

The expanding role of mouse genetics for understanding human biology and disease

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It has taken about 100 years since the mouse first captured our imagination as an intriguing animal for it to become the premier genetic model organism. An expanding repertoire of genetic technology, together with sequencing of the genome and biological conservation, place the mouse at the foremost position as a model to decipher mechanisms underlying biological and disease processes. The combined approaches of embryonic stem cell-based technologies, chemical and insertional mutagenesis have enabled the systematic interrogation of the mouse genome with the aim of creating, for the first time, a library of mutants in which every gene is disrupted. The hope is that phenotyping the mutants will reveal novel and interesting phenotypes that correlate with genes, to define the first functional map of a mammalian genome. This new milestone will have a great impact on our understanding of mammalian biology, and could significantly change the future of medical diagnosis and therapeutic development, where databases can be queried *in silico* for potential drug targets or underlying genetic causes of illnesses. Emerging innovative genetic strategies, such as somatic genetics, modifier screens and humanized mice, in combination with whole-genome mutagenesis will dramatically broaden the utility of the mouse. More significantly, allowing genome-wide genetic interrogations in the laboratory, will liberate the creativity of individual investigators and transform the mouse as a model for making original discoveries and establishing novel paradigms for understanding human biology and disease.

Introduction

Probably no two mammals have shared a longer relationship than mice and humans, even though their evolutionary lineages diverged more than 96 million years ago (Nei et al., 2001). The mouse *Mus musculus* has had an impact on the lives of human beings since the dawn of civilization, from being an agricultural pest to serving as a domesticated pet, and now contributing a more utilitarian role as the foremost genetically tractable model organism for understanding human biology and disease. More so than any other genetically tractable organism, the mouse offers a close glimpse of humankind in terms of similarity in the underlying physiology, tissue structure and organization. In effect, our fellow creature also experiences many of the common diseases we suffer, which have both polygenic as well as mendelian inherited causes. With both human and mouse genomes sequenced, it is no surprise that this kinship is reflected in the 99% of our encoded sequence that we share (Waterston et al., 2002). Furthermore, the evolutionary conservation of large linkage groups within the mouse and human genomes with respect to the nature of the encoded genes and their linear order along chromosomes has been a great asset in the identification of potentially corresponding homologous mutations and disease genes. Innovations in sequencing and mapping technologies have made the once huge effort in identifying disease genes in humans relatively straightforward. Yet, there is a critical gap in our knowledge of how these genes act and what pathways

and processes they regulate in the physiological setting, which can be gained mostly, if not only, through experimental and hypothesis-driven approaches in model organisms. Furthermore, deciphering the roles and the interplay of the genes and the other genomic elements that are not directly mutated in patients is equally important in our effort to understand biology and develop therapeutics. Thus, even with a physical map laid out, the determination of gene function remains one of the most daunting challenges in this post-genomic era. To this end, genetic analysis in the mouse could provide the first functional map for a mammalian genome, allowing us to establish the relevance and causal relationship of genes to human biology and disease.

Initially, functional analysis in the mouse was largely limited to spontaneous mutations and inbred strains. However, the ability to genetically interrogate gene function in mammalian models was slow to advance, in contrast to other lower organisms. This all changed with the revolutionary development of gene targeting technology in the mouse, which has propelled reverse genetics as a means to manipulate the genome and has allowed the analysis of functional knockouts of individual genes. The analysis of approximately 15% of the genes knocked out in mice has already taught us much about biology and disease. The production of a collection of embryonic stem (ES) cells with mutations in the majority of the predicted genes will certainly accelerate the process of discovery despite the significant investment of resources and time required to convert the ES cells into mutant animals. More excitingly, after recent technological advances and emerging genetic tools, we are now in a position to genetically interrogate mammalian gene function in a genome-wide fashion. Based on forward genetics, the strength of these strategies lies in the ability to dissect even the most complex biological process by examining the phenotypic

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outcome of genome-wide genetic perturbations in an unbiased fashion. In this way, new and unexpected discoveries can be made of genetic pathways and mechanisms that regulate developmental and cellular processes, which play significant roles normally as well as in disease. The success of such an approach has been well documented in lower model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans* and yeast.

In this review, we will consider the impact of past genetic approaches and the future possibilities of mouse genetics in defining mammalian gene function with respect to human disease. Indeed, the recent 2007 Nobel Prize in Physiology and Medicine, awarded to Mario Capecchi, Martin Evans and Oliver Smithies for their pioneering work on gene targeting in the mouse, underscores the recognition that the mouse model has finally come to full fruition.

From fancy mouse to model organism

Mouse genetics probably has its roots in China, with what began as a fascination with albinos, coat color varieties and waltzing mice, eventually leading to the enthusiastic breeding of fancy mice in Victorian Europe (Rosenthal and Brown, 2007). Modern day laboratory mice, in fact, are thought to be direct descendants of only a few of these original fancy stocks (Beck et al., 2000; Paigen, 2003). Many of these mutant stocks are inbred as a result of 20 generations of intercrossing of sibling mice, which are derived from the initial single parental pair mating. At the end of this process, the inbred mice would be homozygous at practically all loci on every chromosome (Peters et al., 2007). C. C. Little initially recognized that such uniformity is crucial to obtain reproducibility in genetic experiments. He went on to show, for the first time, the genetic basis of the resistance to the growth of transplanted tumors in inbred mice, since some were resistant to the transplantation (Paigen, 2003). This heralded the beginning of mouse genetics.

For decades, these inbred mice have been an invaluable genetic tool in cancer research and immunology. In fact, it was George Snell who developed the concept of congenic mice, where a single locus can be manipulated between strains to reduce complexity, which enabled him to elucidate the function of the H2 histocompatibility loci. The underlying principle for tumor rejection in Little's transplants was finally revealed to have an immunological basis (Paigen, 2003). This later led to the discovery of the major histocompatibility complex (MHC) in humans and eventually won Snell the Nobel Prize. To date, hundreds of lines of inbred mice have been established and hundreds of spontaneous mutations have been isolated. The collection represents a rich resource of natural genetic diversity and alterations that have provided insights into mammalian biology and human disease (<http://www.informatics.jax.org>) (Paigen and Eppig, 2000). For example, work in mice enabled the discovery of the *obese* (*ob*) and *diabetic* (*db*) gene products as the satiety hormone leptin and its receptor, respectively (Coleman, 1978; Zhang et al., 1994; Chen et al., 1996).

Gene disruption and genomic alterations via mouse ES cells

The analysis of spontaneous mutation and natural variation in inbred mice has provided a wealth of information and a deeper understanding of many disease loci. However, the pace of finding new mutants is limited by the low spontaneous mutation rate, in

addition to the tedious task of mapping the mutations. In the 1980s, the landscape of mouse genetics underwent a dramatic transformation with the development of gene targeting technology in ES cells and transgenesis, where it was possible for the first time to alter any gene in the genome and produce mutant and transgenic animals (Evans and Kaufman, 1981; Gordon and Ruddle, 1981; Doetschmann et al., 1987; Thomas and Capecchi, 1987). The gene targeting process starts with the introduction of mutations or alterations into any desired target genomic sequence by homologous recombination in ES cells. Subsequently, the cells are injected into developing embryos, which are then surgically implanted into foster mothers to generate chimeric progeny and screened for germ line integration. This leap in technology led to an explosion over the past few decades of important discoveries of gene function and roles in mammalian biology and human disease. To date, approximately 15% of the genes have been mutated in mice and many of them model human disease (<http://www.informatics.jax.org/>; <http://www.emmanet.org/>; <http://www.brc.riken.jp/>).

Recently, the International Knockout Mouse Consortium (IKMC) was formed with the goal of establishing a library of mouse ES cells carrying a null mutation for each predicted gene by gene targeting and gene trapping (Collins et al., 2007). By integrating a viral or plasmid vector carrying a promoterless reporter in the genome, the gene-trapping approach can rapidly produce a collection of ES cells, in which individual genes are disrupted and at the same time endogenous gene expression can be reported (for a review, see Standford et al., 2001; Standford et al., 2006). The combined efforts of several large consortia have trapped almost 60% of the total number of genes (Zambrowicz, 1998; Skarnes et al., 2004; To et al., 2004; Nord et al., 2006). Furthermore, a library of mutant ES cells can be generated through mutagenesis with alkylating agents such as *N*-ethyl-*N*-nitrosourea (ENU) (Chen et al., 2000; Munroe et al., 2000). In combination with high-throughput mutation detection technology, multiple alleles in a gene of interest can be generated. Such an allelic series may better reflect the types of point mutation that are often found in human disease genes (Balling, 2001).

Theoretically, mutant mice for every predicted gene can be generated through the ES cell approach. However, the process of converting ES cells into mutant animals remains technically challenging, expensive and time-consuming. Thus, investigators are only converting selected individual ES cell lines in which there are indications that the mutated gene may be involved in the process of interest. Currently, there is no plan to systematically convert all of the mutated ES cells into animals. Nevertheless, these ES cell libraries are an invaluable and widely accessible resource for the research community. The demanding effort and cost associated with generating mutant mice have deterred the sharing of reagents, which results in costly repetitive efforts to mutate the same gene; in one case, more than 20 independent mutant lines have been generated. These ES cell libraries not only speed up the process of generating knockout mice, but also reduce the cost by avoiding repetition. In addition, the ES cell libraries can be used for *in vitro* cell-based screening, particularly for genes involved in ES cell differentiation (Stuhlmann, 2003; Glaser et al., 2005; Wang and Bradley, 2007). Indeed, efforts are underway to produce a library of homozygous mutant ES cells (A. Bradley, personal communication). In combination with high-throughput screening

technologies, such a library will be a great source to conduct forward genetic screens for a variety of cellular processes.

ES-cell-based gene targeting technologies offer unique advantages for a variety of manipulations in the mouse that are not easily accomplished in other multicellular model organisms. For example, tissue- or cell-specific conditional gene knockout can be accomplished with spatially and temporally regulated Cre recombinase (reviewed by Sauer, 1998). The ease of genetic manipulation in ES cells also allows the creation of deletions, duplications and other rearrangements, as well as point mutations, which can precisely model the alterations in human disease syndromes (Yan et al., 2004; Tybulewicz and Fisher, 2006) (for a review, see van der Weyden and Bradley, 2006).

In addition to ES cells, the germline stem (GS) cells isolated from the neonatal mouse testis can also be genetically modified and go through germline transmission upon injection into the testis (Kanatsu-Shinohara et al., 2004). In addition to using a single clone of genetically modified GS cells, it is possible that a population of GS cells with different genetic alterations can be injected into a single male to produce multiple mutant progeny. Finally, somatic cloning provides another novel way to produce genetically modified animals (Aoi et al., 2008; Hanna et al., 2008). These capabilities will further empower the mouse model for addressing a variety of conceptual and therapeutic issues.

Forward genetics by mapping natural variation

Reverse genetic approaches, such as using knockout methodologies can offer in-depth analysis of individual genes. However, such techniques lack the power to predict the phenotypic outcome of a targeted mutation because the particular gene may not be involved in the biological process of interest. It is not surprising therefore that a large proportion of knockout mice do not produce the expected phenotype even after extensive in vitro study (Barbaric et al., 2007). Finally, reverse genetic techniques are too expensive as an approach for genome-wide interrogation of the predicted genes, and cannot yet be used to investigate regulatory elements and the tens of thousands of non-protein-coding RNA genes.

One phenotype-based forward genetic approach for correlating traits and genes is to utilize inbred mouse strains to map causative natural variations (for reviews, see Peltz, 2005; Peters et al., 2007). Two or multiple inbred strains are intercrossed for several generations so that the segregation of traits with the genomic variations can be followed. This approach is particularly successful in the identification of quantitative traits or polygenic loci (Peltz, 2005; Peters et al., 2007). Unlike monogenic traits, which are inherited in a simple mendelian fashion, many human diseases have complex phenotypes that are quantitative in nature, where characteristics are not qualitative, but can have a spectrum of values, such as body weight, hypertension and longevity (Peltz, 2005; Peters et al., 2007). The elaboration of the phenotype is governed by polygenic loci or quantitative trait loci (QTL), which can have numerous genetic interactions among multiple genes or with the environment. Because their natural variations are set in homozygosity, inbred strains, particularly the specialized recombinant inbred (RI) strains (Bailey, 1971; Swank and Bailey, 1973), have been tremendously useful in mapping and identifying QTLs. The development of short sequence length polymorphic

(SSLP) and, subsequently, single nucleotide polymorphic (SNP) markers has refined and speeded up mapping efforts considerably.

To fully capitalize on this tremendous genetic resource, collaborative efforts have been initiated to systematically catalog the phenotypes of numerous common inbred strains to form the mouse phenome database, which contains comprehensive data on physiology, metabolic status, disease susceptibility, morphology and behavioral traits, as well gene expression profiles (Paigen and Eppig, 2000; Bogue and Grubb, 2004; Churchill et al., 2004). Given the enormous heterogeneity observed in the human population, it may be fruitful to involve mice from wild populations in similar studies (Yoshiki and Moriwaki, 2006).

Forward genetic screen using ENU mutagenesis

Instead of relying on natural variation, a more direct forward genetic approach is to randomly mutagenize the genome and to systematically screen for mutants with phenotypes of interest. Such screens may uncover novel and unexpected genes without any bias or assumptions. Mutants sharing a similar phenotype may indeed point to an underlying network of genes that potentially affect the same biological process or genetic pathway. Furthermore, mutations showing differences in phenotypic severity will allow us to identify key players of a pathway. An important aspect of a phenotype-driven screen is that it allows genome-wide interrogation. The strength and broad application of these approaches have been demonstrated in many other model organisms, including yeast, worms, flies, fish and Arabidopsis. For example, the large-scale screen for embryonic lethals in *Drosophila melanogaster* by Eric Wieschaus and Christine Nüsslein-Volhard led to the discovery of genes that regulate embryonic development and many signaling pathways (Nüsslein-Volhard and Wieschaus, 1980).

The discovery of ENU as a powerful mutagen has made phenotype-driven large-scale mutagenesis in the mouse possible. ENU induces mostly point mutations and does so randomly throughout the genome at a density of one point mutation for every 1–2 Mb (Russell et al., 1979; Hitotsumachi et al., 1985). For any single locus, one can expect to recover a point mutation among approximately 1000 mutagenized gametes. Therefore, ENU mutagenesis has a potential to generate an allelic series, which can exhibit a variety of phenotypes reflecting the divergent functional aspects of the encoded protein. While knockout alterations are often null mutations, ENU mutagenesis is more likely to generate hypomorphic mutations that are often found in human disease.

Although most of the mutations induced by ENU are recessive loss-of-function mutations, approximately 25% of the mutations recovered are dominant alleles, which can be due to haploinsufficiency or result from dominant gain-of-function or dominant-negative mutations (Brown and Nolan, 1998; Chen et al., 2000). One of the simplest ENU mutagenesis strategies that has been successfully performed involves the screen for dominant or semi-dominant mutations. This is a relatively fast and straightforward screen because it only requires mating the mutagenized male to untreated females and scoring the G1 progeny. A perfect example of a screen utilizing such a scheme is one carried out by Vitaterna et al. (Vitaterna et al., 1994) to look for mutations that affect circadian rhythms in mammals from which they obtained a semi-dominant mutation in the mouse *Clock* gene. Positional cloning and candidate gene approaches were subsequently used to

identify the gene affected (King et al., 1997). Since then, two major large-scale screens have been initiated to look for dominant mutations causing overt phenotypes (Hrabé de Angelis et al., 2000; Nolan et al., 2000). The combined efforts produced 300 mutants from screening well over 40,000 mice. Beyond the visible phenotypes affecting coat, hair or eye color, for example, were specific defects, which were discovered only after secondary screening using unique phenotyping protocols (discussed below). The analysis reveals that a large proportion of the mutants have phenotypes that are highly relevant to human disease. A few of these mutants have been further characterized to reveal alleles, including beethoven and headturner, that are required for inner ear development, and lead to hearing loss when mutated (Vreugde et al., 2002; Kiernan et al., 2001).

Although dominant mutation screens have been highly successful, the rate of recovery hovers around 2% (Nolan et al., 2000). Thus, many genes would have been missed in these types of screens, since most are likely to mutate to a recessive loss-of-function when induced with ENU (Brown and Nolan, 1998). Indeed, most human diseases are actually caused by recessive loss-of-function mutations. Dominant mutations such as beethoven and headturner have been very informative, especially in cases where details of their molecular nature have been discovered. However, phenotypes arising from dominant mutations are generally hard to envisage mechanistically (Beutler et al., 2007).

In contrast to a G1 screen for dominant genes, recessive screening strategies necessitate additional breeding to a G3 generation in order to achieve homozygosity for expression of the recessive mutation. Such a requirement would entail extensive animal management, effort and resources, which can be prohibitive for any large-scale screen. Thus, most recessive screens that have been carried out are geared to tackle specific phenotypes, such as developmental processes or immunology, rather than to be comprehensive (Kasarskis et al., 1998; Hentges et al., 1999; Anderson, 2000; Herron et al., 2002; Nelms and Goodnow, 2001; Cook et al., 2006). Garcia-Garcia et al. (Garcia-Garcia et al., 2005), for example, successfully carried out a screen for recessive mutants affecting embryonic patterning and morphogenesis in which they recovered mutations in genes of known signaling pathways and also in genes involved in uncharacterized pathways.

To reduce the generation time, an alternative strategy was devised so that the recessive mutations could be screened in the G2 generation (Shedlovsky et al., 1988; Rinchik et al., 1999). This scheme required mating the G1 male mice to a female that carries a chromosomal deletion, such that the recessive mutation would be hemizygous. This type of screen is limited because only mutations that would map to the deleted region can be screened. However, it has now been shown that with the combination of site-specific recombination systems (e.g. Cre/loxP and FLP/FRT) and the piggyBac transposon system, it is possible to systematically generate chromosomal deletions (Ding et al., 2005; Wu et al., 2007; also see below). A set of genome-wide deficiencies will greatly facilitate G2 screens as well as other genetic analyses.

The same tools could be used for generating chromosome inversions for preventing recombination. Chromosomes carrying multiple inversions can be used as balancers, which have been used in *Drosophila* for a variety of genetic manipulations including genetic screens, stock keeping, mapping mutations and epistasis

analysis. Indeed, a chromosome carrying a large inversion has already been used to isolate recessive mutations in a mouse genetic screen (Kile et al., 2003) (reviewed by Hentges and Justice, 2004). The generation of genetically marked balancers carrying multiple inversions for all mouse chromosomes will greatly facilitate genetic analysis in the mouse.

ENU mutagenesis, effective as it is in generating a large number of mutants, bottlenecks at subsequent steps in determining the causative mutation because this requires considerable effort and resources in breeding, mapping and sequencing the mutation. Traditionally, positional cloning has been time-consuming. However, new techniques exploiting highly polymorphic markers such as SSLP and SNP have greatly facilitated mapping efforts (for a review, see Beutler et al., 2007). In addition, the most time-consuming and costly step for mapping ENU-induced mutations is to pinpoint the disrupted gene after the initial localization of the mutation to a small chromosome region. The production of a library of insertional and knockout mutants can dramatically shorten this final step through complementation tests (also see below). Furthermore, evolving high-throughput sequencing technologies, such as 454 pyrosequencing (Margulies et al., 2005) and strategies focusing on exon sequences could allow cheaper and faster determination of the mutations. ENU mutagenesis screens are destined to play a growing role in dissecting components involved in a particular biological or disease process.

Insertional mutagenesis

Insertional mutagenesis has been carried out in a number of model organisms. Retroviral-mediated insertional mutagenesis in the zebrafish has produced more than 20% of the documented lethal mutations (Amsterdam et al., 2004), whereas in mice it has been used to identify oncogenic mutations involved in cancer (Callahan, 1996; Uren et al., 2005; Touw and Erkeland, 2007).

However, the geneticist's most powerful tool for insertional mutagenesis is mobile DNA transposons. Transposable elements can integrate into and transfer between host genomes. By nature of its ability to integrate into the genome, a transposon can serve as an excellent mutagen in genetic screens for interrogating the genome and as a vehicle for transgenesis. Mobilizing a nonautonomous transposon by controlled breeding can rapidly and cost-effectively produce a large number of mutants, which are molecularly tagged. P-elements, for example, have transformed *Drosophila* into one of the most powerful models for molecular genetics, and have played a pivotal role in functionally annotating the *Drosophila* genome through the production of a library of single transposon insertions (Rubin and Spradling, 1982; Cooley et al., 1988).

Ever since the discovery of transposons in maize by Barbara McClintock more than 60 years ago, more than 3000 transposable elements have been identified in a variety of organisms. Generations of geneticists have attempted to develop an efficient transposon system for vertebrates and mammals. The resurrection of the *Sleeping Beauty* (SB) transposon from fish, which was shown to be active in both human and mouse cells, has reignited interest in using transposons for mammalian genetics (Ivics et al., 1997). Transposons, including members of the *Tc1/Mariner* family such as SB, have been successfully used in the mouse for insertional mutagenesis in the mouse germline (Luo et al., 1998; Izsvak et al.,

2000). However, the use of SB for large-scale insertional mutagenesis is restricted by the properties of local and inefficient remobilization (Carlson et al., 2003; Horie et al., 2001; Carlson and Largaespada, 2005), which could be caused by host inhibitory mechanisms. Studying transposition of the reconstructed Harbinger transposon in human cells and the host homologous factors may provide insight into transposition mechanism in mammals (Sinzelle et al., 2008).

In searching for an efficient tool for genetic manipulations in mammals, we succeeded in modifying the *piggyBac* (PB) transposable element from the cabbage looper moth *Trichoplusia ni* to generate a binary PB transposon system for mammalian cells and mammals (Cary et al., 1989; Ding et al., 2005). The system comprises a nonautonomous PB transposon cassette for delivering the exogenous gene(s) of interest flanked by the PB left and right end sequences, and a transgene expressing the PB transposase enzyme (PBase) for inducing transposition in mammalian cells. Using this system, we have shown that PB can efficiently transpose in the mouse germline with each PB cassette carrying multiple transgenes. The PB transgenes exhibit stable inheritance and expression, thus providing a highly efficient means to generate single copy transgenes that can be genetically traced with a visible marker.

One of the most direct applications of the PB transposon system is for insertional mutagenesis. We have recently developed an efficient PB transposition system in mice, which can rapidly produce large numbers of single PB insertional mutant strains (our unpublished data). Insertional mutagenesis is triggered by the simple breeding of a PBase-expressing transgenic line with a line carrying a single nonautonomous PB transposon. This step eliminates the costly and labor-intensive production of mutants by ES-cell-based knockout technology. The single PB transposon in the genome allows the quick and effortless correlation between an insertion, the gene disrupted and the resulting phenotype, and also avoids potential genomic deletions that have been observed for insertional mutagenesis with multiple SB (Geurts et al., 2006). Furthermore, the genotypes of the offspring can be identified visually by the use of PB transposons that label coat color with fluorescent proteins. This achieves two additional goals: (1) PB insertions mobilized into new locations will exhibit a color different from the original insertion; and (2) homozygous mutants can be visibly distinguished from heterozygous or wild-type siblings which carry either one or no copy of the transposon. Visual determination of the genotypes of the mice greatly simplifies phenotypic characterization, colony management and animal breeding, and dramatically reduces the associated cost of animal work. The identities of the disrupted genes can be identified simply by PCR. The transposon can also carry a *LacZ* gene, which reports the expression of the endogenous gene; and the incorporation of loxP and FRT sites into the transposon can be used to generate deletions, chromosome rearrangements and mitotic recombination between homologous chromosomes for mosaic clones. PB has little insertion-site specificity. Finally, PB insertions can be precisely excised from their original insertion sites in the genome, allowing the reversion of insertional mutations to show the correlation of mutant phenotypes with the disrupted genes. As a result, this mammalian PB system provides the genetic and practical means to produce a large number of mutant animals in which genes are

individually mutated and molecularly annotated. A study is underway to generate a genome-wide set of mouse mutants with each strain bearing a single PB insertional mutation.

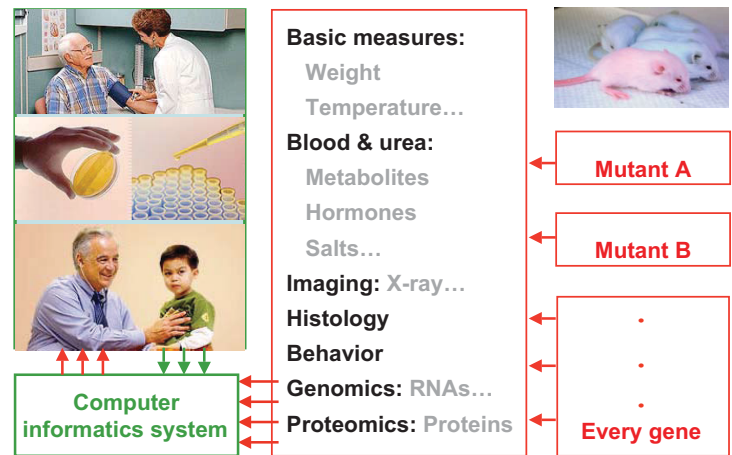
A functional map of the mammalian genome

Genetic innovations used in mutagenesis strategies described above makes it possible to produce a collection of mutants for the majority of genes in the mammalian genome for the first time. A critical task will be to phenotypically characterize these strains in order to correlate the mutated genes with relevant phenotypes. It has been estimated that more than 6000 human diseases are caused by single gene mutations, but the majority of them have not been identified. Progress is continually being made on many common human diseases, such as hypertension and cancer, but for many rare and orphan diseases we still do not know the underlying genetic basis despite clear evidence of the contributing genetic factors. The greatest hope is that through phenotyping libraries of mutants, we will be able to identify many disease genes in mice and provide candidate genes for human diseases. Such a phenotyping effort will also result in the first functional map of a mammalian genome, which will be invaluable for understanding human biology and disease (Fig. 1).

Phenotyping the mutants, however, poses a big challenge. Unlike the cost for raising some model organisms, the high cost associated with raising mice makes it impossible for individual labs to screen the entire collection of mutants for selected phenotypes of interest. However, the only way to gain mechanistic insights is to have the participation of a large number of investigators who are experts in different fields. One possible solution to this dilemma, which is favored by MRC Harwell, the German Mouse Clinic, and us, is to perform a primary screen that can detect a broad array of developmental, anatomic, physiological and behavioral defects (Brown et al., 2005). Individual mutants, once classified in this manner, will then be made available to researchers for in-depth mechanistic studies. Previously, several large-scale ENU screens have successfully used a similar hierarchical screening procedure to enrich for relevant disease mutants (Hrabé de Angelis et al., 2000; Nolan et al., 2000). This approach has several major advantages. First, by screening all of the mutants with the full set of primary assays, biased assumptions about gene function could be avoided and the likelihood of detecting novel phenotypes maximized. This contrasts with the traditional approach of constructing mutants one at a time and subjecting each of them to a small number of assays aimed at testing a specific hypothesis. Second, screening mutants with multiple assays in designated centers would also be a highly efficient strategy, because it would avoid the cost of producing animals and carrying out subsets of phenotyping tests in different laboratories. Third, systematic phenotyping will allow the assembly of the genotypic and phenotypic findings into a database that will be available on the Internet as an indispensable tool for investigators worldwide. By contrast, there is often a substantial lag in the publication of data from individual laboratories, and considerable effort is required to integrate the data among multiple laboratories. Last, the project will foster interactions among researchers in different disciplines and link investigators throughout the biomedical sciences.

Since many of the mutants generated will have morphological and anatomical defects, emerging technologies for high-resolution

Fig. 1. A functional map for the mammalian genome. The combined approaches of knockout techniques, ENU mutagenesis and insertional mutagenesis enable the research community to produce a library of mouse strains in which every gene in the genome is mutated (right panel). Systematic phenotyping of these mutants (middle panel) could identify many disease genes and establish causative relationships to produce the first functional map of a mammalian genome. Such a functional map would not only advance our understanding of human biology and disease, but also allow investigators to query the results of a patient's diagnosis (left panel) for possible underlying genetic causes of illness and to identify possible targets for medical treatment. Diagnostic photos courtesy of Yale School of Medicine.



imaging will be of great benefit for phenotyping. Technologies such as MRI and CT scanning have vastly improved and are already adaptable to small animals that will allow noninvasive examination of tissues and organs (Driehuys et al., 2008). Furthermore, a high-resolution mutant anatomical image database (MAID) for all mouse mutants would be an extremely useful resource. Future image-mining software could reveal all structural defects associated with these mutants. As an extension to imaging solutions, video technologies and motion tracking software can also be used for automated capture and analysis of defects associated with behavioral dysfunction.

Finally, a functional map of the mammalian genome can have a significant impact on medical diagnosis and therapeutic development (Fig. 1). For example, the diagnostic results from patients could be correlated with the possible underlying genetic causes. This could also pinpoint potential targets for medical treatment.

Somatic genetics

The analysis of homozygous mutants has several inherent limitations. For example, mutations causing embryonic lethality would preclude the expression of post-natal and adult gene function. Second, many human diseases are caused by somatic mutations, where only a small number of cells are mutant in otherwise wild-type tissues. To model these diseases and to study the interaction between mutant and surrounding wild-type cells, mosaic animals are necessary. Mosaic animals also allow the examination of tissue- or cell-specific phenotypes that would be masked by pleiotropic effects. Furthermore, it is not possible to include every assay, which must be cost-effective and expeditious, for systematic phenotyping. In addition, many phenotypes can only be addressed with sophisticated and advanced assays requiring in-depth expertise. Thus, to genetically interrogate the mammalian genome for a particular biological and disease process the entire collection of mouse mutants would need to be analyzed. It would be difficult, if not impossible, to apply this approach repeatedly for different biological questions. In fact, the major constraint of the mouse as a genetic model is that investigators could not systematically interrogate the mouse genome for specific biological or disease questions as individual efforts. The power of the mouse is thus significantly under-utilized for making original discoveries

when compared with yeast, worm or fly in which 'off-beat' ideas can be readily tested in individual labs. The solution is to carry out genetic analysis or perform genetic screens using mosaic mice (Fig. 2). Despite the fact that there are millions of cells for any given tissue in a single mouse, a genetic screen in a mosaic animal with somatic cells carrying different mutations can be performed and the genome can be systematically interrogated using as few as 10–100 mice.

Multiple approaches have been developed for generating genetic mosaics in the mouse (for a review, see Rossant and Spence, 1998). Classically, mouse chimeras can be generated by aggregating different cell populations at the eight-cell stage or by injecting them into blastocysts. More recently, conditional gene knockout or expression using Cre/loxP and FRT/FLP recombination systems for conferring temporal and spatial specificity to gene activation or inactivation has been a successful strategy to perform mosaic analysis in mice (Marth, 1996; Rajewsky et al., 1996; Sauer, 1998; Lewandoski, 2001). This approach has been used successfully to mimic the loss of tumor suppressors or activation of oncogenes in modeling sporadic cancer as well as many other conditions (Jonkers and Berns, 2002). Clones of mutant cells can be marked by Cre-mediated deletion or activation of either LacZ or GFP reporter under the control of the ubiquitous ROSA26 locus (Mao et al., 1999; Mao et al., 2001). Alternatively, clones of mutant cells can be positively labeled by fluorescent protein markers that are reconstituted through mitotic recombination between homologous chromosomes (Zong et al., 2005). The development of additional robust cell markers, which would permit the identification of mutant cells in multi-layered tissues or even in intact animals, can further facilitate the general application of mosaic analysis in mice. However, the approach of Cre-mediated conditional knockout mice requires the gene to be genetically re-engineered and the production of animals with such alterations for every gene under analysis. This is a labor- and time-intensive process and cannot be used for genome-wide analysis.

For genome-wide mosaic analysis, one successful strategy has been developed in *Drosophila*, in which a set of strains has been established carrying FRT sites inserted near the centromere of each chromosome arm. High frequencies of mosaic clones for any mutation on the FRT-carrying chromosome arm can be produced by mitotic recombination between the homologous chromosomes

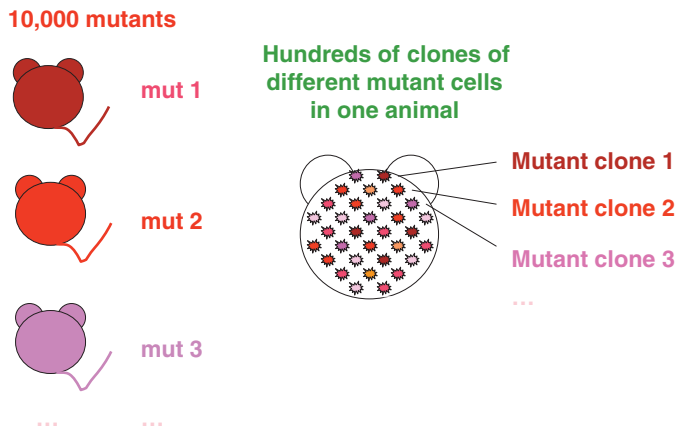


Fig. 2. Genetic screens in mosaic animals. Since mouse tissues contain millions of cells, hundreds or even thousands of somatic clones bearing different mutations can be produced in a single mouse (right), rather than tens of thousands of individual mice (left). Genome-wide genetic screens can therefore be conducted by individual investigators in as few as ten to several hundred mice. Several techniques are now being developed to produce a high frequency of mosaic mutant clones in the mouse, including transposon insertional mutagenesis and Cre/loxP- and FLP/FRT-mediated mitotic recombination between homologous chromosomes. Such a mosaic system for genome-wide genetic interrogation will empower investigators to explore novel and risky ideas in a mammal in individual laboratories.

(Xu and Rubin, 1993). Thus, for any given mutation, a large number of flies carrying homozygous clones can be generated by simple crosses to recombine the mutation onto the FRT chromosome. Furthermore, FRT chromosomes can be mutagenized to produce a large number of mosaic animals, each of which carries clones of cells homozygous for different newly induced mutations (Xu et al., 1995). This allows genome-wide genetic screens in the mosaic animals produced in the first generation without the need to establish individual lines for each of the mutagenized chromosomes. More importantly, it not only permits the recovery of all types of mutations including lethals affecting adult phenotypes, but also dramatically increases the power of genetic screens. This approach has been successfully applied for elucidating the genetic basis of multiple biological and disease processes including recapitulating the loss of heterozygosity and sporadic nature of cancers in flies (for a review, see St Johnston, 2002).

Efforts have been made to create a similar system to produce mosaic mice by mitotic recombination between homologous chromosomes (Zong et al., 2005; Wang et al., 2007; Sun et al., 2008). Currently, mosaics have been observed to be induced at low frequencies in various somatic tissues (less than 1% of mutant cells), which is probably due to the non-pairing of homologous chromosomes in mitosis. However, by recreating intact fluorescent proteins through mitotic recombination to generate positive cell markers, the technique has already enabled investigators to positively label cells for lineage tracing and to analyze gene function in mosaic animals (Zong et al., 2005; Wang et al., 2007; Muzumdar et al., 2007). Furthermore, for selected tissues, this frequency has already permitted the screening and study of the immune system, where FACS analysis can be used

to sort out the mutant cells (Sun et al., 2008). In the germ cells, the frequency of mutant clones is high enough for studying many aspects of germline biology, including maternal effects, stem cell development and gametogenesis (Sun et al., 2008) (our unpublished data). Efforts are ongoing to improve the technology in the mouse. First, loxP and FRT sites are being placed onto every mouse chromosome by targeted recombination or PB transposon insertion, which will allow mosaic analysis for genes throughout the mouse genome (L. Luo, personal communication; our unpublished data). Second, future success in increasing the frequency of mitotic recombination to produce a robust genetic mosaic system in the mouse would permit individual investigators to perform genome-wide forward-genetic screens for a variety of biological and disease processes.

Another somatic genetics approach has been to insert transposons into somatic cells. As proof of concept, Carlson et al. (Carlson et al., 2005) demonstrated the use of the SB transposon system to integrate an activated oncogene in somatic cells to model tumorigenesis. SB has also been successfully used in somatic mutagenesis to identify oncogenes involved in several types of cancer (Collier et al., 2005; Dupuy et al., 2005). Hundreds of copies of SB have been used as mutators in these experiments. Somatic insertional mutagenesis using the more efficient PB transposon system would make it easier to establish causative insertional events for identifying the gene of interest. Furthermore, transposase activities can be regulated by Tamoxifen using a transposase-ER fusion construct (Cadinanos and Bradley, 2007) (our unpublished data). Transgenic mice expressing inducible transposase under the control of a tissue-specific promoter would permit controlled insertional mutagenesis. These technologies could significantly enhance our ability to interrogate the somatic genome in a tissue- or cell-type-specific manner.

Modifier screens

By starting with a mutant of interest, forward genetics can be used to screen for additional mutations that can either enhance or suppress the original phenotype (Fig. 3). These types of modifier genetic screen are a powerful and unbiased means to isolate new and unforeseen components of a pathway because they do not rely on knowing their molecular nature or the underlying mechanism. Extensively used in lower organism models, modifier screens have made a great impact on our understanding and the uncovering of major signaling pathways that regulate developmental and biological processes (Jorgensen and Mango, 2002; St Johnston, 2002).

Most common human diseases are complex, with the symptoms influenced by variations in genetic background and environmental factors (Erikson, 1996; Nadeau, 2001). Notable examples where the genetic background can have a substantial influence over the phenotype come from a series of studies that identify both suppressors and enhancers of the multiple intestinal neoplasia (Min) phenotype in *Adenomatous Polyposis Coli* (*APC*) mutant mice, also referred to as modifiers of Min or Mom mutations. By crossing Min mice to a different inbred strain, mutations in *Mom1* and *Mom2*, which encode a phospholipase and an ATP synthase, respectively, were found to suppress the number of polyps found in *APC/+* mice. By contrast, the number of polyps was enhanced in mice containing an allele of the

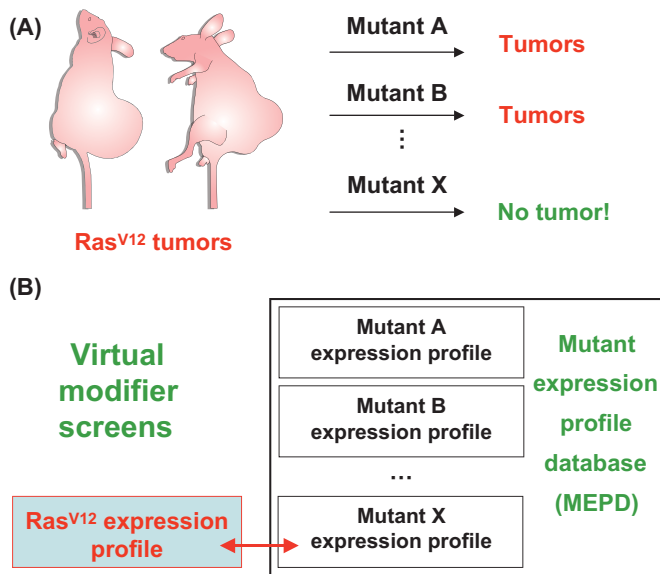


Fig. 3. Database and virtual modifier screens for identifying therapeutic targets. (A) A genetic modifier screen can be performed to search for mouse mutants that can ameliorate a disease phenotype, such as mice with Ras^{V12}-induced tumors. The examination of gene expression profiles for every mutant permits the establishment of a mutant gene expression profile database (MEPD). Such a database would allow one to perform virtual modifier screens to identify candidate mutants, which can completely or partially revert the gene expression profile change caused by a disease condition (B). The small number of identified candidate mutants can then be tested for their ability to suppress the disease phenotype in animal experiments. The MEPD will have universal appeal for identifying potential therapeutic targets and will significantly change the current practice of drug development.

forkhead box L1 gene *Foxl1* (Dietrich et al., 2003; Gould et al., 2002; Silverman et al., 2002; Perreault et al., 2005).

Recently, a large-scale ENU mutagenesis was carried out in a suppressor screen of thrombocytopenia in *Mpl* (myeloproliferative leukemia virus oncogene) mutant mice in order to dissect signaling pathways that regulate platelet production (Carpinelli et al., 2004). From screening nearly 1600 mice, two hypomorphic alleles of the myeloblastosis oncogene *c-Myb* were recovered. This study not only demonstrates the feasibility and strength of a modifier screen in mice, but also highlights several important ideas. First, the deletion of the *c-Myb* gene causes embryonic lethality whereas the partial loss-of-function alleles recovered in this screen result in viable mice with minor hematopoiesis. This suggests that gene-targeting methodology utilized in reverse-genetic strategies would not have been as informative as mutations induced with ENU with respect to the role of *c-Myb* in hematopoiesis. Second, since most pharmacological agents act in the same direction as loss-of-function mutations, that is, both act on reducing gene function, mutations identified in a modifier screen would be excellent candidate drug targets. Adding strong support is the fact that suppressor mutations are those that ameliorate the disease phenotype. In addition, modifier screens, when combined with somatic genetics, can be a very powerful strategy to identify interacting genes or pathways. In *Drosophila*, for example, a screen using flies carrying benign somatic clones expressing activated Ras

has identified genes involved in tumor progression and metastasis (Pagrialini and Xu, 2003). In mice, modifier screens by transposon insertional mutagenesis in somatic cells could have broad applications.

The initial condition or sensitized background on which a modifier screen is based can be extended beyond those that are mediated by a genetically induced phenotype. Many human medical conditions and diseases arise as a result of external challenges to the body. For example, diabetes, obesity and allergy can be correlated with changes in lifestyle and diet, or exposure to environmental pollutants. To understand the biological basis and mechanism of these problems, as well as to find possible therapeutic targets, forward genetics can be used to screen for modifiers in a mouse model that has been challenged in a similar manner to produce the same conditions.

Modifier screens in mice to identify mutations suppressing or blocking disease conditions are one of the most powerful approaches for understanding the underlying mechanisms and for identifying therapeutic targets. However, a large-scale screen of this type would consume a significant amount of resources, especially if a modifier screen was conducted in a disease-causing mutant background. It will be ideal if such a modifier screen could be conducted in silico as a virtual modifier screen (Fig. 3). For any disease, there will be an associated characteristic change in the gene expression profile. It is very likely that a modifying mutation that suppresses the disease state would have reversed a part or the majority of the gene expression profile changes observed. Therefore, if we know the gene expression profile shift caused by the inactivation of each gene in the genome and assemble all the information into a database, we could conduct a virtual modifier screen in silico to identify candidate genes in which those mutations could revert a part or the majority of the gene expression profile change caused by a particular disease. We can then validate only the selected candidate genes for their ability to suppress the phenotype in animal experiments without conducting a genome-wide modifier screen in animals. Such a mutant gene expression profile database (MEPD) will have universal value for identifying potential therapeutic targets for any disease. The production of the library of genome-wide mouse mutants described earlier will make it possible to examine gene expression profile changes in the mutants and to establish the MEPD. Although it will be too costly to examine mutant expression profile changes for all tissues, examinations for a number of selected tissues, such as liver, pancreas, brain, adipose and muscle, which are commonly affected in major diseases, should produce an invaluable and informative MEPD.

Humanized mice

The mouse has been a tremendous in vivo model for understanding many human diseases and exploring clinical therapies in lieu of direct experimentation in humans for ethical and technical issues. The horizon of the mouse as a model for understanding human biology and disease and for developing therapeutics is likely to be further widened by humanizing the mouse, in which human tissues and cells or genes are reconstituted.

One strategy in humanizing a mouse has been to put human genes in mouse ES cells that would give rise to mice expressing the human genes. Individual human genes, chromosome fragments or even an entire human chromosome can be introduced into the

mouse genome (O'Doherty et al., 2005). Furthermore, human bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC) clones engineered with loxP sites flanking the sequence of interest can be used to mediate exchange for the mouse syntenic region (Wallace et al., 2007). Gene trapping and PB vectors have already been developed with integrase recombination sites for introducing human homologs into the corresponding mouse genomic regions. Mice carrying human genes provide experimental models for directly studying the activities of human genes and their gene products and for developing and testing therapeutics against the human gene products.

An alternative strategy to produce a humanized mouse is to utilize immunodeficient mice to allow engraftment of human tissues or cells to repopulate the immune system or tissues (Macchiarelli et al., 2005; Shultz et al., 2007a; Shultz et al., 2007b). Studies have shown the ability to reconstitute the complete human immune system in these immunodeficient mice (Ito et al., 2002; Traggiai et al., 2004; Shultz et al., 2007a; Shultz et al., 2007b; Ishikawa et al., 2005). These mouse models will be invaluable for investigating many questions relating to the human immune system and infectious diseases, and for generating human antibodies against pathogens. Much success has also been seen in chimeric mice, in which 80–90% of the mouse liver was replaced by human tissue (Kato et al., 2007) such that they were able to produce human serum albumin, express human liver-specific genes, as well as many drug-metabolizing enzymes. Importantly, these humanized mice are susceptible to human hepatitis B virus (HBV), which normally does not infect mice. Furthermore, it has been shown that human ES cells can populate, differentiate and incorporate into mouse tissues (Muotri et al., 2005), which provides a unique opportunity for studying human stem cell biology. These humanized mice offer the opportunity to utilize the genetic tools developed for the mouse to directly interrogate the human genome for understanding biology and disease and for developing therapeutics in vivo.

Concluding remarks

The mouse as a genetic model has come a long way since hobbyists began collecting fancy mice over 100 years ago. Unexpectedly, the inbreeding of these mouse strains has evolved into a genetic tool that has been essential for mapping and identifying genes with important implications for human biology and disease. With the development of molecular biology came ES-cell-based knockout approaches and transgenesis, which have revolutionized how we query gene function. Key technological advancement and breakthroughs in forward genetic methodologies such as ENU and insertional mutagenesis allow us to systematically interrogate the mammalian genome for the first time. The combined genetic approaches will give rise to a library of mutants in which every mouse gene is disrupted. It is hoped that phenotyping these mutants to reveal novel and interesting phenotypes and correlating these to genes, will lead to the first functional map of a mammalian genome. The library of mutants has additional benefits. The establishment of an MEPD will have many applications including virtual screens for potential therapeutic targets. The creation of a high-resolution mutant anatomical imaging database (MAID) for the mutants could also provoke the development of software to reveal every structural defect. Innovative genetic strategies, such

as somatic genetics, modifier screens and humanized mice, are increasingly expanding the ability of individual investigators to use forward-genetic approaches to address mechanistic questions in a physiological setting and to identify therapeutic targets. This is the dawn of an exciting era for the mouse as a genetic model for understanding human biology and disease.

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COMPETING INTERESTS

The authors declare no competing financial interests.

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Perspectives in DMM will discuss broad areas of importance to the disease community and define large movements in a scientific field. An important and unique feature of these articles is to provide experts with a voice to anticipate areas of opportunity and future possibilities.