Mutagenesis strategies for identifying novel loci associated with disease phenotypes

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Abstract

The systematic identification of the function of all the genes in the mammalian genome is one of the major scientific challenges for the 21st century. A comprehensive insight into mammalian gene function will illuminate our understanding of the genetic bases of disease. Mouse mutagenesis is a powerful tool for the study of mammalian gene function. Most recently, a number of approaches employing the chemical mutagen ethynitrosourea (ENU) have been utilised by mouse geneticists to deliver a substantial new collection of mouse disease models. The growing mouse mutant archive provides a powerful resource for the identification of novel genes involved with human genetic disease.

Keywords: Mouse mutagenesis, Ethynitrosourea (ENU), Otitis media with effusion (OME)

1. Introduction

With the imminent completion of the human genome sequence and, more recently, the publication of a draft mouse genome sequence we are close to identifying the bulk of the coding potential of the mammalian genome. Comparative sequence analysis of the mouse and human genomes along with the completed sequences of other organisms is proving a powerful tool for the identification and annotation of genes. The available genome annotations are already providing substantial improvements in our ability to identify genes involved with disease, both monogenic and multifactorial. However, the problems of providing a comprehensive determination of the relationship between genetic variation and disease in the human population remain profound. There is no doubt that a systematic effort to provide a functional annotation of a mammalian genome using an experimental model organism such as the mouse would provide a powerful tool for the dissection of gene function often providing significant insights into the relationship between gene and disease. Nevertheless, the available mouse mutant resource, comprising as it does only a few thousand mutants, provides functional information on perhaps only 10% of the total number of genes in the mouse or human genomes. Moreover, considerable added value in terms of functional annotation can be derived by the analysis of multiple allelic variants at any genetic locus. It is clear that we need to increase both the breadth and depth of the mouse mutant resource.

2. Mouse mutagenesis

There are essentially dichotomous strategies in mutagenesis, sometimes labelled ‘forward’ and ‘reverse’, though better described as phenotype-driven and gene-driven, respectively. Gene-driven mutagenesis strategies in the mouse include the construction of transgenics where, for example, overexpression of gene may provide insight into function; knock-out and knock-in technology that allows specific alterations in a targeted gene and, finally, gene-traps. Gene-trap approaches involve the construction of embryonic stem cell libraries carrying gene-trap mutations and this strategy permits the provision of functional information on many thousands of genes [2,3]. These gene-driven approaches have been well described in detail elsewhere and we will not elaborate...
on them further. More recently, however, there has been a resurgence of interest in the use of chemical mutagenesis using the mutagen, ethylnitrosourea (ENU), and in particular its use for systematic and comprehensive mutagenesis of the mouse genome and the identification of novel loci associated with disease phenotypes [4,5]. Consequently we will focus on developments in this area. ENU has particularly been used to date for the phenotype-driven approach whereby mutations are randomly introduced into the mouse genome. Mutant mice are subsequently screened for interesting disease phenotypes and having identified a mutant phenotype of interest, the underlying gene is then identified making the connection between gene and function. The advantage of this approach is that no a priori assumptions are made about the nature of genes involved in any disease pathway and the phenotype-driven approach can be expected to provide a powerful route for the discovery of novel gene function [6]. Nevertheless, the use of ENU can also be adapted to a gene-driven approach and the strategies for ENU gene-driven mutagenesis will also be outlined.

3. Strategies for ENU mutagenesis

ENU is an alkylating agent causing point mutations and acts on spermatogonial stem cells [7]. ENU is delivered intraperitonially to male mice which following a period of sterility will recover fertility and can be bred. Progeny of these mutagenised males carry new ENU mutations. ENU delivers a specific locus mutation rate of around $10^{-3}$, so every 1000 gametes from a mutagenised male might be expected to carry a mutation in any gene of interest.

There are several approaches to generating collections of ENU mutant mice [4] depending upon whether one is interested in acquiring new recessive or dominant phenotypes (Fig. 1). The most complex genetic strategies, not Fig. 1. Several of the approaches that are most commonly used to undertake phenotype-driven mutagenesis in the mouse. (A) Dominant genome-wide mutagenesis. ENU mutagenised males are mated and G1 progeny screened for interesting phenotypes. (B) Recessive mutagenesis targeted to a chromosome deletion region using a two-generation scheme. The basic scheme involves crossing mutagenised males and the progeny G1s are mated to deletion carriers revealing recessive mutations in the G2 generation. There are however several variations on this basic approach, including the marking of various chromosomes with dominant coat-colour markers to aid the discrimination of test, carrier and uninformative classes in the G2 generation. Most importantly, the absence of the test class identified a recessive mutation within the deletion region, which can be recovered and analysed via carrier mice. Alternatively, the test class if viable can be the focus of screens for novel phenotypes. (C) Recessive genome-wide mutagenesis. Mutagenised males are mated and each G1 progeny representing an individual mutant line for analysis is mated again. G2 progeny are backcrossed to the founder G1 and G3 progeny scored for recessive phenotypes.
surprisingly, are associated with the recovery of recessive mutations. Recessive genome-wide screens involve three generation crosses. However, recessive screens can be targeted to recover recessive mutations in particular regions of the genome covered by deletions or inversions. These screens can involve only two generations of crosses and have the added advantage that the identified mutations are already pre-localised to defined regions of the genome in which candidate gene searches can immediately begin. Published figures indicate that up to 30% of lines from a three generation recessive screen will carry heritable mutant phenotypes [8]. Though these figures of course must vary according to the nature of the phenotypes being sought and the screens utilised, they are fairly typical. In contrast, screens for dominant genome-wide mutations require a very simple genetic design whereby mutagenised males are crossed and the progeny are immediately entered into the appropriate phenotype screen. Published figures for major dominant genome-wide screens underway over the last few years, indicate that up to 2% of progeny carry a heritable mutant phenotype [9,10]. Thus dominant screens are a very efficient mode for generating new mutant phenotypes, and there is every reason to suppose that if the phenotype screens were broadened the number of heritable mutants identified could be increased even further. Ongoing, major ENU mutagenesis screens world-wide, both recessive and dominant, have been catalogued in a recent review [4].

Lastly, ENU can be used to undertake sensitised or modifier screens. For the former, mutagenised males are mated to mice carrying mutations in specific genes that may sensitise the mice while not necessarily generating an overt phenotype. The aim is to identify mutations that interact to generate the relevant phenotype and thus help to elaborate a genetic pathway. For modifier screens, mutagenised males are mated to mice carrying mutations (possibly transgenic or knock-out in origin) that demonstrate an overt phenotype. Again the purpose is to identify modifying genetic loci that interact with the mutation. There is every reason to suppose that these approaches will be fruitful given the significant numbers of mutant loci whose phenotype can be modified by genetic background [11]—and these stratagems have exciting potential for the elaboration of the genetic pathways involved with disease.

4. Phenotyping

Critical to the success of ENU mutagenesis approaches is the implementation of efficient phenotyping approaches relevant to the disease area of interest. If large numbers of mice are to be screened, then the screening protocols must be hierarchical employing high-throughput primary screens capable of capturing a large number of potential phenotypes. Relevant heritable phenotypes can then be the focus of more intensive and sophisticated secondary or tertiary screens to further dissect phenotypes of interest. There have been several attempts to define comprehensive and systematic primary phenotyping screens, for example, the SHIRPA protocol [12]. Increasingly, however, the focus of research and development efforts in mouse mutagenesis is focused on improved phenotyping platforms both to develop more efficient primary screens as well as to provide more sophisticated tools to dissect individual phenotypes in diverse disease areas [13]. Important areas for technology development for mouse phenotype screening that are likely to impinge upon a number of disease areas from the neurological to cardiovascular include imaging (such as MRI, CT, ultrasonography) and nanotechnology (microimplants).

5. Disease models—from deafness to development

Once a relevant disease phenotype has been identified and heritability proven, the underlying causal gene needs to be identified. Genetic crosses will be required to finely localise the mutation to a small region of the mouse genome, usually 1 cM or less, representing around 1 Mb of genomic sequence from which candidate genes must be sought. Importantly, genetic mapping helps establish whether the mutant represents an allele of a known locus, or a novel previously undescribed phenotype. There are three possible categories of mutations that can be recovered, mutations that lead to:

- known phenotypes in known or novel genes, i.e. alleles of existing mutations for which the gene may or may not have been characterised;
- novel phenotypes in known genes, for which there is some prior functional annotation;
- novel phenotypes in novel genes, for which there is no prior functional annotation.

The latter two categories are of primary interest, though the value of allelic series and the dissection of subtle phenotypic variation between alleles should not be underestimated in terms of understanding gene function (mentioned later). Equally, it would be easy to misjudge the importance of the second category. Many previously undescribed phenotypes in what might be considered well-characterised genes will be important in providing a comprehensive picture of gene function.

The availability of the draft mouse genome sequence has enormously enhanced the identification and assessment of candidate genes. Moreover, the utilisation of mutation scanning techniques to identify the causal mutation will be greatly improved by the availability of complete finished sequence. Over the past few years, there has been an explosion in new ENU mutant disease models and more recently a noticeable upward trend in reports identifying the underlying gene. It is worth reviewing some of the areas where there has been a noticeable impact of ENU mutagenesis on the identification of new disease models and the identification of causal genes.
5.1. Models of genetic deafness

Both the dominant screens at MRC, Harwell, UK and GSF, Munich, Germany incorporated screens for new deafness mutants [9,10]. Much of the genetic deafness in the human population is sensorineural in origin affecting the hair cells in the inner ear that are the site of auditory transduction. A large number of new deafness mutations were identified and the outcome of these screens, the phenotypes characterised and the underlying genes cloned have recently been reviewed in detail [14]. Importantly, many of the new mutations mapped to regions of the mouse genome where deafness phenotypes had not previously been reported indicating that they represented novel disease phenotypes. Not unexpectedly, a large number of mutants were identified that affect the development or patterning of the hair cells in the inner ear. A number of the mutants have been cloned including the Slalom [15] and Headturner [16] mutants that show an aberrant organisation of hair cells in the inner ear as well as abnormalities of the semi-circular canals in the vestibular apparatus. Both mutants are encoded by the Jag1 ligand, identifying a novel function for the Jagged1 signalling molecule in the patterning of the inner ear. JAG1 mutants in humans result in Alagille syndrome that presents with a number of features including semi-circular canal abnormalities [17,18]. Most exciting, however, was the identification of a number of novel deafness models not previously seen, underlining the value of the phenotype-driven approach. Two mutants, Jeff and Junbo, were shown to have hearing impairment due to a chronic middle ear inflammatory disease and represent genetic models of otitis media with effusion (OME) or ‘glue ear’ [14,19]. OME is the commonest cause of hearing impairment in young children and is known to have a significant genetic component in the human population. The underlying genes for Jeff and Junbo remain to be cloned but both models should give us a new and profound insight into the genetic bases for otitis media in the human population (Fig. 2).

5.2. Screens for vision mutants—models of ocular disease

A major screen has been undertaken for new models of ocular disease [20]. Utilising slit-lamp observation and indirect ophthalmoscopy, a large number of new anterior segment and retinal phenotypes were recovered. Eighteen new dominant inherited phenotypes were recovered. Mapping indicated that eight of these represented novel mutant phenotypes. Four novel mutants were identified that demonstrate phenotypes similar to a number of human syndromes with dilated pupils or iris dysgenesis. For two of these human syndromes, Axenfeld-Rieger Anomaly, AXA (an iridogoniodysgenesis anomaly) and the related Rieger syndrome, the genes have been cloned—FOXC1 and PITX2, respectively. However, there are a number of iris dysplasia or hypoplasia diseases in the human population for which the genetic basis is still unknown, and there are new mutants...
be expected to shed light on the genetic pathways involved. The screen also uncovered a number of additional alleles of known genetic loci, particularly the Pax6 locus which causes aniridia in humans [20]. Three new missense alleles were recovered, one of which alters a conserved arginine in the paired-box domain known to be involved with DNA binding. An identical mutation has been found in humans, but intriguingly, these patients have a mild phenotype with no anterior segment defects in contrast to the mouse.

5.3. Neurological and behavioural disease models

The identification of the clock mutant in a small ENU screen for circadian rhythm mutants and its subsequent cloning remains a paradigm of the phenotype-driven approach and its ability to deliver novel information concerning gene function [21]. Screens for neurological and behavioural disease models have been a significant component of many of the ENU programmes world-wide [4]. The ENU screen at MRC Harwell incorporated a battery of tests to identify mice with neurological or behavioural deficits [9]. Amongst the many new phenotypes identified, three mutants demonstrating tremors were recovered [22,23] that were shown to be allelic to the Trembler locus and to carry mutations in the peripheral myelin protein gene, Pmp22. Comparative analysis of these alleles illustrates well the advantages of allelic series. Two of these mutants alter amino-acids known to be sites for mutation in the severe human peripheral neuropathy Dejerine Sottas syndrome. However, all three mutants show a wide range of severity with respect to phenotype and neuropathology. Interestingly, analysis of the least severe of the mutants shows that Pmp22 mutant protein tends to form large cytoplasmic aggregates, in contrast to the most severely affected mutant where mutant protein occurs in a diffuse perinuclear pattern that co-localises with wild-type protein [23]. It is possible that Pmp22 aggregates may have a protective role.

5.4. Models of developmental disease

Several ENU screens have focused on the identification of new recessive lethal phenotypes representing defects at various stages of development. In addition, dominant screens have often recovered a variety of semi-dominant phenotypes that are viable when heterozygous but show interesting developmental defects in the homozygous state. One recent recessive screen focused on identifying abnormalities in organogenesis at embryonic day 18.5 [8]. Many of these mutations might be expected to survive to term and thus represent potential models of human birth defects. Fifteen mutants were identified from the analysis of 54 lines and the mapping and complementation testing of several of these lines indicated that they represented novel phenotypes. Several of these novel mutant phenotypes indicated that they may be potential new models for human malformation syndromes. For example, the shorty mutation that demonstrates abnormal ribs, vertebrae and post-natal lethality may represent a model of Jeune asphyxiating thoracic dystrophy.

6. Gene-driven ENU screens

It has been proposed for some time that it would in principle be possible to employ ENU in a gene-driven mode and recently this has been demonstrated in practice opening up an exciting new opportunity to generate disease models [24]. It involves the creation of parallel frozen archives of DNA and sperm from male mutant mice generated in ENU mutagenesis programmes. The DNA archive is scanned for mutations in the relevant gene. Having identified a DNA sample carrying a mutation, the mouse is resurrected from the frozen sperm archive and the phenotype examined. The specific locus mutation rate of ENU indicates that a relatively small number of DNA samples need to be screened to have a realistic chance of detecting a mutation in the gene of interest. For example, screening of around 2500 DNAs gives a greater than 90% chance of detecting one mutation. Screening of larger archives provides a significant chance of recovering an allelic series of point mutations at any locus. Proof of principle for this approach was achieved by screening a parallel archive of 2230 samples for mutations in the connexin 26 gene [24]. Mutations in connexin 26 are the most common cause of autosomal recessive sensorineural hearing loss in many human populations. A mouse knock-out of the connexin 26 gene, however, is recessive embryonic lethal due to placental dysfunction, presumably compensated in human by an alternative connexin. The aim was to identify partial loss-of-function mutations in the mouse connexin 26 gene that may be homozygous viable yet show hearing impairment. Three connexin 26 mutations were identified—one stop mutation and two missense mutations. Not surprisingly, the stop mutation was recessive lethal. The two missense mutations remain to be evaluated. One of the most significant advantages of the approach however is that since ENU is a point mutagen it is possible to recover and study the full range of mutational effects that might be expected in any gene—including null, hypomorphic, dominant-negative and gain-of-function mutations. It is also possible to apply gene-driven ENU screens using embryonic stem (ES) cells [25,26]. ES cells can be mutagenised with ENU (or ethylmethanesulphonate; EMS) in vitro. Following screening of ES lines for mutations in target genes, the relevant ES cell line can be transferred to blastocysts and mice recovered and phenotyped.

7. Conclusion

Phenotype-driven and gene-driven ENU mutagenesis are both powerful approaches for gene function studies and the generation of a new mutant map of the mouse. The systematic generation and characterisation of mouse mutants using
ENU approaches that include a variety of phenotypic screens has already generated a large number of exciting new mouse models of human disease. We can expect a continuing expansion of the mouse mutant resource and an increasing number of models for the study of human genetic disease.

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References