Chemical Carcinogenesis Models of Cancer: Back to the Future

Melissa Q. McCreery and Allan Balmain

UCSF Helen Diller Family Comprehensive Cancer Center, San Francisco, California 94115; email: melissa.mccreery@ucsf.edu, allan.balmain@ucsf.edu

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Abstract

Over a century has elapsed since the first demonstration that exposure to chemicals in coal tar can cause cancer in animals. These observations provided an essential causal mechanistic link between environmental chemicals and increased risk of cancer in human populations. Mouse models of chemical carcinogenesis have since led to the concept of multistage tumor development through distinct stages of initiation, promotion, and progression and identified many of the genetic and biological events involved in these processes. Recent breakthroughs in DNA sequencing have now given us tools to dissect complete tumor genome architectures and revealed that chemically induced cancers in the mouse carry a high point mutation load and mutation signatures that reflect the causative agent used for tumor induction. Chemical carcinogenesis models may therefore provide a route to identify the causes of mutation signatures found in human cancers and further inform studies of therapeutic drug resistance and responses to immunotherapy, which are dependent on mutation load and genetic heterogeneity.

INTRODUCTION

For over 250 years, we have known that cancer can be caused by exposure to an environment rich in toxic chemicals, but it is only in the past 50 years or so that the causal mechanistic links among chemical exposures, DNA damage, and cancer initiation have become clear. Following the early observations of scrotal cancers in chimney sweeps exposed to soot and coal tar (Brown & Thornton 1957), Yamagiwa & Ichikawa (1918) developed the first animal model of cancer by painting rabbit skin with coal tar to induce tumors, thus providing a definitive causal link between treatment and subsequent cancer development. In a series of classic experiments, mouse models were refined to show that cancer can develop through specific stages that could be associated with different types of chemicals (Berenblum & Shubik 1947a). A single exposure to a mutagenic chemical could lead to initiation, in which DNA damage permanently disposed the treated tissue to cancer. However, tumors only grew out after subsequent and repeated exposure to other agents, known as tumor promoters, which appeared to act not through mutation induction but by stimulation of proliferation and inflammation (Hecker 1968, Takigawa et al. 1983). These pioneering studies led to the concept of multistage carcinogenesis that is now widely accepted as being applicable to both mouse models and human cancers, particularly in epithelial tissues such as the colon, skin, pancreas, and mammary gland.

We now have a sophisticated view of the mechanisms by which different classes of chemicals interact with DNA, forming adducts or causing base damage that, if improperly repaired, results in cancer-initiating mutations (Lindahl 2016). We have also learned, thanks to the Human Genome Project and the huge databases of normal and tumor genomes it has spawned, that hundreds if not thousands of genes, when mutated, can contribute to cancer initiation or progression. The view of cancer that has emerged is one of increasing complexity and heterogeneity at the cellular and genetic levels, a microcosm of Darwinian evolution leading to selection of those cells that are best suited to their particular environment.

The purpose of this review is to chronicle the development of our present understanding of how tumors are initiated by exogenous chemical agents, how these tumors acquire the capacity for malignant progression, evade the immune surveillance system, and ultimately metastasize throughout the organism. It may be asked why, in this era of precision genomics that has led to development of so many sophisticated genetically engineered mouse cancer models (GEMMs), we would focus on models involving nonspecific mutagens that rarely if ever can be targeted to a specific tissue type or cell population. The reason is simple: Although GEMMs have provided unprecedented opportunities to alter, at will, the germline of mice to induce specific events that increase cancer susceptibility, it is increasingly clear that they portray a vastly oversimplified view of the numbers and types of mutations found in human cancers (McFadden et al. 2014, 2016; Westcott et al. 2014). GEMMs frequently contain only a handful of point mutations, whereas human cancer genomes present a rich tapestry of point mutations, gene copy number changes, and complex genomic events that result in large part from exposure to exogenous agents that mold genome architecture. The analysis of thousands of human tumor genomes by whole-exome or genome sequencing has shown that the frequency of point mutations can vary over several orders of magnitude, from less than 0.1 to greater than 50 mutations per megabase (Vogelstein et al. 2013). Tumors from patients exposed to a high concentration of carcinogens (e.g., lung cancers from heavy smokers or skin cancers due to prolonged exposure to mutagenic UV radiation) have extremely high numbers of point mutations as well as considerable genetic heterogeneity. Other highly exposed tissues such as the esophagus, head and neck, and gastrointestinal tract also suffer many insults that increase point mutation burden, whereas the incidence of these lesions is lower in tumors from other tissues such as the prostate, mammary gland, and brain, which are

in a relatively less exposed environment. Point mutations and large-scale genomic alterations in cancers are important for many reasons: They can confer properties that are critical determinants of individual patient prognosis, responses to therapy, or development of drug resistance (Engelman & Settleman 2008). Chemical carcinogenesis models in the mouse, which like environmentally induced human tumors carry a high mutation burden, are therefore uniquely able to replicate some of these cardinal genetic features of human cancers and provide routes to addressing the many stubborn questions that remain unanswered in the struggle to understand and ultimately prevent or successfully treat human cancer.

THE SMOKING GUN OF CHEMICAL EXPOSURE

Chemical carcinogenesis models also offer us new ways to definitively identify environmental agents that play causative roles in human cancers. The presence of carcinogens in the environment leaves an imprint, a kind of "smoking gun" signature, in the human genome that can act as a chronological record of mutagen exposure (Alexandrov et al. 2013, Petljak & Alexandrov 2016). UV-induced melanomas carry a well-known pattern of C > T mutations predominantly at dipyrimidine sites, which are the target for UV-induced crosslinking (Pfeifer et al. 2005, Viros et al. 2014), whereas lung carcinomas from smokers carry a mutation signature dominated by a G > T mutation pattern, which precisely replicates the trinucleotide context of G > T mutations induced by benzopyrene in vitro in mouse cells (Alexandrov et al. 2013, Kucab et al. 2015). From a total of 31 mutation signatures identified by deep sequencing of human tumors, 11 have been attributed to endogenous repair processes and an additional 7 to known exogenous causative agents such as cigarette smoke or UV exposure (Petljak & Alexandrov 2016). The remaining signatures have unknown origins, suggesting the existence of environmental agents or processes that influence human cancer development in important ways, but that have not yet been identified. These important data have implications for cancer prevention as well as early detection of exposures in the human workplace. Just as seen with the G > T signature of benzopyrene, tumors initiated by carcinogenic agents leave the same genetic imprint in the genomes of mice as they do in humans (McCreery et al. 2015, Nassar et al. 2015, Viros et al. 2014, Westcott et al. 2014). Mouse lung tumors induced by a single treatment with *N*-methyl-*N*-nitrosourea (MNU) show a genome-wide pattern of G > A transition mutations, whereas an alternative initiating mutagen, urethane, induces tumors with either A > T or A > G mutations genome-wide (Forkert 2010, Kurowska et al. 2012, Westcott et al. 2014). Thus once the mutagen signatures are known, the identity of causative agents can be deduced from inspection of tumor sequences. These data offer exciting new possibilities to combine human tumor genome analysis and rodent chemical carcinogenesis models to associate mutation signatures with their causative agents, which could lead to the identification of some of the many unknown environmental factors contributing to human cancer development.

The characterization of mutagen signatures also holds promise for shedding light on the persistent question of whether certain cancer treatments, in particular radiation or chemotherapy agents, contribute to tumor relapses or the development of secondary malignancies. This question was elegantly addressed by Johnson et al. (2014), who identified the characteristic mutation signature of temozolomide (G > A transition mutations) in glioblastoma recurrences in patients treated with this common chemotherapeutic agent. Establishing the mutation signatures associated with a wide variety of therapeutic agents would make it possible to obtain definitive evidence as to whether a tumor that subsequently arises after therapy—whether a short-term relapse or a malignancy that occurs years later—carries the signature of

the therapeutic agent, and thus what role those therapies might play in contributing to future malignancies.

MUTAGENIC ACTIVATION OF DRIVER MUTATIONS BY CHEMICAL CARCINOGENS

The discovery of human *RAS* oncogenes activated by single point mutations (Parada et al. 1982, Perucho et al. 1981, Santos et al. 1982, Shih et al. 1979, Shilo & Weinberg 1981) raised the possibility that chemical mutagens could initiate cancer by targeting and introducing these same mutations. This possibility was first tested using the original mouse skin model of two-stage carcinogenesis involving initiation with a potent chemical mutagen, 7,12-dimethylbenzanthracene (DMBA), and promotion by multiple exposures to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Balmain & Yuspa 2014). Although three members of this gene family (*HRAS*, *KRAS*, and *NRAS*) had been shown to be mutated in human tumors and cell lines, analysis of activation patterns in both early-stage premalignant papillomas and late-stage carcinomas from mouse skin showed remarkably consistent activation of the same gene, *Hras*, in almost all tumors (Balmain & Pragnell 1983). The specific activating mutation was shown to be an A:T > T:A transversion at codon 61 of *Hras* (Bizub et al. 1986, Quintanilla et al. 1986) and was present in over 90% of benign and malignant tumors. Codon 61 encodes glutamine (CAA) and contains two target adenosines, both of which when mutated to T can result in *Hras* activation. DMBA was much less likely to cause Ras activation by mutation at codon 12 or 13 (GGA or GGC, respectively) as adducts with G are much less common. Tumors initiated by an alternative carcinogen (MNU), however, exhibited G:C > A:T transitions, leading to the activation of *Hras* by mutations at codon 12 or 13 (Brown et al. 1990). A similar activating mutation was also seen in mammary carcinomas in rats treated with MNU (Zarbl et al. 1985). These mutation patterns were in complete agreement with prior data showing that DMBA forms adducts predominantly with adenosine residues in DNA (Bigger et al. 1983), whereas MNU methylates guanines, predominantly at GG dinucleotide positions (Kurowska et al. 2012, Sikpi et al. 1990), thus establishing a mechanistic link between carcinogen exposure and specific point mutations in cancer genes.

A series of additional experiments demonstrated the induction of carcinogen-specific mutations in target genes by other chemical mutagens and in other tissues. In chemically induced lung tumors, *Kras* was found to be mutated much more commonly than *Hras*, but the chemical signatures were the same. MNU typically induced mutations in codon 12 of *Kras* (You et al. 1989), whereas urethane, which preferentially causes A:T > T:A and A:T > G:C mutations, predominantly induced mutations in *Kras* codon 61 (Forkert 2010). Polycyclic hydrocarbons such as methylcholanthrene or benzopyrene primarily form adducts with guanine residues, and misrepair of these bulky lesions results in G:C > T:A transversions, the main mutation type seen in *Ras* genes and in the *Trp53* tumor suppressor gene in tumors induced by these agents (**Table 1**) (Brown et al. 1990, Kucab et al. 2015). Additional examples exist of common agents in the human environment that cause cancer through induction of mutations consistent with their known genome-wide mutation signatures. Aflatoxin, a fungal metabolite found in peanuts, binds to guanines, resulting in G > T mutations at a recurrent hotspot in *TP53* (Hsu et al. 1991, Ozturk 1991) as well as throughout the genome (Schulze et al. 2015). Aristolochic acid is a constituent of plant extracts that have been used for medicinal purposes throughout antiquity. This agent acts as a strong mutagen both in humans and in rodent cancer models, primarily by forming adducts with adenosines, resulting in A:T > T:A mutations both in *Ras* oncogenes (Schmeiser et al. 1990) and genome-wide (Poon et al. 2013). These studies provided the rationale for present efforts to identify human environmental carcinogens based on mutation signatures in DNA from a wide variety of tumor types (Alexandrov et al. 2013).

Table 1 Selection of common mouse and other rodent models of cancer

Cancer type	Carcinogenesis protocol	Primary mutation signature	Associated oncogenes	Tumors formed	Selected reference(s)
Skin	DMBA (alternatively, MNU or MCA, single dose) + repeated tumor promoter (e.g., TPA)	DMBA: A > T MNU: G > A MCA: G > T	*Hras*; *Kras* in *Hras*$^{-/-}$ mice	Premalignant papillomas and malignant carcinomas (of either squamous or spindle morphology); surgical resection of the primary tumor leads to metastasis to lymph nodes, lung, and other sites	Balmain & Yuspa 2014, McCreery et al. 2015, Nassar et al. 2015, Quintanilla et al. 1986, Rehman et al. 2000, Wong et al. 2013
Liver	DEN (repeat doses, or in combination with PB promotion)	G > A, T > C	*Hras* (DEN only); *Ctnnb1* (DEN/PB)	Premalignant hyperplastic foci and malignant hepatocellular carcinomas; micrometastases can be observed in lung, particularly with lower doses of DEN in rats	Aleksic et al. 2011, Aydinlik et al. 2001, Da Costa et al. 2014, Verna et al. 1996, Vesselinovitch & Mihailovich 1983
Lung	Urethane or MNU (single intraperitoneal injection)	Urethane: A > T, A > G, and G > A MNU: G > A	*Kras*	Premalignant adenomas and malignant adenocarcinomas	Malkinson 1991, Westcott et al. 2014, You et al. 1989
Sarcoma	MCA (injection into mouse leg)	G > T	*Kras* or *Nras*	Fibrosarcoma	Algarra et al. 1998, Borrello et al. 1988, Foley 1953, Gubin et al. 2014, Riggins & Pilcht 1994
Urothelial	BBN (oral gavage or in drinking water)	G > A	*p53*; less commonly *Hras*	Urinary bladder carcinoma	He et al. 2012, Ogawa et al. 1998, Vasconcelos-Nobrega et al. 2012
Colon	PhIP or MelQx; plus DSS promoter	PhIP: G > T and G > A; −1 base pair deletion (G:C pair)		Adenocarcinoma	Nishikawa et al. 2005
Prostate and colon	PhIP (rat)	G > T and G > A; −1 base pair deletion (G:C pair)	Colon: *Ctnnb1* and *Apc*	Colon adenocarcinoma and prostate carcinoma	Nakagama et al. 2005
Breast	DMBA or MNU (rat)	DMBA: A > T MNU: G > A	*Hras*	Multiple breast carcinoma subtypes	Dias et al. 1999, Faustino-Rocha et al. 2015, Medina & Warner 1976, Zarbl et al. 1985
Pancreas	BOP (Syrian golden hamster)	G > A	*Kras*	Ductal adenocarcinoma	Fujii et al. 1990, Pour et al. 1978

Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; BOP, *N*-nitrosobis(2-oxopropyl)amine; DDS, dextran sodium sulfate; DEN, *N*-nitrosodiethylamine; DMBA, 7,12-dimethylbenzanthracene; MCA, methylcholanthrene; MelQx, 2-aminodimethylimidazoquinoxalin; MNU, *N*-methyl-*N*-nitrosourea; PB, phenobarbital; PhIP, phenylimidazopyridine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

FACTORS LEADING TO INDUCTION AND SELECTION OF ONCOGENIC MUTATIONS

A unique feature of mouse models of chemical carcinogenesis is the insights they provide into the complex mechanisms leading to the selection of specific mutations in oncogenes. As seen above, carcinogens have a propensity to cause mutations at specific locations in the genome. Following the initial chemical insult, a variety of selective processes operating at the level of metabolism, gene target sequence, DNA repair, tissue type, cell of origin, and genetic background all factor into the eventual outgrowth of a tumor carrying a specific set of mutations (**Figure 1**). As we endeavor to show below, chemical carcinogenesis models are therefore not simply a "pepper-spray" approach to cancer modeling, but can be used to address specific mechanisms by which cancers can be initiated or promoted in subsets of tissues or cells by activation of select cancer driver pathways.

Beyond favoring mutations of particular DNA bases in particular trinucleotide contexts, patterns of carcinogen mutations are influenced by the genomic architecture of their target genes and target cells. Carcinogen-induced mutations in human cancers are clustered in regions of closed chromatin, resulting in mutation patterns that depend on the tissue in which tumors arise (Polak et al. 2015). The particular DNA strand—coding or noncoding—on which adducts form can also play a major role in determining mutation specificity, with accumulating data suggesting that coding strand mutations are favored. Mutations in oncogenes and tumor suppressor genes are predominantly caused by the formation of adducts with residues on the nontranscribed coding strand (Nik-Zainal et al. 2015), presumably because similar adducts on the transcribed strand are subject to efficient transcription-coupled repair. A recent elegant study demonstrated how this mechanism can also account for the observation of *Hras* or *Braf* mutations in mammary tumors induced by different carcinogens (Keller et al. 2016). In mammary tumors induced by expression of a *Wnt* transgene followed by carcinogen exposure, treatment of transgenic mice with DMBA resulted in almost 100% of driving mutations being CAA > CTA mutations at codon 61 in the *Hras* gene. In stark contrast, ethylnitrosourea (ENU) treatment resulted in 100% mutations in the *Braf* gene, which can be uniquely activated by altering Val637 (equivalent to human Val600) from GTG > GAG. ENU is a potent mutagen that can cause A:T > T:A mutations in mutagenesis assays (Skopek et al. 1992). Although this mutation is the same as that induced by DMBA, ENU acts through binding to T residues rather than the A residues that bind DMBA. This specificity of observing particular *Hras* versus *Braf* mutations can be explained by the fact that ENU forms thymine adducts on the coding strand of Val637, a mutation that cannot be efficiently induced by DMBA as the target adenosine is on the wrong strand. Such strand bias is seen in mutation signatures genome-wide (Alexandrov et al. 2013) but appears to be particularly important for strongly selected cancer driver genes such as *Ras*, *Braf*, and *Trp53*. Although in the liver model system DMBA has also been shown to cause the same V637E mutation in *Braf*, this is much less frequent than the standard activating CAA > CTA at codon 61 of *Hras* (Buchmann et al. 2008). These data may well be due to tissue-specific variation in transcription coupled repair, but this is clearly an area worthy of further investigation.

INITIATED CELL SELECTION BY TUMOR-PROMOTING AGENTS

The term tumor promotion was first operationally described in the mid-twentieth century (Berenblum & Shubik 1947b, Friedewald & Rous 1994), and various aspects of this process have been reviewed over the past 60 years (Balmain & Yuspa 2014, Boutwell et al. 1983, Yuspa 1994). The consensus view, through the study of agents that promote skin tumors, is that tumor promoters function by nonmutational mechanisms to stimulate the signaling pathways downstream from

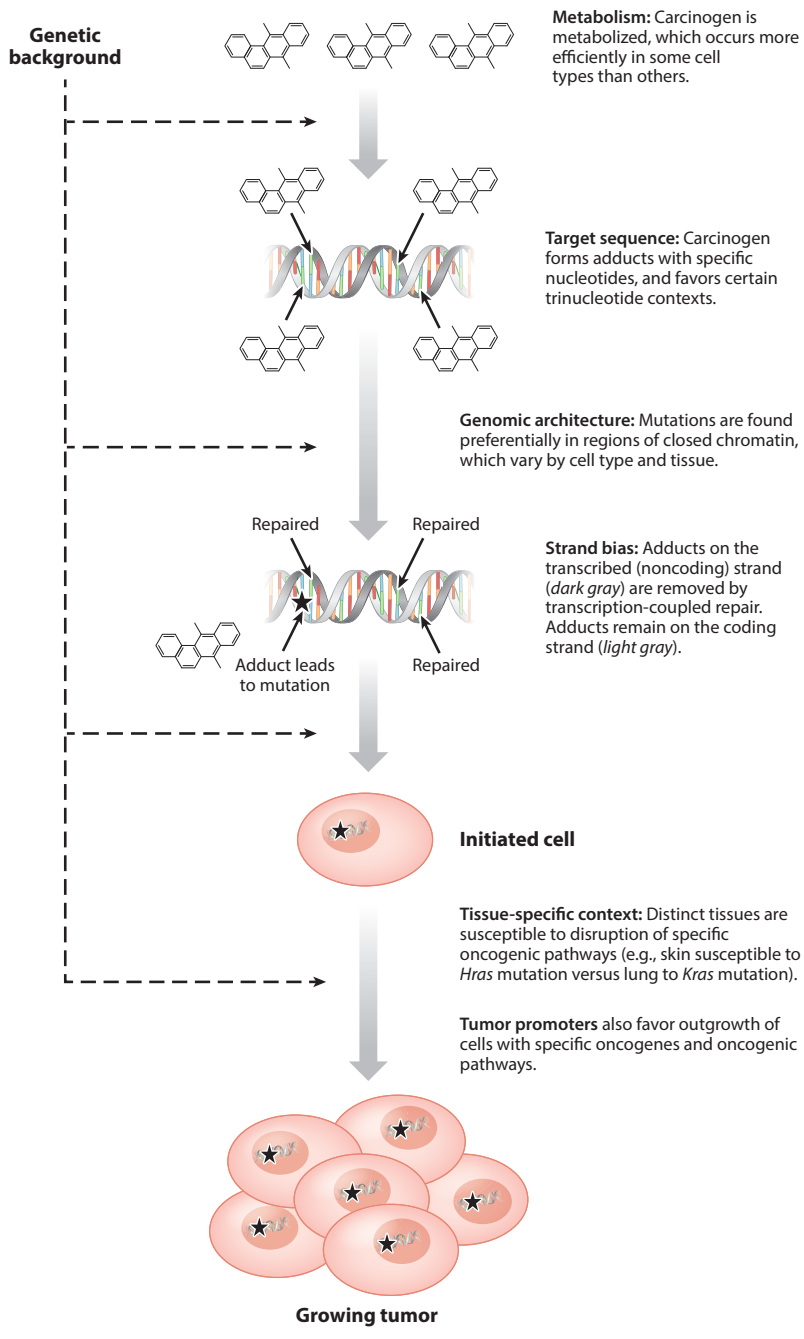

Figure 1

Factors influencing the selection of chemically initiated cells.

protein kinase C (*Pkc*), which acts as the major receptor for the best known tumor-promoting agent, TPA (Castagna et al. 1982, Delclos et al. 1980). A major consequence of pathway engagement is the induction of proliferation, inflammation, and outgrowth of initiated cells, but the precise mechanisms by which these initiated cells are selected remain obscure. Agents exist that efficiently induce both proliferation and inflammation but nevertheless are very inefficient tumor promoters (Ushmorov et al. 1994). Furthermore, distinct promoters exhibit specific selection for cells carrying particular oncogenic mutations. Although the combination of DMBA, MNU, or MNNG as the initiator followed by promotion with TPA in skin tumor induction strongly selects for tumors carrying *Hras* mutations (Brown et al. 1990), treatment with DMBA followed by an alternative tumor promoter (mezerein) (Rehman et al. 2000) or by overexpression of ornithine decarboxylase (*Odc*) (Megosh et al. 1998) instead leads to a higher frequency of *Kras* mutant skin tumors. Some of these differences may result from the selective activation or inhibition of various Pkc isoforms, which could result in either positive or negative effects on selection of *Ras* mutant tumors (Jansen et al. 2001). Some molecules structurally related to TPA have been described that can act as Pkc agonists, but they not only lack tumor-promoting activity (Szallasi et al. 1993, Zayed et al. 1984), but can specifically inhibit the growth of tumors carrying activated *Kras* (but not *Hras*) oncogenes by interfering with noncanonical *Wnt* signaling (Wang et al. 2015).

In other mouse carcinogenesis model systems such as the liver, promoting agents can select one pathway while simultaneously inhibiting another. Phenobarbital (PB) is an antiepileptic drug that can promote liver tumor development when administered subsequent to treatment with a mutagenic initiator, most commonly *N*-nitrosodiethylamine (DEN). In contrast to tumors induced only by DEN exposure, which have a high frequency of *Hras* codon 61 mutations, the PB treatment selects for tumors that lack these alterations, but instead harbor mutations in exon 2 of β-catenin (Aydinlik et al. 2001). Because the DEN treatment precedes promotion with PB, cells carrying *Hras* mutations must be present in the liver tissue but are not promoted by treatment with PB. Clearly, much still needs to be learned about the mechanisms by which promoters act in stimulating or inhibiting the selection of oncogenic pathways.

TISSUE-SPECIFIC SELECTION OF ONCOGENIC RAS MUTATIONS

An early conclusion reached on the basis of chemical carcinogenesis models of cancer was that mutations in the various members of the Ras family of oncogenes were exquisitely tissue-specific as well as carcinogen-specific. Although *Hras* codon 61 mutations were consistently found in DMBA-initiated skin tumors (Balmain & Pragnell 1983), lung tumors caused by systemic injection of carcinogens often carried mutations in *Kras* rather than *Hras* (You et al. 1989). This was the case even in skin and lung tumors from the same animals (Loktionov et al. 1990). Similar conclusions were reached by analysis of a range of tumors induced in rats by transplacental exposure to MNU (Sukumar & Barbacid 1990). All tumor types had activating G > A transitions, but in brain tumors these were in the *Neu* oncogene, whereas mammary tumors and kidney mesenchymal tumors had *Hras* and *Kras* mutations, respectively. Notably, *RAS* mutations in human tumors follow a similar pattern. Lung adenocarcinomas have a high incidence (36%) of *KRAS* mutations, whereas squamous carcinomas of the skin, head and neck, and lung, although they have a low overall *RAS* mutation frequency, tend to have a relatively higher proportion of *HRAS* mutations (Pickering et al. 2014, Stransky et al. 2011, The Cancer Genome Atlas 2012). Interestingly, skin carcinomas are increased in frequency in human melanoma patients treated with the *BRAF* inhibitor vemurafinib, and approximately 60% of these harbor the *HRAS* codon 61 CAA > CTA mutation seen in mouse tumors initiated with DMBA (Su et al. 2012). The short latency for development of these tumors suggests that they pre-exist in normal skin in these patients and are rapidly promoted

by vemurafinib treatment. This particular mutation would therefore appear to be under strong positive selection in squamous epithelia, for reasons that are still not clear. The reader is referred to other more comprehensive reviews of the possible mechanisms that underlie this selection induced by a well-known cancer drug (Poulikakos et al. 2010, Robert et al. 2011).

A trivial explanation of tissue-specific *Hras* and *Kras* mutations would be that *Hras* is expressed in squamous epithelial tissues and *Kras* in the simple epithelia of the lung or gastrointestinal tract. This explanation is, however, untenable, as certain promoting agents such as mezerein or overexpression of *Odc* can promote growth of cancers with *Kras* mutations from normal mouse skin. *Kras* mutations are also seen in a high percentage of skin tumors that arise in *Hras* knockout mice (Ise et al. 2000, Wong et al. 2013). Although *Kras* is commonly activated in lung cancers, it is not in fact essential for lung tumor development. *Hras*, when inserted into the *Kras* locus with concomitant deletion of *Kras*, can lead to rapidly growing *Hras* mutant lung tumors after treatment with a lung carcinogen such as urethane (To et al. 2006).

Although both *Hras* and *Kras* mutations can lead to the transformation of epithelial cells in skin and lung, analysis of skin tumors carrying these mutations highlights some important differences. Skin papillomas carrying *Kras* mutations are less frequent but arise earlier and are more likely to progress to carcinomas than corresponding lesions with *Hras* mutations (Rehman et al. 2000). *Kras* mutant tumors in *Hras* knockout mice are also reduced in frequency, but more likely to give rise to distant metastases (Wong et al. 2013). The reasons for these differences remain obscure, but one model proposed is that *Kras* and *Hras* are expressed in different stem and progenitor cell populations within the skin (Song & Balmain 2015). In this model, mutation of the endogenous genes gives rise to phenotypes that reflect the cell of origin in addition to any biochemical differences in signaling activated by these different oncogenes. This is compatible with results of transgenic experiments in which mutant *Hras* was artificially directed to stem-like and differentiated cell populations in mouse epidermis (Brown et al. 1998), resulting in the development of benign lesions with distinctly different propensities for progression to carcinomas depending on the cell targeted. It is likely that both the cell of origin and biochemical properties underlie these in vivo results, and this is an important area that requires more detailed investigation.

INFLUENCE OF GENETIC BACKGROUND ON MUTATION SELECTION

The selection processes present due to genomic architecture, target tissue, and choice of promoter are further refined by naturally occurring polymorphisms in different mouse strains. Schwarz and colleagues (Buchmann et al. 2008) have shown that although DEN-induced liver tumors in susceptible C3H mice can have either *Hras* codon 61 or *Braf* codon 637 mutations, both of which lead to activation of the Mapk pathway, the relative frequency of *Braf* mutations is significantly higher in C57BL/6 mice that are more resistant to this tumor induction protocol. These results are compatible with other data showing the pleiotropic effects of strain background on multiple factors that influence tumor development in mouse models, ranging from effects on stem cell selection (Popova et al. 2004, Wakabayashi et al. 2007), carcinogen metabolism (Nebert et al. 2004), and inflammation (Quigley et al. 2009) to allele-specific somatic mutations or copy number changes (Chen et al. 1994, Ewart-Toland et al. 2003, McCreery et al. 2015, Nagase et al. 2003). Genetic background can also influence the specific mutation acquired in a given oncogene. Over 90% of urethane-induced lung tumors from wild-type mice carry a Q61R *Kras* mutation, but this switches to Q61L in *Kras* heterozygous ($Kras^{+/-}$) mice (Westcott et al. 2014). The reason for this switch is unknown but implies that the balance between wild-type and mutant *Kras* introduces novel facets of *Ras* biology that influence the selection of specific mutants.

WHAT HAVE WE LEARNED FROM SEQUENCING MOUSE TUMOR MODELS?

The development of cost-effective next-generation sequencing approaches has ushered in a new era of research on chemical carcinogenesis models. For the first time, we are now able not only to study the types of mutations induced in single driver genes by specific carcinogenic agents, but can identify the genome-wide mutation spectra, as well as the patterns of gene mutations at different stages of tumorigenesis. Whole-exome sequence analysis of mutations in chemically induced lung tumors (Westcott et al. 2014) demonstrated that the genome-wide mutation signature exactly replicated the expected carcinogen signatures. MNU produces G > A transition mutations, and this was the predominant mutation seen across the genome in MNU-induced tumors. Tumors induced by urethane, conversely, exhibited genome-wide A > T or A > G mutations in a wide range of target genes, including many known cancer driver genes. In contrast with chemical models, tumors produced by a GEMM—carrying the same primary driver oncogene mutation and on the same genetic background—showed a very different genetic architecture. Two GEMMs of small-cell or non-small-cell lung cancer gave rise to tumors with very few point mutations (McFadden et al. 2014, Westcott et al. 2014). In these models, the sequential acquisition of gross chromosomal events leading to gene copy number alterations would appear to be the major cause of initiated cell selection and tumor progression (McFadden et al. 2014, 2016; To et al. 2011; Westcott et al. 2014). This comparison highlights the importance of making the right choice of mouse cancer model for the research question being addressed, whether it be the effect of targeted drugs, mechanisms of drug resistance, or responses to immunotherapy.

The skin model offers some specific advantages for analysis of the sequential events that influence multiple stages of tumor progression because of the availability of lesions representing benign, locally invasive, and metastatic stages. In agreement with the mutation pattern seen previously in the initiating *Hras* gene, the genome-wide mutation spectrum in all tumors initiated by a single DMBA treatment consisted primarily of A > T mutations in a range of potential cancer driver genes (McCreery et al. 2015, Nassar et al. 2015). Exome sequencing of papillomas, carcinomas, and metastases showed an average of 172 mutations in papillomas compared to 284 mutations in carcinomas and 250 in metastases (McCreery et al. 2015). Whether the reduced number of mutations in papillomas compared to more progressed lesions is significant biologically remains to be determined. All of the A > T mutations were induced at the same time for each tumor, and all papillomas were harvested together with carcinomas and metastases for each mouse. A larger mutation burden may therefore facilitate tumor progression to carcinomas, and papillomas with high mutation load may therefore be underrepresented in this cohort. Alternatively, early-stage papillomas with high mutation load may be recognized by immune surveillance and removed, leaving only progressed lesions or papillomas that have escaped immune recognition. Interestingly, recent analysis of mutation burden in matched melanocytic nevi and melanomas from patients also found a lower mutation burden in the nevi, suggesting that in both mouse and human systems, early lesions, at least in terms of histology, exhibit fewer point mutations (Shain et al. 2015).

Mutations in melanomas and many other human tumors are acquired through chronic exposure to carcinogens (e.g., UV light or cigarette smoking), making it difficult to determine the timing of mutational events just based on signatures. In chemical carcinogenesis models, it is, however, possible to clearly distinguish early from late mutations. In the case of a single initiation with DMBA or MNU, the resulting carcinogen-specific mutations are fixed within a few days of carcinogen exposure, and their timing can be precisely determined. Application of this approach to skin tumor mutation profiles clearly identified patterns of late mutations that were distinct from those initiated by the carcinogen DMBA. In contrast to the signature A > T mutations induced

by DMBA, subclonal mutations acquired during growth and metastasis had a G > T mutation signature that we have presently attributed to oxidative stress (McCreery et al. 2015). Further analysis of these data may help to determine whether the likelihood of progression is encoded in the DNA mutation profile immediately after initiation or is more likely to be acquired through random acquisition and selection of G > T mutations during growth.

The large number of mutations present in chemically induced tumors, and the acquisition of subsequent subclonal mutations over time, also makes chemical models of cancer well suited to studying primary tumor heterogeneity and subclonal behavior, as well as evolution of metastases. Whether metastases arise in a linear fashion by seeding lymph nodes, followed by spread to distant sites, or disseminate in parallel from the primary site, has been debated for many years (Klein 2009, Turajlic & Swanton 2016). Based on the DMBA/TPA model, matching of metastases to their primary tumors using hundreds of specific mutations has shown that metastases generally disseminate from the matched primary tumor in parallel, spreading to all organs at approximately the same time rather than traveling linearly via a regional lymph node (McCreery et al. 2015).

Gene copy number alterations are also observed in skin tumors from the DMBA/TPA model, and these increase quite dramatically in more advanced tumors. Chromosome 7 can be amplified during early papilloma development and, at that time, is the only copy number alteration observed, suggesting that it is the first copy number event to occur. Older tumors will also often have multiple whole chromosome gains or losses, which commonly include gain of chromosome 6. It is likely that these copy number changes reflect increased signaling through the Ras/MapK pathway, as several key genes are located on these chromosomes (*Hras* on chromosome 7, and *Kras*, *Braf*, and *Raf1* on chromosome 6). Approximately 50% of *Hras* mutant squamous cell carcinomas amplify chromosome 1, and such amplification is not seen in papillomas, spindle carcinomas, or squamous cell carcinomas with *Kras* mutations. This may therefore represent a genetic event that is both stage specific and stimulated by signaling through *Hras* but not *Kras*. Other copy number changes seen during progression include deletions of *Cdkn2a* and copy number gains of *c-Met*, both of which are seen predominantly in the most advanced tumors (McCreery et al. 2015, Wong et al. 2013). These changes also replicate some of the most common genomic events seen in human squamous cancers of the lung and head and neck (Stransky et al. 2011, The Cancer Genome Atlas 2012).

OTHER CHEMICAL MODELS OF CANCER

Numerous chemical models exist in addition to the common ones described above, with varying amounts of experimental work done to characterize them (**Table 1**). In some cases, the mouse has proven to be refractory to the development of certain tumor types after chemical exposure, whereas other species, such as the rat or hamster, have provided more tractable models. For example, pancreatic cancer is one of the major causes of cancer-related morbidity in humans, but mice treated systemically with mutagens rarely develop pancreatic ductal adenocarcinomas. The Syrian golden hamster is, however, susceptible to the development of pancreatic cancers after treatment with *N*-nitrosobis(2-oxopropyl)amine (BOP), and these share several genetic features with the equivalent human lesions, including mutations in *Kras* and *Cdkn2a* (Takahashi et al. 2011). The National Toxicology Program has carried out extensive studies of both rats and mice exposed to a range of known and suspected human carcinogens, revealing both tissue-specific and species-specific effects of many of these agents (see Hoenerhoff et al. 2009 for review). These models, as well as others that have led to the identification of important food-borne carcinogens (Nagao et al. 1996, Nakagama et al. 2005), will provide an invaluable resource of tumors for molecular studies aimed at defining the range of chemicals that significantly impact mutational signatures in human cancers.

THE FUTURE OF CHEMICAL CARCINOGENESIS

Mouse models of cancer based on tumor induction by chemical agents were, for the most part, supplanted in the 1980s with more precise genetically based models that allowed manipulation of the mouse germline to induce, in a spatial and temporally defined manner, specific types of genetic alterations found in human cancers. These GEMMs have repeatedly proven themselves to be a font of information, revealing previously unknown facets of tumor biology (Hanahan et al. 2007, Jacks 2005, Kwon & Berns 2013, Nardella et al. 2011). Furthermore, GEMMs have been incorporated into coclinical trials of new targeted drugs, as they allow assessment of the on-target effects of novel drugs designed to inhibit specific signaling pathways (Clohessy & Pandolfi 2015). Such models will continue to be important tools for the discovery and validation of cancer drugs, and their combinations, for the treatment of human cancer.

However, chemical carcinogenesis models have specific advantages for addressing certain important but unanswered questions in cancer biology. The first and most obvious is the question of how different tissues and cell types interact with environmental agents linked to human cancer. The environment has a huge impact on cancer development in humans, and the impact of some of these agents can be seen in the mutation signatures identified by whole genome sequencing of thousands of human cancers (Alexandrov et al. 2013). A large number of mutation signatures have no obvious causal agent, and sequence analysis of tumors in mice or rats induced by known or suspected human carcinogens may help provide this causal link.

Another important question relates to the cell of origin of cancer within different tissues. Different target cells within the same tissue could give rise to tumors of different histological subtypes, or lesions of the same subtype that have different propensities for malignant progression. Both these issues have been addressed largely using GEMMs in which oncogenic mutations are targeted to different cell types within a tissue using specific gene promoters. Skin tumors with a high risk of malignant progression were found to be located within the hair follicle bulge region rather than the more differentiated interfollicular cell population (Brown et al. 1998), whereas in the intestine, adenomas were shown to arise from Lgr5-positive stem cells rather than the committed transit amplifying cell population (Barker et al. 2009). One caveat with these and other similar studies (Peterson et al. 2015, Youssef et al. 2010) is that the target cell population is predetermined by the investigator through the choice of gene promoter used to activate the appropriate oncogenic stimulus in vivo. Although these approaches demonstrate that some cells within a population can give rise to tumors, whether they do so under conditions of environmentally induced cancer formation remains an open question. An alternative approach using a combination of carcinogen models with GEMMs in which target cell compartments are neutrally labeled with a reporter gene offers the potential to reveal which cells are in fact the true cell of origin when an entire tissue is exposed to a carcinogen (Li et al. 2013, Wang et al. 2011).

Finally, tumor heterogeneity has become a major focus for studies of cancer therapy using both targeted approaches and more general chemo- or immunotherapy. Heterogeneity can arise at both the cellular level, through the participation of multiple distinct cells in tumor development, and at the genetic level, through the emergence of subclones carrying particular mutations. The latter can contribute to the development of drug resistance after therapy, and the level of genetic heterogeneity itself is an indicator of poor prognosis in patient samples (McGranahan & Swanton 2015). Chemical carcinogenesis models replicate these features of human tumors more accurately than GEMMs as they have a high frequency of point mutations and substantial intratumoral heterogeneity. As such, they should be valuable tools for analysis of responses to both targeted drugs and chemotherapy, in which resistance is often driven by pre-existing subclones within tumors. The enormous impact of immune checkpoint inhibitors in human clinical trials

highlights the requirement for immunocompetent mouse models that can be exploited for preclinical testing of the many possible combinations of new drugs that might enhance the still relatively poor response rates in cancer patients. Checkpoint blockade therapies have been most effective in patients with tumors carrying high mutation loads (Rizvi et al. 2015), which create neoantigens recognizable to the immune system. These findings suggest that the high mutation load present in chemically induced mouse tumors will make these models particularly suitable for studies of immunotherapy. Notably, both the concept of immunoediting (Dunn et al. 2002, Mittal et al. 2014) and the development of immune checkpoint blockade inhibitors (Gubin et al. 2014, Mitsui et al. 2010) have already exploited the availability of chemically induced mouse tumor cell lines carrying abundant point mutations that stimulate antigenicity. Further development of in vivo chemical carcinogenesis models of disseminated disease may provide the stringent tests required to identify successful combinations of targeted, chemo-, and immunotherapy drugs in preclinical trials.

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