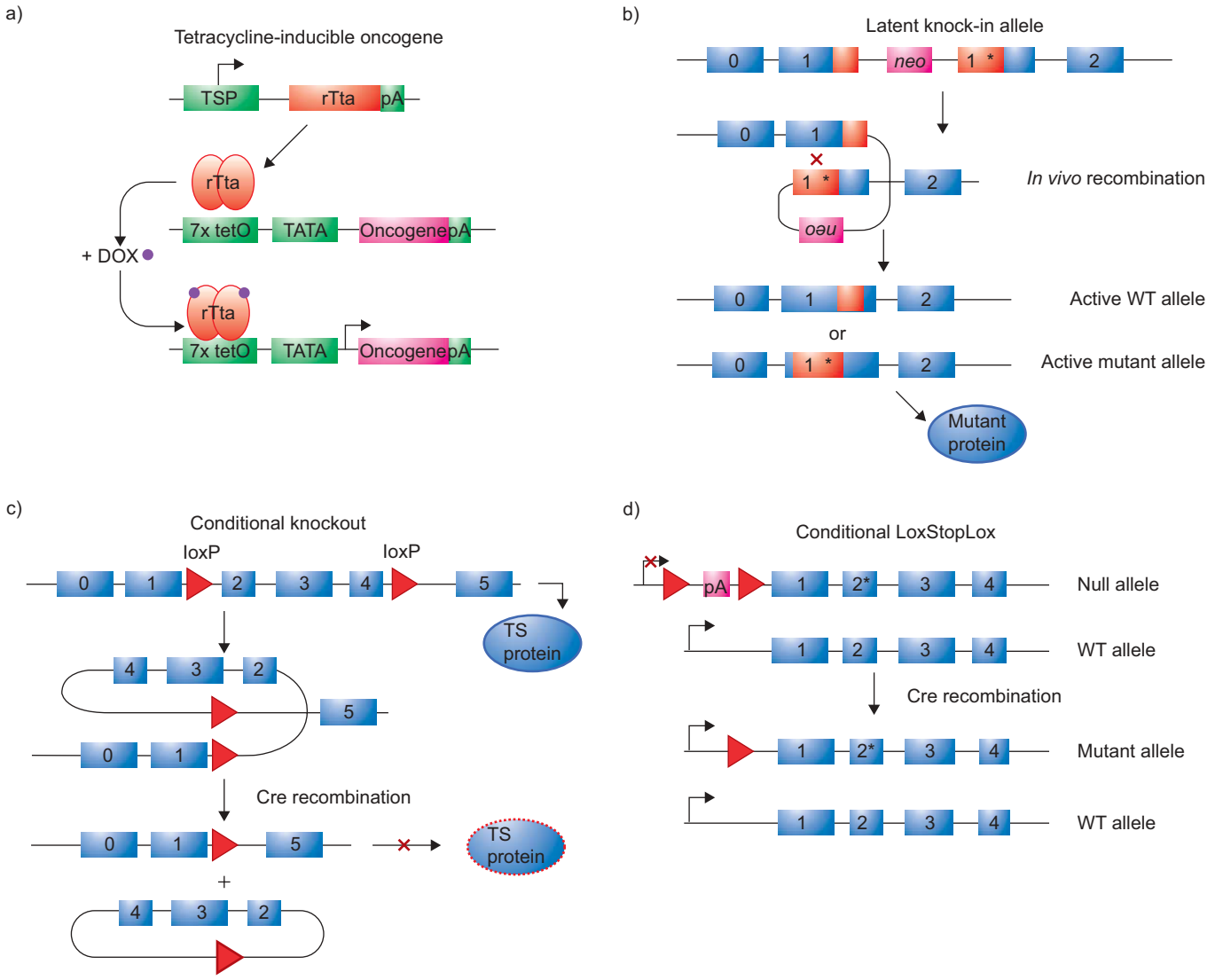


**FIGURE 1.** Overview of systems used in most of the genetically engineered mouse models for lung cancer. a) The tetracycline-inducible bitransgenic system. A tissue-specific promoter (TSP) drives the expression of a Tet-inducible transactivator (rtTA). In the presence of doxycycline (DOX), rtTA dimers can specifically bind to seven *tetO* (7x tetO) sequences and activate oncogene transcription from the minimal TATA promoter. b) The latent activated allele ensures stochastic oncogene activation *via* spontaneous *in vivo* recombination of a silent mutant allele. A neomycin resistance (*neo*) gene separates a wild-type (WT) and a mutated copy of the otherwise identical exons. After recombination, only a WT allele or an allele with an activating mutation is left to exert oncogenic functions. c) Conditional gene inactivation is based on Cre/loxP recombination-mediated deletion of one or more exons. Site-specific Cre recombinase induces mutations through recombination of two direct orientated LoxP sequences. Thus, controlling Cre recombinase activity ensures eventual tumour suppressor (TS) gene inactivation into various tissues of choice. d) Conversely, the conditional LoxStopLox allele makes use of a strong transcription stop, flanked by two lox sites, in front of a coding exon. The resulting null alleles can become an oncogenic mutant allele after removal of the transcription stop element *via* Cre-mediated recombination. pA: polyadenosine.

translational research will be briefly discussed for each of these main lung cancer types.

## MODELS FOR NSCLC

To date, most mouse models generate lung adenocarcinomas with various stages of malignancy. As we have already discussed, a frequently used way of driving lung tumorigenesis is through conditional or spontaneous activating *Kras* mutations [17]. However, it is obvious that more complex mouse models are needed to further our understanding of highly complicated lung cancer genetics (fig. 2). Validation of these mouse models then requires knowledge of how closely mouse models resemble human lung cancer. Several studies used cross-species comparisons of gene expression profiles between murine and human lung tumours to identify similarities in cancer signalling pathways [35, 36]. Integration of gene expression data from a $Kras^{LA2}$ mouse model and KRAS mutated human lung tumours indeed showed a significant overlap but also revealed a gene-expression signature for Kras mutation in human lung cancer itself [35]. These results presented powerful molecular criteria to assess similarities between mouse and human lung cancer, as well as new possibilities to further elucidate important genes that control less well-characterised lung cancer pathways. Since

activating *Kras* mutation models recapitulate some of the human lung tumour phenotypes rather well, closer analyses of early lung tumour initiating events were performed [37, 38]. A combination of both CCSP *CC10-Cre* recombinase and *LSL* $Kras^{G12D}$ alleles [37] resulted in a progressive phenotype of cellular atypia, adenoma and finally adenocarcinoma. These lesions originated exclusively from bronchial epithelia but were accompanied with a strong inflammatory response through expression of various chemokines. Kras activation could, therefore, serve as an excellent model to study the complex interactions between transformed lung epithelial and inflammatory cells, chemokines and early tumour development. Another model for generating early, benign lung tumour lesions makes use of a bitransgenic Tet-inducible $Kras^{G12C}$ allele that can be expressed in both Clara and/or alveolar type II cells [27, 38]. Only mild atypical hyperplasia or benign adenomas were found and even after an induced $Kras^{G12C}$ expression of $\geqslant$12 months, no further tumour progression could be detected. Inhibition of $Kras^{G12C}$ expression through doxycycline withdrawal over 1 month led to a complete reversal of any early proliferative lesion. Thus, both $Kras^{G12C}$ and $Kras^{G12D}$ oncogenes are needed for maintaining a tumour phenotype. One explanation of the low tumour penetrance of $Kras^{G12C}$ alleles could be the absence of activation of Akt and JNK signalling pathways. This latter observation was in strong contrast to the effects of the previously described $Kras^{G12D}$ models.

It is rather puzzling that *Kras* but not *Hras* or *Nras* mutations are predominantly found in lung carcinomas [39]. A plausible explanation for this can either be that differences in the Ras protein isoforms render them different oncoprotein activities, or differences in their respective gene regulatory elements confer tissue-specific expression to the mutated *Ras* gene in its corresponding tumour. Moreover, one has to note that the *Kras* gene encodes two isoforms, 4A and 4B, with differences in their C terminus. This C terminus of the predominant Kras 4B isoform is prone to exclusive post-translational modifications, such as farnesylation or geranylgeranylation [40]. However, Kras 4A has a post-translational modification that includes covalent attachment of a palmitoyl moiety, as do Hras and Nras. The latter modifications give Nras, Hras and Kras 4A distinct signalling activities by facilitating their localisation in specific microdomains in membranes [41]. A recent elegant study elucidates Kras mutation specificity in lung cancer by exchanging either a Hras or Kras 4B cDNA into the original *Kras* gene by generating *Hras* ($Hras^{KI}$) or *Kras 4B* ($Kras^{KI}$) knock-in mice. Surprisingly, the number of urethane-induced lung tumours in $Hras^{KI/KI}$ was much higher compared to $Kras^{KI/KI}$ mice and the latter produced even less tumours than the wild-type (WT) control mice [42]. Interestingly, when mutations were analysed in $Kras^{KI/wt}$ heterozygous mice, they were only detected in the WT allele which expresses Kras 4A, as well as Kras 4B. This led to the conclusion that only a mutated *Kras 4A* allele, which, after all, shares functional C terminus similarity with Hras, can induce lung tumorigenesis and *Kras* regulatory elements provide its specificity for pulmonary epithelial cells [42, 43]. In a different setting, a $Kras^{LA2}$ allele was either combined with WT *Kras*, *Hras* or $Kras^{KI}$ alleles. The number of spontaneous lung tumours was high in $Kras^{LA2/KI}$, but similarly low in $Kras^{LA2/WT}$ and $Hras^{KI}$; $Kras^{LA2}$ mice. This tumour suppressing activity can only be accounted for by an intact WT locus which is capable of encoding an intact Kras 4A isoform and could, therefore, be functionally substituted by a similar $Hras^{KI}$ allele [42]. Thus, there is now compelling evidence that a normal WT Kras allele actually inhibits and that preferentially a mutated *Kras 4A* allele actively induces lung tumorigenesis, respectively, in a strictly tissue specific manner [42–44]. In hindsight, this also explains earlier observations in which vinyl-carbamate treated mice with one *Kras* KO allele produced a higher number of more advanced adenocarcinoma than control WT *Kras* mice [45].

We know that the occurrence of somatic *Kras* mutations in lung cancer is prevalent [46], thus making the understanding of the precise role of Kras pathway effectors very important (fig. 2).

Indeed, mutations in candidate effectors *BRAF* and *PI3K* of activated Ras downstream pathways have been reported in human NSCLC [47, 48]. Activating mutations of *BRAF* would most likely have a stimulating effect on mitogen-activated protein kinase (*MAPK*) pathways. This hypothesis was tested in a lung specific, Tet-inducible, expression of a *BRAFV600E* mutant transgene [46]. In this mouse model, *CCSP-rtTA*;$TetO_7$-*BRAFV600E* showed a development of lung adenocarcinoma with bronchioalveolar carcinoma features. The extracellular signal-regulated kinase (ERK)-1/2 (MAPK) pathway was significantly upregulated. Upon doxycycline withdrawal, the BRAF-mutant lung tumours disappeared in combination with a marked decrease in phosphorylation of ERK1/2. Furthermore, the *in vivo* use of a specific MAPK/ERK kinase (MEK) inhibitor induced lung tumour regression. Interestingly, the latter effect could be reproduced with lung tumours from *CCSP-rtTA*;$TetO_7$-$Kras^{G12D}$ mice [46]. All these results showed that both activated BRAF and KRAS signalling convert on the same MAPK pathway, making this pathway a potential target for lung tumour intervention. These results were confirmed by another independent study in which a floxed BRAFV600E allele was activated in lung epithelium *via* nasal Adeno-Cre delivery [49], which caused a formation of benign adenoma. However, the majority of these latter lesions did not further develop into adenocarcinoma and the abundant nonprogressed adenoma also showed features of oncogene induced senescence. Combining BRAFV600E activation with loss of *Trp53* or *Ink4a/Arf* led, as expected, to marked tumour progression into adenocarcinoma [49]. A close investigation during $Kras^{G12D}$ driven lung tumorigenesis showed an increased phosphorylation of MAPK during which the MAPK antagonist Sprouty-2 (Spry-2) was upregulated. When Cre/lox dependent $Spry\text{-}2^{F/F}$;*LSL* $Kras^{G12D}$ mice were used for lung tumour induction, a clear increase in both tumour load and development was observed in $Kras^{G12D}$;$Spry\text{-}2^{-/-}$ tumours. This clearly suggested a tumour suppressor activity for Sprouty-2 during Kras dependent lung tumorigenesis by impairment of the Ras/MAPK signalling cascade [50].

Another approach to understand the role of the Ras downstream effector pathway led to the simultaneous Cre/lox dependent deletion of $Rac1^{F/F}$ in the presence of activated Kras [51]. Results showed a significant delay in Kras dependent adenoma formation in $Rac1^{-/-}$ *versus* $Rac1^{+/-}$ lesions, which suggest that Kras signalling acts, at least in part, through Rac1 during lung tumour development.

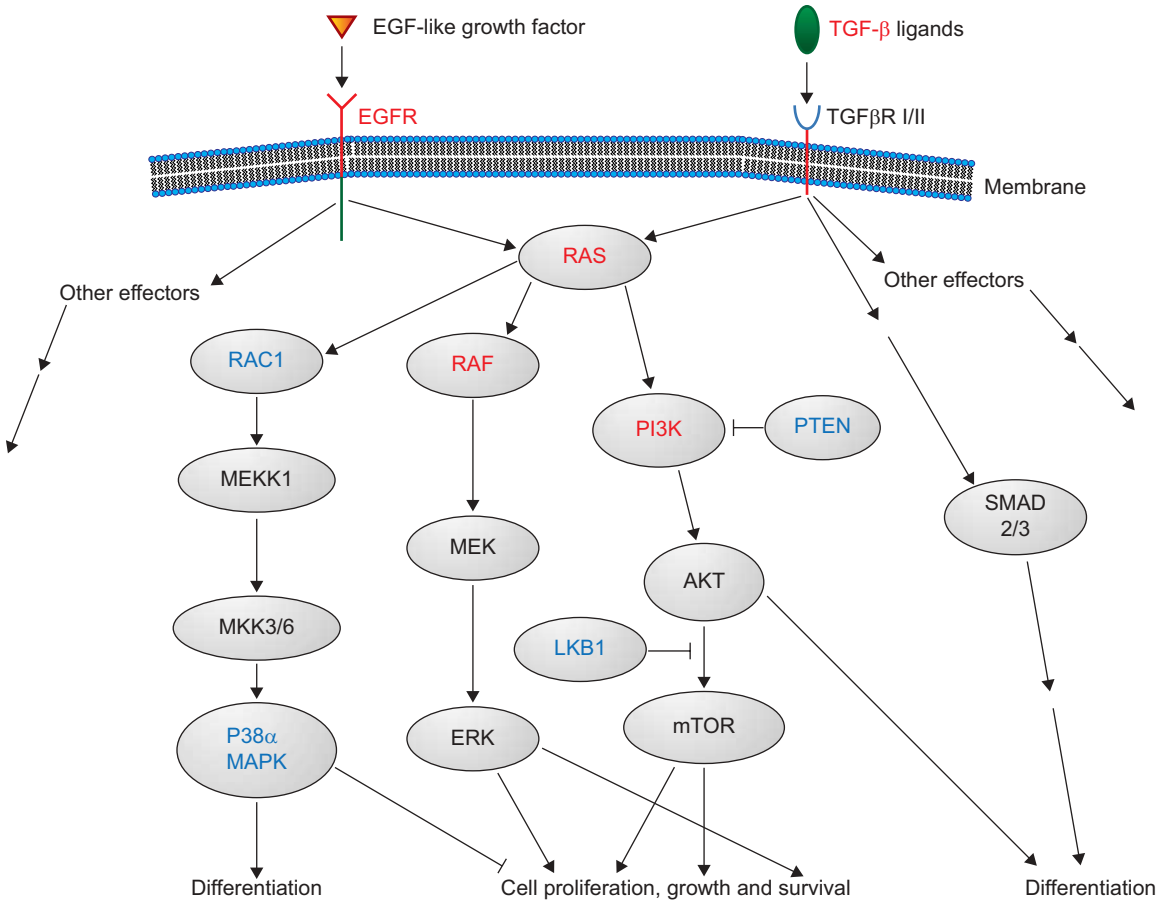


**FIGURE 2.** Schematic overview of the major signalling pathways that are impaired in most of the nonsmall cell lung cancer models. Activation of the signalling network occurs through activating mutations of the oncogenes (shown in red) or inactivating tumour suppressor genes (shown in blue). At the core of the signalling cascade are RAS (rat sarcoma viral oncogene homologue) and its stimulation of the RAF-MEK-ERK (serine/threonine kinase raf-mitogen-activated protein kinase kinase-extracellular signal-regulated kinase kinase) and PI3K (phosphatidylinositol 3-kinase) pathways. Activating mutations in epidermal growth factor receptor (EGFR) serve as the main models for receptor tyrosine kinases upstream of RAS. As for transforming growth factor (TGF)-β, one has to acknowledge its dual role as a tumour suppressor during the early phase of lung tumorigenesis and a pro-oncogenic factor in more advanced and metastatic lung tumours. Note that RAC1 (Ras-related C3 botulinum toxin substrate 1, small GTPase) exerts oncogenic activity in collaboration with RAS, since inactivating Rac1 models showed an impairment of RAS-driven lung tumorigenesis. EGF: epidermal growth factor; TGFβR: TGF-β receptor; Mekk1: mitogen activated protein kinase kinase kinase 1 or MAP3K1, serine threonine kinase; Mkk3/6: mitogen-activated protein kinase kinase 3 or MAP2K3, serine threonine kinase; p38α-MAPK: mitogen activated protein kinase p38α or MAPK14; PTEN: phosphatase and tensin homolog; AKT: serine/threonine kinase also known as protein kinase B; LKB1: serine/threonine kianse LKB1 also known as serine/threonine protein kinase 11, STK11: mTOR: mammalian target of rapamycin, serine/threonine protein kinase; SMAD 2/3: TGF-β-dependent gene transcription factors.

Another downstream effector (fig. 2), interlinked with RAS signalling pathway, is phosphoinositide-3-kinase (PI3K) and downstream targets, such as the protein kinase Akt. In human NSCLC somatic mutations that activate PI3K have been identified in the p110α catalytic subunit (encoded by *PIK3CA*) [52]. Furthermore, selective *PIK3CA* amplification was found in lung squamous cell carcinomas [53]. Therefore, mice were generated with a Tet-inducible expression of p110α, with an activating mutation in its kinase domain (H1047R), and then crossed with CC10 promoter-reverse tetracycline transactivator protein (rtTA) to generate *CCSP-rtTA;TetO$_7$ PIK3CA(H1047R)* mice. Upon doxycycline dependent induction of PIK3CA(H1047R) for 14 weeks, double transgenic mice developed adenocarcinomas, which subsequently completely disappeared after doxycycline withdrawal for 3 weeks [54]. On the contrary, complete ablation of PI3K activity through deletion of both its p85 regulatory subunits (encoded by *Pik3r1* and *Pik3r2*) led to a dramatic decrease in the number of lung tumours in *LSL Kras$^{G12D}$;Pik3r2$^{-/-}$;Pik3r1$^{-/-}$* mice [54]. Many *in vitro* studies showed that Ras proteins directly interact with the p110α subunit of PI3K and introduction of specific mutations in *PIK3CA* blocks this interaction [55]. In order to study the Ras-p110α interactions *in vivo* and its effects on tumorigenesis, *Pik3ca$^{-/-}$* mice were generated and crossed with *Kras$^{LA2}$* alleles. These *Pik3ca$^{-/-}$;Kras$^{LA2}$* mice were highly resistant against lung tumour formation, which suggest that Ras-p110α interaction is needed for Ras-driven tumorigenesis [55].

All these results underline the importance of PI3K signalling not only for lung tumour induction but also maintenance.

Cross talk between Ras-MAPK, transforming growth factor (TGF)-β1 and its type II receptor (TGF-β RII) does exist [56], while TGF-β1 is implicated as a tumour suppressor gene in

human lung cancer [57]. To test this latter hypothesis *in vivo*, lung tumorigenesis was followed in *Kras*$^{LA2}$*;Tgf-β1*$^{+/-}$ *versus* *Kras*$^{LA2}$ mice. The *Kras*$^{LA2}$*;Tgf-β1*$^{+/-}$ lung tumours appeared faster and with a relatively higher amount of adenocarcinomas compared to those from *Kras*$^{LA2}$ mice. Surprisingly, adenocarcinoma that retained WT levels of TGF-β1 had significantly higher angiogenic activity than *Tgf-β1*$^{+/-}$ adenocarcinoma. Therefore, TGF-β1 is a *bona fide* tumour suppressor during onset and progression of lung tumorigenesis, but also needed at the same time for lung tumour-induced angiogenesis and metastatic potential [58].

Another key player driving human NSCLC is EGFR signalling, which is independent and upstream of Kras (fig. 2). In fact, mutations in *EGFR* occur mutually exclusive from those found in the *Kras* gene in human NSCLC [48].

Most mutations in *EGFR* occur around the region encoding the ATP binding pocket of the receptor's tyrosine kinase domain in exons 18–21 [59]. More precisely, these mutations occur as in-frame deletion in exon 19, which eliminate a conserved LREA (lysine, arginine, glutamic acid and alanine protein sequence) motif (EGFR$^{DEL}$), and an L858R substitution in exon 21 [48]. When these specific mutations were engineered in mice by use of the Tet-inducible system, two types of bitransgenic mice were generated: *CCSP-rtTA;Tet-O*$_7$*-hEGFR*$^{L858R}$ and *CCSP-rtTA;Tet-O*$_7$*-hEGFR*$^{DEL}$. Both bitransgenic mice expressed mutated human EGFR in lung type II alveolar cells and following a few weeks of doxycycline administration developed bronchioloalveolar carcinomas [60, 61]. However, tumour development continued when the doxycycline application period was extended beyond 4 weeks, resulting in the formation of invasive adenocarcinomas. However, when doxycycline was withdrawn the absence of mutated hEGFR resulted in a complete regression of lung cancer [60]. Overall, the tumour phenotype showed a remarkable resemblance with human adenocarcinoma. This makes these bitransgenic models excellent tools for the study of EGFR-dependent NSCLC as it confirms that *hEGFR*$^{DEL}$ and hEGFR$^{L858R}$ are dominant oncogenes capable of inducing fully fledged lung cancer. Another distinct *EGFR* mutation, the variant III (vIII) in-frame deletion of exons 2–7, was found in some 5% of analysed human squamous cell carcinoma but not in adenocarcinoma [62]. Bitransgenic *CCSP-rtTA;TetO*$_7$*–EGFR*$^{vIII}$ mice developed adenocarcinoma after 16 weeks of doxycycline application. Lung tumours depended on EGFRvIII expression as doxycycline withdrawal led to complete tumour regression [62]. All *EGFR* mutant mouse models showed the importance of EGFR activity in NSCLC and are highly penetrant in producing advanced adenocarcinoma.

Since expression of phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is often down regulated in NSCLC, several mice models were generated in which *Pten* was inactivated in the bronchial epithelium [63, 64]. *PTEN* is a tumour suppressor gene that acts by blocking the PI3K dependent activation of serine-threonine kinase Akt (fig. 1). Since *Pten*$^{-/-}$ mice are embryonal lethal, one had to make use of floxed Pten alleles (*Pten*$^{F/F}$), combined with *CCSP-Cre* transgene, targeting *Pten* deletion into bronchial Clara cells. However, these *Pten*$^{F/F}$*;CCSP-Cre* mice did not show any aberrant pulmonary development or phenotypic abnormalities even when followed for $\geqslant$12 months [64]. This changed dramatically when the *Pten*$^{F/F}$*;CCSP-Cre* alleles were combined with *LSLKras*$^{G12D}$. Lung tumorigenesis was markedly accelerated compared to that of single *LSLKras*$^{G12D}$ mice. *Kras* mutant, *Pten* deficient tumours were often of the more advanced adenocarcinoma type with more vascularity [64], 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 *SP-C-rtTA;TetO*$_7$*-Cre* [63]. When doxycycline 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 adenocarcinomas. Post-natal doxycycline 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 adenocarcinomas in comparison to WT controls. Prior addition of urethane induced an even higher amount of adenocarcinomas. Interestingly, most *Pten*$^{-/-}$ adenocarcinomas (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.

Recent studies showed that a large fraction of NSCLC cells had germ line mutations and impaired expression of the *LKB1* tumour suppressor gene, a serine threonine kinase also known as *STK11* [65]. Mutations in *LKB1* are found in Peutz–Jeghers syndrome (PJS) patients and are characterised by intestinal polyps (hamartoma) and increased incidence of epithelial cancers [66]. However, somatic *LKB1* mutations have been identified in a minor fraction of these sporadic tumours, such as malignant melanomas, ovaria, breast and pancreatic cancer [67]. An exception of this is formed by lung cancer in which *LKB1* inactivation is a common event for NSCLC [65, 68]. The highest numbers of mutations were found in adenocarcinomas [65] and were significantly more frequent in those adenocarcinomas with *KRAS* mutations [69]. Interestingly, lung cancer does not have the highest incidence among sporadic cancers in PJS patients [67]. This suggests that loss of LKB1 function might not be the primary genetic event *per se* which drives lung tumour initiation, but is rather an important facilitating lesion that ensures tumour progression. Results of a study of *Lkb1* loss in the background of a conditional *LSLKras*$^{G12D}$ dependent *in vivo* mouse model [70] showed that LKB1 inactivation cooperates with KRAS activation, suggesting a role for LKB1 as an active repressor of the KRAS downstream pathway. Moreover, single *Lkb*$^{F/F}$ mice showed no tumour phenotype. Only *Lkb1*$^{F/F}$*/LSLKras*$^{G12D}$ mice showed a broad scale of NSCLCs: the majority were adenocarcinoma but unexpectedly SCCs and large cell carcinoma (LCC) also occurred. Furthermore, 61% of adenocarcinoma progressed into metastases but none were detected for SCC and LCC. Compared to *p53*$^{F/F}$*/LSLKras*$^{G12D}$ and *(Ink4a/Arf)*$^{F/F}$*/LSLKras*$^{G12D}$, the *Lkb1*$^{F/F}$*/LSLKras*$^{G12D}$ mice had a higher tumour penetration and significant higher number of metastases. In addition, no SCC or LCC were detected in *p53*$^{F/F}$*/LSLKras*$^{G12D}$ and *(Ink4a/Arf)*$^{F/F}$*/LSLKras*$^{G12D}$ mice. Results show that LKB1 loss permits squamous differentiation and facilitates metastases but these

are independent events. Adenocarcinoma from *Lkb1*$^{F/F}$/*LSLKras*$^{G12D}$ mice had reduced pAMPK (phosphorylated, adenosyl monophosphate-activated protein kinase) and pACCA (phosphorylated, acetyl-CoA carboxylse α-subunit) levels and activated mTOR (mechanistic target of rapamycin protein) pathway (fig. 2). It is probable that LKB1 loss influences differentiation into the NSCLC subtypes *via* discrete pathways [71]. A large panel of human NSCLC showed *LKB1* mutations in adenocarcinoma (34%), SCC (19%) and LCC (16%) [70]. Concomitant mutations in *p53* and *LKB1* suggest nonoverlapping roles in NSCLC. Moreover, reconstitution of LKB1 in human NSCLC cell lines showed anti-tumour effects independent of their p53 or (INK4A/ARF) status [70]. Finally, loss of LKB1 expression in alveolar adenomatous hyperplasia, precursor lesion for adenocarcinoma, suggests an early role of LKB1 inactivation during adenocarcinoma development [72].

Not only might direct (epi)genetic mutations in oncogenes and tumour suppressor genes affect their respective expression during lung tumorigenesis, but microRNAs (miRNAs) can also perform similar roles. These miRNAs are classes of small non-coding RNAs that post-transcriptionally regulate the expression of target mRNA transcripts. In order to become active small interfering RNA (siRNA), the primary miRNA transcripts 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 has been reported [73]. This 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 *LSLKras*$^{G12D}$;*Dicer1*$^{F/F}$ mice let to a marked increase of tumour development [74]. However, since the 3′ UTR region of Kras transcripts had been shown to be a direct target of let-7 [75], it became especially tempting to increase let-7 expression in *Kras*$^{G12D}$ lung tumours. And indeed, intranasal application of both adenoviral [76] and lentiviral [77] let-7 miRNA caused a severe decrease of *Kras*$^{G12D}$;*p53*$^{-/-}$ lung tumours. These results showed the potential importance of miRNA regulation for the maintenance of NSCLC.

## MODELS FOR SCLC

Contrary to NSCLC, neuroendocrine carcinomas are virtually never found in spontaneous or chemically induced murine lung cancer. One reason for this could be that in these murine lung cancers a combination of both p53 and Rb mutations is almost never found, contrary to the majority of human SCLCs. To avoid this, a Cre/lox based deletion of both conditional alleles for Rb and p53 was performed by intratracheal instillation with Adeno-Cre [78]. After 3 months, various foci of neuroendocrine hyperplasia developed through the proximal as well as distal bronchi. After a further 3 months, these early lesions progressed into massive lung tumours with typical histological features of SCLC. Interestingly, some early type lesions remained even in the presence of extensive SCLC. Consequently it is of importance to determine if the early neuroendocrine lesions are indeed precursors for SCLC and if so, which additional (epi)genetic events are then needed for them to progress. Immunohistological characterisation of the full-blown tumours revealed that they indeed shared neuroendocrine features with human SCLC. All neuroendocrine differentiation markers, such as calcitonin gene-related protein (CGRP), neuron-specific enolase, synaptophysin, neural cell adhesion molecule and achaete-scute homolog-1 (ASH-1) were not expressed in the same way as in human SCLC. Furthermore, the murine SCLC readily metastasised towards similar organs as found with human SCLC [78]. All primary SCLC, as well as their metastases, had all Rb and p53 alleles inactivated. Tumours that retained one WT Rb allele were all invariably adenocarcinomas without any neuroendocrine features. Therefore, the status of Rb most likely determines if tumours do occur with mixed SCLC and NSCLC phenotype, as has also been sometimes observed in patients [79]. No lung tumours could be found in Rb$^{F/F}$ mice, which suggested that loss of Rb alone is not enough to initiate lung tumorigenesis [78] and the additional loss of p53 is needed.

Not only does Rb loss need extra genetic events, the nature of these complementary lesions also determines which type of lung cancer will develop. For instance, RB inactivation and Kras mutations are almost never found together in the same human lung cancer. Moreover, the overall mutation rate of RB in human NSCLC is very low [80]. As we have seen already, Adeno-Cre dependent activation of Kras in a broad range of lung epithelial cells leads exclusively to the onset of NSCLC. However, similar application of Adeno-Cre for inactivating RB (and p53) gives completely different neuroendocrine SCLC. Do these results imply then that Rb loss has no function in Kras driven lung tumorigenesis? Certainly not! When *LSLKras*$^{G12D}$ transgenes were combined with *Rb*$^{F/F}$ and Rb family *p130*$^{F/F}$ alleles for Adeno-Cre dependent lung tumour induction, the results clearly showed much more progressed adenocarcinomas of *Kras*$^{G12D}$;*Rb*$^{-/-}$;*p130*$^{-/-}$ genotypes compared to single *Kras*$^{G12D}$ [81]. Both Rb loss and p130, albeit to a lesser extent, contributed to Kras dependent NSCLC. Clearly, in this genetic context Kras$^{G12D}$ overrules any effect of Rb loss on neuroendocrine differentiation. Another intriguing observation came from *CC10-rtTA;tetO*$_7$*-Cre;Rb*$^{F/F}$ mice that underwent doxycycline application during early embryogenesis, causing a complete Rb ablation in all bronchial Clara cells. However, only an increase of hyper cellular neuroendocrine lesions was detected in these mice and no effect on Clara cell homeostasis could be observed. Contrary to this, when all three Rb family proteins (Rb, p107 and p130) were inactivated by a truncated SV40 large T-antigen oncoprotein (T121) in *CC10-T121* mice, a severe bronchial hyperplasia with complete dedifferentiation of all Clara cells occurred. These results suggested that Rb might be specifically required for determining neuroendocrine cell fate, but only in a strict cellular and genetic context. All this accumulating evidence from mice models makes it unlikely that NSCLC and SCLC do develop from similar target cells. It would be more plausible that separate, non-identical target cells can develop into different lung cancers, although each still depends on specific major genetic pathways.

Apart from the somatic *Rb*$^{F/F}$;*p53*$^{F/F}$ model for SCLC, two other lung cancer models have also been associated with pulmonary neuroendocrine tumours. One model made use of bitransgenic *CC10-hASH1;CC10-SV40 large T* in which progressive neuroendocrine dysplasia and aggressive lung adenocarcinoma develop with both focal neuroendocrine differentiation

(through expression of pro-neural ASH-1 transcription factor) and CC10 expression [82]. These adenocarcinomas closely resembled human NSCLC with neuroendocrine differentiation [83]. The other model made use of inactivating both cyclin-dependent kinase inhibitor p18 (Ink4c) and Men1, a tumour suppressor gene deleted in human multiple endocrine neoplasia. Spontaneous lung tumour formation in $p18^{-/-}$;$Men1^{+/-}$ mice initially led to adenoma formation which steadily grew, after a long latency period of >1 yr, into mixtures of both adenocarcinoma and neuroendocrine carcinomas [84]. These mixed lung tumours were, however, distinct from those that developed in $Kras^{G12D}$ through their neuroendocrine differentiation. And finally, compared with $Rb^{F/F}$;$p53^{F/F}$ mice these lung tumours lacked the typical histology and metastatic pattern of SCLC.

## MODELS FOR SCC

Human lung SCC is closely linked with smoking and shows a distinct sequence of pre-malignant changes in the airway epithelium from hyperplasia, metaplasia, dysplasia and carcinoma *in situ* towards complete invasive SCC [85]. However, normal human or mouse lungs do not contain squamous epithelium. Only under pathological conditions does squamous differentiation (accompanied by high expression levels of keratins such as keratin 14 (K14)) occur in epithelium of conducting airways [86]. This explains why spontaneous SCC virtually never occurs in mice and only a few mouse models reported the onset of SCC, mostly after carcinogen application. The latter models use either intratracheal intubation of methyl carbamate [87] or extensive topical application of *N*-nitroso-methyl-bis-chloroethylurea and *N*-nitroso-tris-chloroethylurea [88, 89]. The whole spectrum of abnormal squamous phenotypes were observed, but only in susceptible inbred mice (NIH Swiss, A/J, Balb/cJ, FVB/J and SWR/J) and not in others (C57BL/6J, AKR/J and 129/svJ) [89]. Besides, specific loci for SCC susceptibility could be identified through linkage analyses in several mice strains [89]. Another study reported a complete different approach for trying to induce SCC through constitutive expression of human K14 *via CC10-hK14* mice [90]. Although *CC10-hK14* mice had a FVB/J background and hK14 was readily expressed in bronchial epithelium, only precursor lesions varying from hyperplasia to squamous metaplasia could be observed [90]. Clearly, the increased K14 expression and onset of squamous differentiation alone is not sufficient to generate fully advanced SCC. However, as we have already described, so far only the $LSLKras^{G12D}$;$Lkb1^{F/F}$ somatic mouse model for NSCLC has been able to generate advanced SCC. LKB1 inactivation results in aggressive and metastatic tumours of which up to 60% have squamous or mixed squamous histology [70], even though precursor lesions were not found in the conducting airways.

## MOUSE MODELS IN TRANSLATIONAL LUNG CANCER THERAPY RESEARCH

Xenograft models have been extensively used for pre-clinical testing of lung cancer therapeutics. This has mostly been performed on human lung cancer cell lines after they had been subcutaneously injected into immunodeficient mice. The more preferred method, however, would be orthotopical transplantation of human lung tumour cells in their natural pulmonary environment. The results to date show that xenograft models have a poor record of accurately predicting the clinical efficacy of anti-tumour drugs. Therefore, a justified question arises as to whether spontaneous and 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 lung physiology between mice and humans, but some of the mouse models that we have already described have a striking phenotypical resemblance, combined with a genetic signature very similar to human NSCLC [35] and SCLC [91]. Importantly, genetically engineered mouse model-derived tumours develop in an innate immune environment and, therefore, use all the tumour-stromal interactions, such as angiogenesis and degradation of the tissue matrix.

Indeed, the first reports have now been published that show very promising predictive capacity of murine lung tumour models in therapy research and we will discuss two of these now.

However, first we want to briefly address some of the findings from studies in A/J mice susceptible for carcinogen-induced lung tumorigenesis. The use of standardised carcinogen-induction protocols in A/J mice generates a highly reproducible amount of well-defined lung adenocarcinoma [92]. Therefore, A/J mice are widely used to test the efficacy of chemo-intervention. For example, a 50–60% tumour mass reduction of adenocarcinomas was obtained after treatment with cis-platinumin combination with metoclopramide and/or indomethacin, while adenoma growth was not affected [93]. Since strain A/J lung tumour models have proved their utility for testing chemotherapeutics, they have also been widely used for evaluating chemoprevention protocols [94]. A broad range of chemopreventive agents, such as farnesyl transferase inhibitors, isothyocynates, nonsteroidal anti-inflammatory drugs and glucocorticoids, as well as natural products like green tea and carotene are just some examples of what has been tested so far [95]. At best, lung tumour growth was impaired by up to 50–60% [94]. Introduction of *p53* and $p16^{ink4a}$/$p19^{ARF}$ mutant alleles into strain A/J mice caused, as expected, a higher lung tumour penetrance but not a dramatic difference on chemoprevention sensitivity [96]. In order to unravel some of the undoubtedly complex genetic traits that cause a chemopreventive effect in the A/J strains, first the genetic signatures for such chemopreventive agents as green tea and budenoside have been established using gene expression arrays [97, 98].

We already described two models for NSCLC in which either the continuous oncogenic activity of Kras [29] or EGFR [61] are prerequisites of tumour maintenance. This not only shows that tumour growth critically depends on the respective active oncogenic pathways, but it also stresses the usefulness of these oncogenic pathways as therapeutic targets. Direct tumour intervention studies with tyrosine kinase inhibitors (TKIs) 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 tumour regression [60–62]. In addition, prolonged treatment with humanised anti-hEGFR antibody (cetuximab) caused a strong tumour regression [60]. Further studies will be needed to investigate the pathways that determine sensitivity and resistance to EGFR related TKI interventions. Other mouse

models for NSCLC were also used for targeted therapies. First, induced over expression of the PI3K p110α catalytic subunit (PIK3CA), mutated in its kinase domain (H1047R) in *CCSP-rtTA;TetO$_7$ PIK3CA(H1047R)* mice, induces adenocarcinomas. Treatment of these lung tumours with NVP-BEZ235, a dual pan-PI3K and mammalian target of rapamycin (mTOR) inhibitor, caused a marked lung tumour regression. Interestingly, when this single agent NVP6-BEZ235 was tested on lung tumours in *CCSP-rtTA;TetO$_7$-Kras$^{G12D}$* mice, no regression was observed. However, after NVP-BEZ235 was combined with MEK inhibitor ARRY-142886, significant regression of *Kras$^{G12D}$* tumours occurred [54]. Obviously, two major RAS (rat sarcoma viral oncogene homolog protein) downstream effector pathways (fig. 2) need to be impaired in order to get an irreversible regression in Ras mutated NSCLC.

Direct targeting of RAS has so far been widely 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 [99, 100]. An explanation for this can possibly be found in the fact that only KRAS4B is farnesylated but not its isoform KRAS4A. Recent results with lung cancer mouse models, as we already described previously, strongly suggest that actually KRAS4A and not KRAS4B is driving the onset of NSCLC. Although we still have to see if KRAS4A is indeed important in human NSCLC, one can imagine the importance of Kras mouse models in testing functional inhibition of KRAS4A [42].

The use of optimised genetically modified mouse models for lung cancer for therapy research necessitates sophisticated noninvasive tools to follow tumour development and response to therapy. Measurement of tumour size as a function of time is the most obvious way of doing this and existing techniques such as positron emission tomography-computed tomography or magnetic resonance imaging are adapted for use on small animals [54, 61]. However, these techniques are time consuming, making them less suitable for high throughput. Other sensitive and reproducible techniques are now being widely used for measuring gene expression or tumour growth *in vivo*: fluorescence imaging and bioluminescence [101, 102]. Expression of fluorescent proteins can be coupled to oncogenes in transgenic mouse models which facilitates fluorescent imaging of tumour growth. In case of bioluminescence, transgenic expression of luciferase allows accurate longitudinal monitoring and good quantification of tumour mass as has been shown in the *LSL Kras* lung tumour model [103]. Feasible imaging techniques will greatly enhance the accuracy and reproducibility of mouse models.

We have shown some of the promising applications of mouse models in therapy research. However, one should not forget that mouse transgenic models are rather simplistic approximations of the human disease, although some of the somatic models do have a striking resemblance to human lung cancer. Lung tumours in mouse models are often driven by limited and defined pathways on which they remain dependent. Whether human lung cancers show similar dependencies on a continued activity of initiating oncogenes remains to be seen. The strength of genetic engineered mouse models is that it can give us insight into the importance of such particular genetic lesions on tumour growth in a therapeutic setting. As such, it can be a valuable first step in more efficient pre-clinical therapy research.

## STEM CELLS AT THE ORIGIN OF LUNG CANCER

Initial mutated cells undergo a series of various genetic alterations before they finally acquire full tumorigenic capacity [104]. Different early stages of tumour development have been identified for human squamous carcinoma and adenocarcinoma [6], which suggested that transformed bronchial and alveolar cells themselves can serve as cells of origin for lung cancer. As we have shown, this has been confirmed for most, but not all, genetically engineered mouse models for lung cancer. Results from NSCLC as well as SCLC models [70, 78, 81] indicated that both distinct types of genetic mutations and the cell-type in which these lesions occur control initial events of lung cancer development. However, no certainty exists about the nature of cells of origin for lung cancer. Knowledge of these cells can have an important impact, not only on our understanding of lung cancer biology but also by offering us new targets for lung cancer therapies. For many years it has been known that human lung cancer of both adenocarcinoma and SCLC contain small populations of cells (<1%) that are able to form colonies in soft agar [105]. Upon transplantation into athymic nude mice, selected soft agar colonies were shown to generate tumours with similar features of the original primary lung cancers [105]. Therefore, human lung cancer clearly consists of heterogeneous cell types of which only a small subset can fully renew the tumour. It is tempting to classify the regenerative or clonogenic subset of lung cancer cells as ''cancer stem cells'' (CSC). Being a CSC would imply that they share features or are directly derived from mutated, transformed normal stem cells. So what are the features of normal stem cells that make them such ideal tumorigenic targets? Stem cells are defined as rare, long-lived cells whose main properties include self-renewal capacity, extensive proliferation *via* transit-amplifying cells, and multipotent capacity through generation of mature, terminally differentiated cells [106, 107]. Self-renewal allows maintenance of an undifferentiated stem-cell pool over the lifetime of the host and together with its longevity makes CSCs ideal candidates for accumulating sufficient genetic lesions needed to initiate tumorigenesis. Multidrug-resistance is an additional property of normal stem cells, which secures their longevity in the face of toxic agents, including many of the well-known drugs used in lung cancer therapy. Functional multidrug-resistance is often mediated by over expression of adenosine triphosphate-binding cassette (ABC) transporter that efflux drugs [108]. Indeed, over expression of ABC transporters have been described in clonogenic subsets and are a probable cause for chemoresistance of the treated tumours [108]. The latter observation could also be of particular interest for human lung cancer since recurrence after chemotherapy and radiotherapy is common in human lung cancer [109]. A defined chemoresistant CSC population could be a potential source of tumour recurrence and thus become in itself a valuable therapeutic target.

However, only recently have similarities between cancer cells and normal stem cells led to the idea of the presence inside epithelial tumours of a small population of CSC or ''tumour-initiating cells'' [108]. CSCs are defined as a rare population of

undifferentiated tumorigenic cells responsible for tumour initiation, maintenance and spreading [106, 108]. These cells have been identified in many types of cancers by sorting cell subpopulations based on surface marker expression patterns, and by transplantation studies into animal models using prospectively isolated tumour cells. Their existence was first documented in the context of leukaemia [110, 111]. Following this, they have been identified in breast cancer [112] and glioblastoma [113]. Lately, ''tumour initiating cells'' have been identified in a wide variety of tumours including prostate, colon, pancreatic and hepatic carcinomas, melanoma and several other tumour types [114]. These studies have shown that these cells present an unlimited renewal potential and that they can undergo differentiation leading to the formation of tumours in immunodeficient mice that recapitulate the heterogeneity of the original tumour at high efficiency [115]. Nevertheless, at present it is still not clear whether CSCs exist inside a solid tumour. However, another possibility for the ontogeny of cancer is the so-called stochastic model. This model foresees the onset and progression of cancer from multiple transformed progenitor cells leading to a polyclonal tumour, in which each individual subclone can, in principle, reconstitute a complete tumour [116]. Thus, the latter model predicts that each transformed, undifferentiated cell has the same tumorigenic capacity and the tumour will comprise of a selection of the most dominant subclones [117]. A relative high percentage of cells from a single tumour should, therefore, be able develop into full-blown cancer after xenotransplantation in immunodeficient mice [118].

Thus, there is evidence of distinct lung cancer cell populations and this necessitates a better understanding of how normal bronchial and alveolar tissues are organised in order to identify lung stem cells.

Formation and renewal of lung tissue is largely endogenous as it is now generally accepted that the formation of lung epithelium by circulating mesenchymal progenitor cells is infrequent and not of physiological relevance [119]. Stem-cell research has progressed rather slowly in the lung compared to other organs due to the anatomical and functional complexities associated with cellular heterogeneity and the low turnover rate of its epithelium. Only after being challenged by environmental and nutritional factors does the lung epithelium show a dramatic increase in its turnover rate. This is in contradiction to the constant rapidly renewing tissues of intestinal and haematopoietic origin. These fast renewing tissues have a classical hierarchical organisation of stem cells, and transit-amplifying progenitors and their differentiated progeny. The pulmonary system, however, contains a variety of nonclassical organised transit-amplifying cells, (facultative) progenitor cells and terminal differentiated cells that are anatomically separated in the conducting airway epithelium and gas-exchange alveolar regions [120]. These different pulmonary epithelial cells were mainly identified by airway injury experiments. Various toxic agents were used to trigger the selective damage response of particular lung epithelial cells after which their increased proliferation rates were followed *in vivo* through [$^3$H] thymidine or bromodesoxyuridine (BrdU). In pulse/chase experiments, only self-renewing stem cells retain the label for an extended period due to slow turnover and are so-called label retaining cells (LRCs).

Although the distal epithelium of the lung is composed of Clara, ciliated and neuroendocrine cells, only [$^3$H] labelled Clara-like cells were found to be able to proliferate and generate *de novo* differentiated Clara cells and ciliated cells after exposure with ozone gas had initially damaged all ciliated cells [121]. This provided evidence that Clara cells are facultative progenitor cells, but it did not provide evidence for a stem cell hierarchy within the Clara-cell population. Another toxic agent, naphthalene, induces ablation of almost all nonciliated bronchiolar epithelial Clara cells due to selective metabolic activation of the toxin. This treatment results in the proliferation of two different populations of LRCs in regions localised to neuroendocrine bodies (NEBs) in the distal airway, namely Clara-like cells expressing CC10 and pulmonary neuroendocrine cells (PNECs) expressing CGRP [122]. Both naphthalene-resistant Clara-like cells (named variant or vClara cells) and the PNECs are able to maintain epithelial renewal and their localisation in NEBs is believed to represent a stem-cell niche [123–125]. vClara cells are resistant to naphthalene injury because they do not express the enzyme Cyp2f2, which is a member of the cytochrome P450 family that converts naphthalene into a toxic compound. Besides this, vClara cells are also able to efflux drug *via* multidrug-resistant receptors and present a side population phenotype [126].

A transgenic mouse model in which the herpes simplex virus thymidine kinase (TK) gene is expressed under the control of the CC10 promoter (*CC10-TK*) was used to test whether PNECs or vClara populations represent the stem cell pool with pluripotent capacity for airway epithelial repair [127]. In this model the CC10-expressing (*e.g.* both vClara and normal Clara) cells can be selectively ablated in the airways of mice through acute administration of ganciclovir. Studies on the regenerating epithelium showed that the ablation of CC10-expressing cells led to PNEC proliferation and hyperplasia but they are unable to effectively repopulate a differentiated ciliated epithelium [127]. PNEC hyperplasia independent of NEB-associated vClara cells may represent a compensatory mechanism to repopulate the airway in the setting of total stem-cell ablation [128]. Hence, these studies lead to the conclusion that either a subpopulation of CC10-expressing cells, but not PNECs, may represent stem cells required for repair of Clara cell injury in the mouse distal airway or that these cells may be required to support the stem cells that give rise to new Clara cells [127].

A second epithelial stem-cell niche has been identified after similar naphthalene treatment [123, 129]. An LRC subpopulation of Clara cells was found near terminal bronchi, at the bronchioalveolar duct junction (BADJs) where airways terminate and form alveoli and only very few NEBs are observed [123, 124]. These cells have been termed bronchioalveolar stem cells (BASCs) [129]. They co-express CC10 and SP-C, which is a major secreted compound of type II alveolar cells but expressed at low levels by early embryonic lung epithelial cells [130]. However, dual expressing CC10/SP-C cells were not found in late embryonal lungs, so it is likely that these BASCs develop after birth in rapidly expanding pulmonary epithelial tissue.

This double positive (CC10 and SP-C) population is quiescent in normal lung [131]. BASCs were found to express cell surface

**TABLE 1** Genetically engineered mouse models for human lung cancer

| Model type | Phenotype | [Ref.] |
|---|---|---|
| **Transgenic** | | |
| CC10-Tag and Sp-C-Tag | Multifocal bronchioloalveolar hyperplasias develop into mixed solid and papillary adenocarcinomas | [10, 12] |
| K5-E6/E7[#] | Adenocarcinomas | [143] |
| MMTV-TGF-β1 DN[¶] | Adenocarcinomas | [144] |
| MMTV-RARβ4[+] | Alveolar hyperplasia | [145] |
| CC10-Tag; CC10-hASH1 | Adenocarcinomas with focal NE differentiation | [82] |
| CC10-hASH1 | Bronchial hyperplasia | [146] |
| CC10-hK14 | Squamous differentiation hyperplasia with occasional squamous metaplasia | [90] |
| Sp-C-IgEGF | Alveolar hyperplasia | [147] |
| Sp-C-cMyc; SpC-IgEGF | Bronchioloalvealor adenocarcinomas | [147] |
| Sp-C-cMyc | Mixed bronchioloalveolar adenomas and adenocarcinomas | [147] |
| Sp-C-cRaf-1 | Adenomas | [148] |
| MMTV-RARβ2[§] | Adenomas and adenocarcinomas | [149] |
| CC10-cMyc | Bronchioloalveolar hyperplasia | [14] |
| CGRP-H-Ras | NE hyperplasia and non-NE adenocarcinomas | [150] |
| **Conditional transgenic** | | |
| Using Cre/lox system[ƒ] β-actin-lox GFP lox-K $Ras^{V12}$-IRES-hPLAP | Alveolar hyperplasia, adenomas and adenocarcinomas | [31] |
| Lox-stop-lox-K $Ras^{G12D}$[##] | Epithelial hyperplasia of bronchioles, adenomatous hyperplasia, adenomas, both solid and papillary adenocarcinomas | [33, 34] |
| **Doxycycline regulatable** | | |
| CC10-rtTA; $(tetO_7)$CMV-FGF7 | Epithelial cell hyperplasia and adenomatous hyperplasia | [28] |
| CC10-rtTA; $(tetO_7)$CMV-K $Ras^{G12D}$ | Bronchogenic adenocarcinomas. Phenotype is completely reversible upon Dox removal | [29] |
| CC10-rtTA; $(tetO_7)$CMV-K $Ras^{G12D}$ in a $Trp53^{-/-}$ or $Ink4a^{-/-}$ background | Bronchogenic adenocarcinomas. Phenotype is completely reversible upon Dox removal | [29] |
| CCSP-rtTA; $TetO_7$ PIK3CA(H1047R) | Adenocarcinomas | [54] |
| CCSP-rtTA; $TetO_7$-BRAFV600E | Mixed adenocarcinoma and bronchioalveolar carcinoma | [46] |
| SP-C-rtTA; TetO7-Cre; $Pten^{F/F}$ | Impaired alveolar epithelial differentiation and hyperplasia | [63] |
| CCSP-rtTA; Tet-$O_7$-$hEGFR^{L858R}$ | Adenocarcinoma with bronchioalveolar carcinoma features<br>Local invasive adenocarcinoma after longer incubation | [60, 61] |
| CCSP-rtTA; Tet-$O_7$-$hEGFR^{DEL}$ | Adenocarcinoma with bronchioalveolar carcinoma features<br>Local invasive adenocarcinoma after longer incubation but with longer latency | [60, 61] |
| Spontaneous activatable knock-in latent allele K $^{RasG12D\ LA}$ | Epithelial hyperplasia of bronchioles, adenomatous hyperplasia, adenomas, both solid and papillary adenocarcinomas | [23] |
| K $Ras^{G12D\ LA}$ in $Trp53^{-/-}$ background | Epithelial hyperplasia of bronchioles, adenomatous hyperplasia, adenomas, both solid and papillary adenocarcinomas above but with shorter latency | [23] |
| K $Ras^{G12D\ LA}$; TGF-$\beta^{+/-}$ | Increase of adenocarcinoma formation | [58] |
| **Conditional knockout** | | |
| Using Cre/lox system | | |
| Trp53 | Adenocarcinomas | [78] |
| $Rb^{F/F}$; $Trp53^{F/F}$ | NE hyperplasia, SCLC with metastases | [78] |
| Compound conditional knock out and transgenes | Phenotypes compared with LSLKrasG12D control | |
| Spry-$2^{F/F}$; $LSLKras^{G12D}$ | Increased number of adenocarcinoma | [50] |
| $Rac1^{F/F}$; $LSLKras^{G12D}$ | Adenoma formation with long latency | [51] |
| $Pik3r2^{-/-}$; $Pik3r1^{F/F}$; LSL $Kras^{G12D}$ | Strong decrease of adenoma and adenocarcinoma formation | [54] |
| $Dicer^{F/F}$; $LSLKras^{G12D}$ | Increased number of adenocarcinoma | [74] |
| $Trp53^{F/F}$; $LSLKras^{G12D}$ | Progressed adenocarcinoma with lymph node metastases | [151] |
| $Trp53^{LSL\ R172H/-}$; $LSLKras^{G12D}$ | Idem | [151] |
| $Trp53^{LSL\ R270H/-}$; $LSLKras^{G12D}$ | Idem but with higher penetrance | [151] |
| $Lkb1^{F/F}$; $LSLKras^{G12D}$ | Shorter latency with mixed adenocarcinoma/squamous cell carcinoma with occasional large cell carcinoma; frequent metastases | [70] |
| CC10-Cre; $Pten^{F/F}$; $LSLKras^{G12D}$ | Bronchioalveolar lesions followed by adenocarcinoma with local invasion and stromal interactions | [64] |
| $Rb^{F/F}$; $p130^{F/F}$; $LSLKras^{G12D}$ | Shorter latency and higher number of adenocarcinoma<br>No neuroendocrine tumours | [81] |

TGF: transforming growth factor; RAR: retinoic acid receptor; Ig: immunoglobuling; EGF: epidermal growth factor; NE: neuroendocrine; Dox: doxycycline; SCLC: small cell lung cancer. [#]: E6/E7 fusion protein was generated; [¶]: dominant-negative form of TGF-β1; [+]: RARβ4 giving sense transcript; [§]: RARβ2 giving antisense transcript; [ƒ]: sporadic inactivation of the conditional alleles occurred after intratracheal instillation of Ad-Cre virus; [##]: intranasal application of Ad-Cre virus.

marker Sca-1 and CD34, which are established stem-cell surface markers in other mouse tissues [132]. Viable (Sca-1+, CD34+) BASCs were able to be isolated by flow cytometry after excluding haematopoietic (CD45+) and endothelial (CD31+) cells [129]. They appear to be crucial for the regeneration of epithelial components of terminal bronchioles and alveoli. Moreover, BASCs are capable of several passages *in vitro* while either maintaining their (CC10+/SP-C+) status or developing into Clara (CC10+/SP-C-), alveolar type II (CC10-/SP-C+) or alveolar type I (SP-C+/aquaporin5 +) cells. Thus, BASCs were shown to be capable of multipotent differentiation as well as self-renewal. At present, we still do not whether these cells function as true stem cells as the evidence for their self-renewal and multipotency *in vivo* is, as yet, preliminary. Clearly better characterisation of BASCs is needed to identify more unique surface markers, as has been shown by several isolation studies [133, 134] but also to investigate how similar or identical vClara cells and BASCs are to each other. Indeed, all the

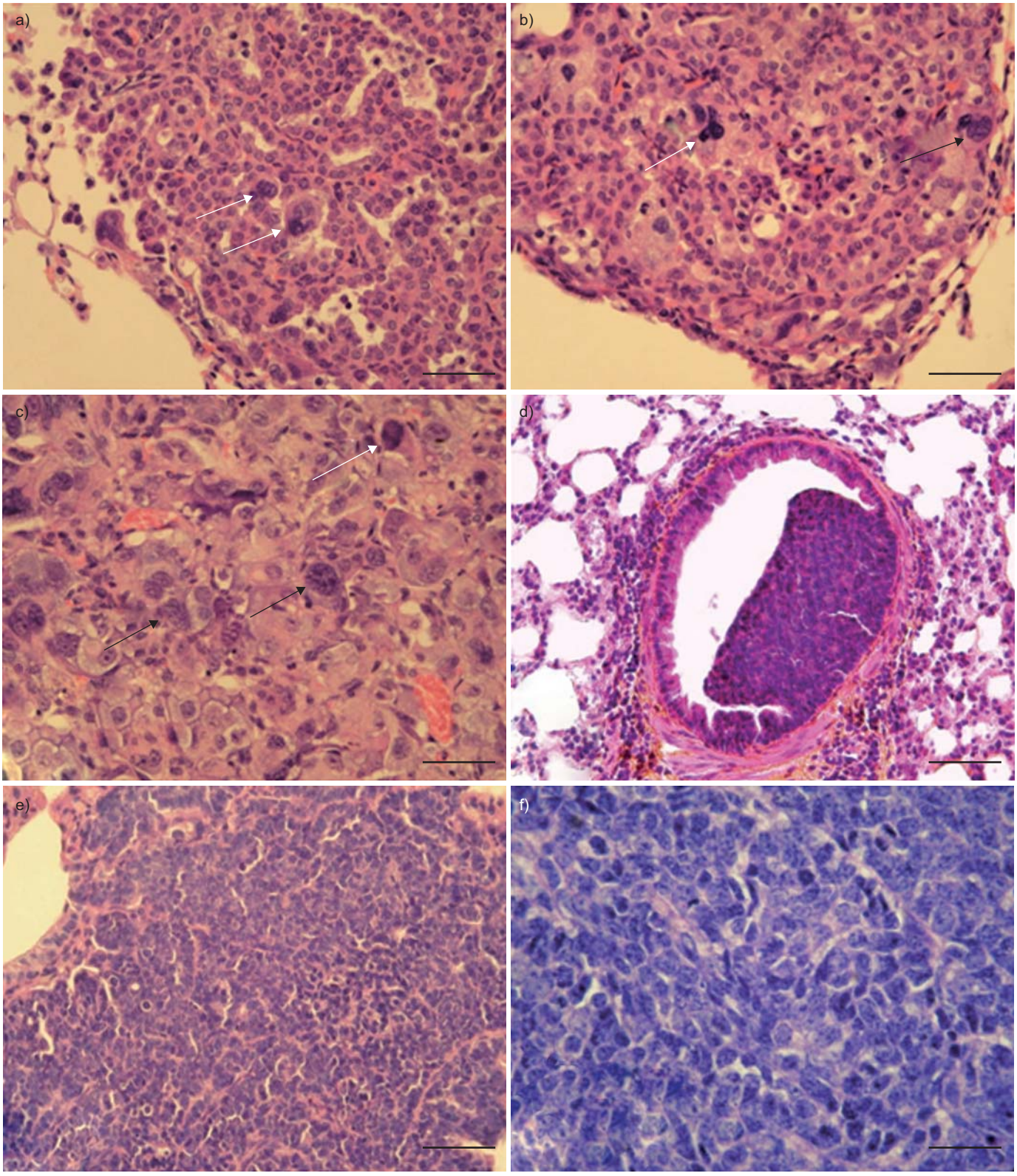


**FIGURE 3.** Representative lung tumours from mouse models for advanced nonsmall cell lung cancer and small cell lung cancer. The progressed adenocarcinoma from *Trp53*$^{F/F}$;*LSLKras*$^{G12V}$ mice 3 months after Adeno-Cre treatment are shown (a–c; unpublished data). a) Adenocarcinoma in which some tumour cells have irregular nuclei (arrows). b, c) Further advanced adenocarcinoma showing tumour cells with pleiomorphic nuclei (white arrows) and giant cells (black arrows). d) Early neuroendocrine hyperplastic lesions inside bronchi of *Rb*$^{F/F}$;*Trp53*$^{F/F}$ mice 3 months after Adeno-Cre treatment which, after a further 3 months, progressed into small cell lung cancer (e, f). Note the characteristic moulding of nuclei of adjacent tumour cells and the sparse amount of cytoplasm. Haematoxin and eosin stain. a, b, d, e) Scale bars=25 μm. c, f) Scale bars=10 μm.

evidence so far indicates that both vClara and BASCs are candidates for bronchial stem cells and normal Clara cells are facultative transit-amplifying cells.

Another putative lung stem-cell population was found through the use of a serum-free culture system for primary neonatal pulmonary cells. The arising colonies were positive for CC10 and Sca-1 but negative for SP-C and CD34. In addition, cells were found to express octamer-binding transcription factor 4 (Oct4) and stage-specific embryonic antigen-1. BrdU pulse-chase experiments indicated that slow-cycling label retaining Oct4 positive cells are localised at the BADJ [135]. These Oct4 positive cells were able to differentiate into both alveolar type 2 or 1 cells. Again it remains to be seen which similarities this candidate bronchial stem cell shares with the other two candidates. Studies on the finding of CSC in human lung cancer were mostly directed on isolating distinct cell populations from primary tumours. To date, no clear robust markers have been characterised that could be exclusively linked with a clonogenic CSC populations. Some of these results have been reviewed elsewhere [136]. Furthermore, since there is no known human homolog of Sca-1, it becomes obvious that BASCs but also vClara cells need further characterisation in order to find their human counterpart. Recently, it has also been demonstrated that both human SCLC and NSCLC contain small populations of undifferentiated cells expressing the cell surface marker prominin-1 (CD133) [137]. Cd133 has been found to be expressed in cancer-primitive cells as well as normal cells from neural, endothelial, epithelial and haematopoietic lineages [138]. Lung cancer CD133 positive cells are able to grow indefinitely as tumour spheres in particular culture conditions and they can generate tumour xenografts in immunocompromised mice, phenotypically identical to the original tumour, while CD133 negative cells do not [137]. Lung cancer contains a rare population of CD133 positive cancer stem-like cells that are able to self-renew and generate an unlimited progeny of nontumorigenic cells [137]. Chemoresistance was observed in the tumour spheres and was associated with increased expression of ABC transporter and embryonal stem cell markers Oct4 and NANOG. Interestingly, after naphthalene treatment in normal mice, a rare population of CD133 positive cells increased. Therefore, it would be very tempting to suggest that those expanding CD133 positive cells are BASCs. Follow-up studies are needed to substantiate this and to translate some the other findings from the mouse models into a better characterisation of human lung CSC.

The bronchial stem cell population could be an ideal candidate for lung cancer progenitors. As these lung stem cells are already endowed with the capacity for self-renewal, they are excellent candidates for the accumulation of deleterious mutations. In fact, accumulating evidence already exist for BASCs that this is indeed the case.

Cells expressing both SP-C and CC10 were first described in the precursor lesions of murine lung tumours initiated by oncogenic Kras [33]. Expression of the oncogenic protein KRAS in these double positive cells induces their proliferation *in vivo* [33]. Moreover, induction of tumorigenesis in naphthalene-treated *LSL-Kras$^{G12D}$* mice induced an increase in the number and size of tumours [129]. Furthermore, *Kras$^{G12D}$* expression causes BASCs *in vitro* expansion and generates hyperproliferation at the BADJ *in vivo* [129]. Similarly, BASCs from p38α MAPK$^{-/-}$mice were also highly sensitised to *Kras$^{G12D}$*-induced lung tumorigenesis, leading to an immature and hyperproliferative lung epithelium, thus permitting earlier induction and faster progression to adenocarcinoma [139]. Abrogations of major signalling pathways were shown to be important for the proliferation and maintenance of BASCs. Conditional deletion of PTEN or PI3K in mice result in increased numbers of BASCs and increased propensity to lung tumour development [63, 140]. In p27$^{-/-}$ mice, BASCs expand first at the BADJ and progress from hyperplasia to adenocarcinomas through dysplasia and adenomas [141]. In contrast, deletion of *Bmi1* inhibits tumorigenesis through inhibition of BASC expansion [142]. Taken together, these studies show that BASCs may play a role in the initiation of tumorigenesis but there is still no direct proof of being the initial target cell *per se*. Indeed CSC may arise from bronchial stem cells but we cannot exclude the fact that they develop from transit-amplifying cells, such as PNECS and Clara cells with each having acquired the ability to self renew as a result of oncogenic mutations. In addition to the acquisition of genetic and epigenetic mutations, interactions between tumour cells and their microenvironment (also called niches, which are composed by the stroma, inflammatory cells and recruited vasculature), has a profound influence on the tumorigenic process. More and more evidence shows that tumour growth can be sustained not only by rare CSC populations but also through dominant (sub) clones or a mixture of both [117]. Although the importance of characterising CSC of lung cancer is of obvious importance, it would be too optimistic as to say that targeting of CSC would cure lung cancer. Most likely a combined targeting of all the different populations is needed to impair tumour growth and prevent long-term recurrence.

## PERSPECTIVES AND NEW DIRECTIONS

The use of mouse models is helping us to understand lung cancer biology by uncovering the critical molecular pathways that govern each stage of tumour formation and progression. It will be important to adjust mouse models for each different human lung cancer type. Progress on genome-wide expression profiling and genomic hybridisation will be helpful in cross-species analysis of similarities in cancer signalling pathways that govern both mouse and human lung tumorigenesis. These studies will be very useful for the design of inhibitors, which can impair lung tumour growth and can be tested in pre-clinical mouse models. New, better adapted mouse models with complete human genes could lead to the development of humanised mice that recapitulate human lung cancer at en even higher degree [32]. Rapid advances of lung cancer genome analysis will reveal many additional novel mutations that could be complemented into mouse models for lung cancer.

Metastatic mouse models for both NSCLC and SCLC are now well-established and it will be a challenge to use these models to unravel the mechanism by which lung cancer is often refractory to chemo- and/or radiotherapy. Defined early stages of lung cancer development in combination with noninvasive imaging technologies in these mouse models will be very

helpful for testing chemoprevention protocols. Another very interesting aspect of the conditional lung tumour models is that they can yield promising biomarkers for lung cancer. Advanced mass spectromy-based proteomics will allow scrutinising analysis of the mouse serum proteome, under both normal and cancerous conditions. There is a clear need for clinically relevant biomarkers of early lung cancer and both somatic mouse models for NSCLC and SCLC show distinct stages in lung tumour development. Combining gene expression profiling with proteomics of these early murine lung tumours will hopefully lead to the discovery of new, relevant biomarkers that can be translated into diagnostic use. A major challenge will not be the discovery but the evaluation of candidate biomarkers through proper validation on patient samples.

The extra-ordinary power of mouse genetics will continue to help us identify lung tumour precursor cells by selective targeting of lung epithelial cell compartments. Candidate precursor cells could then be tagged with reporter genes so one can follow whether they behave like cancer stem cells during tumour growth.

Much progress has been achieved on mouse models for human lung cancer (table 1, fig. 3), but further characterisation is still needed for most NSCLC and SCLC models. It is also important to note that the development of mouse models for SCC clearly lacks behind. A better knowledge of the cell of origin of lung SCC, together with identifying the most suited genetic lesions that cause this lung cancer type, might facilitate proper design of a mouse model.

Finally, we have seen that mouse models for lung cancer have already helped us to further our insight in basic lung cancer biology and test lung cancer therapies. These models still hold many promises within these fields and should be expanded by finding new markers for lung cancer diagnosis. Results will undoubtedly fulfil some of these promises and be importance in the fight against human lung cancer.

## SUPPORT STATEMENT

S. de Seranno was supported by a Postdoctorate grant (Instiut National du Cancer, Paris, France) and R. Meuwissen was supported by a Jeune Chercheur fellowship (INSERM, Paris).

## STATEMENT OF INTEREST

None declared.

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