Genetically Modified Mouse Models for Biomarker Discovery and Preclinical Drug Testing

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Abstract

The ability to grow pluripotent mouse embryonic stem cells in culture, and to introduce precise genetic modifications into those cells through gene targeting, has greatly facilitated the generation of mouse models of human disease. This technology is playing a particularly important role in cancer research. In addition to their use in elucidating the role of individual genes or combinations of 2 or more genes, the genetically engineered mouse models are being used to develop biomarkers and for preclinical drug testing. By examining plasma samples from tumor-bearing mice from mice carrying specific mutations in tumor suppressor genes and/or oncogenes, investigators can identify tumor-specific biomarkers that are overexpressed in the tumor cells. These markers are directly relevant to the corresponding human cancer. The ability to generate tumors at the correct anatomical site within the normal cellular environment is augmenting the use of xenografts in drug testing in a preclinical setting. *Clin Cancer Res; 18(3); 625–30.* ©*2012 AACR.*

Introduction

This work is one of a series of articles (1–4) that stem from discussions at the AACR Clinical and Translational Cancer Research Think Tank meeting (5) held in San Francisco in early 2010. It synthesizes some of the opinions and issues considered at that meeting, principally as they apply to the use of genetically modified mouse models and their use in biomarker and drug development. In the process of cancer drug development, studies are often performed in animal models before human clinical studies are conducted. However, in such studies, investigators have primarily focused on introducing human tumor cells obtained from established cell lines, injecting them subcutaneously into immunocompromised mice, and testing the efficacy of a drug in the xenograft model. The availability of mice with defined genetic modifications that are known to be involved in human malignancies, reliably develop tumors at the correct anatomical site, and have properties very similar to those of their human counterparts is changing the landscape. Two technologies developed in the 1980s enabled targeted genetic manipulation of mice. The first enables researchers to isolate mouse blastocyst cells and grow them in cell culture. These cells, which are referred to as embryonic stem cells, retain their pluripotency even after many generations of growth *in vitro* (6). The second technology allows the genetic modification of embryonic stem cells through homologous recombination (7, 8). The combination of these 2 technologies has revolutionized our ability to understand gene function through genetic modification of embryonic stem cells, generate mice that carry the desired genetic modification, and examine the resulting phenotypes. Such mice are referred to as genetically engineered mouse models (GEMM). The genetic modifications can be introduced into the germ line or into specific tissue or cell types by means of a conditional knockout strategy (Fig. 1). GEMMs have found an important place in cancer research. Many known or suspected cancer genes have been modified in many different ways, and mice carrying these genetic changes have been studied extensively. These mouse models for human cancer have provided many new insights into the biology of cancer and are also serving as tools for the development of cancer biomarkers and drugs. This article provides a few examples of the utility of mouse models in imaging and biomarker studies. The Mouse Models for Human Cancer Consortium and the Jackson Laboratory maintain stocks of many genetically modified mouse models, and information about the particular models can be obtained from http://mouse.ncifcrf.gov/ and http://jaxmice.jax.org/index.html, respectively.

Use of GEMMS for Biomarker Development

It is well established that detection of cancer during its early stages provides a significant survival advantage. For many cancers, the common modes of detection rely heavily on imaging modalities such as computed tomography scans for lung cancer, mammograms for breast cancer, colonoscopies for colorectal cancer, and pelvic ultrasounds for ovarian cancer. The costs, lack of compliance, and lack of

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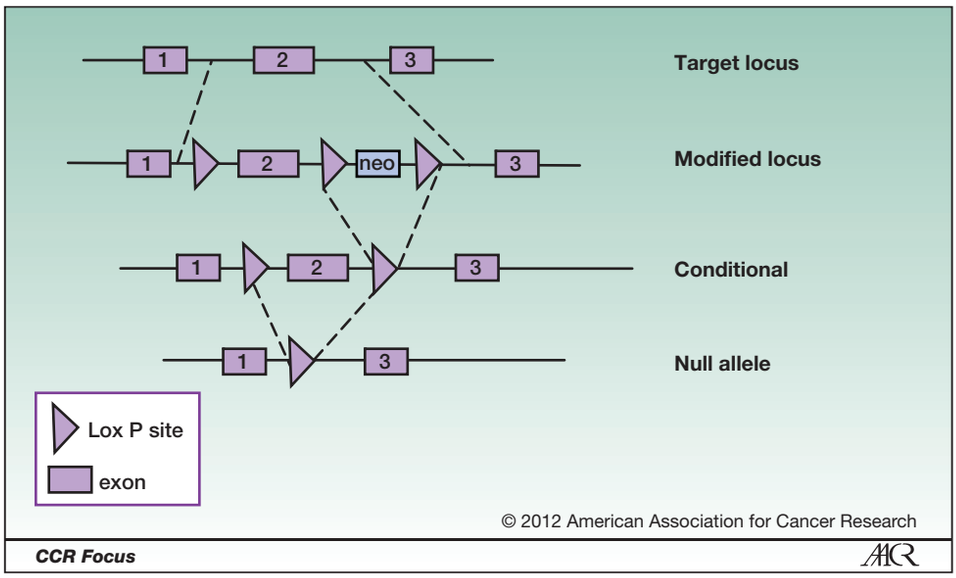

Figure 1. Strategy for generating tissue-specific gene modifications.

complete accuracy of these methods in effectively detecting cancers is preventing the effective detection of tumors in their early stages. There is a substantial need for additional noninvasive methods to detect cancers in their early stages. Blood-based biomarkers have the potential to fill this gap. Blood-based markers can also be used to monitor recurrence and the effectiveness of therapies. Although several blood-based markers are available, such as CA125 for colorectal cancer and prostate-specific antigen for prostate cancer, there is a clear-cut need to find additional markers. Mouse models are proving to be very valuable for identifying such markers.

Much of cancer drug development involves the use of animal testing; however, the models that are most often used are cell-line–derived tumors in immunocompromised mice. These models are not always accurate predictors of drug response in humans. Therefore, investigators are beginning to use genetically modified mice more extensively for drug development and testing.

Applications

There is extensive literature on the use of mouse models for biomarker discovery and drug testing. I will use a few examples to illustrate such use. In an attempt to determine whether proteins present in plasma samples would enable detection of cancer, Hung and colleagues (9) used mass spectrometry to assess the protein profiles in tumor-bearing mice and their corresponding normal controls. Instead of using gel-based separation of proteins, these investigators used liquid chromatography/tandem mass spectrometry of peptides derived from trypsin-digested proteins to assess this feature. In plasma, the concentrations of different proteins can vary by as much as 10 to 12 orders of magnitude. Mass spectrometry allows even low-abundance proteins to be detected with greater accuracy. In their initial set of experiments, Hung and colleagues used plasma samples from tumor-bearing Apc^{Min} mice that carry a point mutation in the Apc gene and develop multiple adenomas within the first 4 months of life. Using principal component analysis, they were able to distinguish between tumor-bearing and normal samples with as few as 19 different proteins. Thus, they showed that it is possible to use such panels of markers to detect and monitor many different types of cancers.

To identify specific proteins that might serve as good cancer biomarkers, Hung and colleagues (10) used a different mouse model for colon cancer. This mouse, designated Apcδ580, resulted from deletion of exon 14 in the Apc gene that results in development of multiple intestinal tumors during the early stages of life. Pooled plasma samples from tumor-bearing mice and their normal littermates were immunodepleted for the most abundant proteins, such as albumin. Proteins from the normal samples and tumor samples were differentially labeled, mixed, extensively fractionated, and examined by mass spectrometry. This analysis yielded 51 different proteins that are expressed at higher levels in samples derived from tumor-bearing mice. Cathepsin B and cathepsin D were 2 of the proteins that were identified at increased levels in plasma from tumor-bearing mice. They were indeed tumor-specific because their levels of expression were found to be higher in tumor tissue by immunohistochemistry. Both of these proteins are cysteine proteases that have been implicated in cancer pathogenesis. To assess their biological activity in tumors compared with normal mucosa, Hung and colleagues (10) injected tumor-bearing mice with the Prosense imaging agent, a normally inert substrate that contains a fluorophore that is activated when cleaved by cathepsins. They detected the activated fluorophore using near-infrared imaging (Figs. 2 and 3). These studies revealed that only the tumor, and not the surrounding normal mucosa, was positive, indicating that cathepsin activity was tumor-specific. To assess the significance of these observations with regard

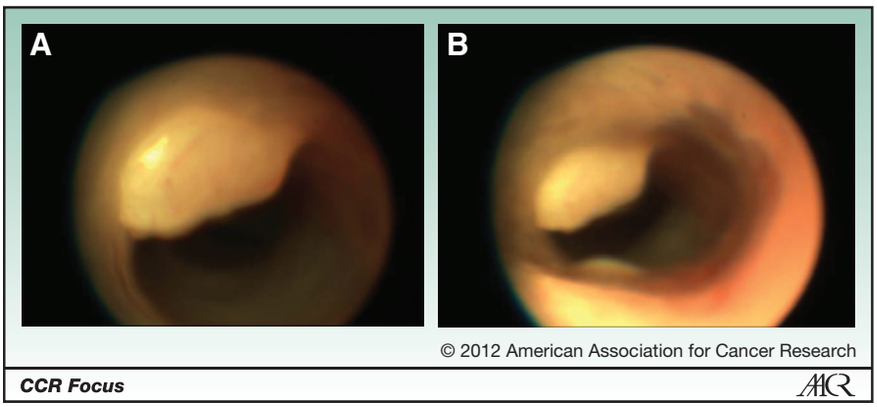

Figure 2. Images of mouse colonic tumors obtained with a colonoscope. Mice containing a conditional knockout allele at the Apc locus were injected with Adeno-cre, and images were obtained by colonoscopy 9–12 weeks after injection. A and B, images from 2 different mice.

to detecting human colorectal cancer, Chan and colleagues (11) examined the levels of expression of cathepsin B in human colorectal cancer samples. They observed that of the 558 tumor samples examined, 82% were positive for cathepsin B, and the increased level of expression correlated with a higher risk for mortality. They also observed that as much as 91% of early-stage adenomas expressed this marker. These results unambiguously show the utility of mouse models for detecting cancer biomarkers that have direct relevance to human cancer.

The critical role played by mice in identifying cancer biomarkers is exemplified by a study of pancreatic cancer by Faca and colleagues (12). In this study, the authors used a mouse that carries mutations resulting in *Kras* oncogene activation and inactivation of the *Ink4a* and *Arf-p53* genes. These mice develop early-stage focal pancreatic neoplasia that progresses to invasive pancreatic ductal adenocarcinoma. Plasma samples from tumor-bearing and normal mice were subjected to immunodepletion and fractionation in a manner similar to that described above for colon cancer samples. A detailed analysis of the protein compositions revealed several proteins whose levels of expression had increased substantially in samples derived from tumor-bearing mice, and some other proteins that were exclusively detected only in the tumor-bearing mice. A total of 45 proteins were chosen for further study. Several of these proteins were confirmed by immunohistochemical analysis or ELISA to have relevance to pancreatic cancer. The proteins included CD166 antigen precursor (ALCAM); receptor-type tyrosine-protein PTPRG, TIMP1, tenascin C (TNC), and ICAM1. A number of these proteins, including ALCAM, ICAM1, LCN2, TNFRSF1A, TIMP1, REG1A, REG3, and WFDC2, were found to be elevated in human samples from patients with pancreatic cancer, suggesting that these markers might be used to detect early stages of human pancreatic cancer.

A third example is the application of biomarker discovery approaches in a study of lung cancer by Taguchi and colleagues (13). The authors examined 3 well-characterized mouse models for lung cancer: *TetO-EGFR*^{L858R}*/CCSP-rtTA* [Lung-EGFR (14)], *TetO-Kras4b*^{G12D}*/CCSP-rtTA* [Lung-Kras (15)], and urethane-treated [Lung-Urethane (16)]. They also compared the plasma proteomic profiles of these different samples with those obtained from other mouse tumor models. The comparators included breast cancer, pancreas cancer, colon cancer, ovarian cancer, and prostate cancer. Hierarchical clustering of the data revealed that the profiles of these tumor types are distinct, and the tumor types can be easily distinguished from each other and from the lung. Although the 3 lung tumor types cluster together,

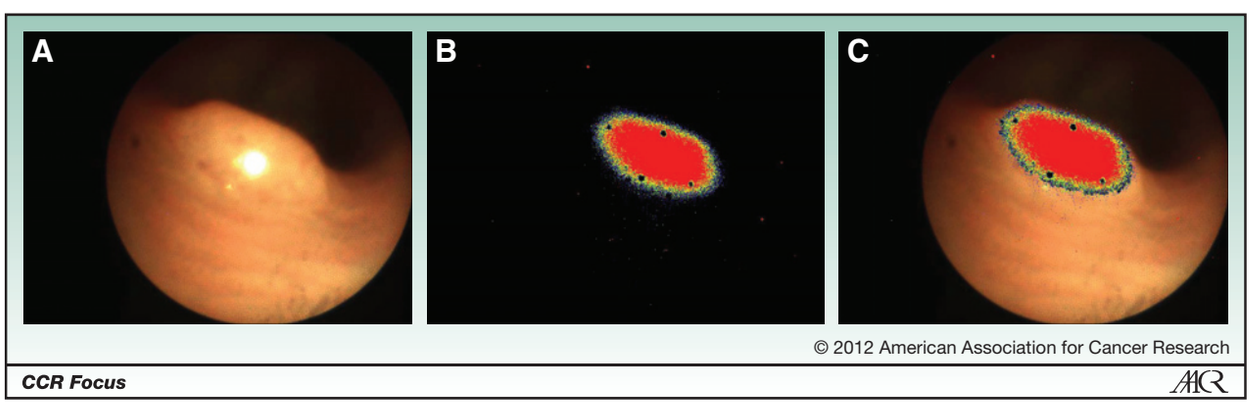

Figure 3. Mouse tumors imaged with Prosense. A, image of a mouse tumor obtained with white light. B, the same mouse tumor after injection of Prosense, obtained with near-infrared light. C, superimposition of images A and B.

they are also distinguishable from each other. To assess whether the proteins detected from the lung-tumor–bearing mice are tumor-derived, the investigators examined culture media from cell lines derived from the 3 different tumor types. They found that 25 of 39 proteins (64%) were detectable in the spent culture media. To assess the significance of the proteins identified with the use of mouse models, they also tested several of the proteins in human samples. They performed ELISA assays for SFTPB, WFDC2, and ANGPTL3; EGFR assays for non–small cell lung cancer; and ROBO1 assays for small cell lung cancer. These analyses not only confirmed the presence of these markers in human samples but also revealed that their addition to previously known markers enhances the sensitivity and specificity of detection of cancer.

All of these examples clearly show that mouse models can be used as effective tools for discovering biomarkers that are directly applicable to human samples. Given the large sets of markers that have been identified, we can now consider initiating clinical trials with human samples. One of the difficulties of translating this type of protein-based information from mice to humans is the lack of robust antibodies that are capable of recognizing human proteins with a high degree of specificity. Larger efforts to create such reagents are necessary.

Use of GEMMS for Drug Testing and Development

In most drug-development strategies, cells that are derived from human tumors and have been maintained in cell culture for many generations are transplanted into immunocompromised mice, and the mice are then challenged with therapeutic agents. However, cell-line–based models are not truly predictive of response in human patients because they are derived from tumor cell lines that may have acquired many novel properties during the time in culture, and are implanted into ectopic sites that bear no resemblance to the normal microenvironment in which tumors develop and grow. GEMMs circumvent these shortcomings, which makes them an attractive platform for preclinical therapeutic trials. As a result, the use of genetically modified mice for drug testing and drug development is on the rise. Here are 3 examples of how different models are being used for this purpose:

The first example involves the development of an accurate model for human sporadic colorectal cancer and use in drug testing. Hung and colleagues (17) described a mouse model for sporadic colorectal cancer. A large proportion of human sporadic colorectal tumors originate from the acquisition of an inactivating mutation in the APC gene. Following the initial APC mutation, cells that lose the wild-type copy or suffer a mutation in the second copy of APC develop into adenomas. To mimic this series of events, Hung and colleagues developed a mouse in which exon 14 of the Apc gene is flanked by loxP. Cells that contain this allele will lose exon 14 in the presence of the protein cre. To modify the Apc gene, these investigators introduced Adeno-cre into the colon or rectum by enema or through a surgical incision of the distal region of the gastrointestinal tract. Under these conditions, the mice typically developed a single tumor at the site of introduction of the adeno-cre. These investigators also used a colonoscope that was specially built for examining mouse colons, which enabled them to image all tumors developed by the mice and follow their progression in time without having to kill the mice (Fig. 1). When mice that have the conditional inactivation mutation of the Apc gene are subjected to treatment with adeno-cre, they develop tumors that are detectable by the colonoscope as soon as 6 weeks after adeno-cre treatment, and become large enough to completely occlude the lumen by 12 to 14 weeks. If the mice contain a conditional activation mutation of KRAS in addition to the APC mutation, they develop more tumors, and some of the tumors metastasize to the liver in a manner that is common to human colorectal tumors.

Tumors that were derived from the Apc mutant mice did not have spontaneous KRAS mutations. An examination of these adenomas revealed that they had activated the mTOR pathway. On the basis of these results, Hung and colleagues (17) tested the ability of an mTOR inhibitor, rapamycin, to inhibit tumor growth. They showed that these particular tumors are indeed susceptible to this inhibitor, and tumor regression was achieved. However, the mice that contained both the Apc mutation and the activated KRAS mutation did not respond to the mTOR inhibitor. Because KRAS activates the mitogen-activated protein kinase (MAPK) pathway, it is understandable that tumors from mice that carry the 2 mutations do not respond to mTOR inhibitors. These results suggest that either a combination of MAPK and mTOR inhibitors or stratification of patients based on these different mutations may be required to obtain an effective therapeutic index.

The second example involves the combination of conditional knockout mutations in Apc and Pten, as performed by Wu et al. (18). The authors injected adeno-cre into the ovarian bursae of mice that are homozygous for conditional mutations in Apc and Pten. Simultaneous inactivation of these 2 genes has been shown to result in ovarian cancer (19). The mice developed small tumors in as little as 4 weeks after injection of adeno-cre. The mice were then treated with rapamycin (an mTOR inhibitor), API-2 and perifosine (2 AKT pathway inhibitors), or the chemotherapeutic agents cisplatin/paclitaxel. They showed that all of the treatments resulted in significantly smaller tumors that did not metastasize as compared with controls. Images of the mice obtained at different times after drug treatment confirmed the observations. A biochemical analysis revealed that inhibition of the AKT pathway resulted in increased activity of the MAPK pathway. As is the case for the colorectal tumors described above, it may be necessary to consider treating ovarian tumors that result from deregulation of the Wnt and AKT signaling pathways with a combination of MAPK and AKT inhibitors.

In the third example, Meylan and colleagues (20) used a mouse model for lung cancer to evaluate therapy with NFκB inhibitors. They showed that activation of the NFκB

pathway is a critical event in lung tumorigenesis. Based on observations that activation of the KRAS oncogene results in activation of the NFκB pathway, and that wild-type p53 antagonizes this pathway, Meylan and colleagues (20) developed mice with mutations in both of these genes. When the lungs of the mice were exposed to adeno-cre, the authors were able to show that the mice developed tumors and these tumors could be inhibited by treatment with NFκB antagonists.

Challenges and Recommendations

Cancer is a somatic disease. With the exception of cancer predisposition syndromes, human cancers arise as a result of mutations in somatic cells. Many of the early GEMMS involved mutations in the germ line of the mouse. Such mice were good models for studying human cancer susceptibility syndromes but did not provide good models for sporadic cancer. The use of conditional modifications and the ability to modify somatic cells at the desired organ site enabled the development of models for sporadic cancer. These procedures also reduced the time required to generate tumors for studies. Nevertheless, the construction of genetically modified mice is a time-consuming process. Although researchers are attempting to generate mouse embryonic stem cells with each of the mouse genes inactivated, for cancer studies it may be necessary to introduce specific mutations that require a significant effort. A single organ-site–specific human cancer may result from one of many initiating genetic events. Each GEMM represents one such event. To reproduce the diversity of human tumors, we may need to generate many mouse models, each containing a different genetic change. Because of possible modifying genes in different mouse strains, it is necessary to conduct GEMM studies in a congenic background. Breeding the mice to have the genetic mutations in a uniform genetic background is time-consuming. An alternative approach is to generate tumors in chimeric mice that contain mutant and normal cells, and such technologies should be promoted. Many of the initial embryonic stem cell experiments were conducted with cells derived from strain 129 mice. The current availability of embryonic stem cells from other genetic backgrounds is making this process more efficient. It is not difficult to generate cell lines with multiple changes; however, generating mice with combinations of genetic changes can add additional constraints. Newer technologies that can more quickly introduce specific genetic changes into particular mouse tissues are required.

The biomarkers that are discovered in mice have to be validated in human samples. If they are validated, it is necessary to develop appropriate human reagents (e.g., monoclonal antibodies directed against human proteins) and test them in a clinical setting. A bank of high-quality antibody reagents directed against many of the protein biomarkers that have been discovered would be very useful.

Conclusions

GEMMs are becoming increasingly useful for preclinical drug testing. However, studies are usually limited by the lack of appropriate drugs for such testing, at least within an academic setting. Mechanisms to provide broader access to new chemical entities or biological molecules directed at specific cancer targets would benefit drug development. As the usefulness of this type of testing becomes better appreciated, it is possible that there will be widespread use of such models in drug discovery and development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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