Mouse Models of Lung Cancer

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Abstract Human lung cancer is responsible for ~30% of all cancer deaths worldwide with >160,000 deaths in the United States alone annually. Recent advances in the identification of novel mutations relevant to lung cancer from a myriad of genomic studies might translate into meaningful diagnostic and therapeutic progress. Towards this end, a genetic model animal system that can validate the oncogenic roles of these mutations *in vivo* would facilitate the understanding of the pathogenesis of lung cancer as well as provide ideal preclinical models for targeted therapy testing. The mouse is a promising model system, as complex human genetic traits causal to lung cancer, from inherited polymorphisms to somatic mutations, can be recapitulated in its genome via genetic manipulation. We present here a brief overview of the existing mouse models of lung cancers and the challenges and opportunities for building the next generation of lung cancer mouse models.

Lung cancer is the leading cause of cancer-related mortality for both men and women in the United States and in the world (1). Despite advances in cytotoxic drug development, radiotherapy, and patient management, the cure rate for advanced non–small-cell lung cancer (NSCLC) remains dismal. A comprehensive understanding of the genetic alterations involved in the initiation and progression of human lung cancer would facilitate progress in clinical treatment and early diagnosis. The recent genome-scale effort to identify lung cancer–relevant genes has generated many candidate genes of potential therapeutic relevance. Success with a systematic genome resequencing approach, which has identified mutations in the *EGFR* kinase domain in human lung cancer and showed their correlation with patient response to the kinase inhibitors gefitinib and erlotinib (2–6), has revolutionized targeted therapy in lung cancer. Similarly, complementary genome-wide analytic approaches such as comparative or array genomic hybridization to detect copy number alteration (7, 8), as well as single-nucleotide polymorphism array analyses (9), have proved highly productive in discovering novel lung cancer–specific genomic alterations. *In vitro* studies have begun to validate some of these gene candidates of potential clinical relevance to lung cancer. In particular, the recently identified somatic mutations in *EGFR*, *BRAF*, and *PI3K* kinases have been shown to possess transforming activity. However, although these *in vitro* cell culture studies are informative, they cannot fully recapitulate the complex nature of *de novo* tumorigenesis or *in vivo* responses to therapy. Thus, the development of animal models harboring these mutations will likely yield additional insights into the underlying pathophysiologic perturbations. With such models in hand, it will also become possible to generate preclinical models to yield histopathologic and molecular surrogates for a specific mutation-directed activity in a mature tumor, and thus better evaluate the efficacy and specificity of emerging agents directed against such lesions.

The ability to easily manipulate the mouse genome, along with the anatomic and physiologic similarity of the mouse to humans, makes the mouse an ideal system to model human diseases. Different generations of mouse models have evolved employing diverse innovative strategies to model lung cancer as summarized in Fig. 1. Although they have all been informative and further propel our understanding of human lung cancer, they still do not fully recapitulate the complexities of human lung cancer. The existing models and the strategies for the development of better mouse models will be discussed in this review (for additional review, see ref. 10). Although we have attempted to be as comprehensive as possible, our list of mouse models is likely to be incomplete.

Evolving Mouse Models for Human Lung Cancer

Classic transgenic models for ectopic expression. The development of methods for manipulating the mouse genome, combined with the sequencing of the mouse genome, has advanced our ability to generate new mouse models of human diseases. Some of the earliest lung cancer mouse models were based on the introduction of a transgene under the control of heterologous promoters. To generate a transgenic mouse, a linearized plasmid vector carrying a transgene is integrated into the genome of the fertilized oocytes following microinjection

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and later implanted into pseudopregnant mothers. In addition to constitutively expressed promoters such as keratin, genes have also been targeted to specific subsets of lung epithelial cells using alveolar type II cell–specific surfactant protein C (*SP-C*) promoter (11), the nonciliated secretory cell–specific Clara cell secretory protein (*CCSP*) promoter (12, 13), and calcitonin gene–related peptide (*CGRP*) promoter (14). Examples of transgenic mice that model lung cancer include models with expression of human papillomavirus-16 E6/E7 under the keratin-5 promoter, which developed lung adenocarcinoma (15). Similarly, simian virus large T antigen (Tag), targeted with *SP-C* (16, 17) or *CCSP* (18, 19) promoter, resulted in adenocarcinoma of alveolar type II cells and distal Clara cells of the lung, respectively. Furthermore, *SP-C* specific expression of c-*myc*, epidermal growth factor (EGF; ref. 20), *c-met*-related tyrosine kinase, *Ron* (21), *Raf-1* (22), and dominant-negative p53 (23) transgenes developed bronchioloalveolar adenocarcinomas.

These transgenic mice are ideal for overexpression of the modeled oncogenes, a phenomenon that is frequently observed due to amplification or epigenetic modification in human cancer. However, a major biological caveat to this strategy is that heterologous promoters might not recapitulate the true expression pattern of the gene during development, adult life, and/or tumorigenesis. Further, oncogenes in such transgenic mice are constitutively expressed and cannot be regulated to assess the role of the transgene in tumor maintenance after the initiation and establishment of the tumor.

Classic knockout/knock-in models. In a knockout technology, a tumor suppressor gene in embryonic stem cells (24, 25) is inactivated or replaced by site-specific homologous recombination between the chromosomal DNA and recombinant DNA flanked by homologous DNA sequences (the targeting vector). Recombinant DNA could code either for an antibiotic marker or for any intervening sequence that interrupts the coding region of the target gene to knock out or to knock in a novel sequence. The embryonic stem cells with the modified genome are injected into a pre-implantation embryo (blastocoele of 3.5-day-old embryo). The incorporation of the modified embryonic stem cell into the murine germ line of the chimeric mouse gives rise to genetically modified loci, enabling comparative studies with wild-type mice littermates.

The knockout strategy allows preferential deletion of selective tumor suppressor genes implicated in lung cancer. In particular, targeting genes deleted early during human lung tumor development, such as the nonessential genes on chromosome 3p, *FHIT* and *VHL*, is likely to closely recapitulate a cancer phenotype. For example, 44% of *Fhit-Vhl* doubly deficient mice were found to develop spontaneous lung adenocarcinomas (26). However, this strategy cannot be used to study essential tumor suppressor genes like *Rb-1* or *WT-1*, which are embryonic lethal if deleted (27, 28). On the other hand, other tumor suppressor knockout mutant animals that are not embryonic lethal, such as p53, p16, p19, or p16/p19 mutant mice, either do not develop lung adenocarcinomas or succumb to other tumors like lymphomas and sarcomas early on, thus precluding the development of lung cancer. Given the complex genetic alterations in human lung cancer, it is unlikely that deletion of a single tumor suppressor gene is sufficient to phenocopy human lung cancer in the mouse.

The knock-in approach, in contrast, has been applied to activate oncogenes. In one study, one allele of mouse K-*ras* was replaced with an oncogenic K-*rasG12D* allele, which could be expressed only if somatic recombination occurred between the targeted allele and the wild-type allele (29). This strategy bypasses potential alterations in development due to the early expression of the mutant allele. Subsequently, following a spontaneous somatic recombination event, the endogenous promoter gets juxtaposed to express the mutant allele and, thus, physiologic levels of expression are obtained. Mice with the targeted K-*ras* mutation developed pulmonary adenocarcinomas because of the sporadic activation of the mutant allele. However, these mice also developed other tumor types in addition to lung adenocarcinomas.

Conditional bitransgenic tetracycline-inducible mouse models. In lung cancer, most oncogenic activating mutations are somatic whereas the classic transgenic mouse carries transgenes of which the expression is limited by the inherent temporal-spatial expression pattern of the chosen heterologous promoter. The next generation of transgenic mice was designed to overcome this drawback by conditionally regulating the expression of a gene in a specific somatic tissue, providing leverage to researchers in generating inducible mouse tumor models. The binary transgenic conditional model is an inducible model that offers to overcome basic deficiencies of the classic model by sequentially splitting temporal-spatial regulation into individual events (30, 31). Based on the principle of gene activator protein–mediated positive regulation of the lac operon in bacteria, a gene of interest is cloned downstream to a regulator element that requires the cooperation of at least two different molecules to initiate transcription, a regulator protein and its cognate binding partner (32). The expression of the regulator protein under a tissue-specific promoter defines the spatial regulation of the gene of interest whereas the temporal regulation is achieved by exogenously supplementing the binding partner.

One of the classic examples of a bitransgenic inducible system that has been frequently exploited is the reverse tetracycline transactivator (rtTA) inducible system. In this system, the bitransgenic mouse harbors two different transgenes. One transgene, the *rtTA* gene linked to a tissue specific promoter, drives the expression of the rtTA protein in a specific cell type. In the presence of tetracycline/doxycycline, the rtTA protein activates the expression of the second transgene, the targeted gene linked to the tetracycline-responsive promoter element (*Tet-Op*; refs. 33, 34), as shown in Fig. 2. This bitransgenic regulatory system has been successfully employed to generate different inducible models of lung cancer. For example, bitransgenic CCSP-rtTA/Tet-Op-FGF-7 mice (35) and CCSP-rtTA/Tet-Op-K-Ras4G12D mice (36) developed epithelial cell hyperplasia, adenomatous hyperplasia, and eventually adenocarcinomas after administration of doxycycline. More interestingly, these tumors regressed following withdrawal of doxycycline, showing that the continual expression of these genes is necessary for tumor maintenance. Similar experiments done in a *Tp53*- or *Rb1*-deficient background resulted in an even faster tumor onset. Thus, by combining the inducible transgenic system with the traditional knockout model, the complex mutant mice showed that the expression of oncogenic K-ras in combination with tumor suppressor deficiency is synergistic in lung tumorigenesis.

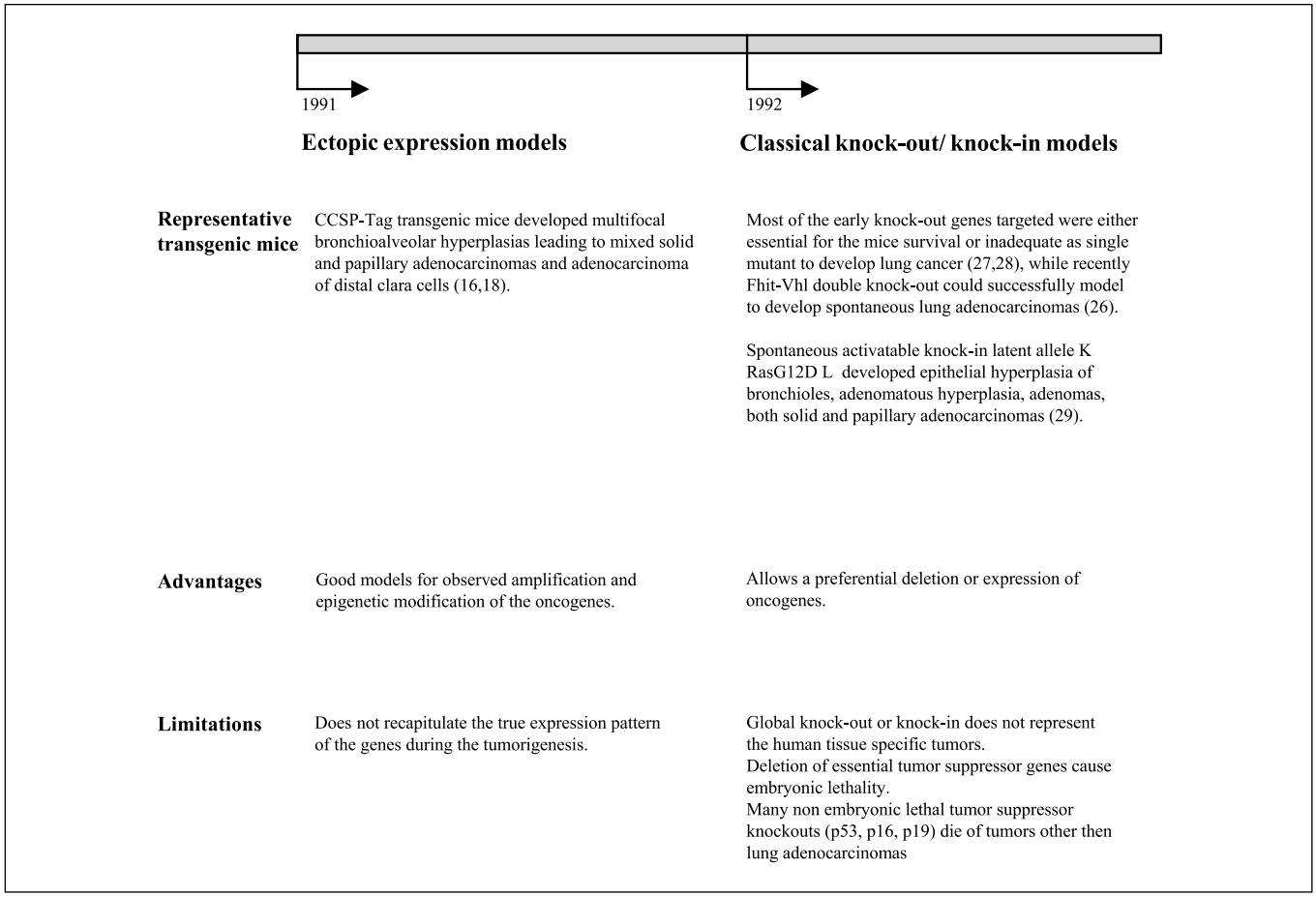

Fig. 1. Evolving mouse models of human lung cancer.

This bitransgenic system provides a high level of tight, inducible, and reversible gene expression in the appropriate tissue *in vivo* to recapitulate the somatic mutation at a defined stage of life in an otherwise normal mouse. The most striking feature of this system is its ability to switch off oncogene expression following the withdrawal of doxycycline, which allows the assessment of the role of these alleles in the maintenance of established lung tumors. However, these murine lung tumor models develop pulmonary adenocarcinomas with limited metastatic potential, albeit with a striking histopathologic similarity to human adenocarcinomas. Finally, considering the high complexity of lung cancer genetics, this regulatory system is an approach that likely needs to be combined with other conditional genetic systems to fully recapitulate human lung cancer.

Conditional bitransgenic mouse models involving Cre/loxP system. As it is becoming increasingly clear that mammalian gene expression is regulated by multiple complex mechanisms, the generation of conditional knockout models using tissue-specific expression of recombinases of the Cre/loxP and Flp/FRT systems has revolutionized lung cancer modeling, whereby mutation at a specific locus can be directed to occur strictly in a set of differentiated cells while the genome of the adjacent cells remains unaltered (37–39). The versatility of this robust system makes it an efficient tool with dual applicability to generating conditional alleles of both tumor suppressor genes and oncogenes.

A target region to be deleted in the gene locus can be marked for deletion by signal sequences of loxP or FRT that are identified by their site-specific recombinase Cre or Flp, respectively. The expression of the recombinase leads to the precise removal of the stretch of DNA between the recombinase signal sequences. To generate a conditional null, hypomorphic mutation or a reduction-of-function allele of a tumor suppressor gene, an appropriate sequence of a coding or noncoding region is flanked by a loxP or FRT site (commonly referred as the region being floxed). Similarly, as a complementary approach, to conditionally activate an oncogene, one could flox the region that prevents its expression. To add an additional level of maneuverability to regulate the expression of the target gene, the recombinase is expressed under a tissue-specific or inducible promoter like the tetracycline-inducible system (40), *Protamine-Cre* (41), and *CMV-Cre* (42).

An alternative approach to Cre-mediated recombination in the lung is to employ engineered viruses (such as adenovirus) via nasal instillation (43). This strategy allows closer recapitulation of lung cancer events by localized and timely regulation of target gene expression in a relatively small number of cells. As a proof of principle, adenoviral-mediated expression of mutant alleles of oncogenic *K-RasG12D* or *K-RasG12V* in bronchioloalveolar cells produced multiple adenomas and adenocarcinomas, revealing the precise role of *RAS* oncogenes in the development of human lung cancer (43, 44). And more

Inducible bitransgenic models	Conditional cre/loxP models	Compound conditional models
Inducible CCSP-rtTA/Tet-Op-FGF7 mice developed epithelial cell hyperplasia and adenomatous hyperplasia and CCSP-rtTA/Tet-Op-K-Ras4G12D developed bronchogenic adenocarcinomas (35, 36).	Conditional transgenic Lox-STOP-Lox-K RasG12D developed epithelial hyperplasia of bronchioles, adenomatous hyperplasia, adenomas, both solid and papillary adenocarcinomas (43, 44)	Mice with conditional activation of Kras2 along with conditional inactivation of Rb or p53 developed hyperplasia that developed into NSCLC, while development of SCLC required inactivation of both genes in addition to conditional activation of Kras2 (47). Similarly, conditional knock-in of mutant K-ras LSL-K-rasG12D and p53 LSL-p53R270H allele aggressively developed lung cancer with stromal desmoplasia (48), while conditional mutations in the K-ras proto-oncogene and three alleles of p53 could recapitulate advanced human pulmonary adenocarcinoma along with desmoplastic response and metastasis (49).
Temporal-spatial expression of a gene in a somatic tissue can be conditionally regulated to recapitulate the *in vivo* situation.	Target gene can be expressed by nasal instillation of adenovirus expressing cre. This situation closely resembles the human lung cancer events.	Allows study of the combined effect of many factors and mutations in the initiation and progression of cancer. Of all the available mouse models, these most closely simulate the *in vivo* situation of tumor initiation and progression.
Full representation of the complex genetics of the lung cancer can only be done by combining this system with the other conditional genetic systems.	Variation in results due to mode of delivery and expression of cre recombinase by nasal instillation	Complex genetics of human lung cancer necessitates complex transgenic mice, though it remains an issue of concern about how far can one keep transforming and retransforming the genome of an organism and still maintain recapitulation of natural genetic events.

recently, Olive et al. and Lang et al. independently generated conditional knock-in mice with dominant-negative p53 modified with two point mutations, *p53R270H* (45) and *p53R172H* (45, 46), at regions analogous to human p53 found to be mutated in patients with Li-Fraumeni syndrome. Following adenoviral-mediated activation of the mutant allele, 19% of *p53R270H*/+ mice developed lung adenocarcinomas with malignant characteristics and metastasis that were found to be similar to human lung adenocarcinoma. However, one of the major downsides of this gene activation system is that the variability in delivery and infection with the virus adds to the heterogeneity of results and experimental inconsistency.

Compound conditional mouse models. A natural extension of the new transgenic technology has yielded a newer generation of compound conditional mouse models that integrate varied features from the above models. The conditional knock-in and knockout genetic systems, for example, allow the ability to simultaneously activate and/or delete multiple genes in a temporal- and tissue-specific manner. In a recent report, compound mouse mutant models with conditional activation of a K-ras transgene (*Kras2*), along with conditional inactivation of the alleles of *Rb* or *p53* by Cre/loxP system, showed involvement of specific factors in regulating non–small-cell lung cancer and small-cell lung cancer (SCLC). The activation of *KRas2* could induce hyperplasia that developed into non–small-cell lung cancer. Development of small-cell lung cancer instead required inactivation of two tumor suppressor genes, *Ink4A/Arf* and *Tp53* (47). Similarly, a mouse carrying conditional alleles of both mutant K-*ras* LSL-K-rasG12D allele and mutant *p53* LSL-p53R270H allele has been generated that developed aggressive lung cancer with induction of stromal desmoplasia, local invasion, and distant metastases. These compound mutant mouse models more faithfully recapitulate the causal genetic phenotypic correlation for human lung cancer (48). Another compound mouse with conditional mutations in the *K-ras* proto-oncogene and different alleles of *p53* tumor suppresser gene (a contact mutant, a structural mutant, or a null allele) has recently been reported. It recapitulates several aspects of advanced human pulmonary adenocarcinoma with development of tumor growth and progression, along with induction of a desmoplastic response and metastasis (49).

Complex Genetics of Lung Cancer Necessitates Complex Mouse Models

The current era of genetically engineered compound conditional mice has generated models that more methodically recapitulate the sporadic forms of cancers in human. These new model systems create an opportunity to evaluate how different combinations of mutations would affect the initiation and progression of lung cancer. In addition, these novel mouse models would allow for the dissection of how different combinations of oncogene or tumor suppressor genes might differentially affect the responses of the lung tumors to novel targeted therapeutics. It is interesting that with the exception of one elegant small-cell lung cancer mouse model (50), all the other mouse lung cancer models to date only develop adenocarcinoma. Presently, there is no

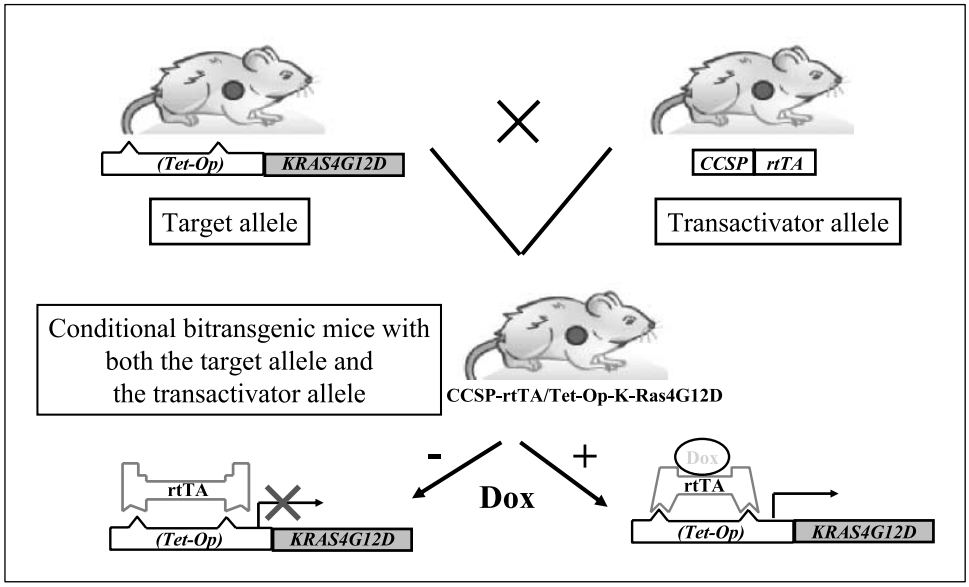

Fig. 2. Example of a conditional bitransgenic inducible mouse using the tetracycline regulatory system.

genetic mouse model of squamous cell carcinoma of the lung. A better understanding of the cell of origin that give rise to lung squamous cell carcinoma and identification of unique gene alterations that are specific to lung squamous cell carcinoma might help the squamous cell carcinoma mouse model development.

The recently discovered *EGFR*, *BRAF*, and *PI3K* mutations involved in human lung cancer are yet to be exploited with appropriate murine models to determine their role in lung tumorigenesis. Murine lung cancer models with inducible expression of these mutations in the lung are in the process of being generated in several laboratories. In combination with the existing oncogene and tumor suppressor mutant alleles that are relevant to lung cancer, these novel alleles will help further our understanding of human lung cancer pathogenesis and aid in the development of novel therapeutics. Lastly, with the rapid advances of lung cancer genome analyses, many additional novel mutations will no doubt be uncovered. Mouse modeling will play a role in the elucidation and validation of these new potential therapeutic targets.

Open Discussion

Dr. Thomas Lynch: What do you think the ultimate significance of this EGFRvIII mutation is going to be in lung cancer biology?

Dr. Wong: We need to screen additional adenocarcinomas to see if these mutations are only found in squamous cell carcinomas. That would then be a very interesting question as to why this mutation is only in squamous cell carcinomas versus the kinase mutations, which are predominantly found in adenocarcinomas. That may suggest that there are different biological forces driving it.

Dr. Lynch: Question for Dr. Meyerson or Dr. Haber: Have either of your labs looked for the vIII mutation in other groups of lung cancer patients?

Dr. Daniel Haber: We have. We looked by RT-PCR at gefitinib responders, nonresponders, and in broad samples. We found it in brain tumors but not otherwise.

Dr. Matthew Meyerson: We have found it. We know they are erbB3 mutations because we see novel genomic DNA rearrangements that we can't imagine would be artifacts.

Dr. Wong: Just to go back to the question of whether there is any clinical relevance to the vIII mutation, Dr. Meyerson and I have been talking about what patient populations to look at, among patients who initially responded to erlotinib or gefitinib. One interesting population would be the BR.21 trial in which there was the 4% to 5% response rate in the nonadenocarcinoma group. One might think that some of those patients might harbor the EGFRvIII mutation.

Dr. Glenwood Goss: Ian Lorimer, who works in my institution, was one of the original investigators on the vIII mutation [Clin Cancer Res 1995;1:859–64]. So we can do it in our lab; we will look at the mutation.

Dr. Pasi Jänne: If you look at the vIII mutations in mice in the adenocarcinomas versus the kinase mutations, do they look histologically the same or different?

Dr. Wong: They do look histologically the same. We have some preliminary evidence in our laboratory suggesting that additional tumor suppressor mutations can actually transform an adenocarcinoma into a squamous cell carcinoma, at least in mouse.

Dr. Bruce Johnson: Do you see histologic evidence of apoptosis in the treated mice? Also, in your controls versus your experimental group, is it actually regression or is it a growth delay?

Dr. Wong: It is actually regression. When you treat the mice with erlotinib, after 2 days of treatment there is striking apoptosis. After a week, there is a big hole with evidence of scarring where the tumor should be. So there is active remodeling taking place where the tumor was.

Dr. John Heymach: These tumors were reversible when you took away the doxycycline—was it with just one of the alleles or more? It is intriguing and reminiscent of the work by Lynda Chin and Ron DePinho [Nature 1999;400:468–72] that driving Ras you get tumors, but take it off and those tumors regress. Do you see any cytogenetic abnormalities in your tumors yet,

and do you think that it is going to be reversible until you start to get additional cytogenetic abnormalities accumulating?

Dr. Wong: With the Ras experiments that you describe in the lab of Ron DePinho and Lynda Chin, when they look at those cell lines there are not a lot of cytogenetic abnormalities. We are doing those kinds of experiments now, trying to culture out these primary tumors. We find it very difficult to do at the moment, unless we put p53 deficiency in these tumors; then it becomes a lot easier. We are going to be looking at genomic instability in these tumors.

Dr. Jeffrey Settleman: I have a general philosophical question about mouse models in lung cancer. These are models where in a few months, you have a single gene-driven event that results in a rapidly growing tumor. How does that compare to what is happening in human lung cancer patients where maybe it is 30 years that a tumor is developing? What do you think will ultimately be the value of drug studies in that setting?

Dr. Wong: Obviously, the human lung tumors will have many more mutations. They are a lot more complex, with aneuploidy and multiple chromosomal aberrations. In the mouse model, you don't have any of that when it is just driven by a single oncogene. With the other tumor suppressor alleles that we have, we can try to recapitulate the human condition by introducing p53 deficiency, pTEN deficiency, or telomere dysfunction into this model. I think the proper number of alleles that you can put into a mouse and start rebuilding what you see in people as tumor progression we may be able to recapitulate the condition. Presently, this is a very simplistic model and we are going to try to build on top of that.

Dr. Heymach: One of the values of doing mouse models as opposed to *in vitro* studies is that, in mouse models, you have the capability of understanding interactions between different cellular compartments and using that as a drug screening strategy. If the only question is how well does a target get inhibited, *in vitro* studies allow you to do much higher throughput studies. I have seen *in vivo* data that match what is seen *in vitro*, but I haven't seen anything that gives us qualitatively new information. When you are looking for specific targets and asking biochemical and pathway questions, I think those studies can be better done in cell lines.

Dr. Paul Bunn: In my opinion, an orthotopic model with human tumors is a much better model because it has all the genetic changes. Having a single genetic change and then trying to do drug testing in that model is almost ludicrous. You can learn about that one gene but it is not going to help you with human therapy.

Dr. Johnson: Let me respond to that. If a genomic study has identified some mutated gene that is important in both initiating and driving the tumor, then perhaps you can learn something about its role as a necessary but not sufficient step. To find out if, by inhibiting that particular genetic lesion, there is an impact on tumor growth is a worthwhile initial step. Then following up with orthotopic models or whatever you think is important to actually verify its relevance in humans is a second step.

Dr. Bunn: I don't think providing scientific value is the question. Is it a good way to screen for drugs? I don't think it is. But I think you learn something.

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