Refinement and reduction in production of genetically modified mice

Sixth report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement

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1 Preface

Wherever animals are kept or used for scientific purposes, the objectives of minimizing any pain or distress they may suffer and promoting high standards of welfare should be as important as achieving the experimental results. This is important for humanitarian reasons, for good science, and for satisfying broad legal principles (e.g. European Directive on animals used for experimental and other scientific purposes, European Community 1986) and specific national legislation (e.g. UK Animals (Scientific Procedures) Act 1986, Home Office 2000).

Significant and immediate improvements to animal husbandry and scientific procedures can be made in a number of ways. To facilitate this, up-to-date information on all aspects of animal use and care are essential. The need to provide such information led the British Veterinary Association Animal Welfare Foundation (BVAAWF), the Fund for the Replacement of Animals in Medical Experiments (FRAME), the Royal Society for the Prevention of Cruelty to Animals (RSPCA) and the Universities Federation for Animal Welfare (UFAW) to establish a Joint Working Group on Refinement. The aim was to set up a series of working parties to define and disseminate ways in which husbandry and scientific procedures can be refined to improve animal welfare and reduce suffering. Thus far, a series of working groups have considered a range of issues, including blood removal (Morton *et al.* 1993a); rabbit (Morton *et al.* 1993b), mouse (Jennings *et al.* 1998) and bird husbandry (Hawkins *et al.* 2001); and the administration of substances (Morton *et al.* 2001).

This report is the sixth in the series. The topic—refinement and reduction in production of genetically modified[1] (GM) mice—reflects both the increasing prominence of GM mice in biological research and the concerns for the welfare of these animals and those used in their production. The Working Group comprises members from the scientific community and from animal welfare organizations. It is important to note that some of the organizations participating in the Working Group are opposed to the use of animals in scientific procedures that cause pain, suffering, distress or lasting harm. However, they share with many in science the common aim of ensuring that where animals are used, every effort should be made to avoid or minimize suffering and to improve animal welfare. This report is intended to help achieve this aim.

2 Introduction and aims of the report

Since the results of creating GM mice were first reported in 1980 (Gordon *et al.* 1980), GM mice have been used extensively in a wide range of scientific disciplines to try to answer basic and applied biological questions relating to the regulation of gene expression, the generation of animal models of human genetic diseases, and the evaluation of potential therapies. Many within the scientific community claim that the use of GM mice has revolutionized the life sciences and will continue to do so. However, from an animal welfare standpoint, the use of GM mice is of serious concern because of the numbers of animals involved, the surgery and other invasive procedures used, and the deleterious effects that genetic modification can have on animal welfare.

(i) *Number of animals*

The use of GM mice differs from that of other animals in scientific research in that large numbers of animals are used to generate those animals that are of actual scientific 'value'. Current transgenic technologies are inherently inefficient in terms of the numbers of mice used in relation to the numbers of founder GM mice that are ultimately obtained, prior to these animals being conventionally bred. Many of the manipulated embryos do not survive and of those mice eventually born relatively few, on average 15%, are genetically modified—although a typical range is between 1–30%. The

[1]Genetically modified or transgenic refers to cells or organisms containing integrated sequences of cloned DNA (transgenes) being transferred using techniques of genetic engineering (which may include gene transfer or gene substitution) (Beardmore 1997).

remaining mice may be surplus to requirements and consequently culled. Thus, to ensure the production of a sufficient number of founder GM mice, large numbers of females are used to provide fertilized eggs and embryos for manipulation, or as embryo recipients and foster mothers, and large numbers of males are produced in the breeding of these females and are often surplus to requirements.

(ii) *Procedures*
Females used to provide fertilized eggs or as embryo recipients undergo procedures including surgery that can cause pain, suffering and distress. Similarly, techniques used to obtain biopsy material for genotyping or to identify individual mice may also have an adverse effect on animal welfare.

(iii) *Effects of genetic modification on animal well-being*
Genetic modification can compromise animal welfare by causing or predisposing animals to pain, suffering, distress or lasting harm. This may be intentional as a result of the genetic modification introduced, or unintentional through the disruption of gene function by random integration of the transgene into the genome.

The production of GM mice and their subsequent management and care poses new challenges for, and obstacles to, the implementation of the principles of reduction and refinement, i.e. the *reduction* in the number of animals used and the *refinement* of procedures to minimize any suffering caused. This is due, in part, to the nature of the current technology. There is, however, also a lack of awareness among some researchers of the animal welfare issues and best practices associated with the generation, management, and care of GM mice that needs to be addressed if the principles of reduction and refinement are to be consistently and effectively applied. The Sixth BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement was therefore convened to develop recommendations on how the procedures used can be refined, and the welfare of GM mice improved. Given the large numbers of mice used and the associated potential for wastage, the Working Group has also considered how the number of mice used can be reduced.

The Working Group has focussed on the two most commonly used methods of producing GM mice, namely pronuclear microinjection and gene targeting in embryonic stem (ES) cells. Recommendations of current best practices have been proposed by drawing on both published material and the practical experience of the Working Group's members. The Working Group hopes that by raising the issues and opportunities for reduction and refinement, current best practice for GM mice will be disseminated and greater attention focussed on the animal welfare implications associated with transgenic technologies.

The Working Group does not intend its report to be used as a technical guide. All steps in the generation, management, and care of GM mice have been considered and it has been necessary to detail many of the scientific and technical issues in order that the welfare concerns and opportunities for reduction and refinement are set in context. Recommendations of best contemporary practice are highlighted at the end of each section in **bold**. The report is aimed at all those involved in producing, managing, and using GM mice, and indeed at those concerned with reviewing research involving these animals. Some parts of the report will have greater relevance than others, depending on the reader. The Working Group hopes, however, that the report will be read in its entirety in order to achieve maximum benefit for the mice and its readers. Although the focus of the report is on GM mice, many of the principles of best practice proposed also apply to GM rats. GM rats are discussed specifically in Section 23.

A glossary of the terms most commonly used in the report is provided in Appendix A.

3 Methods of producing GM mice

Genetically modified mice can be produced using a number of different methods, namely pronuclear microinjection, gene targeting in ES cells, nuclear transfer cloning (Wakayama

et al. 1998), sperm mediated DNA transfer (Lavitrano *et al.* 1989, Perry *et al.* 1999), integration of proviral DNA into germline cells (Stewart *et al.* 1987), lentiviral vectors (Lois *et al.* 2002), and transposons (http://www.tosk.com). Although the Working Group has focussed exclusively on pronuclear microinjection and gene targeting in ES cells, the principles of best practice proposed in this report apply to all of these methods.

The processes of pronuclear microinjection and gene targeting in ES cells are shown schematically in Figs 1a and 1b. Decisions as to which technique to use depend primarily on the type of genetic modification required (see Table 1). Whatever method is used, unnecessary repetition of the production of GM mice is clearly wasteful of animals, and it is essential to confirm that the same mice are not already available elsewhere. A list of

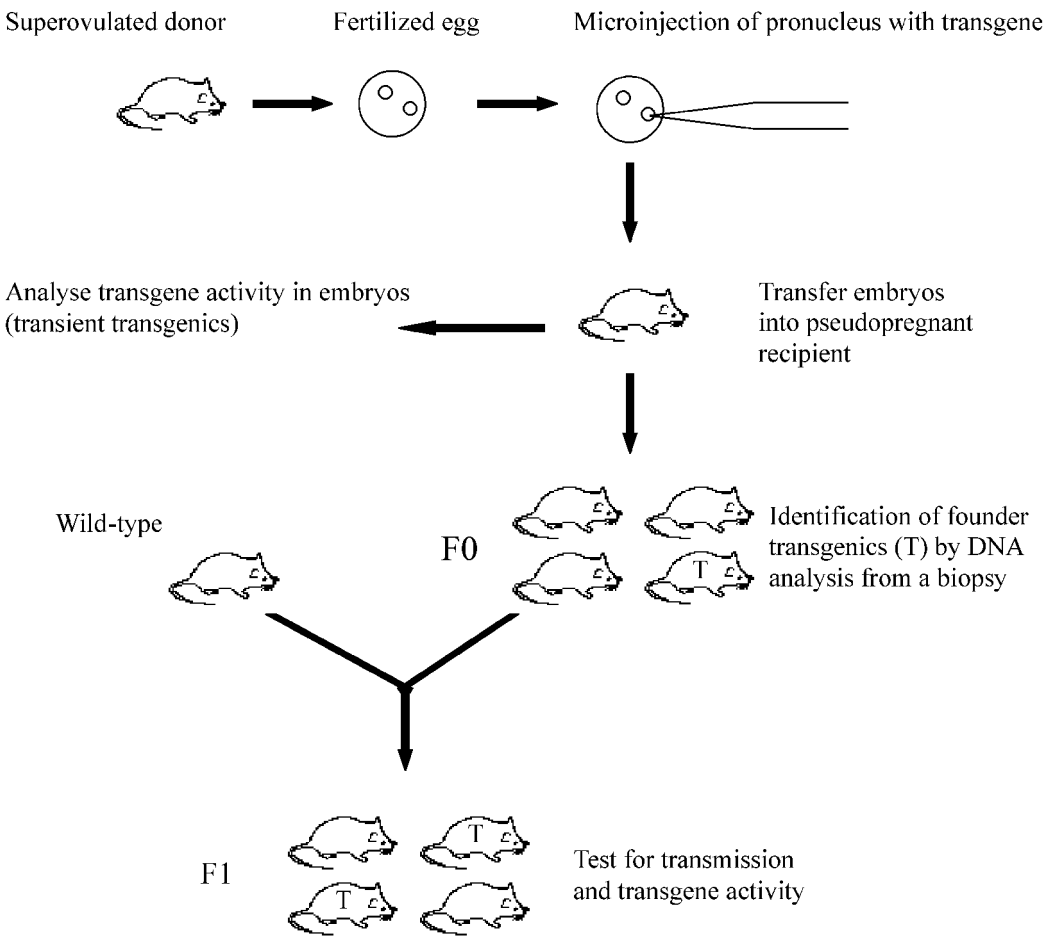

Fig 1a Mice are superovulated to increase the number of fertilized embryos available for microinjection. After microinjection of the transgene into one of the two pronuclei, the embryos are transferred into a recipient mouse that has been rendered pseudopregnant by mating with either a vasectomized or genetically sterile male. In some cases, the embryos are collected from the recipient at a defined point in gestation and analysed for transgene expression (transient transgenics). In others, the recipient is allowed to give birth to pups which are subsequently screened for the presence of the transgene by analysis of a tissue biopsy. Founder transgenics (F0) are then bred with wild-type animals to produce the next generation (F1). Analysis of these offspring, tests transmission of the transgene and they can then be used to assess transgene activity

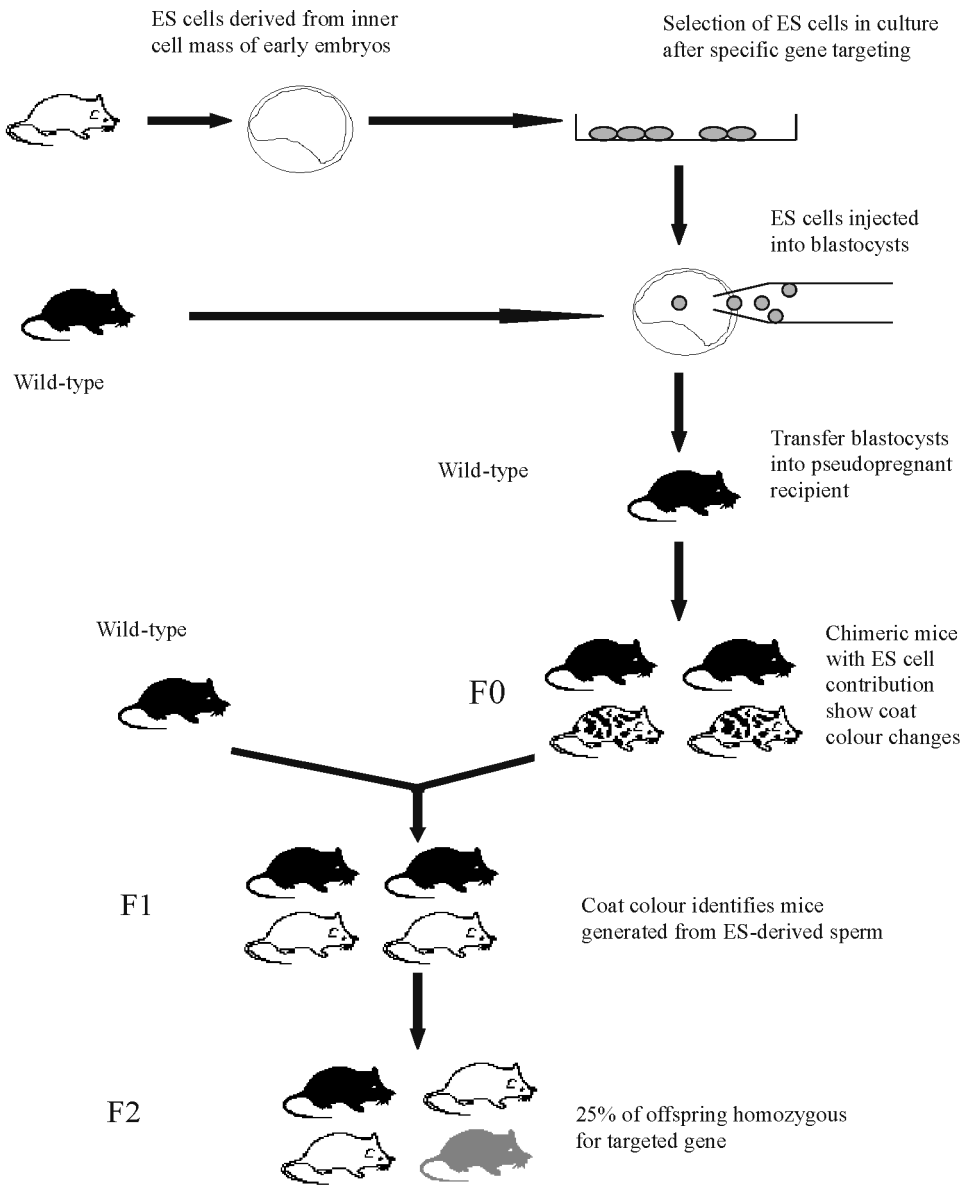

Fig 1b Manipulation of endogenous genes (knock-out/knock-in) is carried out using pluripotent embryonic stem (ES) cells derived from the inner cell mass of blastocysts. Gene targeting is achieved by adding manipulated highly homologous specific DNA sequences to the cells and selecting those incorporating the construct. Selected clones can then be introduced into a developing wild-type embryo by injection of the modified ES cells into a recipient blastocyst. The manipulated blastocysts are then transferred into pseudopregnant recipients. Mice developing from embryos where the ES cells have been incorporated are usually identified by coat colour changes and are often chimeric. These founder chimerics (F0) mice are then mated with wild-type mice. Chimeras are usually male as most ES cell lines are derived from male embryos. Offspring from male chimeras are generated from wild-type or ES-derived sperm and again coat colour is usually used as a convenient marker. ES-derived mice are screened for the presence of the modified gene. As sperm only carry one of the two alleles and as only one allele is usually targeted in culture, approximately half of the ES-derived offspring will carry the modified gene. Crossing mice carrying the modified gene should produce 25% of pups homozygous for the modified gene. Note that in the F2 generation coat colour will not be a reliable guide to genotype

Table 1 The relative merits of different approaches to the generation of genetically modified mice

	Pronuclear microinjection	Gene targeting in ES cells
Advantages	Potentially rapid generation of results, i.e. transient transgenics. Good for promoter analysis and ectopic expression studies.	Allows defined genetic manipulation (knock-out or knock-in). Avoids position effect problems. Potentially less mice used in the generation of modified embryos.
Disadvantages	Integration site is random and this may effect both the level and pattern of transgene expression. A significant number of animals may have to be used to produce scientifically informative GM mice.	Transgene design may be more complicated. Excellent cell culture techniques are required to maintain ES cell pluripotency. Failure to achieve germline transmission can mean mice are wasted. Usually only one allele is targeted. Several generations of breeding are required to produce homozygotes.
Refinements	Inducible promoters, for example the tetracyclin-dependent (Tet-on/Tef-off) system allows for temporo-spatial control of gene expression. Use of locus control regions, insulators or YACs may reduce position effects.	Conditional knock-outs using Cre/*loxP* technology allows for temporo-spatial control of gene expression. Inducible promoters to drive Cre expression offer even finer control of transgene expression.

ES = embryonic stem; YACs = yeast artificial chromosomes

websites detailing databases of some of the existing GM mice is given in Appendix B.

Developments in conditional transgenic technologies such as the Cre/*loxP* and Flp/*frt* recombination systems (see Fig 1c), and inducible transgenes (e.g. the tetracycline-dependent system) increasingly allow greater control over the temporal and spatial pattern of gene deletion or expression (e.g. Metzger & Feil 1999, Gorman & Bullock 2000, Lewandowski 2001). Such control can provide a mechanism for minimizing any adverse effects on animal welfare, and for limiting pre- or post-natal deaths, that may be associated with constitutive knock-outs or overexpression of the transgene. For example, GM mice used to study the role of the oncogene *K-ras* in the initiation and progression of lung cancer succumb at a young age to respiratory failure caused by a large number of lung lesions. The use of the Cre/*loxP* system to regulate expression of oncogenic *K-ras* has enabled the lung tumour burden on the mice to be reduced (Jackson *et al.* 2001).

There are a number of issues that should be considered before using conditional transgenic technologies:

(i) It may be necessary to generate two different lines, one with the targeted *loxP* (or *frt*) sites and the other with the recombinase under the control of a spatially-restricted or inducible promoter. In order to minimize the number of mice used, it should be confirmed as far as possible that GM mice with the necessary recombinase expression pattern are not already available.

(ii) Where an inducible promoter is used, the promoter should be well characterized in order to avoid deleterious effects arising from unexpected expression of the transgene.

(iii) It is important to be aware that topically applied inducing agents may not be restricted to their initial site of application. This is illustrated by the activation of the c-MycER oncoprotein in the epidermis of GM mice. The *c-mycER* transgene is comprised of the *c-myc* cDNA fused in frame to the hormone-binding domain of a modified oestrogen receptor. The encoded MycER protein is inactivated by the binding of heatshock protein 90 (HSP90) to the hormone-binding domain. Administra-

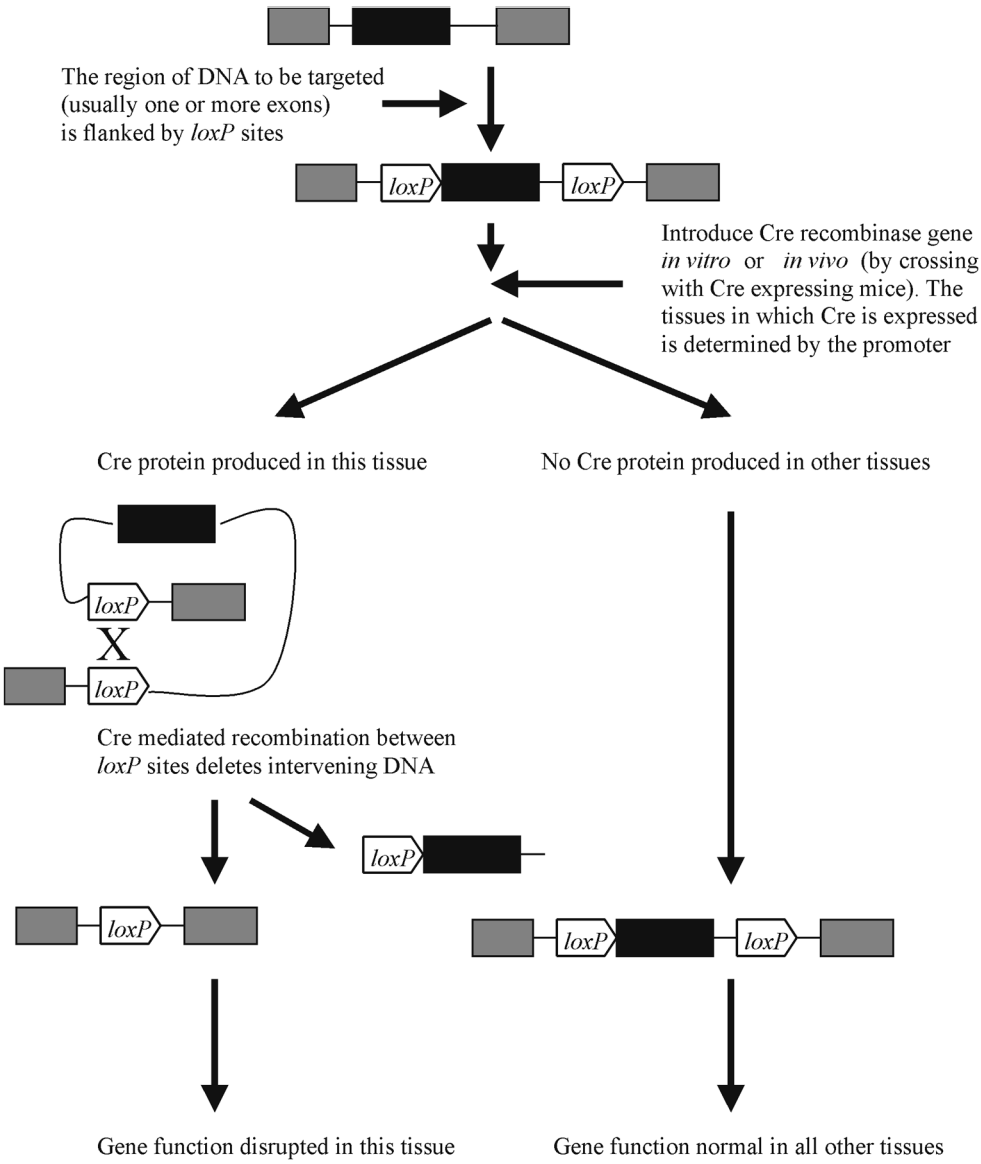

Fig 1c It may be desirable to knock-out a gene only in a subset of tissues or at a defined point in the life of the mouse. Such conditional knock-outs are most readily generated by taking advantage of Cre/*loxP* technology. This system relies on the deletion of DNA sequences flanked by *loxP* sequences through the action of bacteriophage Cre recombinase. Highly homologous DNA is used and the region to be deleted is flanked by *loxP* sites. These *loxP* sites are usually placed in intronic sequences where they should not affect the normal function of the gene. Correctly targeted clones can be treated with Cre recombinase in culture but more commonly, mice carrying the targeted gene are mated with GM mice expressing Cre recombinase. The spatial and temporal specific expression of the Cre recombinase is determined by the promoter used to drive the Cre. In those tissues where Cre is expressed, the DNA between the *loxP* sites is deleted leaving a single *loxP* site, and the function of the targeted gene is disrupted. In contrast, in tissues where there is no Cre expression the gene function remains normal. This system, therefore, has the potential to allow very specific patterns of gene disruption

tion of 4-hydroxytamoxifen (4OHT) results in the release of the HSP90 protein and the activation of the c-Myc protein. In this case, 4OHT was applied to the dorsal skin of *c-mycER* GM mice, but was transferred to the face during self- and group-grooming. This resulted in the activation of the c-Myc protein and the formation of dry scabby lesions on the cheeks and around the mouth, which caused a reduction in food intake. To prevent this, the mice had to be housed singly and protective collars had to be used (S. Pelengaris, personal communication).

Recommendations:

- **Confirm that the desired GM mice are not already available before commencing production.**
- **Consider the use of conditional transgenic technologies to regulate harmful effects arising from undesirable spatial and temporal transgene expression.**

4 Pronuclear microinjection: factors affecting the design, expression and transmission of the transgene

Transgene integration following pronuclear microinjection is a random event with respect to the chromosomal locus. This can influence both the expression of the transgene and the survival of the microinjected embryos, such that it is necessary to use relatively large numbers of embryos in order to produce relatively few GM mice with the desired level, and temporal and spatial pattern, of transgene expression. Large numbers of females, therefore, have to be used as embryo 'donors' or recipients. In most cases, failure to achieve expression or subsequent transmission of the transgene means that the mice used in the process have been wasted. The only exceptions to this are promoter analysis experiments where a failure to express the transgene can provide useful information, or where the intention is to use transient transgenics.

The term 'transient transgenics' refers to the collection of microinjected embryos following re-implantation but prior to birth. Transient transgenics are often used in the assessment of patterns of promoter activity during embryogenesis. This has the advantage that information can be rapidly collected without having to breed each GM mouse to assess expression in the offspring. However, the collection of only one piece of information per GM embryo may necessitate the use of greater numbers of donor and recipient females.

There are a number of technical variables relating to the design, preparation, expression, integration and transmission of the transgene that should be considered in order to optimize the number of GM mice produced with the required transgene expression and to minimize the overall numbers of mice used to achieve this. Many of the details have been published elsewhere but the main principles are given below. Performance targets and intervention levels for the generation of GM mice by pronuclear microinjection are presented in Table 2.

4.1 Transgene design

Most transgenes will have been manipulated in one or more bacterial plasmids. The presence of plasmid sequences can inhibit the expression of the integrated transgene and it is advisable to remove the vector backbone prior to microinjection. Linear DNA integrates at a higher frequency than circular or supercoiled molecules (Brinster *et al.* 1985). Therefore, constructs should be designed so that the transgene can be excised to yield linear molecules for microinjection. To avoid recircularization, restriction enzymes should be selected that produce incompatible ends.

The purity of the DNA preparation is one of the essential elements for successful transgenesis. It should be free of chemical contaminants and debris. Ultra-clean and filtered solutions should be used in the preparation of DNA solutions.

Transgenes based on genomic DNA are more likely to be expressed than those based on the corresponding cDNA. The presence of introns can substantially improve expression, either as a result of a splicing event or due to the presence of enhancers within introns (Brinster *et al.* 1988).

Table 2 Targets and intervention levels for the production of genetically modified (GM) mice by pronuclear microinjection

Process	Average performance	Level requiring review	Possible problems
Donor females	At least 80% mated after superovulation.	60% or less mated after superovulation.	Poor stud male. Females not in oestrus.
Embryo yield	30 embryos per female with at least 70% normal.	Less than 15 embryos per hybrid female with less than 70% normal.	Hormone quality, timing of hormone treatment. Strain of mouse (lower yield in inbred strains). Age of female.
Microinjection	At least 60% survive microinjection and if cultured overnight 80% of injected embryos reach 2-cell stage.	Less than 50% survive and less than 60% of those develop with overnight culture.	Poor microinjection technique, poor-quality DNA preparation, too high a concentration of DNA, incorrect media (try culturing uninjected eggs to test media).
Recipient pregnancies	At least 70% mice are pregnant.	50% or less pregnant.	Poor transfer technique. Transfer of too few embryos. Environmental disturbances or diseases.
Recipient litters	At least 5 pups per female following transfer of 20–25 embryos.	Average less than 3 pups per pregnant recipient.	DNA construct causes embryonic lethality. Poor transfer technique. Transfer of too few embryos. Environmental disturbances or diseases.
Percentage GM	5–30% is the normal range.	Less than 5%.	Poor-quality DNA preparation or insufficient DNA injected. Aspiration of media into microinjection pipette is not uncommon so change the pipette regularly during a microinjection session.

These targets are average guidelines for hybrid mice and actual figures may vary according to mouse strain

Construct size does not seem to be critical. GM mice have been generated using cosmids (35 kb), double cosmids (70 kb), and artificial chromosomes (100–400 kb) e.g. bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs) and yeast artificial chromosomes (YACs). It is, however, important to note that rearrangements and deletions can occur frequently in the larger constructs. Giraldo and Montoliu (2001) provide a recent review of the use of artificial chromosomes.

Recommendations:

- **DNA constructs should be linearized and plasmid sequences removed prior to microinjection.**
- **High-quality DNA preparations, free from chemical contaminants and debris, are essential. Use ultra-clean and filtered solutions in the preparation of DNA solutions.**
- **Transgenes should be based on genomic rather than cDNA sequences, wherever possible.**

4.2 Expression of the transgene and its site of integration

Apart from poor construct design and preparation, the most common reason for a failure to achieve expression is the random integration of the transgene into an area of chromatin that is transcriptionally inactive. Conversely, integration near to powerful enhancers can increase transgene expression but may modify the expected pattern of expression.

The effects of the integration site may be avoided by including intronic sequences in the transgene (Webster *et al.* 1997), or

sequences that are reported to have insulating properties (Krnacik *et al.* 1995, Namciu *et al.* 1998). Locus control regions (LCRs) which confer copy-number dependent and position-independent transgene expression have been described for a number of genes (Grosveld *et al.* 1987, Lang *et al.* 1991, Montoliu *et al.* 1996, Kushida *et al.* 1997, Bennani-Baiti *et al.* 1998, Baker *et al.* 1999, Ortiz *et al.* 1999). In particular, the β-globin LCR has been used widely for position-independent erythroid-specific expression in GM mice. Constructs such as YACs appear to be relatively insensitive to the site of integration within the genome (Fujiwara *et al.* 1997).

Perhaps most significantly, it has been demonstrated that the multiple transgene copy number, which is commonly observed in GM animals, can in itself lead to silencing of the transgene (Garrick *et al.* 1998). DNA integration usually occurs at one random site in the genome, but may do so as one to 1000+ tandem copies of the transgene. It is, therefore, advisable to deliver a lower number of transgene copies during the DNA microinjection process. This can be achieved by reducing the concentration of DNA or reducing the volume injected. The optimal concentration of DNA for microinjection is generally 1–3 ng/µl in 10 mM Tris and 0.1 mM EDTA at pH 7.4 (Brinster *et al.* 1985).

Recommendations:

- **Consider including in the transgene sequences that avoid the effects of integration site on transgene expression.**
- **The optimal concentration of DNA for microinjection is generally 1–3 ng/µl in 10 mM Tris and 0.1 mM EDTA at pH 7.4.**

4.3 Transmission of the transgene

With the exception of transient transgenics, most research programmes using GM mice are dependent on the establishment of GM lines through the transmission of the transgene from the initial founder animals to subsequent generations. Germline transmission may, however, be problematical because some, and perhaps most, GM founder animals are to some extent mosaic (Whitelaw *et al.* 1993). Consequently, founder mice may only contain the transgene in a proportion of their gonadal cells, and in some cases few, if any, of the offspring produced will be genetically modified. Once the transgene has been transmitted, future generations should transmit the transgene to approximately 50% of their offspring, unless the transgene interferes with development.

Initially, in most cases, the transgene is integrated at one site on one chromosome. These mice are heterozygous for the transgene (hemizygous) and their breeding with wild-type mice results in 50% of the offspring being non-GM. These mice may be surplus to requirements and consideration should be given to using them as experimental controls or for other scientific purposes such as a source of tissues. Alternatively, their generation should be avoided where possible by the breeding of homozygous lines. Maintaining homozygous lines also obviates the need for genotype analysis and thus for the removal of tissue biopsies. However, it is important to note that determination of the homozygous state can be difficult and may involve test mating using large numbers of mice. Moreover, in some cases, a harmful phenotype may arise in homozygotes and not heterozygotes. This is generally a result of the site of transgene integration causing a disruption of normal gene function, and in such circumstances the line should be maintained as heterozygotes.

Transgene integration may occasionally occur at more than one chromosomal site and this may lead to transmission rates of greater than 50% from the breeding of founder mice. Multiple integration sites usually segregate in subsequent generations.

Some transgene insertions can lead to sterility if they disrupt genes whose products are required for normal physiological or behavioural responses. Similarly, the transgene product itself may cause mortality before the mouse reaches reproductive age. Empathetic and observant husbandry is essential to minimize the potential for pain, suffering or distress in these mice. Those managing and caring for GM mice need to be able to recognize possible reductions in

fertility and/or libido, post-implantation losses, and developmental or birth defects so that appropriate actions can be taken immediately.

Recommendations:

- **All personnel involved with the breeding of GM mice should be aware of the potential for mosaicism in founder animals.**
- **GM lines should be maintained as homozygotes provided that an adverse phenotype is not observed, or compounded in the homozygous state.**
- **Where the GM line is maintained as heterozygotes, consideration should be given to using the non-GM mice produced as experimental controls, or for other scientific purposes, such as a source of tissue.**
- **Those responsible for managing and caring for GM mice should look for reductions in fertility and/or libido, and developmental or birth defects so that appropriate actions can be taken.**

5 Gene targeting: improving the efficiency of chimera formation and germline transmission

Gene targeting by homologous recombination in ES cells allows exact precision over the site of transgene integration. Whilst ES cells are most commonly used to ablate gene expression ('knock-out'), they can also be used to introduce mutant alleles at the locus of interest ('knock-in'). By selecting and microinjecting only those ES cells that have the desired modification, it is possible to avoid the wastage of mice that is inherent with conventional transgenesis. However, the ES cell route is technically more demanding and its efficiency is only better when high rates of germline transmission of the ES cell genome are consistently achieved. Failure to achieve germline transmission means that mice are often wasted and that female mice used to provide host blastocysts or as surrogate mothers have been used unnecessarily.

There are a number of issues relating to ES cell production, growth, transfection and microinjection that should be considered if efficient germline transmission is to be achieved. Performance targets and intervention levels for the production of GM mice using ES cells are shown in Table 3.

5.1 Transgene design for gene targeting

A number of factors should be taken into account when designing the transgene for gene targeting to ensure that the frequency of targeting is high, and only those clones with the desired gene targeting are selected for microinjection into blastocysts. Transgene design is described in detail in Hasty and Bradley (1992) and Plagge *et al.* (2000).

The construct should be based on genomic DNA that is isogenic to that of the strain from which the ES cell is derived. The length of the regions of homology affects the frequency of targeting, and the larger the region of homology the greater the frequency of gene targeting events. Large constructs can, however, be difficult to manipulate and it is advisable to aim for a total region of homology of approximately 5–10 kb. The 'arms' of the regions of homology should be reasonably equal.

The neomycin resistance gene is commonly used to positively select for clones that have the targeting construct incorporated. The frequency of homologous recombination can be increased by positive–negative selection, although exposing ES cells to complex selection schemes may affect their pluripotency and thus the likelihood of subsequently achieving germline transmission.

The screening method used to identify ES clones with the desired gene targeting event should be reliable, so that clones with random transgene insertions can be distinguished from those with homologous recombination events. Sequence or restriction sites located outside the regions of homology are required for the identification of legitimate recombination events. Screening should also be rapid so as to minimize any loss in the pluripotency of the ES cells.

Table 3 Targets and intervention levels for the production of genetically modified mice by embryonic stem (ES) cell technology

Process	Average performance	Level requiring review	Possible problems
Donor females	25% plug rate (natural mating).	Less than 10%.	Poor stud males.
Embryo yield	2–6 injectable blastocysts.	Consistently less than 3 injectable blastocysts.	Using mice that are stressed, unhealthy or old can reduce blastocyst yields.
Micro-injection	All blastocysts survive.	Less than 70%.	Blastocysts are fairly robust and easily survive injection. Poor survival may be due to inappropriate media or injection of poor quality blastocysts.
Recipient pregnancies	>50% pregnant.	Less than 50%.	Aberrant ES cell clone chromosome counts lead to poor embryo survival. Chromosome count ES cell clones prior to injection. Poor transfer technique. Environmental disturbances or disease can reduce pregnancy numbers.
Recipient litters	30–50% of blastocysts lead to a birth.	Consistently less than 30%.	Environmental disturbances or disease can reduce the numbers that are born and weaned. If there are a number of good/transmitting chimeras in the litters, although birth rates are poor, intervention is not necessary.
% Chimeras	50% of mice born are chimeric.	Less than 50%.	Good ES cells and injection techniques should produce a high proportion of chimeric animals. As the ES cells used are by choice male, most chimeras should be male.
% Coat colour	More than 70%.	Less than 70%.	Chimeras should have a high proportion of donor colour in their coats. In most situations it would be wasteful to breed low (<50%) contribution chimeras.
Germline transmission	50% of paired chimeras.	0%.	If the ES cell line used has previously transmitted well, failure to achieve germline transmission may be attributable to poor ES cell culture techniques.

Figures are based on the injection of early passage (<11) 129 ES cells into C57BL/6 blastocysts produced by natural mating

Recommendations:

- **Constructs for gene targeting should be isogenic to the strain from which the ES cells are derived.**
- **A total region of homology of approximately 5–10 kb should be sufficient for most gene targeting purposes.**
- **Screening strategies to identify ES cells with the desired gene targeting should be reliable and rapid.**

5.2 Embryonic stem cells

Embryonic stem cells should be cultured under optimal conditions to ensure that there is a high degree of pluripotency. Wherever possible, ES cells should be grown using established protocols for the line in question. It is important to ensure that cellular differentiation is minimal and a steady growth rate is maintained. With increased passage numbers, ES cells accumulate mutations and

chromosomal abnormalities which may prevent germline transmission. Early passage (<13) cells should be used (Fedorov *et al.* 1997). It is advisable to test-inject ES cells of a passage close to that to be generated in the targeting experiment to confirm that the cells are capable of germline transmission. In such a case, more than 50% of offspring should be strongly chimeric and most chimeras should achieve germline transmission in 1–2 litters.

Embryonic stem cells from the 129 strain are the most commonly used and widely available. The use of 129 ES cells may, however, be problematical because 129 mice are generally poor breeders and this can necessitate the use of large numbers of mice for subsequent breeding. The outcrossing of 129 substrains has led to extensive genetic variability among substrains and ES cells derived from them. This can have a negative impact on gene targeting efficiencies, on the generation of inbred lines and on the availability of suitable controls (Simpson *et al.* 1997). The use of ES cells from other strains such as C57BL/6 and BALB/c may, therefore, seem preferable. However, ES cells from the 129 strain are more stable in culture than those from other strains and there is thus a greater likelihood of achieving germline transmission by using them. It is, therefore, advisable to balance the efficiency of the initial gene targeting events and the likelihood of achieving germline transmission, with subsequent breeding performance to ensure the minimum number of mice are used.

Embryonic stem cell cultures are a potential source of pathogenic infection in mice (Nicklas & Weiss 2000) and this may subsequently affect animal welfare as discussed in Section 8. A mouse antibody production (MAP) test should be used to screen for the presence of pathogens.

Recommendations:

- **Culture ES cells under conditions optimal for the maintenance of pluripotency.**
- **Use early passage ES cells.**
- **Screen ES cell cultures for the presence of mouse pathogens.**

5.3 Microinjection of ES cells into blastocysts

Embryonic stem cell clones selected for microinjection should be karyotyped and fully characterized for the desired genetic modification. In the case of gene targeting, it is important to confirm that there are no additional random transgene insertions.

The strain of blastocyst host greatly influences the number of offspring obtained and the rate of germline transmission. Inbred strains achieve the highest rates of germline transmission—C57BL/6 is commonly used as a host strain. The production of blastocysts is discussed in Section 11.2.

It is advisable to generate mice from two independent clones so that any underlying mutation in the ES cell line, which may modulate any phenotype subsequently produced, can be identified. In the event of a discrepancy, a further independent clone should be microinjected.

Recommendations:

- **ES cells should be characterized prior to microinjection.**
- **Host blastocysts from inbred strains such as C57BL/6 should be used in order to maximize the likelihood of achieving germline transmission.**
- **Two independent clones should be used to confirm that any phenotype subsequently observed is not attributable to an underlying mutation in the ES cells.**

5.4 Breeding for germline transmission

Embryonic stem cell lines in common use are derived from male embryos. A bias in the number of male chimeras, together with a high percentage of donor coat colour (>70%), indicates that the ES cell clone has contributed to the germline.

To avoid wastage of mice, limits should be set on the number of chimeras to be used for breeding. As a general rule, the best three or four male chimeras should be used and transmission should occur in the first litters of these pairs.

Having produced germline chimeras, it is usual to breed away from the commonly used 129 background, as these mice are poor

breeders, and to establish the mutant on a mixed genetic background. C57BL/6 is a standard background for knock-out mice (Battey *et al.* 1999), although most publications are based on phenotypes seen in mice of the 129/C57BL/6 mixed genetic background (Hogan *et al.* 1994). Genetic background can influence the phenotype. A segregating and mixed genetic background can reveal background specific phenotypes without the need for extensive backcrossing (Doetschman 1999), although this can complicate the analysis of the phenotype.

Recommendation:
- **Limits should be set on the number of chimeras used for breeding.**

6 Training and competence

The generation of GM mice requires expertise in diverse areas including recombinant DNA technology, *in vitro* techniques, microinjection of embryos, surgery, mouse husbandry, and colony management. Specialist knowledge and skills are required, and the efficient production of GM mice is consequently dependent on a combined effort between researchers, and animal care and veterinary staff. It is important that all those involved understand their responsibilities and roles in the process, and have the necessary motivation, attitude, training, supervision and competence to carry them out.

6.1 Husbandry and welfare

A good understanding of laboratory animal science is essential if the number of mice used is to be minimized and animal welfare optimized. Breeding programmes need to be carefully considered to ensure that mice of the desired genotype are produced and that animals are not wasted. Good husbandry practices are essential to ensure that the animals used in the production of the GM mice are healthy, and that the females used to provide embryos for manipulation or as embryo recipients are plugged.

The generation of GM mice raises the possibility of deliberately or unintentionally altering their morphology, physiology or behaviour. Staff should be able to recognize any deviation from normality so that appropriate action can be taken to minimize suffering, develop humane endpoints, and provide appropriate housing and care. The effects of genetic modification should be carefully documented, including the age of onset and prevalence of the abnormality. Assessing the welfare of GM mice is discussed in Section 17.

Training should be provided to all relevant personnel on a range of animal husbandry issues. A brief check-list is indicated below:

(i) Handling and restraint.
(ii) Genetics and breeding programmes.
(iii) Record keeping.
(iv) Welfare assessments.

Recommendation:
- **All staff should have a good understanding of laboratory animal science and husbandry practices.**

6.2 Procedures

The generation of GM mice involves a number of invasive procedures which can cause pain, suffering or distress. Adverse effects on animal welfare should be minimized by ensuring that all staff carrying out procedures have appropriate training and supervision, and that their performance and competency is regularly assessed. A level of competency should be attained before procedures are carried out on live mice, even with supervision. For surgery, other invasive procedures, or physical methods of euthanasia, training should be carried out using cadavers. Competence should be confirmed prior to any procedure being carried out without supervision, and should subsequently be regularly assessed.

A brief check-list of procedures where technical competence is necessary is shown below.

(i) *In vitro* techniques, e.g. ES cell culture.
(ii) Administration of substances by injection.
(iii) Humane killing.
(iv) Harvest of fertilized eggs and blastocysts.

(v) Pronuclear and ES cell microinjection skills.
(vi) Anaesthesia, analgesia and post-operative care.
(vii) Embryo transfer.
(viii) Vasectomy.
(ix) Tissue sampling for genotyping and animal identification.

Recommendations:

- **All staff carrying out procedures on mice should receive appropriate training and supervision. Competency should be ensured and subsequently regularly assessed.**
- **For surgery, other invasive procedures, or physical methods of euthanasia, training should always be carried out using cadavers.**

6.3 Performance reviews

It is important to distinguish the inherent wastage of mice due to the inefficient nature of the technology from that associated with poor technique, lack of skill, or carelessness, so that appropriate remedial action can be taken. Good records of all stages in the transgenic process should be maintained and regularly compared with the suggested figures in Tables 2 and 3, and with the performance of colleagues. The actual number of mice used will vary depending on the DNA construct or the ES cell clone used, and average figures should be collated from three microinjection sessions. New trainees should show improving performance even if the levels shown in Tables 2 and 3 are not achieved initially.

Recommendation:

- **Records of the transgenic process should be maintained and compared with colleagues and benchmark figures (see Tables 2 and 3) so that performance can be reviewed and appropriate remedial action taken where necessary.**

7 Husbandry

The increase in the numbers of GM mice produced poses potential problems for animal facilities and animal care staff in terms of available space, time and resources. It is critically important that this does not have an adverse impact on standards of husbandry and care, particularly in terms of the amount and quality of the space that the mice are provided with, and the time and attention staff are able to devote to animal welfare. It is beyond the scope of this report to include rodent husbandry practices; this has already been covered in detail by Jennings *et al.* (1998). It is, however, important to reiterate that regardless of the husbandry system used, all mice should be provided with sufficient space to allow them to be maintained in social groups wherever possible, they should be able to perform a wide range of normal behaviours, and they should be provided with environmental enrichment. All mice should be provided with substrate and nesting material as a minimum requirement. Apart from the inherent benefits of a good-quality environment, the provision of adequate nesting material and environmental enrichment can increase pre-weaning survival.

Genetically modified mice are increasingly maintained in specialist bioexclusion systems such as isolators or individually ventilated cages. The effects of these systems on animal welfare have not been widely documented. It is, therefore, particularly important to closely monitor mice to ensure that their behaviour, breeding, growth and welfare are at least consistent with those observed in conventional cages.

Genetically modified mice can require specialist care depending on the nature of their phenotype. Husbandry practices and care should be optimized to meet the particular needs of the mice or to ameliorate any suffering. For example, mouse models of cystic fibrosis which lack CFTR function die shortly after weaning of intestinal obstruction, and a liquid diet is required to extend their life span (Grubb & Boucher 1999). Many GM mice are smaller at weaning than their wild-type siblings and they may not be able to reach the food hopper. Food should be made easily available, for example, by providing soft food pellets on the cage floor. Some GM mice may have facial or maxillary malformations that also necessitate a

specialist diet, and sometimes require frequent teeth trimming.

Recommendations:

- **All mice should be provided with the highest standards of care and with a good quality and quantity of space to allow them to be maintained in social groups, they should be able to perform a wide range of behaviours, and they should be provided with environmental enrichment.**
- **Husbandry practices should be modified to provide the specialist care required by some GM mice.**

8 Health status

It is widely accepted that natural infection with many commensal or pathogenic microorganisms can have a deleterious effect on laboratory animal health and welfare, and can jeopardize the scientific validity of the experiments in which they are being used, even if the infection is subclinical. The effects of infection can range from subtle biochemical or immunological changes through clinical disease to death. Incidental infection is a particularly important issue with GM mice since they may be, to some degree, immunocompromised, either directly as a result of the genetic modification itself, or indirectly through the random disruption of genomic sequences. For example, *Pneumocystis carinii* is of marginal pathogenicity in most mice but can cause pneumonia and death in immunocompromised GM mice (J. F. A. Carver, personal communication). In addition to compromising animal welfare, the presence of incidental infections may also modify or exacerbate an existing phenotype.

Every effort should be taken to prevent or minimize the microbiological contamination of GM mice. This is best achieved by ensuring that the mice used in their generation are free from unwanted microorganisms (that is, specified pathogen free (SPF) animals) and that the GM mice produced are subsequently maintained in appropriate husbandry systems. Various bioexclusion husbandry systems (such as filter top cages and individually ventilated cage racks) can assist in achieving this objective. It is also important to remember that biological materials, such as cell lines, can be contaminated and that the inoculation of such materials can result in mice becoming infected.

An informed assessment of the microbiological status of a colony of GM mice is only possible if a properly designed health-screening programme is implemented. Such programmes range from the very extensive (e.g. Federation of European Laboratory Animal Science Association 1996) to in-house customized schemes that concentrate on the pathogens of interest. Where mice are immunocompromised, careful thought should be given as to how health monitoring can be achieved. Possible strategies include the use of sentinel animals that are exposed to soiled bedding from experimental cages, or which are housed in the same cage as the mice under investigation.

Genetically modified mice from external sources may be infected and every effort should be made to prevent them from posing a threat to the existing colonies. Options range from isolation using bioexclusion husbandry systems (such as isolators) to rederivation of the GM line. Rederivation is discussed in Section 21.

Recommendations:

- **Every effort should be made to prevent or minimize the microbiological contamination of GM mice, especially if they are immunocompromised.**
- **High health status animals should be used in the production of GM mice.**
- **Cell lines and colonies should be regularly screened for the presence of pathogens.**
- **The safest approach to the introduction of GM mice into a host establishment is to assume that they are infected and to plan their housing and husbandry accordingly.**

9 Euthanasia

The generation and management of GM mice requires large numbers of mice to be killed. All animals have intrinsic value and the ethical implications of taking their lives,

regardless of the purpose, should be considered by all those involved with producing and using GM mice, as well as by those who authorize and review research.

The number of mice killed should be kept to an absolute minimum by following the principles of best practice set out throughout this report. The method of killing must be as humane as possible. All those required to kill mice should receive appropriate training and supervision, and their competency in all methods they are required to use, and their willingness to euthanize mice, should be ensured. The distress caused to staff from having to kill large numbers of unwanted or surplus mice should not be underestimated (Arluke 1992).

National legislation on the use of animals in scientific procedures (e.g. Schedule 1 under the Animals (Scientific Procedures) Act 1986 in the UK) or local institutional guidelines specify permissible methods of euthanasia. For mice, the most commonly used methods are dislocation of the neck or exposure to a rising concentration of carbon dioxide. Provided that it is carried out by a competent person, death by cervical dislocation is very rapid and is considered to be a humane method. Where large numbers of mice are to be killed at any one time, cervical dislocation may be impractical and exposure to carbon dioxide is commonly used. There is, however, evidence to indicate that exposure to carbon dioxide, even at low concentrations, may cause distress to mice and rats prior to their loss of consciousness (Leach *et al.* 2002a). It has therefore been recommended that carbon dioxide should only be used for euthanasia once the rodents have first been rendered unconscious with an anaesthetic agent such as halothane (Leach *et al.* 2002b).

Recommendations:

- **The number of mice killed must be kept to an absolute minimum.**
- **The method used for euthanasia must cause the minimum distress to the mice.**
- **Staff should receive appropriate training and supervision to ensure that they are competent before they are allowed to carry out euthanasia. Competency should subsequently be regularly assessed.**

10 Anaesthesia, analgesia and perioperative care

Some of the procedures involved in the generation and management of GM mice have the potential to cause pain, suffering and distress. Adverse effects can be kept to an absolute minimum by the use of appropriate surgical techniques, general anaesthesia, analgesia and perioperative care. This section primarily considers the mouse, although the general principles also apply to the rat. Further information on anaesthesia, analgesia and surgery in mice and rats can be found in Flecknell (1996) and Flecknell and Waterman-Pearson (2000).

10.1 Anaesthesia

General anaesthesia is necessary for vasectomy and embryo transfer procedures in the generation of GM mice. Subsequently, it may also be required for the removal of tissue biopsies and identification. A number of different injectable and inhalation general anaesthetic agents are available. Veterinary advice should be sought when selecting the most appropriate anaesthetic regimen, particularly as this may also influence the choice and action of analgesia that is given (see Section 10.4).

The response to an anaesthetic agent may vary considerably depending on the strain, age, sex and weight of the mouse. It is, therefore, vitally important to proceed with caution when using an anaesthetic for the first time or in a different strain. Invasive surgery should never commence until a state of surgical anaesthesia is confirmed by the absence of the pedal withdrawal reflex, following the application of moderate pressure to the hind paw (e.g. using fine forceps). The depth of anaesthesia and the animal's vital signs should be monitored throughout the period of anaesthesia.

A brief description of general anaesthetic regimens appropriate for the surgery used in the production of GM mice is given below. Further details on anaesthesia for mice and rats can be found in Flecknell (1996).

(i) Fentanyl/fluanisone ('Hypnorm', 0.4 ml/kg, Janssen, Oxford, UK) in

combination with midazolam ('Hypnovel', 5 mg/ml, Roche, Basel, Switzerland) produces good surgical anaesthesia lasting 20–30 min. The two compounds should be mixed (1 part Hypnorm, 2 parts water, 1 part Hypnovel) and administered intraperitoneally at the rate of 0.1 ml/10 g. Recovery can be accelerated by administering buprenorphine ('Vetergesic', Alstoe Veterinary, York, UK) or butorphanol ('Torbugesic', injectable, Fort Dodge Animal Health, Southampton, UK), which also provide post-operative analgesia.

(ii) Medetomidine ('Domitor', 1 mg/ml, Pfizer, Sandwich, UK) in combination with ketamine hydrochloride ('Vetalar', 100 mg/ml, Pharmacia Animal Health, Corby, UK) produces surgical anaesthesia lasting 20–30 min. The two compounds should be mixed (0.5 ml Domitor with 4.12 ml water and 0.38 ml Vetalar) and administered intraperitoneally at a rate of 0.1 ml/10 g. To reduce the recovery time, anaesthesia can be reversed with the medetomidine antagonist, atipamezole ('Antisedan', 5 mg/ml, Pfizer, Sandwich, UK) administered subcutaneously at the rate of 1 mg/kg.

(iii) Tribromoethanol in tertiary amyl alcohol ('Avertin') provides rapid surgical anaesthesia after intraperitoneal injection (dose 375 mg/kg as a 2.5% weight/volume solution). Surgical anaesthesia lasts for 15–20 min with full recovery after 1.5–2 h provided that the mouse is kept warm. Tribromoethanol causes peritoneal irritation (Zeller *et al.* 1998), particularly if it is not correctly prepared or stored. On this basis it may be preferable to use an alternative anaesthetic agent. Where it is used, tribromoethanol should be stored at 4°C in the dark and only freshly prepared and filtered solutions should be used. Administration of tribromoethanol for a second time is associated with high mortality rates in mice and should be avoided. In rats, tribromoethanol causes peritonitis and is associated with high mortality rates, at the first time of use (Reid *et al.* 1999).

(iv) Inhalation agents such as the fluorinated hydrocarbons (halothane, isoflurane and enflurane) induce anaesthesia rapidly. The level of anaesthesia is easily controlled and recovery is rapid. These inhalation agents are particularly useful for relatively quick procedures, such as the removal of tail biopsy, in addition to lengthier procedures.

It is unacceptable to use ether, chloroform, or carbon dioxide as anaesthetic agents.

Recommendations:

- **Veterinary advice should be sought on the choice and dose of anaesthetic agent.**
- **Care should be taken, particularly when using a different strain or anaesthetic for the first time, to ensure that the mice are closely monitored for adequate surgical anaesthesia and that vital functions are not compromised.**

10.2 Basic surgical procedures

The risk of infection should be minimal provided that surgery is carried out aseptically through the use of sterile instruments and techniques. An appropriate and separate area should be set aside for conducting surgical procedures. Solutions to be administered parenterally should be prepared and stored so that their sterility is maintained. Where surgery is to be conducted on more than one mouse, either separate sets of sterile instruments should be available or instruments should be decontaminated with disinfectant or dry heat between mice. It is best practice to clip the fur around the incision site and wipe with a skin antiseptic or 70% ethanol. Care should be taken not to soak the mouse with excessive amounts of fluid as this will lead to hypothermia.

Incisions should be closed in two layers, with the muscle being sutured with Vicryl (Polyglactin 910, Ethicon, Edinburgh, UK) or cat gut (4–5/0), and the skin closed with skin clips, surgical staples or a non-capillary attractant suture such as Prolene (4–5/0) (Polypropylene, Ethicon, Edinburgh, UK).

Skin clips can be painful as they pinch the skin and it is advisable to avoid using those, such as Michell clips, which can be over-tightened. Provided that sutures and surgical staples have been properly applied, it is unlikely that the wound will re-open. Should this happen, however, the mouse should be re-anaesthetized and the wound debrided and re-closed.

Sutures, skin clips or staples should be removed after 7–10 days when the wound has healed.

Recommendations:

- **To minimize the risk of infection all solutions and instruments should be sterile.**
- **When cleaning the site of the incision, care should be taken to avoid soaking the fur or skin as this has a high risk of hypothermia.**
- **Incisions should be closed in two layers.**
- **Skin clips that can be over-tightened should not be used.**
- **Remove sutures, skin clips or staples 7–10 days after surgery.**

10.3 Perioperative care

Good perioperative care is vitally important if mice are to make a rapid and full recovery. Mice have a large surface area to volume ratio and are thus susceptible to hypothermia. Their body temperature should be maintained both during and after anaesthesia. During surgery, supplementary heat should be provided, for example, through the use of a temperature-controlled platform or pad. Subsequently, mice should be transferred to a quiet, warm area until they have fully recovered consciousness as indicated by the return of the righting reflex and ambulation. The recovery temperature should be 25–30°C. Care should be taken to avoid over-heating during the recovery period and it is advisable to use a thermostatic device to control the local ambient temperature.

Mice can become dehydrated following surgery. This can be avoided by administering sterile isotonic fluids (e.g. 0.3 ml of a warm 0.9% saline solution administered subcutaneously or into the peritoneal cavity) after suturing is completed. Fruit, fruit-flavoured gelatin blocks (Jelly/Jello) or food pellets moistened with water and left on the floor of the cage can help ensure that a mouse's fluid and calorific intake are maintained in the days immediately following surgery. To prevent the eyes drying during surgery, the use of eye drops or ointment is advisable.

Mice recovering from anaesthesia should be placed on synthetic fleece bedding (e.g. 'Vetbed', Kennel Needs and Feeds, Morpeth, UK) or paper tissue. Sawdust should not be used as it may stick to the incision, eyes and/or nostrils. Provided that they are at a similar stage in the recovery process, mice can be kept together. Fully conscious mice should not be left with those still recovering from anaesthesia.

Changes from normal behaviour, locomotion, posture or a continuing loss of body weight are strong indicators that the mouse is failing to make a full recovery from surgery. Any mouse showing a change in behaviour or a significant reduction in body weight (i.e. more than 10%) should be thoroughly examined to determine the cause so that appropriate action can be taken.

Recommendations:

- **To avoid hypothermia, body temperature should be maintained by providing supplementary heating both during surgery and in the recovery period prior to the mouse regaining consciousness. Care should be taken to avoid over-heating.**
- **Dehydration should be avoided by fluid therapy or by providing fruit, jelly or water-soaked food pellets in the days immediately proceeding surgery.**
- **All mice should be closely monitored following surgery to ensure they are making a full recovery.**

10.4 Pain management

The recognition, assessment and management of pain are integral components of good post-operative care. All of the surgical procedures discussed in this report may result in some degree of pain. Pain causes suffering and distress, slows recovery and healing, and

can reduce food and water intake (Flecknell & Waterman-Pearson 2000). Provided, however, that the induction and maintenance of anaesthesia and surgery are performed competently, and perioperative care is properly implemented, any pain should be temporary and ameliorated by the use of analgesics.

The majority of mice do not show any overt signs of suffering after surgery or other procedures, despite the fact that there may be some degree of pain. Mice are prey species and are adapted to conceal or suppress behavioural indicators of pain, suffering and distress. Recognizing subtle indicators of pain is, therefore, difficult, particularly as monitoring is generally carried out during working hours when mice are often least active and least likely to show behavioural signs of pain. Consequently, analgesia may not be provided when it is needed. In order to avoid this, a precautionary approach should be adopted so that all mice undergoing surgery or other potentially painful procedures receive analgesia.

Analgesics are most effective at controlling pain if they are administered before the animal actually experiences pain, thus avoiding central sensitization and the phenomenon of 'wind-up' (Woolf & Chong 1993, Dobromylskyj *et al.* 2000). Central sensitization can be prevented by providing analgesia pre-emptively before the animal recovers from the anaesthetic, and subsequently as required. Depending on the anaesthetic agent and the analgesic used, these may be given simultaneously, or separately with an appropriate interval before or after the anaesthetic. Veterinary advice for suitable combinations and timings should be sought.

A brief description of analgesics suitable for mice and rats is given below. Further details can be found in Flecknell and Waterson-Pearson (2000).

(i) Buprenorphine ('Vetergesic', Alstoe Veterinary, York, UK) provides pain relief for 4–6 h and is the most commonly used analgesic in mice. Opioids such as buprenorphine can interact with anaesthetic agents. For example, buprenorphine interacts with the fentanyl component of Hypnorm, reversing surgical anaesthesia. In this case, buprenorphine should only be given once surgery is completed but before the animal regains consciousness. It is administered subcutaneously at a dose of 0.01 to 0.1 mg/kg.

(ii) The non-steroidal anti-inflammatory drugs, carprofen ('Rimadyl', Pfizer, Sandwich, UK) and flunixin ('Finadyne', Schering-Plough Animal Health, Welwyn Garden City, UK), provide long-lasting analgesia for up to 12 h. They are generally administered subcutaneously, immediately after the induction of anaesthesia at a dose of 5 mg/kg for carprofen and 2 mg/kg for flunixin. They can subsequently be administered at the same rate in the animal's drinking water, assuming a daily water intake of 10% of body weight per day for 2–3 days.

(iii) Local anaesthetics such as bupivacaine ('Marcain', Astra, Kings Langley, UK) can be applied topically to augment the analgesic regimen. Provided that the local anaesthetic infiltrates both the skin and underlying connective tissue, pain relief should last for approximately 6 h. Care should be taken to avoid the muscle, as local anaesthetics such as bupivacaine are myotoxic (Benoit 1978, Foster & Carlson 1980).

Recommendations:

- **Mice undergoing procedures such as embryo transfer or vasectomy should always be provided with pre-emptive analgesia, and top-ups as required.**
- **Always seek veterinary advice regarding the optimum analgesic regimen.**

11 Females used to provide fertilized eggs and blastocysts

11.1 Superovulation of mice

Superovulation is used to reduce the total number of mice required to provide eggs for manipulation, to minimize variability in the quality of the eggs obtained, and to

synchronize the production of a sufficient number of embryos at a given time. Even with superovulation, approximately 20 female mice may be required to generate three to four GM founders. Superovulation is less commonly used to increase the number of blastocysts available for ES cell microinjection (see Section 11.2).

The number of females used as egg 'donors' depends on the success of superovulation. This is influenced by a number of factors including the strain, age and weight of the female, the quality of the gonadotrophins, and the hormone regimen used. The age of the females and the administration of the gonadotrophins also raise animal welfare issues, as discussed below.

11.1.1 Age and weight

The age at which superovulation can most effectively be induced varies from strain to strain and from user to user. Traditionally, prepubescent female mice between 3–5 weeks of age have been used. There are, however, concerns about the welfare of these young mice at mating. Reports of injury occurring, particularly to the reproductive tract, are anecdotal. Nevertheless, it is not unreasonable to assume that there are circumstances where young female mice may be injured; for example, where the female is significantly smaller than the male or where the male is over-aggressive. To minimize the likelihood of this occurring, the size of young females should be optimized and only appropriate stud males should be selected. Alternatively, the use of sexually mature females should be investigated.

The use of immature females has often been considered unavoidable on the basis that superovulation is less effective in mature females and substantially more mice may, therefore, be required to provide a similar number of fertilized eggs. Nonetheless, there is evidence to show that sexually mature females can produce a comparable number of eggs to females aged 3–5 weeks. Using C57BL/6 × CBA F1s, it is possible to obtain approximately 28–45 injectable embryos from females aged 8–9 weeks and 30 embryos from those aged 10–11 weeks (B. Doe, personal communication). As is the case with prepubescent females, the number of embryos obtained is dependent on the strain and the quality of the hormone batch, and success may vary between users. Nevertheless, the feasibility of using superovulating mature females as an alternative to prepubescent females should be investigated.

Where prepubescent females are used, mice below 12–13 g should not normally be superovulated and mated. This should minimize the likelihood of injury occurring to females during mating. In addition, the number of mice used should be reduced because females that are retarded in their development, as evidenced by lowered body weight at the start of the superovulation protocol, tend to yield reduced numbers of eggs (Lang & Lammond 1966, Gates 1971). The body weight of young females can be optimized by maintaining high standards of nutrition and disease control. Moreover, the removal of male litter-mates which are not intended for use in other studies, in the week following parturition when lactation is fully established, ensures that the remaining females receive a better level of nutrition and their body weight at weaning can be increased by 10% (J. F. A. Carver, personal communication).

For rederivation programmes, it may be desirable to superovulate GM females prior to mating in order to reduce the number of mice used. Given the inherent problems of reduced fertility that can be observed in GM mice, it may be difficult to establish optimum conditions for superovulation and this can result in the mating of undersized mice. To avoid this, the possibility of *in vitro* fertilization (IVF) should be investigated as an alternative to natural mating. IVF is discussed in Section 20.

Strategies to optimize the size of prepubescent females should be combined with the careful monitoring of stud males. Females should be checked for signs of damage to their reproductive tract during the collection of the fertilized eggs. It is also advisable to note any external injuries so that males persistently causing harm can be readily identified and removed from the programme. Similarly, over-sized males should

not be used. It is difficult to be prescriptive about the maximum size ratio for males to females. Males should be, as far as possible, size-matched to the females. This may be difficult to achieve when using small prepubescent females, and common sense should prevail so that they are not mated with males with which they are clearly incompatible in size.

Recommendations:
- **Investigate the use of sexually mature female mice as an alternative to prepubescent females.**
- **Mice weighing less than 13 g should not be used.**
- **Over-aggressive or over-sized stud males should not be used.**

11.1.2 Strain

The number of eggs produced after superovulation varies from strain to strain. For many studies certain outbred (particularly, hybrid) strains are used. Using hybrid strains such as (C57BL/6 × CBA) F1s it is routinely possible to obtain 30 eggs per mouse. Hybrid strains also have the advantage that they demonstrate good hybrid vigour, their eggs are relatively easy to culture *in vitro* and they produce good litter sizes.

Having generated GM mice on an outbred or hybrid background, it may however be necessary to minimize genetic heterogeneity and breed onto an inbred background. This can involve extensive breeding programmes involving large numbers of mice and it may therefore be desirable, as an alternative, to use inbred mice as embryo donors. Inbred strains such as C57BL/6, FVB/N and BALB/c have been used successfully to produce GM mice, but differ in the number of eggs that can be induced by superovulation (McLaren 1967, Gates 1971, Hogan *et al.* 1994), and also in their ability to develop *in vitro*. Subsequent reproductive performance can be poor in GM inbred strains.

Clearly, a balance has to made based on the choice of strain of donors and the subsequent need for backcrossing. To reduce the overall number of mice used, careful thought should be given at the outset to the research programme's long-term requirements. Included in this decision should be the benefits of selecting a strain which superovulates well, whose gametes/embryos can be successfully cryopreserved, which breeds well, which produces large litters and which has good mothering ability.

Recommendation:
- **Consider the long-term requirements of the research programme when selecting the strain of embryo donor.**

11.1.3 Administration of gonadotrophins

Superovulation is achieved by the sequential administration of pregnant mare's serum (PMS) and human chorionic gonadotrophin (hCG) which mimic the activities of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively. There are a number of issues to address for the administration of hormones to ensure that the number of mice used is minimized and animal welfare is not compromised.

For most strains, the recommended dose of PMS is 5 I.U. (in a total volume of 100 μl) injected either intraperitoneally or subcutaneously. Generally, 5 I.U. hCG (in a total volume of 100 μl) is administered by intraperitoneal injection, although a dose of 2.5 I.U. is sufficient to ensure ovulation in most strains. Intraperitoneal injections can be difficult to perform in mice because of their small size, and care should be taken when injecting into the peritoneal cavity to avoid puncturing the abdominal viscera. The potential problems and refinement of this route of administration are discussed in Morton *et al.* (2001).

The developmental uniformity and number of eggs obtained, and thus the number of females used, are dependent on the timing of PMS and hCG administration relative to each other and to the light–dark cycle of the animal room. In practice, for most strains optimal egg yield is achieved by allowing a 42–48 h interval between the administration of PMS and hCG. Regular checks on the light–dark cycle in the animal room should be made, and females that are purchased commercially or moved between rooms should be allowed at

least one week to adjust to the light–dark cycle prior to hormone administration.

Following administration of hCG, one female should be placed in a cage containing one stud male. The following morning, she should be checked for the presence of a semen-derived plug in the vagina. Plugged females should be humanely killed by a competent trained person and their eggs collected as described in Hogan *et al.* (1994).

Recommendations:
- **Care should be taken when administering gonadotrophins intraperitoneally to ensure that the abdominal viscera are not punctured.**
- **Regularly check the timing of light–dark cycle of the animal room.**
- **Allow mice to adjust to the light–dark cycle before administration of gonadotrophins.**

11.1.4 Quality of media

The quality of the embryo culture medium can vary depending on the batch. Poor-quality media can affect the development and viability of eggs. It is advisable to test the media used in every session by culturing as controls a few eggs that have not been microinjected.

Recommendation:
- **Use fresh, quality controlled, embryo culture media.**

11.2 Blastocysts for microinjection of ES cells

11.2.1 Superovulation and mating

The success of superovulation in increasing the number of blastocysts obtainable, for the microinjection of ES cells, varies from strain to strain and user to user. Inbred strains such as C57BL/6 are commonly used to provide blastocysts. For reasons that are unclear, many users find that blastocysts from superovulated C57BL/6 or BALB/c females are abnormal, poorly expanded and show low survival rates when transferred to the uterus of pseudopregnant females. The number of usable blastocysts may not be sufficiently increased to justify the superovulation of some strains, and it is common practice to mate sexually mature females that are 6–8 weeks of age and have been demonstrated to be in oestrus (by examining vulval changes).

Where outbred strains are used, they are usually superovulated.

Recommendation:
- **The number of usable blastocysts obtained from strains such as C57BL/6 or BALB/c may not be sufficiently increased to justify superovulation.**

11.2.2 Collection of blastocysts

To maximize the yield, blastocysts should be collected before they have hatched from the zona pellucida and become attached to the uterus. In practice, this means that the blastocysts should be collected, from humanely killed females, 3.5 days *post-coitum* (where 0.5 day *post-coitum* is equivalent to the fertilized egg stage) as discussed in Hogan *et al.* (1994).

Recommendation:
- **Collect blastocyts 3.5 days *post-coitum*.**

12 Fertile stud males

To obtain the maximum number of fertilized eggs or blastocysts from the minimum number of mice, it is important to use stud males with good vaginal plugging rates and high fertility. The stud male should be of the same strain as the females with which he is intended to mate.

Male mice reach sexual maturity at 8 weeks of age. Those intended for use as stud males should be separated from their litter-mates at least one week before they are required to mate, as the dominant male can suppress fertility in his litter-mates. Stud males should be housed singly to avoid fighting and injury. The provision of environmental enrichment (e.g. nesting material, plastic pipes, shelter, etc) is, therefore, particularly important to prevent boredom and distress.

For fertilized egg production, a super-ovulated female should be placed in a cage with one stud male, usually in the afternoon

following hCG injection, and the female should be checked for the presence of a vaginal plug the next morning. For blastocyst production, one or two females should be placed with each male. In both instances, the females should be taken to the male's cage as this prevents the male having to re-establish his territory and minimizes aggression. Following successful mating, the male should not be used for 2–3 days. If a vaginal plug is not present, the male can be re-used the following night.

The performance of individual males should be monitored by recording the presence or absence of a vaginal plug. Males of hybrid or outbred strains that have a vaginal plug rate of less than 70% of the time the male is presented with a female should be replaced. The vaginal plug rate is more unpredictable for inbred mice; for example, C57BL/6 males can be expected to produce a vaginal plug 25–50% of the time they are presented with a female.

To avoid harming females, over-sized or over-aggressive stud males must not be used. It is important to note that aggressive behaviour is not synonymous with good vaginal plugging ability. Stud males can be used successfully from 8 weeks of age onwards. As reproductive success tends to decrease after 6 months, it is recommended that males are replaced accordingly. Indeed, by 6 months the male is likely to be large and as such may be incompatible in size with young females.

Recommendations:

- **Use male mice that are more than 8 weeks and less than 6 months old.**
- **Over-aggressive or over-sized males must not be used.**
- **Maintain a record of male's plugging performance. Outbred or hybrid males with a vaginal plugging rate of less than 70% should be replaced.**

13 Embryo transfer and pseudopregnant recipients

The transfer of embryos into the reproductive tract of recipient females, following pronuclear or ES cell microinjection, involves surgery under general anaesthesia and every effort should be taken to minimize any subsequent pain and suffering through appropriate perioperative care as detailed in Section 10.

Optimizing the transfer of embryos into the recipient is critical if the number of females used is to be kept to an absolute minimum and the survival of the embryos is to be maximized. Consideration should be given to the strain of the female host, her stage in the reproductive cycle and the number of embryos transferred.

13.1 The reproductive cycle and the induction of pseudopregnancy

The maintenance of manipulated embryos in the reproductive tract is dependent on conditions being favourable for their implantation. Such conditions are created by mating females with sterile males to induce pseudopregnancy. The formation of a vaginal plug in this case is taken as an indicator of pseudopregnancy.

Females are only receptive to mating at the oestrous stage of their reproductive cycle. Selecting only those females that are in oestrous for mating, therefore, increases the likelihood of obtaining the required number of plugged females and prevents microinjected embryos from being wasted. Females that are not in oestrus, and thus are not receptive to mating, can be injured by vigorous males.

Females in oestrus are most commonly identified by examining the appearance of their vulva (Hogan *et al.* 1994). In general, females are sexually mature at 6 weeks of age and enter oestrus and ovulate every 4–5 days. Embryo transfer at the appropriate stage provides the embryonic signal that leads to the maintenance of the corpora lutea and prevents a return to cyclicity. Unused pseudopregnant females can be mated again.

Recommendation:

- **Select females in oestrus for mating to increase the likelihood of obtaining a sufficient number of pseudopregnant embryo recipients.**

13.2 Strain of pseudopregnant embryo recipients

To optimize the survival of microinjected embryos, it is important that the strain selected for embryo transfer recipients has

good reproductive performance and makes good mothers. Random-bred or F1 hybrid mice [e.g. (CBA × C57BL/10), (C57BL/6 × CBA)] are suitable recipients. Females of some random bred stocks (e.g. ICR) have very large ampullae and infundibula which make oviduct transfers easier, and they are generally good mothers. F1 hybrid females similarly make exceptionally good mothers, rearing both large and very small litters equally well. Where embryo donors and recipients are F1 hybrids, using the same colony for both can reduce the number of mice bred.

If the sterility of vasectomized males has been assessed by test mating, the same pool of pseudopregnant recipients can be used for all embryos, regardless of strain.

Recommendation:

- **The strain used for embryo recipients should have the characteristics of both good reproductive performance and good maternal behaviour.**

13.3 Embryo transfers

Embryo recipients should weigh 20–30 g. Mice below 20 g do not support pregnancy efficiently and this can result in microinjected embryos, embryo donors, and embryo recipients being wasted. In mice weighing more than 30 g, the presence of additional fatty tissue can lead to problems with both anaesthesia and surgery.

For pronuclear microinjection, embryos 0.5–2.5 days *post-coitum* should be transferred into the ampullae of 0.5 day *post-coitum* pseudopregnant recipients. Embryos can be cultured until a recipient female is available. For uterine transfer of microinjected blastocysts, 2.5 day *post-coitum* pseudopregnant recipients should be used. Since *in vitro* manipulation and culture have the effect of delaying embryonic development, asynchronous transfer gives the embryo time to 'catch-up' in its development before being exposed to conditions favourable for implantation.

Embryo transfers can be carried out unilaterally or bilaterally. Many laboratories perform bilateral oviduct transfers but unilateral uterine transfers. Where bilateral transfer is carried out, it should be refined so that it involves only one dorsal midline skin incision. There are no published data to prove that transuterine migration occurs where unilateral transfers are carried out, although there is plenty of anecdotal information. Regardless of possible migration effects the number of live pups seems to be unaffected, whichever method is used.

Recommendations:

- **Embryo recipients should weigh 20–30 g.**
- **Bilateral transfer of embryos should only involve one dorsal midline skin incision.**

13.4 Number of embryos transferred

The number of embryos transferred should be sufficient to give a litter size of 5–10 pups. If only one or two embryos survive in the uterus they can grow too big to be born without damage to the mother or pup. Mothers of some strains may also not take care of small litters. If litters are too large (>10) then some mice may be underweight, and this can lead to sterility.

For oviduct transfers, up to 90% of unmanipulated embryos develop to term. This figure is markedly reduced following DNA microinjection. The actual number depends on factors such as the nature of the transgene, its site of integration, the concentration at which the transgene is injected and optimization of the embryo transfer procedure. Approximately 20–40% of manipulated embryos develop to term following oviduct transfer and as a general rule 20–25 embryos should be transferred.

For uterine transfers, 50–80% of unmanipulated blastocysts develop to term. Blastocysts that have been microinjected with ES cells should produce a similar proportion of births, although this can be dependent on the ES cell clone. For an expected birth rate of 50–80%, approximately 12 embryos should be transferred.

Provided that two recipients have received embryos from the same experiment, they can be housed together. In this case, the foster mothers will jointly raise their litters.

Recommendations:
- **Sufficient embryos should be transferred to give a litter size of 5–10 pups.**
- **Embryo transfer recipients receiving embryos from the same experiment should be housed in pairs.**

14 Sterile males for the induction of pseudopregnancy

Pseudopregnancy is induced by mating with either vasectomized or genetically sterile males. There are a number of issues relating to surgery and how it is carried out, and the production of surplus mice that should be considered when deciding whether to use vasectomized or genetically sterile males. Failure to ensure that a good supply of pseudopregnant females is available can result in microinjected embryos being wasted. Sterile males should therefore have good vaginal plugging rates.

14.1 Vasectomized males

Producing vasectomized males requires surgery under general anaesthesia and the likelihood of any suffering occurring subsequently should be minimized by the use of appropriate perioperative care and analgesia as detailed in Section 10. The surgery involved can also be refined to minimize its impact on animal welfare.

Vasectomized mice are produced by cutting and/or cauterizing the vas deferens such that reconnection is unlikely. The vas deferens can be exposed either via a midline ventral incision in the abdominal body wall (Hogan *et al.* 1994) or an approximately 5 mm incision into each scrotal sac as shown in Fig 2 (Rafferty 1970). The scrotal sac method is a refinement and should be used instead of a laparotomy, for the following reasons.

(i) There is no incision into the abdominal musculature. This is important as the abdominal musculature (and the central midline tendinous structure), especially in the quadruped, is weight bearing for the abdominal contents (and these can weigh up to 30% of the body weight). This is in contrast with the scrotal sac where there is little, if any, weight bearing. It is therefore likely to be less painful.

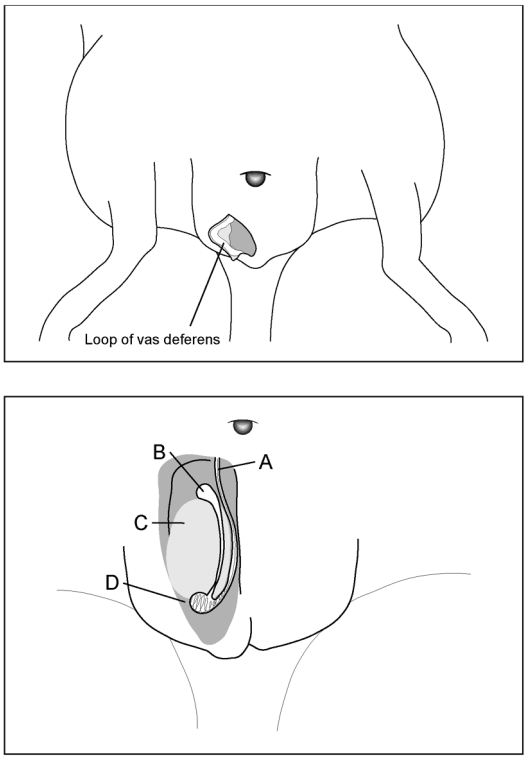

Fig 2 The vas deferens on each side can be accessed through a single 5–7 mm midline incision in the skin of the scrotum. A small area subcutaneously is blunt dissected to either the left or right of the incision such that an outline of a testis can be seen through the body wall. A 5 mm incision is then made in the body wall to one side in line with the base of the testis. The cauda epididymis is located and the pale vas deferens with a prominent blood vessel leading from it is dissected away such that a loop is formed. A 5–7 mm section of vas deferens is cauterized or cut away, and the two ends are either cauterized or tied. A single stitch is made to the body wall and the procedure is repeated for the second side. The skin is stitched. A = vas deferens; B = caput epididymis; C = testis; D = cauda epididymis

(ii) Although both approaches strictly enter the body cavity, the scrotal sac route is essentially only a minor opening whereas the abdominal route potentially exposes all of the abdominal viscera to infection.

(iii) The vas deferens can be exposed without exteriorizing the testis.

(iv) The body wall and skin incisions require only a single suture on completion.

(v) Males recovering from a vasectomy via a scrotal sac incision appear less

hunched than those that have had a laparotomy, suggesting that less discomfort is caused by this method (K. Mathers, personal communication).

Following surgery, males should be allowed a recovery period of 3 weeks. This also ensures that no residual sperm remain in the proximal part of the vas deferens. Vasectomized males can then be test mated with two females to confirm the success of the vasectomy. Pregnancy is normally apparent within 2 weeks, and if not visible after this time the male can be used. Depending on the strains of embryo donor, embryo recipients and vasectomized males used, test mating may not be necessary as with some strains differences in coat colour can be used to identify any offspring resulting from a failure of the vasectomy.

The vaginal plug rate should be unaffected by the vasectomy but it is good practice to keep a plugging record for each male to monitor performance. Vasectomized males can be used daily and can be expected to plug regularly for one year or more.

Surplus males used in breeding programmes for embryo donors, and recipients can be used as a source of males for vasectomy. Strains commonly used are either F1 hybrids or random-bred, which have the characteristics of high vaginal plugging rates and good behaviour.

Recommendations:

- **Surgery should be refined so that the vas deferens are accessed via a small incision in the scrotal sac, rather than by laparotomy, as this avoids cutting the abdominal body wall musculature and is, therefore, likely to be less painful.**
- **Following surgery, vasectomized males should be allowed a recovery period of 3 weeks.**
- **Surplus males produced in routine breeding programmes for embryo donors and recipients can be used as a source of males for vasectomy.**

14.2 Genetically sterile males

The use of genetically sterile males, instead of vasectomized males, obviates the need to subject male mice to the stress of surgery. Sterility is guaranteed and males are available for use without the need for test mating. The overall number of mice used may, however, be increased because the breeding of genetically sterile males will be accompanied by the production of females, some of which may be surplus to requirements. In contrast, the use of vasectomized males does not require the maintenance of a specific colony because sufficient males, produced as surplus from routine breeding programmes for embryo donors and recipients, can be obtained.

Only strains of genetically sterile males with good plugging rates should be used. One strain of mouse that has successfully been used to induce pseudopregnancy is the T145H-Re strain. T145H-Re sterile males are obtained by crossing females carrying a chromosomal translocation between chromosomes 7 and 19 (and linked to the pink-eyed locus on chromosome 7) with wild-type males. Males produced carrying this chromosomal translocation are sterile and can be identified non-invasively by their dark eye colour (Searle 1989). The vaginal plugging performance of F1 hybrid vasectomized males has been compared with that of the genetically sterile T145H-Re males in matings with randomly selected C57BL/10 × CBA F1 hybrid females. In each case, the plugging frequency was very similar: vasectomized males had a 22% plugging rate (1575 pairings) and T145H-Re males had a 21% plugging rate (689 pairings) (D. J. Wells, unpublished data). These plugging rates are close to the theoretical 25% rate for randomly mated mice, based on a 4-day oestrous cycle.

Recommendation:

- **The potential effects on animal welfare of the surgery necessary for vasectomizing males should be carefully balanced against the production of surplus mice associated with the use of genetically sterile males.**

15 Tissue biopsy collection for genotyping

It may be necessary to determine or confirm the genotype of potentially GM mice by the analysis of DNA extracted from a tissue

biopsy or blood. The removal of tissue has the potential to cause pain, suffering and distress. To ensure that suffering is minimized, it is important to consider the source of tissue, the age of the mice to be biopsied, the size of the sample taken, and the requirement for local or general anaesthesia.

A number of methods of tissue sampling have been developed. The age of the mice is an important factor when deciding on the choice of tissue sampling method, as summarized in Table 4. As general rules, the least invasive method should be used and the amount of tissue taken should be kept to an absolute minimum. The most commonly used methods (e.g. tail biopsy) involve the surgical removal of tissue. Less invasive methods are available, although their use is not currently widespread, despite the fact that the methodologies have been published in refereed journals. The method chosen for tissue biopsy collection should not be justified on the basis that it has historically always been used. Techniques should be regularly reviewed to take advantage of any advances in scientific techniques that allow smaller samples to be taken or less invasive procedures to be used.

The screening system used to distinguish GM from non-GM mice should seek to minimize the amount of tissue required. Southern blot hybridizations require more DNA, and concomitantly more tissue, than analysis by polymerase chain reaction (PCR). While the use of Southern blotting may be unavoidable in some circumstances, for example, for determining the transgene copy number, the use of PCR should always be considered, particularly for the routine genotyping of breeding colonies.

Whichever method is used for obtaining biopsy material, it is important to avoid the carry-over of tissue between successive samples as this can lead to the identification of 'false' GM mice, particularly where PCR is used in the analysis, and to the spread of microbiological infections between biopsied animals. All instruments should be decontaminated or replaced between samples.

Consideration should be given to the identification system that enables cross-reference between the biopsies and the individual mice from which the samples were taken. Some methods such as ear notching serve the dual purpose of both providing a biopsy sample and identifying individual mice, thus negating the need for a further identification procedure. Methods of identification are discussed in Section 16.

Recommendations:

- **Use the least invasive method of tissue sampling.**
- **Keep the amount of tissue taken to an absolute minimum.**
- **Techniques should be regularly reviewed to take advantage of any advance in scientific techniques that allow smaller biopsy samples to be taken or less invasive procedures to be used.**
- **Care should be taken to avoid cross-contamination between biopsy samples, particularly where PCR is to be used in the subsequent analysis.**

15.1 Non-invasive biopsy methods

A number of methods for providing biopsy material for genotyping by PCR have been described that negate the requirement for

Table 4 Choice of biopsy method depending on age of mice

	Less than 2 weeks	3–4 weeks	Over 4 weeks
Saliva or faecal samples	✓	✓	✓
Tail biopsy	✓/✗	✓	✓/✗
Ear notching	✗	✓	✓
Blood	✗	✓	✓
Toe amputation	✗*	✗	✗

✓/✗: Unless there is good scientific justification to the contrary, tail biopsies should not be taken from mice significantly younger than 3 or older than 4 weeks of age (See Section 15.2.2)
✗: Blood should not be taken for genotyping from mice less than 2 weeks old unless it is absolutely unavoidable
✗*: Toe amputation must not be used except in exceptional circumstances (see Section 15.5)

surgical biopsies. These include the collection of rectal epithelial cells (Lahm *et al.* 1998), oral epithelial cells (Irwin *et al.* 1996, Zimmermann *et al.* 2000), faecal pellets (Broome *et al.* 1999), and hair follicles (Schmitteckert *et al.* 1999). Although described as less invasive than tail biopsies, these methods are not without problems. For example, extreme care is necessary for the collection of rectal epithelial cells to ensure that the rectum is not damaged, whereas with the use of hair follicles there are concerns about the possibility of cross-contamination between litter-mates. The use of saliva or faecal samples does, however, present the opportunity to refine the collection of biopsy material for genotyping using PCR.

Small samples of saliva containing oral epithelial cells and lymphocytes have been used to provide DNA for the genotyping of GM mice by PCR (Irwin *et al.* 1996, Zimmermann *et al.* 2000, I. Rosewell, personal communication). Saliva samples can be collected using a pipette (Irwin *et al.* 1996) or an oral swab (Zimmerman *et al.* 2000). Using this latter method, sufficient DNA can be prepared using the 'HotSHOT' protocol (Truett *et al.* 2000) from saliva samples taken from mice as young as 10 days old (I. Rosewell, personal communication). Although, the forcible opening of the mouth may be stressful, provided that the mice are competently handled, this latter method should not cause more than momentary stress.

The collection of faecal samples, containing intestinal epithelial cells, from adult or young mice also provides an easy and non-invasive method of obtaining material for genotyping by PCR. Stools are usually produced when mice are handled and they can be collected directly into a tube (Broome *et al.* 1999). The technique is readily repeatable and, other than the stress of handling, should not compromise animal welfare.

Neither saliva or faecal samples are currently routinely used as methods of providing biopsy material for genotyping by PCR. It is recommended that their use as humane alternatives to the commonly used more invasive methods should be investigated.

Recommendation:

- **Investigate the use of oral or faecal samples as non-invasive sources of DNA for genotyping.**

15.2 Tail biopsy

The surgical removal of the tip of the tail is frequently used as a source of tissue for DNA extraction. It has previously been assumed that the tip of the mouse's tail is cartilaginous, however, the caudal vertebrae start to ossify between 2–3 weeks of age and there is bone mineralization and clear evidence of vertebrae, even within the last 1 mm of tail (D. J. Wells, unpublished observations; J. Wallace, A. Sebesteny & R. Raymond, personal communication). The skin and periosteum of the mouse's tail is well supplied with nervous tissue, and the removal of even a small section of tail is likely to be very painful, particularly where the bone is cut. Furthermore, the long-term effects of a tail biopsy on animal welfare (e.g. the possibility of phantom pain) have not been thoroughly investigated, although the removal of a large section of tail has been demonstrated to cause thermal and mechanical hyperalgesia in the remaining part of the tail (Zhuo 1998). The removal of a tail biopsy may therefore be acutely and chronically painful for the mouse, and the use of less invasive or more humane methods such as ear notching/punching or blood samples should always be investigated first.

Where the use of tail biopsies is absolutely unavoidable, appropriate anaesthesia and analgesia should be used. The tip of the tail can be removed using a sterile scalpel blade or surgical scissors. A balance should be struck between the haemostatic benefit of crushing the tissue and the reduced tissue trauma associated with a 'clean' cut. If bleeding occurs, the cut surface should be sealed with a coagulant such as silver nitrate or a surgical wound adhesive such as 'Vetbond' (3M Corporation, USA).

The size of the biopsy and the age at which it is taken are important considerations for ensuring that where tail biopsies are unavoidable the procedure is as humane as possible. The Working Group discussed these

factors at length. The main points are set out below and summarized in Table 5.

Recommendation:

- **Tail biopsies should not be the first choice for providing DNA for genotyping. The use of non-invasive and more humane methods should be investigated first.**

15.2.1 Size of tail biopsy

The tail is used extensively in the mouse's behavioural repertoire and the size of the biopsy taken should be kept to an absolute minimum. The amount of tail required to provide sufficient DNA for analysis is dependent on the strain and age of the mouse, and the method used for subsequent genotype analysis. In practice, it should not be necessary to remove any more than 5 mm of tail to provide enough DNA for the majority of analyses. The size of the biopsy should reflect what is actually required, and preferably less than 5 mm should be taken. Removal of more than 5 mm of tail must be avoided, as the bone is thicker in more proximal parts of the tail and this increases the likelihood of causing tissue trauma and suffering. Indeed, larger sections of tail will consist mainly of bone and consequently proportionately less DNA.

If necessary, tail biopsies should be divided into two and one half should be stored as a reserve to avoid repeat biopsies. Where on rare occasions a second biopsy is required, a further tail biopsy should be avoided by using one of the other methods described.

Recommendation:

- **No more, and preferably less, than 5 mm of the tail should be taken. Repeated tail biopsies must be avoided.**

15.2.2 Age at tail biopsy

Whilst it is possible to recommend limits on the size of the tail biopsy, determination of the most humane age to take tissue is difficult. The Working Group has considered the removal of tail biopsies from mice that are approximately 2 weeks, 3–4 weeks, and over 4 weeks of age, and the provision for anaesthesia and analgesia at these ages.

Approximately 2 weeks: In mice approximately 2 weeks of age, the bone and nervous tissue of the tip of the tail are relatively undeveloped, although there is evidence of bone deposition (D. J. Wells, unpublished observations; J. Wallace, A. Sebesteny & R. Raymond, personal communication). At this age topical anaesthetics such as ethyl chloride should penetrate sufficiently to minimize any pain caused by the removal of a tail biopsy. Tail biopsies at this age may, nevertheless, be problematical. The removal of 5 mm of tail represents a significant proportion of the tail—the long-term effects of such an amputation on animal welfare are unknown. Moreover, it is possible that the mice may be in some degree of pain once the effects of a short-acting anaesthetic such as ethyl chloride

Table 5 Age and tail biopsies

Age	Potential harm	Opportunities for refinement
Approximately 2 weeks	Possibility of excessive shortening of the tail—possibility of long-term effects on animal welfare.	Can be performed using local or general anaesthesia.
	Identification may be difficult.	
	Potentially painful.	Use of analgesia—although this may be difficult.
3–4 weeks	Possibility of additional stress if performed close to weaning.	Should local or general anaesthesia be used.
	Potentially painful.	Use of analgesia.
		Use ear notching as an alternative.
4 weeks and older	Potentially more painful as the bone is thicker and there is increased likelihood of tissue trauma.	Use general anaesthesia.
		Analgesia can be readily provided.
		Use ear notching as an alternative.

have worn off. Administering analgesia to such young mice is, however, difficult. Finding suitable methods for identifying young mice may also be a problem. Taken together, these factors indicate that unless there is good scientific justification to the contrary, tail biopsies should not routinely be performed on mice significantly younger than 3 weeks of age. Where it is unavoidable, it is important to address how analgesia can be effectively and safely provided.

Three to four weeks: Tail biopsies are commonly taken at 3 weeks of age either shortly before or after weaning. Whether the removal of a tail biopsy at the time of weaning adds to the stress of weaning itself is debatable. It is important to consider whether, from the mouse's standpoint, it is preferable to have the combined stress of weaning and tail biopsy at the same time or whether the two should be separated by a few days. The potential distress associated with weaning should be reduced by the group housing of litter-mates, at least until their transgenic status is confirmed.

The Working Group was divided as to whether tail biopsies at 3–4 weeks of age should be performed using local or general anaesthesia. Some members of the Group were concerned that local anaesthetics such as ethyl chloride may not penetrate sufficiently to alleviate the pain caused by the removal of a tail biopsy and therefore advocated general anaesthesia using inhalation agents, particularly as this also avoids the stress of manual restraint. Conversely, other members were of the opinion that the use of general anaesthesia has the potential to cause more distress than the tail biopsy itself, and, provided that local anaesthetics are given the appropriate time to take full effect, animal welfare should not be adversely affected by the procedure (e.g. no squeaking or flinching should be observed at the time the tail is cut). In the case of ethyl chloride, for example, this means that the section of tail to be removed should appear blanched, and should be placed in a pool of ethyl chloride at the time of cutting.

In the absence of firm data to support either the use of local or general anaesthesia for the removal of a tail biopsy at 3–4 weeks of age, the Working Group strongly recommends that local practices for tail biopsies are critically reviewed and that research is undertaken to assess the relative merits of using local or general anaesthesia for tail biopsies at this age.

It is reasonable to assume that there may be some degree of pain following the removal of a tail biopsy from 3–4-week-old mice, once the effects of either the local or general anaesthesia have worn off. Preliminary investigations have shown that young mice will drink carprofen-medicated water for 2 days after tail biopsy (D. Fleary, J. Kelly & D. B. Morton, personal communication). The use of pre-emptive analgesia should be considered as best practice. In most cases, a single injection of a low dose of an analgesic such as buprenorphine or carprofen may be sufficient. However, the Working Group recommends that the provision of analgesia, in terms of the type, dose and duration, are further investigated.

Over 4 weeks: The removal of tail biopsies from mice older than 4 weeks of age should be avoided because by this age the tail is fully ossified and there is greater risk of causing tissue trauma and pain. Where the removal of a tail biopsy is absolutely unavoidable, general anaesthesia using inhalation agents such as halothane should always be used, since suitable anaesthesia will be extremely difficult to achieve using local anaesthesia. Pre-emptive analgesia should also be provided to minimize any subsequent pain that may arise. If any significant haemorrhage occurs, haemostatic measures such as thermal cautery under general anaesthesia or a tissue adhesive should be used.

Recommendations:

- **The removal of a small section of tail, at any age, is likely to cause pain. This should be minimized by the use of appropriate anaesthesia and analgesia.**
- **The most humane age to perform tail biopsies in mice is between 3 and 4 weeks**

of age. Unless there is good scientific justification to the contrary, tail biopsies should not be taken from mice significantly younger than 3 weeks old or older than 4 weeks.
- **In-house practices for tail biopsies should be regularly reviewed to determine whether the type, dose and duration of anaesthesia and analgesia used are effective.**

15.3 Ear notching/punching

Ear notching can be used as a source of tissue for genotyping, particularly where PCR is used in the subsequent analysis (D. Tucker, personal communication; Ren *et al.* 2001). Experience suggests that the PCR results obtained are cleaner and more reproducible than those obtained from tail biopsy material (M. Maconochie, personal communication). Moreover, using a sample of ear tissue of approximately 2 mm in diameter, it is possible to routinely perform at least 50 PCR reactions (M. Maconochie, personal communication). From a welfare standpoint, ear notching is far preferable to tail biopsies as less tissue is removed, the pinna is entirely cartilaginous and the risk of haemorrhage occurring is minimal. Ear notching can also serve the dual purpose of being used to distinguish individual litter-mates.

Ear notching/punching can be performed using either an ear punch or fine-tipped straight scissors to remove a sample of tissue, of approximately 2 mm in diameter, from the perimeter of the pinna where the tissue is thinnest. Care should be taken to avoid tearing the pinna.

Ear notching/punching should not be used in mice of less than 2 weeks of age because the removal of even a small piece of tissue can represent a significant portion of the pinna. Moreover, the small size of the pinna makes the removal of tissue difficult and the procedure may thus be more stressful for the mice.

Recommendations:
- **Ear notching should be used as an alternative to tail biopsies, particularly where PCR is used in the subsequent analysis.**
- **Ear notching/punching should not be carried out in mice less than 2 weeks of age because the removal of even a small piece of tissue can represent a significant proportion of the pinna.**

15.4 Blood

Sufficient DNA for PCR analysis can be obtained from small blood samples of 20–50 μl obtained by venepuncture of the tail vein or other superficial veins. When performed competently blood sampling should cause minimal stress to the mice. It also has the further advantage of being easily repeatable.

There is a well established series of techniques for tail bleeding in mice which include venepuncture of the lateral or ventral tail veins, nicking of lateral tail veins with a scalpel blade, and removal of the distal 1 mm of the tail with consequent collection of the resulting blood drop (Diehl *et al.* 2001). Use of the latter technique should be avoided in mice that have previously had a tail biopsy.

Whichever method of blood sampling is used, no more than 10% of the total blood volume should be taken at any one time and no more than 15% in a 28-day period. The removal of blood is discussed in detail in Morton *et al.* (1993a).

The removal of blood for genotyping, from mice less than 2 weeks old should be avoided because of the risk of hypovolaemic shock—at this young age 50 μl of blood represents 10% of the total blood volume. Where it is absolutely necessary to take a blood sample extreme care should be taken to remove only a small volume of blood and to control haemorrhaging.

Recommendations:
- **20–50 μl of blood should provide sufficient DNA for genotype analysis by PCR.**
- **No more than 10% of the total blood volume should be taken at any one time and no more than 15% in a 28-day period.**
- **Blood should not be taken for genotype analysis from mice less than 2 weeks of age.**

15.5 Toe amputation

Toe amputation is likely to cause pain and may impair the mouse's ability to grip

and groom. It must not be routinely used to identify mice or as a source of tissue for genotyping. Very rarely, however, its use may be unavoidable—for example where there is good scientific justification for identifying and genotyping mice, housed in isolators, that are less than 14 days old. In such circumstances, toe amputation may be the only practical way to identify the mice, given their small size and the possibility of compromising the biosecurity of the barrier. Toe amputation should only be used as a last resort where no other less invasive method is available. In such circumstances only a single toe should be removed from one hind paw, using local anaesthesia. The excised toe should be used as a source of DNA for genotyping and mice must not be subjected to an additional procedure for biopsy purposes.

Toe amputation must not be used in mice older than 14 days that are housed in isolators, as other methods such as ear notching are available to identify mice and provide tissue for genotyping.

Note: The RSPCA is opposed to many of the procedures such as toe amputation that are described in this report.

Recommendation:

- **Toe amputation must not be used except as an absolute last resort.**

16 Identification of GM mice

The identification of individual GM or potentially GM mice is necessary for many research and breeding programmes. Careful consideration should be given to the method of identification which allows individual mice to be easily recognized and cross-referenced; for example, with tissue biopsy samples taken for genotype analysis. The age of the mice is an important consideration when deciding on the method of identification, as summarized in Table 6. Non-invasive methods should be used wherever possible. Where invasive procedures are unavoidable, care should be taken to minimize the likelihood of any pain or distress occurring by the use of anaesthesia and analgesia, and by ensuring that those carrying out the marking are competent to do so. The number of mice identified using invasive methods should be minimized by also using easily observable variations such as coat colour or sex to distinguish cage or litter-mates.

It may be necessary for scientific purposes or for welfare assessments to identify individual mice prior to weaning. In such circumstances, the use of invasive methods should be avoided. Care should be taken when marking neonates as disruption of the nest and handling of the pups may lead to rejection or cannibalism by the mother.

Recommendations:

- **Non-invasive methods of identification should be used wherever possible.**
- **Avoid using an invasive method to identify mice less than 3 weeks of age.**

16.1 Non-invasive methods of identification

Non-invasive methods of identification are temporary and should be checked regularly to ensure that markings are still visible, so that they can be repeated as necessary.

(i) Depending on the colour of the mouse, spirit-based pens can be used to apply a circular band at varying positions on the tail such that individual mice can be identified by the number and position of

Table 6 Choice of identification method depending on age of mice

	Less than 2 weeks	3–4 weeks	Over 4 weeks
Non-invasive methods	✓	✓	✓
Ear notching	✗	✓	✓
Ear tags	✗	✓	✓
Microchips	✗	✓	✓
Tattoos	✓	✓	✓

the bands. Non-toxic correction fluid, for example 'Tipp Ex' (BIC, Société BIC, France), can be used as an alternative to mark the tails of black mice.

(ii) Once hair has started to grow at approximately 10 days of age, histological stains such as malachite green can be used to mark a small area of the coat. Stains should be allowed to dry before returning the mouse to his/her cage mates. Generally such stains persist for approximately 2 weeks. The potential toxicity of any marking substance should always be considered prior to its use.

(iii) Mice can be identified by clipping patterns in the fur using fine scissors or clippers. Such marking will be visible for approximately 3 weeks.

Recommendation:

- **Regularly check that markings are visible.**

16.2 Invasive methods of identification

16.2.1 Ear notching

A series of notches on the outer edge of the ear, or small holes punched within the pinna can be used to identify a reasonable number of mice. Simple codes should be used to minimize the number of notches required to identify each mouse. Codes should be sufficiently robust to avoid confusion should the ear be damaged by fighting.

Care should be taken to avoid tearing the pinna. Ear notching can be performed using either an ear punch or fine-tipped straight scissors to remove a sample of tissue of no more than 1–2 mm in diameter. Due to the small size of the pinna, ear notching should not be carried out on mice younger than 2 weeks of age.

The excised ear tissue can be used as a source of DNA for genotyping (see Section 15.3).

Recommendations:

- **Minimize the number of notches required to identify each mouse by using simple codes.**
- **Ear notching should not be used in mice younger than 2 weeks of age.**
- **The excised ear tissue should be used as a source of DNA for genotyping wherever possible, thus avoiding subjecting the mouse to an additional procedure to remove biopsy material for genotyping.**

16.2.2 Ear tags

Mice can be identified by the insertion into the ear of an approximately 5 mm long metal tag marked with a predetermined code. The tag should be placed low in the pinna so that it rests against the mouse, and does not bend the ear, cause the mouse to hold his/her head in a lopsided manner, or catch on the cage. Care should be taken when applying ear tags to ensure that the pinna is not torn.

Ear tags may cause discomfort as a result of a local tissue reaction around the site of insertion. In some circumstances this may extend to malignancy. Mice should be regularly inspected for signs of inflammation or damage and alternative methods of identification used where necessary.

Recommendations:

- **Tags should be placed so that they do not bend the pinna, interfere with movement or catch on the cage side.**
- **Check for tissue damage or inflammation caused by the ear tag and remove if necessary.**

16.2.3 Microchipping

Small microchips can be implanted subcutaneously between the shoulders using a purpose-built implanter or trochar. Given the relatively large size of the needle, the microchip should be implanted using appropriate anaesthesia and analgesia.

The smallest microchip possible should be used to minimize any discomfort. Microchips should not be used prior to weaning because the relative size of the microchips to the pups may interfere with the mouse's posture and movement.

Recommendations:

- **Use the smallest microchip possible. Implant using appropriate anaesthesia and analgesia.**
- **Microchips should not be used prior to weaning.**

16.2.4 Tattoos

Depending on their coat colour, mice can be identified by tattooing the tail using either an electric tattooing gun or a fine gauge needle attached to a syringe containing ink. A local anaesthetic spray should always be applied prior to tattooing the tail.

Small numbers of mice can be identified by tattooing the footpad, or by injecting small dots of Indian ink subcutaneously using a 29-gauge needle. Although this technique may be carried out on conscious mice, inhalation anaesthesia is advisable for the purposes of restraint and to avoid distressing the animals. No more than one footpad per mouse should be tattooed.

In all cases, needles should be sterile and sharp.

Recommendations:

- **Local anaesthetic spray should always be applied prior to commencing tail tattooing.**
- **No more than one footpad should be tattooed on any mouse.**
- **All instruments should be sterile and sharp.**

16.2.5 Toe amputation

See Section 15.5.

17 Assessing the welfare of GM mice

Assessing the welfare of GM mice is an integral and central component of the scientific investigation of the phenotype. Genetic modification can result in morphological, physiological, biochemical and/or behavioural abnormalities that compromise animal welfare by causing, or predisposing mice to, pain, suffering, distress or lasting harm. Animal welfare can be affected even if the phenotypic effects of the genetic modification are subtle. Estimating the number of GM mice with deleterious phenotypes is difficult, although some researchers put the figure at less than 10% (K. R. Humphreys, personal communication). Regardless of the prevalence, it is important that GM mice are comprehensively assessed and monitored for indicators of suffering and reduced welfare so that any harm can be identified and minimized, humane endpoints for the mice determined, and housing and husbandry tailored to meet the specific requirements of the animals.

The effects of transgenesis on animal welfare are not wholly predictable and can depend on a range of factors including the nature of the genetic modification, the genetic background, the mouse's age, health status and environment, and whether the animal is homozygous or hemizygous for the transgene (e.g. Wolfer & Lipp 2000). Likewise, it is not possible to reliably predict the effect of crossing different transgenic lines as the actual phenotype obtained may not necessarily reflect what is observed in either of the two parental lines.

A number of studies have examined the welfare of selected transgenic lines (Costa 1997, Francis 1997, van der Meer *et al.* 1999). However, given the fact that the effects of transgenesis are variable, unpredictable and influenced by many factors, it is not possible to extrapolate the results of this limited number of studies to all GM mice. Rather, a best practice approach to welfare assessments should be adopted. General health and welfare assessments should commence at birth; continue through the mouse's entire lifespan and, where appropriate, for several generations; include a range of anatomic, behavioural, physiological and biochemical indicators; and involve scientists, veterinarians and animal technicians who have appropriate expertise. Where abnormalities are detected, assessments should be tailored accordingly and humane endpoints refined.

Assessing the welfare of neonates can be difficult not least because of problems associated with their small size. A simple system has been proposed (Lloyd *et al.* 2000) for assessing the welfare of mice in the first few days after birth using criteria such as colour, surface temperature, natural activity, reflexes/responsiveness to touch, and the presence or absence of milk in the stomach. The likelihood of the mother cannibalizing or rejecting the litter should be minimized

by ensuring that all litter-mates and not just selected mice are handled.

Many parameters can be used as indicators of welfare (e.g. Morton & Griffiths 1985, Dennis 2000). While actual assessments will vary, depending on the age of the mice, they should include observation and where appropriate objective measures of the following:

- Developmental abnormalities (e.g. cleft palate).
- Number of live births, pre- and post-weaning mortality.
- Suckling ability (e.g. presence of a milk spot).
- Litter size and mothering ability.
- Morphological characteristics (e.g. tooth development and overgrowth, skeletal abnormalities, skin colour and coat texture).
- Clinical signs of poor health (e.g. discharge from the eyes or nose; or diarrhoea).
- Movement and posture (e.g. hunched, gait, ataxia).
- Reproductive performance (e.g. libido and fertility).
- Behaviour and stereotypies (e.g. alertness, grooming, head weaving and circling).
- Immune status.
- Neurological parameters (e.g. elements of the SHIRPA test (Rogers *et al.* 1997)).
- Growth rates and body weight.
- Clinical biochemistry (e.g. glucose and protein levels).
- Microbiological status.
- Post-mortem analysis and histopathology of target tissues and primary organs.

Regardless of the parameter(s) used, for any assessment of welfare to be meaningful, equivalent observations and tests should be carried out in the background strain in order to establish baseline measurements with which to compare the GM mice. The phenotype may be affected by the animal's environment and husbandry (e.g. Carter *et al.* 2000) and mice used as controls should also be exposed to the same environmental conditions and husbandry practices as the GM mice. Similarly, *in utero* effects can influence some parameters and it is a good idea to use non-GM litter-mates as controls. For long-term comparative assessments, it is advisable to select a small representative group of GM and wild-type mice rather than to attempt to assess the whole colony.

The results of all analyses should be meticulously recorded and regularly evaluated, for example using scoring systems (e.g. Mertens & Rulicke 2000, van der Meer *et al.* 2001), and reported to those involved in using and caring for the mice as well as those involved in regulating the research, so that appropriate actions can be taken where necessary. Where GM mice are distributed to other establishments, they should be accompanied with information regarding the nature of the phenotype and any specialist care required.

Care should be taken when altering the environment of GM mice, as they can respond unexpectedly and differently to the wild-type strain. For example, mice carrying a null mutation in the gene for the neuropeptide galanin die if the temperature of the animal room is not maintained at normal levels (R. J. Francis, personal communication).

Recommendations:

- **The health and welfare of GM mice should be thoroughly monitored to identify any abnormalities in order that action can be taken to minimize any suffering, to ensure housing and husbandry is provided which meets the specific requirements of these animals, and that humane endpoints are appropriate.**
- **General health and welfare assessments should commence at birth; continue throughout a mouse's entire lifespan and, where appropriate, for several generations; and include a range of indicators. Where abnormalities are detected more detailed investigations should be undertaken.**
- **Results of all welfare assessments should be recorded and disseminated to all relevant persons involved in using and caring for the mice, together with those involved in regulating the research.**
- **Information regarding the nature of the phenotype and any specialist care required should be provided to the relevant personnel (scientists, and animal care and**

veterinary staff) whenever GM mice are distributed to other establishments.

18 Reducing the number of surplus mice

Every effort should be made to minimize the number of 'unused' or 'surplus' mice that arise from the production of mice for embryo manipulation or from the subsequent establishment and maintenance of GM lines. The careful management of breeding colonies is a primary factor in achieving this. Responsibility for maintaining the colonies may not necessarily lie with those who are using the mice, and it is critical that there are good lines of communication so that supply of, and demand for, mice can be closely coordinated.

The production of some surplus mice, for example those that fail to integrate the transgene following pronuclear microinjection, is an intrinsic and unavoidable characteristic of the technology. These mice should be used as experimental controls rather than as breeding animals specifically for this purpose.

18.1 Surplus mice arising from embryo manipulation

Matching the supply of embryo donors or recipients to demand can be difficult, particularly where the production of GM mice is intermittent. Consideration should be given as to whether it is more efficient to maintain in-house colonies or to purchase females as and when required from a commercial supplier. Where females are produced in-house, using the same strain for both embryo donors and recipients can help reduce the numbers of mice bred. Inevitably, large numbers of males are produced in the breeding of the females and these should be used for other scientific purposes, as stud males, or following vasectomy for the generation of pseudopregnant females.

Where there are surplus freshly fertilized embryos available at the end of a microinjection session, these should be used for testing the quality of the media, or cryopreserved and subsequently used for microinjection training purposes or media testing.

Recommendations:

- **Careful management of colonies is required in order to match supply to demand and to avoid the production of surplus mice.**
- **To avoid wastage, use mice that have not integrated the transgene as controls or for the provision of tissues.**
- **Use surplus males for other scientific purposes, as stud males, or vasectomize and use for the production of pseudopregnant females.**

18.2 Surplus mice arising from the establishment and maintenance of GM lines

Having generated GM founders by pronuclear microinjection or chimeras by gene targeting in ES cells, it is necessary to establish a GM line; and this in itself may result in the production of surplus mice which are not of the required genotype. Provided that adverse effects on animal welfare are not observed or compounded in the homozygous state, GM mice should be maintained as homozygotes wherever possible, as this prevents the production of surplus mice that are not of the desired genotype. Scientific objectives should be regularly reviewed so that GM lines that are presently not required or are subject to sporadic use can be archived by cryopreservation of gametes, embryos or ovarian tissue rather than from being maintained as 'tick-over' colonies (see Section 19).

There may be scientific reasons for requiring the genetic heterogeneity of a GM line to be minimal. In such circumstances, it is necessary to use the inbred strain of choice as embryo donors, or to establish a congenic line by breeding the transgene onto an inbred background. In deciding which is the most appropriate strategy, it is important to consider the overall numbers of mice that are anticipated to be required and wherever possible to use the method requiring the minimum number.

The number of generations that are required to produce a congenic strain may be significantly reduced using 'speed congenics' (Markel *et al.* 1997, Wakeland *et al.* 1997). Speed congenics uses microsatellite markers

to follow the inheritance of the genomic sequences of interest. In this way, it is possible to select only mice with the desired genotype for breeding. The overall number of mice that are used, however, may not always be reduced, as large cohorts may have to be analysed at each generation in order to select appropriate mice for breeding.

Recommendations:

- **Regularly review scientific objectives so that GM lines subject to sporadic use, or which are no longer required, can be archived by cryopreservation where possible rather than maintained as 'tick-over' colonies.**
- **As long as an adverse phenotype is not observed, or compounded in the homozygous state, GM lines should be maintained as homozygotes.**

19 Cryopreservation

The cryopreservation of gametes, early stage embryos and ovarian tissue can be an important aid in the management of GM mice and associated scientific programmes. Many GM lines are subject to sporadic use and their maintenance by continuous breeding can result in the production of surplus mice. Cryopreservation, therefore, provides the opportunity to archive GM lines until required, and in addition, provides a strategic reserve in case of genetic contamination or 'drift', pathogenic infection, and natural disasters. Cryopreservation can also be used to avoid the potential logistical, and animal health and welfare problems associated with the transport of live mice; and wherever possible GM mice should be distributed as frozen embryos or gametes. Techniques for the cryopreservation of mouse germplasm and the subsequent re-establishment of GM mice are well established (e.g. Glenister & Rall 2000).

Decisions as to whether to cryopreserve embryos or gametes depend on a number of factors including the strain, the specific requirements of the research programme, and the scientific 'value' of the mice. Reliable records of the cryopreservation protocol used, and the location and genotype of each sample, are essential. There may be circumstances where it is necessary to re-determine the genotype of cryopreserved embryos or gametes. Clearly, it would be wasteful of mice to re-establish a line only to find that it was not the desired genotype. It is, therefore, recommended that for each line archived, a sample of tissue (e.g. liver) is stored which can be used as a source of DNA for genotyping if necessary.

Prior to any cryopreserved GM line being re-established, it is essential to ensure that consent is obtained from the appropriate institutional and regulatory authorities.

Recommendations:

- **Cryopreservation of embryos and gametes should be used to archive GM lines that are presently not required or subject to sporadic use, and to protect against genetic drift, disease and natural disasters.**
- **GM lines should be distributed as frozen embryos or gametes wherever possible in order to avoid the animal health and welfare problems associated with the transport of live mice.**
- **For each line archived, it is a good idea to freeze a sample of tissue (e.g. liver) which can be used as a source of genomic DNA for genotyping if necessary prior to re-establishing the line.**

19.1 Cryopreservation of embryos

The number of embryos frozen will depend on the scientific and operational objectives, including whether the embryos are homozygous or heterozygous for the transgene. In general, 300–500 embryos of a single GM line should provide an effective archive for most situations. It is advisable to freeze embryos at the eight-cell stage as they are more robust than earlier stage embryos and are more likely to survive the freezing and thawing process. The production of a sufficient number of embryos for cryopreservation may require superovulation and care should be taken to avoid the mating of undersized females as discussed in Section 11.1.1. For some GM strains, natural matings may be preferable and thought should be given to the most appropriate time to freeze the line so that additional breeding programmes, to

provide a sufficient number of embryos, are avoided.

The efficient recovery of cryopreserved embryos is dependent on the strain. The viability of the frozen embryos should be assessed by transferring a small number of thawed embryos into one or two foster mothers to confirm that the embryos are capable of development to either live birth or late fetal stages. Once the success of the embryo cryopreservation has been confirmed, it is not necessary to routinely recover the GM line as the embryos should remain viable indefinitely. To prevent embryo recipients being used unnecessarily when re-establishing a GM line, it is a good idea to culture thawed embryos *in vitro* to the blastocyst stage so that only 'healthy' embryos are selected and transferred into recipients.

Cryopreserved fertilized eggs can be used for pronuclear microinjection. Using five different DNA constructs, the efficiency of production with cryopreserved fertilized eggs has been shown to be close to that obtainable from freshly collected fertilized eggs (Leibo *et al.* 1991). Establishing a bank of cryopreserved fertilized eggs for pronuclear microinjection can help avoid problems of matching the supply of donor females to demand, and this may substantially reduce the number of females bred for this purpose (Topps & Bussell 1999, S. Topps, personal communication).

Any spare fresh eggs that are remaining at the end of a microinjection session should be cryopreserved and used for training purposes or for media testing.

Recommendations:

- **The viability of frozen embryos should be confirmed prior to culling the GM line.**
- **Spare fertilized eggs remaining at the end of a microinjection session should be frozen and used for training purposes or media testing.**

19.2 Cryopreservation of sperm and oocytes

The cryopreservation of sperm or oocytes from GM mice allows for both the archiving of lines and the rapid generation of novel genotypes without lengthy breeding programmes involving large numbers of mice.

Sperm cryopreservation presents the opportunity to substantially reduce the number of mice used to both store and re-establish a GM line. Sperm collected from a single male can potentially give rise to large numbers of offspring following IVF of oocytes collected from a minimal number of females. The success of sperm freezing is, however, strain-dependent. It is possible to cryopreserve and subsequently recover viable sperm for IVF from many outbred and hybrid strains. However, sperm from inbred strains such as C57BL/6 and 129 are sensitive to freezing, and this can significantly reduce their fertility post-thaw. Some laboratories have begun using techniques such as intracytoplasmic sperm injection, which use the sperm head and are not dependent on the sperm having normal physiological activity (Szczygiel *et al.* 2002).

Oocyte cryopreservation is less commonly used than either embryo or sperm freezing to archive GM mice. The freezing of a sample of wild-type oocytes can, however, be an invaluable tool in maintaining a GM line where the males have a poor reproductive performance as a result of old age, or die unexpectedly. In such cases, particularly where the sperm is not amenable to cryopreservation, sperm can be taken from the vas deferens and cauda epidymis of humanely killed males and used in conjunction with cryopreserved oocytes for IVF without having to wait for fresh eggs to become available. Live births following IVF have been reported using sperm recovered from dead refrigerated mice or dead mice kept at room temperature for 24 h (Songsasen *et al.* 1998).

In vitro fertilization is discussed in Section 20.

Recommendation:

- **The cryopreservation of gametes should be used as a reduction strategy to minimize the number of mice used for the archiving of lines, and for the rapid generation of novel genotypes.**

19.3 Cryopreservation of ovarian tissue

Ovary cryopreservation can be used to bank mouse germplasm when embryo or sperm freezing are not possible. Sztein *et al.* (1998)

have reported a successful combination of appropriate techniques for ovary cryopreservation coupled with half-ovary transplantation. Provided that elaborate breeding programmes are not required to ensure histocompatibility between donors and recipients, this approach provides the opportunity to reduce the number of mice required to guarantee gamete preservation, compared to when embryos are used. However, mice that have had an ovary transplant can take a significant length of time to conceive and may produce small litters. Thus, rederivation may require large numbers of transplants to be carried out. This procedure should only be used when the cryopreservation of embryos or gametes is not possible.

The use of ovary transplantation to maintain sub-fertile GM lines is discussed in Section 22.

Recommendation:

- **The cryopreservation of ovarian tissue should only be used where the freezing of embryos or gametes is not possible.**

20 In vitro fertilization

In vitro fertilization can be used for both reduction and refinement in the production and management of GM mice. Although developed in recent years primarily as a means to recover live born mice from frozen sperm, IVF of mouse oocytes can also be used for: rederivation; the rapid production of novel genetic combinations without the need for elaborate breeding programmes involving large numbers of mice; as an alternative to the mating of undersized females where their use would otherwise be unavoidable; and to rescue lines from males that are poor breeders or that die unexpectedly.

The success of IVF is dependent on both the strain and media used. It is generally very effective with outbred and hybrid strains, although it can be more difficult with some inbred strains. Some strains require specific formulations of media or supplements for good fertilization *in vitro*.

Protocols for IVF can be found in Glenister and Rall (2000). It is common practice to superovulate the females to increase the number of eggs obtained (see Section 11). Oocytes should be collected 12–14 h after hCG has been administered. If the eggs are too 'old' many will not fertilize or may undergo parthenogenic activation. Fresh sperm should be cultured for 1–2 h prior to adding the oocytes in order to allow the sperm to become competent for fertilization (i.e. the process of capacitation). Where cryopreserved sperm is used, a culture period is not necessary as the cooling and freezing/thawing process results in capacitation-like changes.

Recommendations:

- **Depending on the strain, IVF can be used for rederivation and breeding programmes, or to rescue lines where the males are poor breeders or die unexpectedly.**
- **Consider IVF as an alternative to mating where the use of undersized females would otherwise be unavoidable.**

21 Rederivation

Incidental microbial infection of GM mice should be avoided as it can compromise both animal welfare and the validity of the scientific data. This is best achieved by using SPF mice in the generation phases and by subsequently maintaining GM mice in appropriate husbandry systems. However, there are occasions where it may be necessary to eliminate the presence of an infectious agent; for example, when importing GM mice. The process by which this elimination is effected is known as rederivation.

Rederivation can be achieved either by hysterectomy and subsequent fostering of the pups, or by embryo transfer. There are animal welfare issues associated with both methods, and both involve the death of the 'biological' mothers of the pups. Embryo transfer requires surgery, which has the potential to cause pain and discomfort. With hysterectomy, there are the welfare issues associated with the timing of the hysterectomy, the resuscitation of the pups, and the subsequent fostering process. In terms of animal welfare, there is, therefore, little to separate the two methods. However,

from a biosecurity standpoint and the effective elimination of microbiological contaminants, embryo transfer is the preferred method for rederivation. While surface microbiological contaminants can be removed from the uterus, rederivation by hysterectomy may not be effective at eliminating microorganisms capable of trans-ovarian or trans-placental infection. Any laboratory that is already competent in transgenesis should have the equipment and expertise necessary for successful rederivation by embryo transfer.

For both rederivation by embryo transfer or hysterectomy, it is advisable to maintain the foster mothers and pups in bioexclusion husbandry systems until it can be proven, via health screening, that the process has been successful and that the pups are not infected with the target microorganism(s).

Recommendation:

- **Rederivation by embryo transfer or hysterectomy can affect animal welfare. From a biosecurity standpoint, rederivation by embryo transfer is recommended in preference to that by hysterectomy.**

21.1 Rederivation by embryo transfer

For rederivation by embryo transfer, frozen or freshly collected embryos (up to the eight-cell stage) are washed thoroughly in sterile embryo culture medium to remove any microbiological contaminants derived from the maternal tissue fluids. The embryos are then transferred into the oviducts of a pseudopregnant female, of appropriate health status, under general anaesthesia; and provided that implantation occurs, the embryos should develop to full-term. The effects of surgery on animal welfare should be minimized through the use of appropriate perioperative care and analgesia as detailed in Section 10. The number of transferred embryos resulting in live births should be greater than when using microinjected embryos, and the number transferred should reflect this. The actual number will depend on the strain of the recipient, and the stage and strain of the embryos.

It may be necessary to superovulate GM females used for rederivation by embryo transfer. In some cases it may not be possible to establish optimum conditions for superovulation, and this can result in the use of very small females that may be harmed during mating. In such circumstances, the possibility of fertilizing the eggs *in vitro* should be investigated as an alternative to mating. The embryos thus produced can then be washed and transferred into a recipient female. Similarly, depending on the strain, IVF using sperm collected from a single male of the line to be rederived can be used to generate large numbers of offspring.

21.2 Rederivation by hysterectomy

For rederivation by hysterectomy, a pregnant female is humanely killed just before the fetuses reach full-term and the uterus is removed intact. The uterus is then washed in a solution of warm disinfectant to remove any microbiological contaminants that may be present in the maternal tissue fluids. It is then opened and the fetuses are removed and resuscitated by gently rubbing with a cotton bud until breathing commences. The survival of the fetuses is very much dependent on the timing of the hysterectomy relative to the expected date of parturition, and it is vital that the fetuses are as close to full-term as possible. Good record keeping of timed-matings and an accurate knowledge of the gestation time of the strain used are vital.

The resuscitated pups are subsequently fostered with a lactating female whose own pups have been removed. Normally, there should be a minimum of three foster mothers available that are of a strain with good mothering ability, of appropriate microbiological status, and that are lactating well. It is advisable to use foster mothers that have given birth in the previous 12–24 h; however, females up to 48 h *post-partum* can be used where necessary. Preferably, the foster mother's own pups should have a different coat colour to the transferred pups. This avoids any confusion arising from a failure to remove all of her own offspring or late births. Care should be taken when

substituting the foster mother's pups to prevent her rejecting the transferred progeny.

Recommendations:

- **The survival of the pups is dependent on them being as close to full-term as possible. Good record keeping of timed-matings and an accurate knowledge of the gestation time of the strain are essential.**
- **Foster mothers should have good mothering ability; be lactating well and of the appropriate health status; and preferably have given birth within the previous 12–24 h.**

22 Ovary transplantation

Orthotopic ovary transplantation can be used to maintain sub-fertile GM lines in cases where the phenotype may be lethal before puberty or where females may be unable to carry embryos to full-term. The procedure should not be used to maintain transgenic lines that are poor breeders.

Ovary transplantation requires surgery under general anaesthesia, and every effort should be taken to minimize the effects on animal welfare through the use of appropriate perioperative care and analgesia (see Section 10). The usual procedure is to transplant one-half of an ovary unilaterally, into the empty bursal cavity of a histo-compatible ovariectomized female. Thus one donor serves four hosts. Residual pieces of recipient ovary can hypertrophy and function normally. Consequently, mating systems should be designed so that offspring of the donor and recipient ovaries can be distinguished by coat colour or other genetic markers.

Recommendation:

- **Ovary transplantation should only be used as a last resort to overcome severe fertility problems. It should not be used as a routine procedure to maintain transgenic lines that are poor breeders.**

23 The production of GM rats

The principles of best practice set out throughout this report also apply to GM rats produced by pronuclear microinjection. There are, however, a number of specific factors, relating to superovulation and embryo transfer that should be considered for the generation of GM rats.

23.1 Production of fertilized eggs

The superovulation of rats presents additional technical challenges to that with mice in terms of the timing and route of gonadotrophin administration. A number of different strains have been superovulated including the inbred Fischer F344 and Lewis, and the outbred Wistar and Sprague-Dawley (SD) strains. As with mice, it is important to optimize the size of the young females to minimize the likelihood of them being harmed by mating with large stud males. Normally, female rats below 125 g should not be used. The use of sexually mature females should be investigated as an alternative to prepubescent females.

A number of superovulation protocols have been established for rats as shown in Table 7. The number of usable embryos may not be increased by administering high doses of gonadotrophins. High doses of PMS can lead to a variable ovulatory response and abnormal embryo development (Walton *et al.*

Table 7 Examples of superovulation protocols for production of genetically modified rats

Strain	Superovulation	Egg yield	Reference
SD-WKY	FSH(mini-pump)/LH-RH	60–85	Mullins *et al.* (1990)
Lewis/Fischer 344	FSH(mini-pump)/LH-RH	NR	Hammer *et al.* (1990)
Wistar	PMS (20 I.U.)/hCG (20 I.U.)	40	Hochi *et al.* (1990)
Wistar	PMS (20 I.U.)/hCG (7.5 I.U.)	50–80	K. E. Mathers (unpublished)
Wistar*	PMS (150 I.U./kg)/hCG (75 I.U./kg)	28–78	Mukumoto *et al.* (1995)
SD	PMS (40 I.U.)/hCG (5 I.U.)	NR	Charreau *et al.* (1996)

*Adult rats. NR = not reported

1983, Yun *et al.* 1987, Armstrong & Opavsky 1988, Charreau *et al.* 1996), and thus the females may be used unnecessarily. Hormones can be administered either by intraperitoneal injection or continuously via an osmotic mini-pump implanted subcutaneously (see Morton *et al.* 2001). Surgery is required to implant the mini-pump and, wherever possible, this should be avoided by administering the gonadotrophins intraperitoneally. Care should be taken to avoid damaging the abdominal viscera when injecting intraperitoneally (see Morton *et al.* 2001). Where the use of osmotic mini-pumps is absolutely unavoidable they should be of an appropriate relative size, and the impact of surgery on animal welfare should be kept to an absolute minimum by the use of appropriate general anaesthesia and analgesia (see Section 10).

Stud males can be used from 10 weeks of age. Care should be taken to avoid using over-large or aggressive males. On the morning following mating, the females should be examined for the presence of a vaginal plug. In some strains, vaginal plugs are not readily retained and it may be desirable to confirm mating by examining vaginal smears for sperm (Szabo *et al.* 1969). Alternatively, rats can be kept on a gridded floor overnight and the tray examined for plugs the next morning. Up to 80 embryos can be harvested for pronuclear microinjection from one female. Rat embryos do not culture well *in vitro*, and embryo-tested media should be used.

Recommendations:
- **Female rats below 125 g should not normally be used.**
- **Do not use over-sized or aggressive males.**
- **Wherever possible, administer gonadotrophins by intraperitoneal injection rather than using an osmotic mini-pump.**

23.2 Embryo recipients

Pseudopregnancy is not as stable in rats as it is in mice and a lower percentage of females will actually carry the embryos to full-term. Suitable recipients include Wistars, AS and Sprague-Dawleys.

Embryo transfer in rats is similar to that in mice, but there are differences. These are as follows:

(i) The rat's ovarian bursa is highly vascularized and significant bleeding can occur when it is opened. Bleeding should be minimized by applying a droplet of a vasoconstrictor such as epinephrine (adrenaline) to the ovarian bursa prior to accessing the infundibulum.

(ii) The presence of a microbial infection, even at sub-clinical levels, can affect the success of pregnancy in the rat and it may be necessary to administer antibiotics following embryo transfer (Mullins *et al.* 1997).

Recommendations:
- **Minimize bleeding from the ovarian bursa by applying a vasoconstrictor prior to accessing the infundibulum.**
- **It may be necessary to administer antibiotics to embryo recipients to optimize the success of pregnancy.**

24 Transport of GM mice

Increasingly, large numbers of GM mice are transferred between research establishments, nationally and internationally. Transporting live mice can cause considerable distress and this can be compounded in some GM lines by the nature of the phenotype. Wherever possible, the transport of live mice should be avoided through the use of fresh embryos, or cryopreserved embryos or gametes. Fresh embryos should remain viable for 24 h provided that they are stored in buffered embryo culture medium.

Where live mice are transported, every effort should be made to ensure that they are not subjected to environmental stresses. Live animals should only be transported by approved animal couriers and according to the guidelines set out by the Laboratory Animals Breeders Association (LABA) and the Laboratory Animal Science Association (LASA) (1993). There are laws regulating the movement and importation of animals, embryos and gametes, and due regard should

be paid to these and approval secured from the relevant authorities (e.g. in the UK these are the Department for the Environment, Food and Rural Affairs, and the Home Office).

Confirmation should be given by those sending the mice that they have been despatched and those receiving the mice should confirm receipt. All GM mice, or fresh or cryopreserved embryos, should be accompanied by information detailing the microbiological status, the nature of the phenotype and any specialist management the mice require in terms of housing, husbandry and veterinary care. These arrangements should be discussed prior to the transport of the mice so that appropriate measures are in place. Prospective discussions should include all of those involved in using and caring for the mice.

Recommendations:

- **The transport of live mice should be replaced, wherever possible, by the use of fresh embryos, or cryopreserved embryos and gametes.**
- **Live mice should only be transported by approved animal couriers and in accordance with LABA/LASA guidelines.**
- **The nature of the phenotype and any specialist care required should be discussed prior to the transport of the mice, and detailed in written information accompanying the GM mice.**

25 Summary

Despite the extensive use of GM mice, relatively little has been published regarding applying the principles of reduction and refinement to their generation, management, and care. This reflects in part the nature of the technology—the large numbers of mice used both in the generation and breeding of GM mice that are of scientific 'interest', the surgery and other potentially painful procedures that are involved, and the difficulties associated with predicting the effects of genetic modification. While these factors represent significant hurdles, progress has also been limited by a failure to consider, implement and disseminate best practices wherever possible. With such considerations in mind, the Sixth BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement set out to identify and document the areas of concern and how they might be addressed. In doing so, the Working Group has identified current best practices that should ensure the number of mice used is kept to a minimum and their welfare improved, without compromising scientific objectives. The main recommendations are summarized below.

25.1 Refinement

Proper design of constructs and choice of animals can refine the production of GM mice. The use of inducible promoters and conditional transgenes can minimize the effects of the genetic modification on animal welfare (see Section 3). The use of SPF mice prevents incidental infection with pathogenic microorganisms and thus avoids the possibility of clinical disease and death (see Section 8).

Breeding and husbandry considerations can refine methods in the production, maintenance and transport of GM mice. Optimizing the size of prepubescent egg donors and the careful selection of stud males can help prevent the females being harmed during mating (see Sections 11.1.1 and 12). Pseudopregnant females receiving embryos from the same microinjection experiment can be housed together after embryo transfer, thus avoiding having to keep social animals singly (see Section 13.4). In this case, the females will help each other raise the joint litter. The distribution of cryopreserved embryos, sperm or ova, rather than live mice, avoids the potential welfare problems associated with transport (see Section 24).

Refinements in surgical techniques are possible. Vasectomies performed by a scrotal sac incision rather than laparotomy are a refinement as they avoid cutting the abdominal body wall musculature and are, therefore, likely to be less painful (see Section 14.1). The use of genetically sterile males as an alternative removes the need to subject males to surgical vasectomy (see Section 14.2). This refinement, however,

conflicts with the principle of reduction since a separate colony of mice has to be maintained. The surgery required for vasectomy and embryo transfer can cause pain. Recognizing subtle indicators of pain can be difficult in mice and it is recommended that a precautionary approach is adopted for the management of pain, with pre-emptive analgesia being given to all mice undergoing invasive procedures (see Section 10.4).

The removal of biopsies for genotype analysis can cause pain, suffering and distress. It is important that the least invasive method is used and the size of the biopsy taken is kept to an absolute minimum (see Section 15). The screening system used to distinguish GM from non-GM mice should seek to minimize the amount of tissue required. Southern blot hybridization requires more DNA and concomitantly more tissue than analysis by PCR, and therefore the use of PCR for genotyping should always be considered. Tail biopsies are commonly used as a source of tissue for genotyping. The removal of even a small section of tail is likely to be painful and the use of more humane sources of biopsy materials, for example, ear tissue, and oral, faecal or blood samples, should be investigated as alternatives, particularly where the intention is to use PCR (see Sections 15.1, 15.3 and 15.4). Where the use of tail biopsies is unavoidable, no more than 5 mm of tail should be taken and appropriate anaesthesia and analgesia should be used. Unless there is scientific justification to the contrary, tail biopsies should not be taken from mice significantly younger than 3 weeks of age or older than 4 weeks of age (see Sections 15.2.1 and 15.2.2).

Mice should be marked using non-invasive methods, where possible. Readily observable variations such as sex and coat colour should be included in order to minimize the number of mice that require marking (see Section 16).

Genetic modification can have a deleterious effect on animal welfare. All GM mice should be carefully monitored so that appropriate action can be taken to minimize any harm, to develop humane endpoints and to ensure that husbandry and care are optimal (see Section 17).

25.2 Reduction

Unnecessary production and use of GM mice should be avoided. This requires a thorough search of subject-specific and specialized databases such as the Trangenic/Targeted Mutation database (TBASE, http://tbase.jax.org/), and cryopreservation banks, to ensure that the GM mice are not already available. Comprehensive searches are required to determine whether the transgene is suitable in terms of its promoter specificity and potential levels of expression (see Section 4).

The design of the transgene, in addition to the strains used for generating a new GM line, requires careful consideration in order to minimize the production of unwanted mice. Inclusion of insulator or intronic sequences in the transgene can help avoid the effects of random transgene integration, thus increasing the likelihood of producing scientifically 'informative' GM mice (see Section 4.2). Selection of an appropriate strain as a source of eggs for microinjection can reduce the overall number of mice used to generate a line (see Section 11.1.2). The selection of males with high and consistent plugging rates should minimize the numbers of mice required to generate pseudopregnant females (see Section 14). Careful choice of the strain of host blastocysts increases the likelihood of obtaining germline transmission of the ES cell genome (see Section 5).

The number of animals used to produce GM mice can be minimized by ensuring that all staff are given appropriate training and have the specialist skills and knowledge required (see Section 6). Monitoring the efficiency of transgenic production by using benchmarks figures as an indicator of performance should identify where remedial action is necessary (see Section 6.3). A good understanding of laboratory animal science and husbandry practices are essential for the careful management of colonies, to match supply to demand, and to avoid the production of surplus mice.

Cryopreservation of gametes, embryos and ovarian tissue provides the opportunity

to archive transgenic lines until required, and thus avoids the potential wastage associated with their maintenance by continuous breeding (see Section 19). The cryopreservation of sperm is itself a reduction initiative as it substantially reduces the numbers of mice required to store and regenerate a transgenic line compared with the cryopreservation of embryos (see Section 19.2).

Provided that no adverse welfare problems have been identified, homozygotes should be bred (see Section 18). Such breeding programmes prevent the production of unwanted genotypes, as well as negate the need to genotype and identify mice (a refinement). Care should be taken, however, as the random integration of transgenes can cause mutations with no phenotypic effects in heterozygotes but which lead to poor welfare in homozygotes.

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Appendix A Glossary

Allele: One of several alternative forms of a gene occupying a given locus on a chromosome.

Bioexclusion: Strategies to prevent the introduction of unwanted microorganisms.

Biosecurity: General term for measures to control the transmission of microorganisms into or out of a specified area or population.

Blastocyst: An early stage embryo that consists of a ball of cells enclosing a fluid filled cavity.

cDNA: A DNA copy of a mRNA.

Chimera: An animal that is a mixture of cells from two different embryonic sources.

Chromatin: Complex of DNA and protein that make up the chromosomes.

Conditional transgenic technology: Allows gene targeting to be restricted to certain cells or tissues, or to occur in response to an exogenous induction system.

Congenic: An inbred strain that contains a small genetic region from another strain, but which is otherwise identical to the original inbred strain.

Construct: A piece of artificially created DNA containing the transgene of interest.

Copy number: The number of copies of the transgene integrated into the host genome.

Cryopreservation: The process of freezing cells or tissue at very low temperatures that ensure the viability of the material is maintained.

Donor females: Females used as a source of fertilized eggs or blastocysts for microinjection.

Ectopic expression: Expression of a gene that does not correspond with its normal temporal or spatial pattern.

Embryonic stem cells (ES cells): Cells derived from blastocyst stage embryos which can differentiate into any type of cell when incorporated into a host blastocyst.

Enhancer: DNA sequences that regulate the expression of a gene.

F1 hybrid: The first generation produced by the crossing of two different parental strains.

Genotype: The genetic constitution of the organism.

Germline: Spermatozoa or eggs (ova).

Founders: Animals of the first generation, arising from a transgenic experiment, that are proven to have the transgene of interest.

Gonadotrophins: Hormones released by the anterior pituitary gland, which stimulate growth of the gonads and release of sex hormones.

Hemizygous: Having a gene present in only one copy in a diploid cell.

Heterozygous: Having different alleles at corresponding loci on homologous chromosomes.

Histocompatibility: The state where due to genetic identity, grafts between different animals do not stimulate immunological rejection.

Homozygous: Having the same allele at corresponding loci on homologous chromosomes.

Homologous recombination: Reciprocal transfer of information between DNA sequences that have a high degree of similarity.

Hybrid vigour: The phenomenon where the offspring arising from a mating of two different strains exhibit better physiological performance than that of their parental strains.

Inducible transgenes: A method of gene targeting that allows the activation or inactivation of the target gene to be temporally and spatially controlled by an exogenous induction signal.

Intron: A segment of DNA that is transcribed but subsequently removed from the transcript.

Isogenic: Having the same genotype.

Karyotype: The entire chromosome complement of a cell or species. It is characterized by the number, size and configuration of the chromosomes.

Knock-in: The introduction by gene targeting of DNA sequences at a specific locus.

Knock-out: A mutation in which the target gene is inactivated.

Laparotomy: Surgical incision into the abdominal cavity.

Microinjection: Process by which, using a fine needle, DNA or ES cells are injected into fertilized eggs or blastocysts.

Mosaic: An individual consisting of cells of two or more genotypes.
Nuclear transfer cloning: Transfer of a nucleus into an enucleated egg cell.
Null mutation: The complete elimination of the function of a gene.
Ovarian bursa: The thin layer of tissue that encloses the ovary.
Ovariectomy: The surgical excision of one or both of the ovaries.
PCR: The polymerase chain reaction is a technique that enables the *in vitro* amplification of target DNA sequences.
Phenotype: The physical manifestation of the genotype.
Plasmids: Bacterial, autonomous, self-replicating, extrachromsomal circular DNA molecules.
Pluripotent: Refers to cells that give rise to more than one type of differentiated cell.
Position effect: The expression of a gene can be influenced by its position in the genome and the sequences that surround it.
Promoter: The region of DNA involved in the initiation of transcription.
Pronucleus: Either of the two haploid gamete nuclei, just prior to their fusion in the fertilized egg.
Pseudopregnant: A term used to describe females that have been mated with sterile males. The stimulus of mating results in physiological conditions appropriate for implantation and the maintenance of pregnancy.
Recipient females: Pseudopregnant females into which fertilized eggs and blastocysts are transferred to continue their development.
Southern blot hybridization: The transfer of DNA from an agarose gel to nylon or nitrocellulose membrane. Once immobilized on the membrane, specific sequences are identified by hybridizing with labelled DNA probes.
Superovulation: Administration of gonadotrophins to females to increase the number of eggs that are ovulated.
Transfection: The incorporation of exogenous DNA into eukaryotic cells.
Transgene: Integrated sequences of exogenous DNA.
Transgenic: Genetically modified or transgenic refers to cells or organisms containing integrated sequences of cloned DNA transferred using techniques of genetic engineering.
Transient transgenics: Microinjected embryos following re-implantation but prior to birth.
Vaginal plug: A visible mass in the vagina, which develops *post-copulation*, following the coagulation of seminal proteins.
Vector: A plasmid used to 'carry' foreign DNA.
Wild-type: The phenotype (or allele) that is considered to be the 'normal' type.

Appendix B Useful websites

The following databases provide information on existing strains of genetically modified mice.

- The Transgenic/Targeted Mutation Database: http://tbase.jax.org/
  Contains a database of genetically modified mice and a glossary of technical terms.
- Transgenic and Targeted Mutant Animal Database: http://www.ornl.gov/TechResources/Trans/hmepg.html
  Contains a database for genetically modified mice, a system for naming transgenics and links to other useful sites.
- Andras Nagy's Cre Transgenic Database: http://www.mshri.on.ca/nagy/cre.htm
  Contains a list of Cre transgenic lines with details of contacts and references for each line.