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Large-scale production of mouse phenotypes: The search for animal models for inherited diseases in humans

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Abstract

This paper is aimed principally at bioinformaticians and biologists as an introduction to recent advances in mouse mutagenesis, concentrating on genome-wide screens utilising the powerful mutagen *N*-ethyl-*N*-nitroso-urea (ENU).

It contains a brief background to the underlying genetics as well as details of the practical aspects of organisation and data capture for such projects.

INTRODUCTION

In biology, a paradigm shift has occurred, where the analysis of biological material is approached more and more in a large-scale, high-throughput manner. This shift has been especially true within the field of molecular biology, exemplified by the Human Genome Project, where the acceleration in technology has made approaches such as whole genome shotgun sequencing and DNA microarray gene expression a reality.

The use of model organisms (eg yeast, *Drosophila* or mouse) has been a fundamental part of the Human Genome Project since its inception. These organisms are used not only to provide genomic sequence data for comparative analysis but also for generating phenotypic models to allow the dissection of gene function in a whole organism or biological system context. It is clear that the paradigm shift seen in the use and generation of genomic data has only been mirrored to a limited extent in the generation of model organism phenotypes. This was true, until fairly recently, with the exception of a few species (eg *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*) where short generation times, easy experimental manipulation and storage make large-scale phenotype generation feasible. However, several groups have reported methodologies for the generation and screening of large numbers of novel mouse phenotypes, increasing the utility of the mouse. This will further promote the mouse as the model of choice for the study of human disease and mammalian biology.

Reviewed in this paper are recent advances in mouse mutagenesis, concentrating on genome-wide screens utilising the powerful mutagen *N*-ethyl-*N*-nitroso-urea (ENU). A brief background to the underlying genetics as well as details of the practical aspects of organisation and data capture for such projects are given.

CHALLENGES FOR THE POST-SEQUENCE ERA

In 1997 the complete sequence of the yeast genome was published through a collaborative effort of many laboratories.[1] Upon the completion of the yeast genome 'Eurofan' was initiated, again as an internationally coordinated, large-scale project, trying to produce at least one loss of function mutant yeast strain for each of the 6,000 discovered open reading frames.[1] Can such an approach also be used for the functional analysis of the

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human genome? The answer is a clear 'Yes'. However, experimental induction of mutations is, for ethical reasons, not possible in humans. For this reason model systems are required which help to provide insight in understanding human gene function.

'phenotype gap'

The mouse has successfully become the most profound model organism system to investigate the biology, genetics and pathogenesis of human diseases. Homologous recombination in embryonic stem cells allows the systematic production of mouse mutants for any gene that has been cloned. Gene trap strategies have been designed to interrupt even unknown genes that are tagged by the inserted vector and can be characterised structurally and functionally. Complementary to such a 'gene-driven' approach, in which mutants are produced for those genes that we already know, we will also need 'phenotype-driven' approaches, in which new genes or gene products are identified through a search for new mutants with specific phenotype. The study of mouse mutants that develop the same disease but have mutations in different genes is essential for our understanding of the molecular mechanisms involved in the pathogenesis of different diseases. Furthermore, the full power of a genetic analysis of gene function requires the availability of multiple alleles of the same gene such as hypomorphs, alleles of different strength, and gain of function alleles. Genetic heterogeneity can be a result of allelic or non-allelic heterogeneity and is one of the central matters currently addressed in human clinical genetics.

'phenotype-driven' approach

assessment of mutant phenotypes

The necessity to study different alleles of mutants has been convincingly demonstrated in *Drosophila* genetics, in which null alleles often do not reveal all functions of genes, as the corresponding mutants are early embryonic lethal. Many of the clinically relevant human diseases are the result of a partial but not complete loss of gene function. Having access to mouse models for every known inherited disease in humans would be of great help towards the understanding of underlying molecular mechanisms, eventually leading to diagnostics and cure. So far we have only a very small fraction of mouse genes available in a mutated form. The term 'phenotype gap' was created to focus attention on the gulf between the mouse mutants accessible and the entire range of phenotypes that is essential to use the full power of the mouse as a model organism.[2] To narrow this gap we need to recover mutations for unknown loci and have to produce new alleles for known genes.

PHENOTYPE-DRIVEN APPROACH

The phenotype-driven approach identifies new genes, gene products and their relevant biological pathways by recovering novel mouse mutants. Random mutagenesis has a long history in mouse genetics and encompasses induction of a variety of different types of mutations or lesions by both chemical- and radiation-mediated mutagenesis. In general radiation treatment (eg X-ray or γ-ray radiation) leads to larger chromosomal deletions or rearrangements, while some chemical mutagens generally induce smaller deletions (eg chlorambucil) and others predominantly point mutations (eg ENU). The responsible genes are then identified through conventional mapping and positional cloning strategies.

The main interest in such a phenotype-driven strategy is the establishment of appropriate procedures to assess the mutant phenotypes of interest and to obtain animal models of human diseases or gene functions. To date several protocols meet these demanding requirements. Comparatively high-throughput screening and phenotyping protocols have been developed for pathophysiological parameters to assess mutant phenotypes for specific, pre- and postnatal abnormalities, comprising congenital malformations, clinical chemical, biochemical, haematological, immunological defects and complex traits such as allergy and behaviour (for

phenotype assays see: *Mammalian Genome*, Vol. 11, No. 7, July 2000).

Two of the early large-scale ENU mutagenesis projects were launched at the GSF Research Centre in collaboration with the LMU in Munich[3] and at the MRC in Harwell.[4] These screens employed high-throughput, phenotype screening techniques and have characterised over 500 medically relevant new mouse mutants. These projects showed proof of principle that large-scale, genome-wide mutagenesis screens are feasible in mammals.[5,6]

ENU: The most potent mutagen in mice

ENU is an alkylating agent and has turned out to be one of the most powerful mutagens for the production of mutations in mice.[7–15] In contrast to X-rays, ENU mainly creates point mutations, ie A-T base pair substitutions as well as small intragenic lesions as opposed to large deletions.[16,17] In many cases this leads to mutants with hypomorphic (partial loss-of-function) mutations. In addition, hypermorphic as well as total loss of function alleles can be induced and recovered.[18,19] The injection of ENU in a male mouse mutagenises, among other tissues, premeiotic spermatogonial stem cells. Thus a single treated male can produce a large number of F1 animals carrying different mutations.[7,15] Protocols were developed that allow a very efficient mutagenesis rate in mice.[12,20,21] On average the frequency of mutant recovery is about 1/1,000 for the recessive SLT genes (specific locus test) that can be scored phenotypically, but strain, dosage and treatment regimen do influence the mutagenesis rate.[8,13,14,20,21]

hypomorphic mutations

The logistics of ENU-mutagenesis

Male mice are injected with ENU and then mated to females to produce F1 founders. These F1 animals are analysed for dominant traits or bred further to screen for recessive phenotypes (Figure 1). Large numbers of F1 animals can easily be screened for dominant mutations. With respect to their mutations, every F1 animal carries a unique set of altered alleles. If screens are used where F1 animals might die, N2 animals have to be produced and analysed. F1 animals are kept for breeding the potential mutants.

Two generations of breeding are required to produce homozygous mutant alleles in order to detect recessive mutations. F1 founder males are mated to wild-type females to produce N2 animals (Figure 2). Half of the latter are heterozygous for the induced mutations.

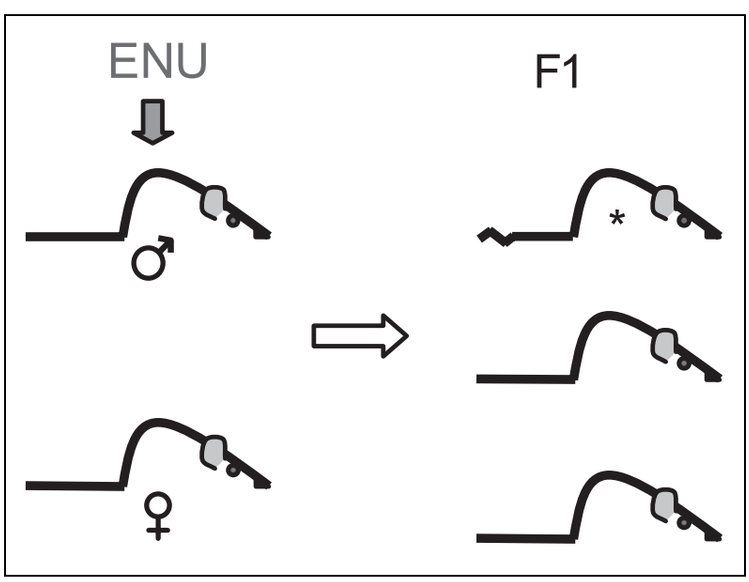

Figure 1: Dominant breeding scheme for ENU mutagenesis. F1 animals will undergo phenotype screens ('*' – individual bearing induced mutation)

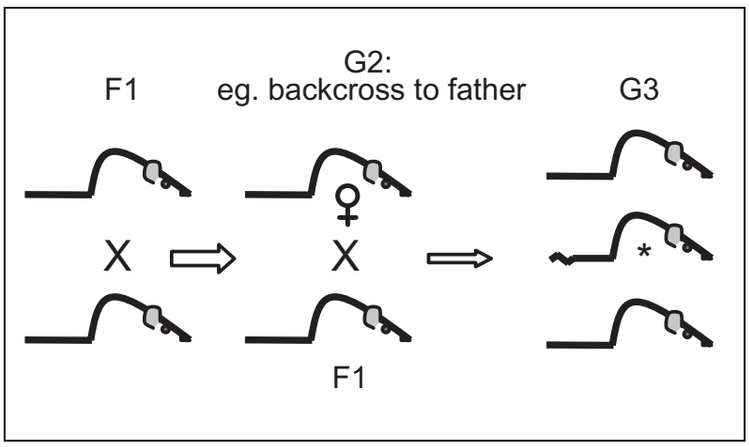

Figure 2: Recessive breeding scheme. F1 males are crossed to wild-type females to produce G2 generation. One possibility is to backcross G2 females to their fathers ('*' – individual bearing induced mutation)

All N2 animals are backcrossed to the F1 founder males or intercrossed with their siblings. The recessive mutants can be identified among the offspring from these crosses. A genome-wide scan for a certain recessive phenotype requires a tremendous effort. The evaluation of only 100 pedigrees would request screening of approximately 5,000 animals (given the screening procedure is not lethal to the animal). These numbers show clearly that a genome-wide saturation mutagenesis for recessive traits is very hard to achieve in the mouse.

region-specific scan

The clear advantage of the region-specific scan is the higher number of mice that can be analysed for recessive mutations at a known chromosomal position. Eugene Rinchik[15,22] has pioneered this strategy and saturated the *albino* deletion complex with ENU-induced mutations. This region spans about 6–11 cM and is particularly suitable for a saturation mutagenesis screen owing to the availability of a coat colour marker (albino) in the deletion interval. A rich collection of mutants was identified, among them many with medically relevant phenotypes, such as cleft palate, sterility or fitness. Of course the availability of well-defined deletions is a limiting factor for many region-specific mutagenesis screens. The development of site-specific recombinases such as the Cre-recombinase system will be one way to circumvent this problem. Lox-P sites can be introduced into specific sites in the genome and deletions produced by the transient expression of CRE-recombinase.[23] Another problem in maintaining mouse mutants is the absence of suitable balancer stocks, which are routinely used in *Drosophila* genetics. Also this could be changed in the near future using site-specific recombinases for the construction of defined inversions or translocations.[24]

DATA CAPTURE, ANALYSIS AND PRESENTATION

Large-scale phenotype-driven mutagenesis screens require a considerable commitment in terms of resources and personnel especially with regard to the issues around data recording and storage. It is clear that with the diversity (observational, semi-quantitative and quantitative) and volume of data generated from many phenotype screening projects that a paper-based recording approach is simply inappropriate, providing significant obstacles to subsequent analysis and presentation of data. However, the decision to implement or install an electronic animal management and data capture system requires a number of strategic decisions to be made:

strategic decisions for data capture

- Is there sufficient commitment in terms of funds and personnel before and after the project to develop, install and support such a system? The frequent nightmare of IT directors or

bioinformaticians is to be told that a data capture system is required 'next week'! The planning of large-scale phenotyping projects requires the involvement of computer scientists and bioinformaticians from the outset of financial planning through to project design and finally through the execution phase of the experiments.

- Is there a commitment to apply the system comprehensively across the project? One of the greatest dangers of an electronic system is that there are so many data or process holes in it (ie data that are not properly integrated or under the control of the data management system) that subsequent data validation and analysis become very problematic at best.

commitment to IT resourcing

Although it requires significant commitment in both time and resources there are a number of major advantages in the installation of an electronic data management system for animal production and screening:

- A system that combines the functions of animal breeding management and workflow removes much of the guesswork from activities such as routine colony census, forward planning and provision. Retrospective analysis of previous workflow patterns and activities can be fed forward into future plans, allowing fine tuning of colony requirements based on easily acquired facts as opposed to anecdotal experience.

- A centralised data repository is ideal for online data interrogation and analysis. This raises the possibility of integrating data from many screens or related projects to provide a much more powerful analysis of new phenotypes. In contrast, downstream analysis is more difficult when data are dispersed in different formats on multiple machines.

barriers to AMS provision

- Centralising the data makes it comparatively easy to transfer the data to collaborators (eg via private intranet WWW sites) or to publish data via the Internet to the wider scientific community.

In order to capitalise on the advantages of deploying an online data capture and analysis system, it should have the following basic components:

- Animal management system (AMS) – for tracking mating, breeding, weaning and disposal of animals within a project or colony.

- Sample tracking system (STS) – this allows samples of blood or tissue to be tracked accurately and for storage space to be used efficiently (eg liquid nitrogen dewers or freezers).

- Result documentation system (RDS) – the accurate recording of observational material and quantitative analysis by named individuals is essential if confident downstream analysis is to be carried out.

- Data analysis system (DAS) – this capitalises on the other components to provide facilities to interrogate data from a wide variety of sources, with confidence, for statistical analysis and reports.

These components should be represented in some degree in order to provide a comprehensive system and avoid 'black holes' associated with non-computerised tasks or activities.

Data capture

One of the principal barriers to providing an effective AMS is the simple fact that most animal breeding facilities have simply not been designed to have computer equipment installed in the areas where breeding and screening are carried out. This intimate installation of computer data input stations is essential to a data management solution to avoid having intermediate paper-based data

recording solutions that are both slow and error prone.

Fortunately there are now a number of different hardware technologies that can be applied, in combination, to provide offline or online data recording solutions. These include the following:

personal digital assistants

- Hand-held devices – there are an increasing range of rugged-cased, hand-held computing devices based on a number of domestic personal digital assistants (PDAs). These can be used as offline data collection devices (eg for animal census operations) or use radiofrequency networking technologies to provide a limited online capability to central servers on a local area network (LAN). Some products also have built-in barcode scanners allowing cage numbers or breeding names to be entered without transcription errors.

centralised data storage

middleware component

- Laptop and rugged-cased computers – these are more capable versions of the PDAs running mainstream operating systems and a wider range of software. The drawbacks are principally those of increased cost per unit (both in terms of initial outlay and servicing) and lower battery life. However, many laptops now include wireless networking solutions (eg radiofrequency or infrared) and their ability to run many mainstream applications (eg standard WWW browsers) and greater input–output flexibility (ie ability to connect to printers, barcode scanners and other instruments such as laboratory balances) may outweigh the other limiting factors.

user interface

- Fixed computer installations – this is the cheapest online system but provides least flexibility in terms of siting data input stations. However, with the increasing popularity of flat-panel monitors and the use of video and keyboard extension systems it is possible to provide fixed data input stations with larger base units located elsewhere in easily serviceable areas.

As with any hardware option, it is usually down to a price versus flexibility equation using the most appropriate hardware solution in the most appropriate way. For example a mixture of solutions may be most appropriate, using fixed data input stations for a screening area or a static instrument compared with a PDA-based system for animal census carried out in a number of different areas.

The second provision for a project data management system is some type of animal management software. In general most commercial and academic AMSs attempt to provide access to a central data 'server' from a number of physically distributed access stations, or 'clients', where data entry or interrogation can be carried out. This client–server system is common to many different distributed computing environments and in general is composed of three major components:

- Centralised data storage system – usually a proprietary relational database management system (RDBMS) such as Oracle 8i, Sybase Adaptive Server Enterprise, Microsoft SQL server or MySQL.[25]

- Middleware component – this optional component usually provides a generic system providing communication between distributed clients (where data entry is carried out) and the central data storage system. For example this could be WWW server software if the client's user interface is provided through a standard WWW browser or Java Applets.

- User interface – this is the component of the system that presents data and interrogation tools to the user. This is a critical part of any computer-based animal management system both in terms of user-acceptance and in terms of the power the system is able to deliver to the user. Any user interface is a delicate balance between providing a rich set of controls and features required by a wide range of users and

keeping the interface accessible enough so as not confuse or inhibit its use.

All systems will require some combination of these components although the exact implementation of these systems is possible using any number of combinations of client–server and middleware technologies. For example, both of the systems employed by the Harwell[26] and Munich Mutagenesis screens[5] used WWW and Java client interfaces (Figure 3), communicating to back-end databases from server-executed software using JDBC (Java Database Connectivity) to execute SQL queries to the RDBMS. However, there are many other two- and three-tier systems that could be used to provide similar functionality such as CORBA (Common Object Request Broker Architecture)[27] or commercial 'e-commerce' systems such as Allaire's ColdFusion product.[28]

JDBC

Data analysis

One of the core advantages of centralising data in a relational database is the ability to take various datasets and subject them to analysis and display the data in a comprehensible format to the user. This analysis and presentation can take place in real time, that is producing analysis results as per user request or offline by a scheduling system producing prepared reports and digests. Similarly the level of analysis can be varied from simple data extraction and presentation (eg pedigree reports – Figure 4) through to complex statistical analysis (such as multivariate or principal component analyses).

All data entered into a data management system have the advantage that they have been validated by the user interface (to exclude at least incorrectly entered or out of range data) and put into a data storage system where they are easily integrated with other data. For example in a clinical blood-biochemistry screen, data cannot be entered into the system if the subject is not already recorded in the AMS but when entered it would immediately be related to other screens (eg body fat index, bone mineralisation or organ pathology). This gives the user the ability to form complex queries that encompass a range of screens and individuals – in this example 'is there a relationship between blood cholesterol and body fat index?' or 'is there a relationship between bone mineralistion and blood metal ion concentrations?'.

Another form of analysis is possible

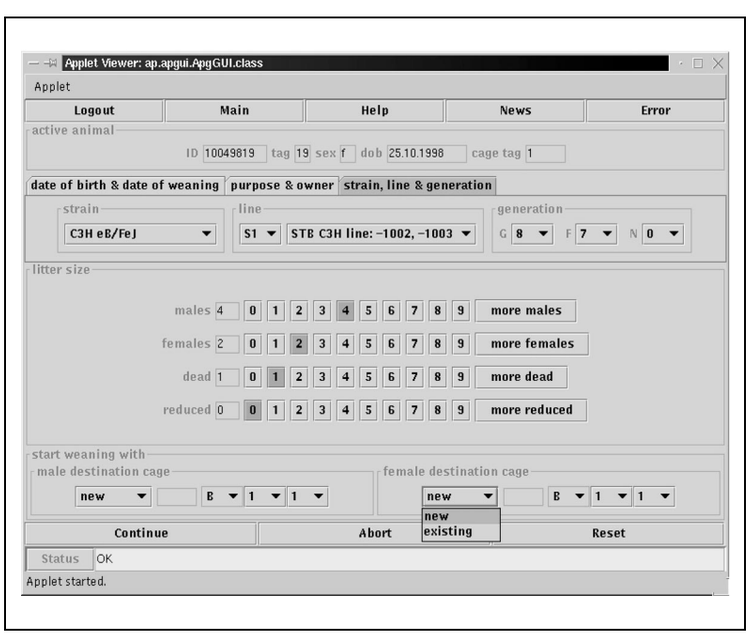

Figure 3: The weaning panel as an example of several panels available from the user interface of the Mousenet © database (Munich Screen[5]). The selection of possible weaning procedures is supported and stored in the Mousenet © database tables and can be updated and/or extended online. This panel is implemented in Java to provide an easily accessible graphical user interface

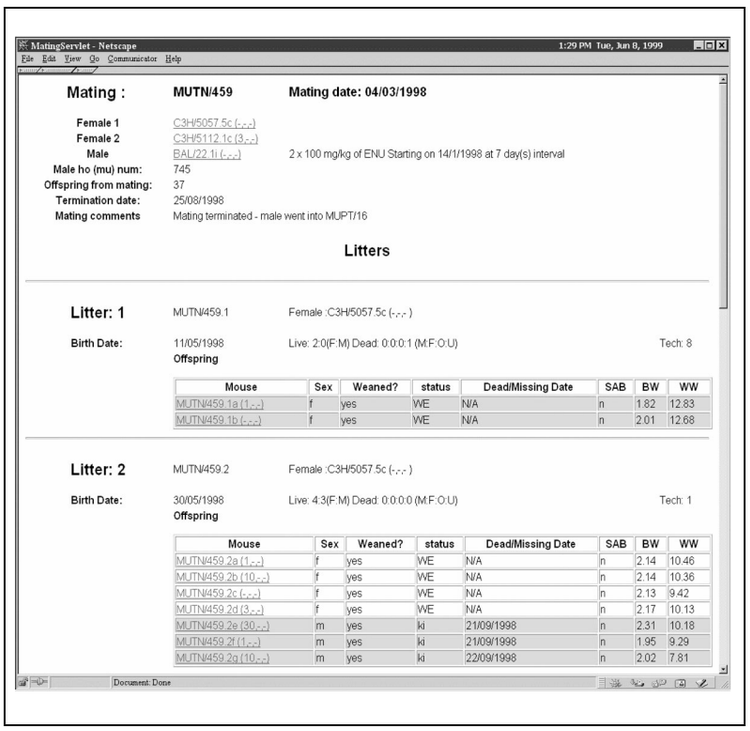

Figure 4: Online pedigree reporting features for Mutabase[26] – the initial breeding pair is listed at the top of the page. Subsequent litters are then listed in litter tables below the breeding pair, in date order, showing the live born individuals. Links from each individual on each litter table allow you to view further information about that individual

offline statistical analysis

using offline statistical packages, whereby data screens on a range of individuals are dumped into data files and then utilised by a package (eg Nag, Genstat[29] or SPSS Science, SPSS[30]) for more complex statistical analysis. To aid this type of processing many packages provide a scripting language to allow the analysis to be executed without user intervention with data being dumped into a specific file for reimportation to the data management system.

Data presentation

It is increasingly important for both commercial and academic groups to be able to provide data in a comprehensive and intelligible fashion to collaborators and customers. This has become considerably easier with the development of a number of technologies, not least being the advent and expansion of the WWW.

Increasingly many groups dump flat files of research data on to their WWW server where they can be searched or formatted for user requests using simple scripts written in a range of languages (eg Perl,[31] Java,[32] Python,[33] PHP[34]). An alternative approach is to provide limited access via the WWW to the central data management system using some type of middleware (eg CORBA) or communication protocol (eg JDBC). This allows access to the data as and when they are produced which has the benefit of the user always seeing the most up-to-date information. However, if using a commercial database server there can often be significant licensing costs associated with providing access to the database via the Internet as well as possible security risks when writing access scripts for unknown user access.

The global picture

As more of the programmes for the large-scale identification and characterisation of novel mouse phenotypes come to fruition (this includes programmes in the UK, Europe, Japan and a new National Institutes of Health (NIH) initiative in the USA) it is increasingly important to share both information and methods of

describing information to the wider research community. There are efforts underway to produce ontologies, search tools and baseline data for inbred strains, increasing the utility and availability of mouse phenotypes to the research community. These efforts include the following:

ontology

- Phenotype ontology – a group of existing and new mutagenesis centres are collaborating to work on a mouse phenotype ontology (J. Eppig, personal communication) – this is a controlled series of terms and relationships to allow the description of novel and existing phenotypes. The application of such an ontology in the description of phenotypes will be an essential component when providing search tools capable of looking at the phenotypes produced and stored by many programmes in many countries.

IMSR

- IMSR – the International Mouse Strain Resource[35] provides an electronically searchable set of mouse strains and mutants available worldwide (live or cryopreserved), via the WWW. The IMSR goal is to assist the international research community in locating and obtaining the necessary mouse strain resources to carry out fundamental research. The project has, at present, over 2,700 strains catalogued, with data being derived from the original two participating centres (The Jackson Laboratory, Bar Harbor, USA, and the MRC Mammalian Genetics Unit, Harwell, UK). Plans are in progress to widen this to other major centres with significant mouse mutant resources.

phenome

- Phenome project – this project is an international collaboration aimed at providing comprehensive phenotypic information on inbred mouse strains. There data are urgently needed if full use is to be made of the wide diversity of inbred mouse strains as models for human disease and of fundamental mammalian biology. It is hoped that contributors will be experts within their various fields and that all the contributed data will be integrated and assimilated at a central WWW site.[36] The data can then be used as a baseline on which to assess new mouse phenotypic traits given a specific genetic background.

MUTANT ARCHIVE – SPERM FREEZING

With the increasing number of new mutants it has become necessary to preserve genetically valuable strains. It is relatively easy to establish and increase the number of mice in certain projects starting with spermatozoa from a single male. Spermatozoa can be collected from the epididymides of euthanised males. For a repeated collection from one male, ejaculated spermatozoa can be used. Having oocytes and foster females available, a single male is sufficient to build up a colony of over a hundred animals within a few weeks. For this purpose it does not matter if the spermatozoa are used directly or after a freezing–thawing procedure. The vision is to receive frozen sperm samples from your strain of choice, perform *in vitro* fertilisation or artificial insemination and have a well-sized mouse colony within a few weeks. Application of this method can accelerate various crossing procedures resulting in, for example speed-back, speed-inter or speed-outcrosses. Using a Petri dish for the fertilisation procedure rather than expensive and limited mouse space will change mouse work dramatically. Sperm freezing will make it much easier to exchange mutants between different laboratories worldwide, as the shipment of live animals would not be necessary any more.[37,38]

in vitro fertilisation

Because of the highly conserved linkage of genes among mammalian species (conserved synteny), the mapping of the mutant loci to a mouse chromosomal region allows the prediction of where a corresponding human disease gene would map. Very soon we will have

an almost complete transcript map of the human and mouse genome. This will speed up the process of mutation detection.

OUTLOOK

The success of this and future screens will depend heavily on the exploitation of interdisciplinary collaborations, ie in the design of new phenotyping assays, that are fast, cheap, robust and preferably non-invasive. Furthermore, ENU-mutagenesis is not necessarily restricted to large screening centres, but can be set up efficiently in smaller laboratories.[39,40] The combination of region-specific deletion screens with the use of dominant coat colour markers and balancer stocks should further improve the overall efficiency of mutant recovery. Even when chromosomal mapping has reduced the candidate region to a few hundred Kb or a few candidate genes, one of the major rate-limiting steps is still the actual identification of the individual causative mutations. However, once cheap high-throughput mutation detection technology becomes available, the DNA of offspring from mutagenised mice could be directly analysed for mutations in specific genes, permitting the convergence of phenotype- and gene-driven mutagenesis. One of the major long-term goals of the human genome project is to understand multigenic and multifactorial diseases. In most cases, the mutants arising from ENU mutagenesis programmes will probably be monogenic. However, crossing them onto different genetic backgrounds will reveal modifying genes and permit us to study their corresponding complexities. Suppressor and enhancer screens will then become routine tasks in mouse genetics as they are in other model organism systems.

high-throughput mutation detection

We are confident that current and future ENU mutagenesis programmes will be important tools in providing resources for gene function discovery within the worldwide efforts of genome research.

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