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Gene Knockout Protocols

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Overview

Gene Knockouts

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1. Introduction

The ability to generate a mouse with a targeted mutation in a desired gene has been one of the most important advances in understanding the function of gene products. Not only can gene disruption demonstrate the function of genes, but by disrupting cell development or survival it can also demonstrate the function of specific cell types. Some knockout mice are murine models of human genetic diseases and have proven that a single gene defect is capable of causing disease. In addition, the crossing of mice that are heterozygous or homozygous for specific gene mutations provides important information on human diseases that are caused by multiple genetic defects. In genetic terms, targeted gene disruption is directly complementary to (and arguably more direct than) using mutant strains of mice with a known phenotype to map and identify the disease-causing genes.

The major benefit of gene knockout technology is that it enables the analysis of the function of a protein produced from a specific gene in vivo. Thus, the function can be determined in all normal cell types and the complex interactions between molecules and/or between cells are taken into account. In mice with a targeted mutation, it is possible to determine the function of gene products in the "resting" state of the body, in different physiological states and also in various pathological conditions. Thus, gene knockouts have generated important lessons for all areas of biomedical research and opened new doors to the potential future of medicine.

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2. Historical Perspective

Pioneering work during the 1980s in the areas of the molecular biology of homologous recombination; the derivation, culture, and manipulation of embryonic stem (ES) cell line, and micromanipulation/microinjection of embryos, together resulted in the first knockout mouse line over ten years ago. Key work included the demonstration that mammalian somatic cells could mediate homologous recombination ***(1,2)***. During this time, embryonic stem cell lines were generated from murine blastocysts ***(3,4)*** and shown to generate germline chimeras even after extensive manipulation and selection in vitro ***(5,6)***. This work enabled the first homologous recombination experiments to be performed in murine ES cells, the targeting of the *hprt* gene ***(7,8)***. Since that time, this technology has been applied in various forms to generate null mutations in thousands of genes, and the lessons learned about gene function and cellular systems are enormous.

At the time of writing this overview, the number of citations concerning gene knockouts in mice had risen from about 254 in the period 1990–1994 to 4345 in the period 1995–1998. The imminent completion of the sequencing of the human genome will create an even greater demand to identify the function of each gene and hence an increase in activity in this area. The availability of sequence for the approximately 100,000 genes that make up the human genome will present new challenges to streamline the process of generating mice with targeted mutations in specific genes and the analysis of their phenotype.

It therefore appeared timely to bring together a collection of chapters from practitioners of various aspects of gene targeting to document the current status and future directions of this field at a practical level. It is hoped that this will provide a useful reference for those in the field, or those contemplating entering the field, and help underpin the progress in this field that will accompany the "genomic era" in biomedicine.

3. Conceptual/Theoretical Framework

The basic concept of establishing gene function through gene targeting is that a mouse with a null mutation in a specific gene is generated and whatever phenotype was observed indicates where the gene function was important. However, in general, it is important to remember that when a gene is knocked out in a mouse, the resultant phenotype is due to two major factors — first, the loss of function of the targeted gene and second, the reaction that the organism may initiate to compensate for that loss. This section considers such theoretical issues and the lessons we have learned from gene knockouts to date.

As mentioned earlier, there have been thousands of genes targeted in mice in the past decade and there are now available several compendia of these genes and the resultant phenotype ***(9)***. The purpose of this book is to focus on the

practical issues in generating gene knockout mice, nevertheless it is worthwhile discussing some of the general principles we have learned from those generated so far.

The first step in gene targeting is the design of the targeting construct and the nature of mutation that is desired. If a true null mutation is required, then it must be demonstrated that in the resultant mouse, no functional protein is made. Indeed, as it is not always possible to know all of the activities that reside in different functional domains of a protein, it is perhaps safer to demonstrate that no protein is made at all. There are several examples where the targeting strategy has resulted in a truncated polypeptide being made, and this might have some biological effects by virtue of a functional domain that it contains. This situation can complicate the interpretation of gene function in the simplest sense. Nevertheless, these mutations can give important insight into the functional domains of proteins and how they interact in vivo. Indeed, planned targeting of specific domains or important amino acid residues of proteins has become a valuable, arguably the preferred, means of examining the structure/function relationship within proteins. For example, the so-called "hit-and-run" strategy was developed to introduce subtle mutations (e.g., a nucleotide change) into a gene in ES cells and the derivative mouse. This strategy has been successfully applied to a homeobox gene ***(10)***, making collagen resistant to collagenase ***(11)*** and demonstrating the function of a signaling molecule associated with a transmembrane receptor ***(12)***. The advantage of this approach is that it enables the analysis of function in vivo using normal cells rather than transformed cell lines and it enables the comparison of function in different cell types.

Another important issue regarding the targeting event is that the expression of only the targeted gene is affected. It is possible that the targeting strategy could interfere with the regulation of the expression of a neighboring gene. This possibility has prompted some to favor the hit-and-run strategy mentioned before. Alternatively, it is important to undertake appropriate measures such as a "rescue" experiment to reintroduce the functional gene into the mouse and test for reversal of the phenotype.

Thus, what have we learned in general terms from the phenotype analysis of gene knockout mice that have been generated so far? Some mice with targeted genes have had predictable phenotypes, based on prior experimental data. For example, the *p53* gene product had been demonstrated to have tumor suppressor activity in vitro, it mediated apoptosis, and it was the most common mutation identified in a range of human tumors ***(13)***. Mice with a null mutation in the *p53* gene are highly susceptible to the development of spontaneous tumors. Perhaps a surprising result was that in the absence of such a molecule, which plays a key part in the cell function, mice could develop at all. Even mice which are heterozygous for the mutation demonstrate increased incidence of

spontaneous tumors compared with normal mice, but these are less frequent and of longer latency than in mice which are homozygous for the mutation.

It is perhaps more usually the case that gene targeting has resulted in unexpected or surprising phenotypes. For example, there was a large body of evidence to demonstrate an important role for colony-stimulating factors (CSF) in hemopoiesis of myeloid lineage cells. Indeed, when G-CSF was knockedout, mice were neutropenic and had decreased hemopoietic progenitors in bone marrow and spleen ***(14)***. By contrast, mice with a null mutation in GM-CSF (which acts earlier in myeloid differentiation than G-CSF) surprisingly showed no impairment of steady-state hemopoiesis. However, other phenotypes were observed, notably impaired pulmonary homeostasis ***(15)***. This raises a frequent and major question for gene-targeting experiments, i.e., whether other factors compensate for the loss of gene function, or whether we had oversimplified the predicted function of a gene. There are other examples where the unexpected result has been a virtual lack of phenotype. For example, the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) is a key regulator of many important genes, and mice lacking a functional *CREB* gene were expected to have a severe phenotype. However, CREB knockout mice were apparently normal except for impaired memory ***(16)***. It was further shown that a related member of this gene family is overexpressed and probably compensates for the absence of CREB in many organs ***(16)***.

In other circumstances, where gene function has evolved to deal with environmental stress or disease situations, the phenotype of the knockout mouse may not be apparent until the mouse is exposed to a particular stimulus. For example, mice with a null mutation in a component of the type I interferon receptor are essentially normal, but demonstrate extreme susceptibility to acute viral infection ***(17,18)***. Similarly, mice with a null mutation in glutathione peroxidase, an enzyme involved in the detoxification of reactive oxygen species, are essentially normal. However, when these mice are exposed to oxidative stress, they demonstrate dramatically increased susceptibility compared to controls ***(19)***.

Many gene knockouts have resulted in embryonic lethality, indicating that the gene plays an important role in embryonic development. This phenotype obviously precludes analysis of gene function in the adult. For example, the *Rb* gene is a tumor suppressor whose lack of function leads to the development of retinoblastomas in humans. However, mice with a homozygous null mutation in the Rb gene die in utero ***(20)***. Also, mice with a targeted mutation in many of the *ETS* family of transcription factors also die *in utero* ***(21)***. In vitro experiments have led to indications that ETS family members are important in immune responses, stress response, apoptosis, and bone development, therefore it would be desirable to study adult mice in which the gene was deleted ***(21)***. An important technological development in this regard is the CRE-loxP

system, which enables the generation of "conditional" knockouts that can overcome the fetal lethal phenotype and enable the analysis of adult mice with a targeted mutation in the gene of interest (*see* Chapters 6 and 7).

Therefore, one of the most important things we have learned is to interpret the initial or obvious phenotype with caution. Perhaps the greatest limitation in gene knockout technology at present is our ability to analyze all possible phenotypes. Although our ability to analyze in depth the components of some organ or cell systems is advanced, our ability to analyze other systems is not. For example, the hemopoietic/lymphoid system, with its range of cell surface markers and its suitability for flow cytometric analysis, can be analyzed in great detail with respect to the precise development of cell lineages, their migration within an organ and between organs in the immune system, and the response of cells to disease situations such as inflammatory and immune responses. However, our ability to analyze most other cellular/organ systems is primitive by comparison. Consequently, many subtle phenotypes have been noted in the hemopoietic system. For example, mice with a null mutation in a component of the type I interferon receptor are essentially normal, but on closer examination of the hemopoietic system demonstrate elevated levels of a subset of myeloid lineage cells ***(17)***. Without the reagents to analyze the hemopoietic system in fine detail, a subtle phenotype would be missed.

Mammals have undoubtedly evolved many genes whose function it is to cope with changes in our environment, to protect us from pathogens, noninfectious diseases, or modify behavior or emotions. Some of these endpoints are currently difficult to measure. Therefore, an apparent lack of phenotype in a gene knockout should be regarded with caution and may simply reflect the limitations in our ability to measure/assess/detect some phenotypes.

The absence of phenotype (or an expected phenotype) in a knockout mouse has often been cited as evidence of redundancy in the genome. That is, if the absence of a gene results in no phenotype, then there must be another gene whose protein product can perform the same function. There are apparent examples of this phenomenon such as the CREB gene knockout cited previously ***(16)***. However, it must be noted that there are at least two major objections to this generalization. The first, as stated previously, is the limitations on our ability to determine phenotype, so that the "absence" of phenotype may not be real. The second is that a gene deficiency may be compensated in many ways. Compensation might not involve performing the identical function. For example, there might be alternative pathways of achieving the same end so that the "compensation" comes from amplification of an alternate route rather than performance of the same function. This phenomenon of functional redundancy is difficult to predict or measure. For example, it might be expected that targeting a gene that is a member of a large family might readily lead to

compensation by other family member(s). For example, the *ETS* family of genes contains about 30 family members with highly homologous sequences in the DNA binding domains that apparently many sequences bind to the same element in promoter regions of regulated genes ***(21)***. It was therefore expected that when one of the ETS genes was targeted, others might compensate for its absence. However, most knockouts of *ETS* family members so far have produced a detectable phenotype, many fetal lethal ***(21)***. This demonstrates that at least some individual ETS proteins perform a unique, nonredundant function in the body and furthermore, that some members are essential for embryonic development.

4. Advancements in Gene Targeting Technology

The theme of this book is to document the current state of the art for the genetic manipulation of the mouse. There have been many technical advancements that have been discovered since the first gene targeting experiments were conducted, and these are in all aspects of the technology: molecular biology, ES cell biology, and micro-manipulation of embryos and mice. These innovations have improved the scope of the type of mutation that can be introduced into the genome, the efficiency of targeting, the time taken for these experiments, and our ability to analyze gene function in vivo.

The number of knockouts that have now been conducted means that there is considerable collective experience on the optimal design features for the targeting construct, although this knowledge is currently diluted across many laboratories with few examples each. Although there can still be no absolute guarantees, the nature of the targeting site, size, and composition of flanking genomic DNA are all important elements whose consideration will improve the frequency of specific targeting events, and therefore the amount of work involved in screening.

The ability to generate "conditional" gene knockouts has had a dramatic impact, particularly in cases where a conventional knockout results in a fetal lethal phenotype. The essence of this approach is to use homologous recombination to insert Lox P recognition sites, for a bacterial recombinase enzyme, CRE, flanking a length of DNA that is planned to be excised. Mice are generated with this (silent) modification to their genome. These mice can be mated with transgenic animals (or cells transfected with constructs) expressing the CRE recombinase driven by a particular promoter. Depending on the desired outcome, the promoter could be inducible in the case where a gene deletion is fetal lethal and it is required to analyze gene function in the adult. Alternatively, the promoter could be tissue/cell specific in cases where it is required to study the consequences of loss of gene function in a particular cell type in isolation. There is a growing collection of CRE transgenic mice using

different promoters to "drive" expression of the transgene and also a growing list of innovative applications of this system (*see* Chapter 7).

The area of ES cell technology has also seen important advances in the past decade. There are many new ES cell lines available, mostly from the 129Sv strain of mice plus a few from other strains; some of these are claimed to give better rates of germline transmission, are more robust in the face of extensive in vitro manipulation and grow faster and more reproducibly. This facilitates the establishment and maintenance of routine and transferable protocols for gene targeting in ES cell lines. ES cell lines have now been developed from different species other than 129Sv. As will be described in a later chapter, there are certain knockout phenotypes that are strain-dependent and for these instances the ability to generate knockout mice that are homozygous in strain and perhaps different to 129Sv would be a necessity (*see* Chapter 8).

Another improvement in technology has been the ability to generate ES cells that are homozygous (rather than only heterozygous) for the targeted event (*see* Chapter 16). This technique enables the study of the gene function in ES cells or in cell lineages that can be generated by in vitro differentiation of ES cells (*see* Chapters 17,18). This can also simplify the breeding of knockout mice, because if "double knockout" mice are microinjected into host blastocysts, then the appropriate colored offspring of the resultant chimeras should all contain a targeted allele. Thus, mice numbers would be reduced and screening of the pups would not be required.

As mentioned previously, the strain of mice is an important consideration, because as we know from conventional mouse genetics, the genotype–phenotype relationship can differ markedly in different mouse strains. This is well known in the fields of immunology, neurology, and behavior, but could apply equally as well in other fields.

5. The Future

The ability to manipulate gene expression in the whole animal has a key role in the future of biomedicine. Not only will knockout and transgenic technologies be important for biomedical research and to generate murine models of human genetic diseases, but it will also enable the identification of novel diagnostics, therapeutics and also form the basis of gene therapy in human disease.

By 2001, it is anticipated that all expressed human genes will be identified. This is already bringing a new era to biomedical research in which gene identification/cloning will not be necessary. The challenge will be to identify the function of the products of the genes in vivo, the diseases in which each gene is involved, and the therapeutic benefit to be gained from this information. This abundance of genetic information will enable the more rapid mapping of disease phenotypes or mouse mutants to determine genotype–phenotype

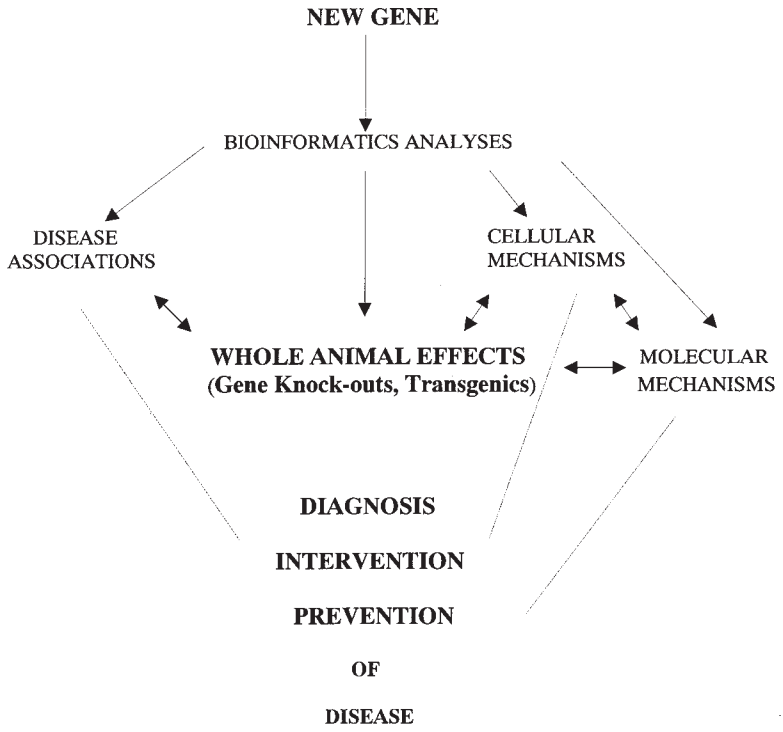

Fig. 1. Advancements in technology and gene therapy leads to better disease prevention, sound disease diagnosis, and novel disease treatments.

associations. In addition, gene knockout/transgenic technology will have a central role to play because it enables the link from new gene to in vivo function and disease association to be made in a direct and timely manner. Given the size of the task (approx 90,000 genes of unknown function), the timelines are a major obstacle.

6. Disease Diagnosis, Intervention, and Prevention

This new era of functional genomics will entail the integrated use of gene identification, bioinformatic analyses, disease associations, gene knockouts or overexpression, and cellular and molecular mechanisms to gain insights into gene function (*see* **Fig. 1**). The use of differential gene expression analyses and gene array technology, together with advancements in proteomics, are beginning to be used to analyze genetically modified mice to determine "upstream" and "downstream" factors involved in the function and mechanism of action of a particular gene product. This is an important step toward the discovery of new diagnostic tools or novel drugs for the treatment of disease.

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2

Isolation and Maintenance of Primate Embryonic Stem Cells

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1. Introduction

Primate embryonic stem (ES) cells are derived from preimplantation embryos and are capable of prolonged undifferentiated proliferation in culture. Under particular conditions, these cells differentiate into derivatives of endoderm, mesoderm, ectoderm, and trophoblast ***(1,2)***. In mammals, many developmental events are studied using mouse embryos or ES cells, but some aspects of development differ significantly between humans and mice, such as the timing of embryonic genome expression ***(3)***, fetal membrane and placental structure and development ***(4–6)***, and the formation of an embryonic disc instead of an egg cylinder ***(7,8)***. These and other features of human development are better studied using a primate model.

Recent embryological investigation in primates chiefly has addressed gamete interactions and preimplantation development. Primate ES cells provide an opportunity to use techniques that have never been developed in primates, such as lineage analysis, chimera formation, and transgenesis to study postimplantation events. Primate ES cell lines offer exciting possibilities for establishing a robust experimental primate embryology, and provide a powerful new model for understanding human development and disease.

Murine ES cells, unlike primate ES cells, have characteristics that make them relatively easy to culture. They can be readily passaged with a reasonable cloning efficiency, allowing large numbers of cells to be propagated for uses such as transfection or homologous recombination. Additionally, murine ES cells can be maintained in an undifferentiated state in the absence of feeder layers when culture medium is supplemented with leukemia inhibitory factor

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(LIF) ***(9)***. In contrast, primate ES cells differentiate or die in the absence of fibroblast feeder layers, even in the presence of LIF ***(1,2)***. Primate ES cells require regular and meticulous attention to detail in all aspects of the culture process. Here we present a concise summary of the methods we use to isolate and maintain primate ES cells in vitro.

2. Materials

2.1. Immunosurgery

1. 0.5% pronase E (Sigma) in Milli-Q water.
2. Rabbit anti-rhesus or anti-marmoset spleen cell antiserum: Antiserum is raised as described previously ***(10)***, except primate spleen cells were used.
3. Guinea pig complement diluted 1:10 (Gibco-BRL; *see* **Note 1**).

2.2. Culturing Primate ES Cells

1. Irradiated mouse embryonic fibroblasts (MEF) plated on 0.1% gelatin ***(11)***.
2. Embryo culture-grade water (*see* **Note 2**).
3. Dulbecco's modified Eagle medium (DMEM) with D-glucose (4500mg/L) and L-glutamine but without sodium pyruvate or sodium bicarbonate (Gibco-BRL).
4. Primate ES cell culture medium: 79% DMEM, 20% FBS, 1% nonessential amino acid stock, 0.1 m*M* 2-mercaptoethanol, and 1 m*M* L-glutamine. Combine and filter (0.22 μm) before use.
5. Sodium bicarbonate (Sigma).
6. Fetal bovine serum (FBS; *see* **Note 3**).
7. 2-Mercaptoethanol (Sigma).
8. L-glutamine (Gibco-BRL).
9. 50X MEM nonessential amino acid stock without L-glutamine (Gibco-BRL).
10. Ethylenediamine tetraacetic acid (EDTA; Sigma).
11. Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; Gibco-BRL).
12. Dimethyl sulphoxide (DMSO; Sigma).

2.3. Handling Primate ES Cells

Primate cells should always be considered biohazardous because some viruses such as herpes B, which can be carried by rhesus macaques without noticeable clinical signs, are potentially fatal when transmitted to humans. Never use a mouth pipet for handling primate cells, and dispose of used equipment (pipets, test-tubes, and the like) according to local regulations.

1. 15 mL polystyrene tubes.
2. Glass pipets (1 mL, 5 mL, 10 mL, 25 mL).
3. Glass Pasteur pipets (9 in. borosilicate).

4. Micrometer syringe apparatus ***(12)***.
5. 4-well and 6-well tissue culture plates, 35mm tissue culture dishes (Nunc), T25 and T75 polystyrene tissue culture flasks (Becton Dickinson).
6. Cryogenic vials (Nalgene).

3. Methods

3.1. Preparation of Mouse Embryonic Fibroblasts

Instructions for the isolation, preparation, and plating of MEF can be found elsewhere ***(11)***. MEF should be isolated and frozen in quantity well before required.

1. Culture MEF to 70–80% confluence. To keep differentiation of ES cells to a minimum, passage MEF regularly, and do not allow MEF to reach confluence immediately before irradiation.
2. Mitotically inactivate by exposure to 3000 rads γ-radiation.
3. Plate at 5×10^4 cells/cm^2 at least 2 h (preferably 12 h) prior to immunosurgery or ES cell passage.

3.2. Isolation of the Inner Cell Mass

All solutions used for the immunosurgical procedure must be made fresh (from frozen stocks) on the day of the procedure, and allowed to equilibrate in an incubator at 37°C for at least 1 h.

1. Incubate blastocyst **(Fig. 1A)** briefly in 0.5% pronase until the zona pellucida disappears. This takes approximately 30 seconds, so constant attention is required.
2. Immediately remove the zona pellucida-free embryo and wash three times in DMEM + 20% FBS.
3. Incubate the blastocyst in antibody for 30 mins at 37°C in 5% CO_2 in air.
4. Wash three times in DMEM + 20% FBS.
5. Incubate in guinea pig complement for 30 mins at 37°C in 5% CO_2 in air.
6. Wash in DMEM + 20% FBS.
7. Attach to the micrometer syringe apparatus a finely drawn glass pipet that has an internal diameter slightly larger than the inner cell mass (ICM).
8. Draw the embryo into the pipet and expel. If the immunosurgery was successful, the trophoblast cells will lyse, leaving the ICM as a small clump of tightly bound intact cells **(Fig. 1B)**.
9. Using the pipet, transfer the ICM onto the prepared MEF feeder layer. The ICM will usually attach within 24 h and after approximately 72 h, the ICM will have flattened on the feeder layer **(Fig. 1C)**. Four to six days after immunosurgery a small colony will be evident **(Fig 1D)**, and the first passage should be performed (*see* **Note 4**).

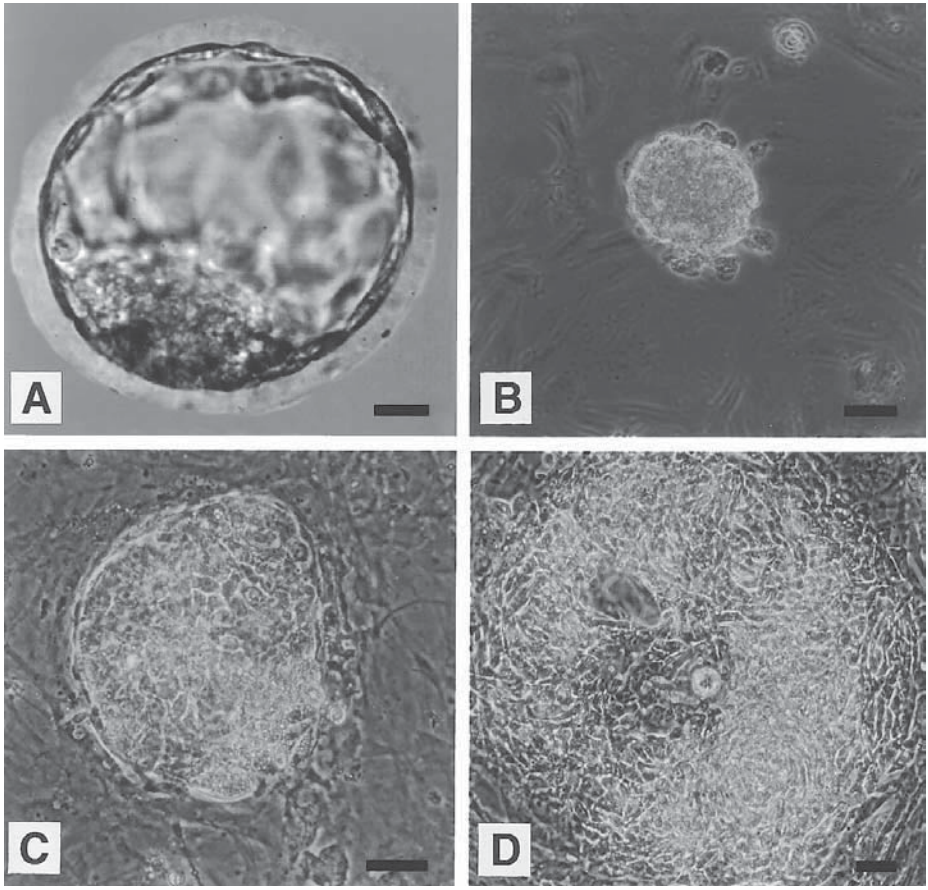

Fig. 1. Primate embryonic stem cell isolation. **(A)** Rhesus blastocyst. Bar = 25 μm. **(B)** Rhesus inner cell mass (ICM) immediately following immunosurgery. Bar = 50 μm. **(C)** ICM 3 d postimmunosurgery, attached to feeder layer. Bar = 50 μm. **(D)** ICM 7 d postimmunosurgery, immediately prior to initial dissociation for ES cell isolation. Bar = 50 μm.

3.3. Passaging Primate ES Cells

1. Remove culture medium.
2. Wash with Ca^{2+}/Mg^{2+}-free PBS with 0.5 m*M* EDTA and 1% FBS.
3. Reapply Ca^{2+}/Mg^{2+}-free EDTA/FBS and observe cells under phase contrast microscopy.
4. When cells show signs of individualization (3–5 min), immediately either:
   a. Aspirate ES cells with a small bore glass pipet attached to a micrometer syringe apparatus and expel onto fresh feeder layers (*see* **Note 5**), or

b. Scrape ES cells with the tip of a glass 5 mL pipet and aspirate. Expel cells into a 15 mL centrifuge tube. Centrifuge at 1000*g* for 5 min in a benchtop centrifuge. Resuspend ES cells in culture medium and plate onto prepared feeder layers.

3.4. Maintenance of Primate ES Cells in Culture

Primate ES cells are difficult to maintain in vitro **(Fig. 2)**. Differentiation of primate ES cells can be minimized by careful attention to detail in all aspects of the culture process:

1. Feed every 2 d and more often as colonies grow.
2. Eliminate differentiated cells from the continuing culture when passaging by selecting individual undifferentiated colonies, as described in **Subheading 3.3., step 4a.** Failure to remove most of the differentiated cells from the culture will result in rapid loss of the culture to complete differentiation.
3. Try to keep time in suspension minimized during all procedures. Primate ES cells fragment and die rapidly when removed from feeder layers.

3.5. Freezing Primate ES Cells

1. Remove cells from the culture plate as for passaging.
2. Spin in a 15 mL tube in a benchtop centrifuge at 1000*g* for 5 min.
3. Remove supernatant.
4. Resuspend in 0.25 mL 20% FBS: 80% DMEM. Add an equivalent volume of 20% DMSO: 20% FBS: 60% DMEM dropwise into the tube, mix and transfer to a 1.5 mL cryogenic vial.
5. Place the cryogenic vial between two polystyrene racks and freeze at –70°C overnight.
6. Transfer to liquid nitrogen for long-term storage.

3.6. Thawing Primate ES Cells

1. Remove cryogenic vial from liquid nitrogen.
2. Gently swirl vial in 37°C water bath until thawed and wash vial in ethanol.
3. Pipet contents of vial up and down once to mix.
4. Place contents of cryogenic vial in a 15mL centrifuge tube.
5. Add an equal volume of ES medium and mix.
6. Spin cells for 5 min at 1000*g* in a benchtop centrifuge.
7. Remove supernatant and resuspend cells in ES medium.
8. Place cell suspension on a culture plate previously plated with irradiated MEF.

3.7. Primate ES Cells for Tumor Formation (see Note 6)

ES cells can be injected into severe combined immunodeficient (SCID) mice for tumor formation. In this environment, undifferentiated ES cells can differenti-

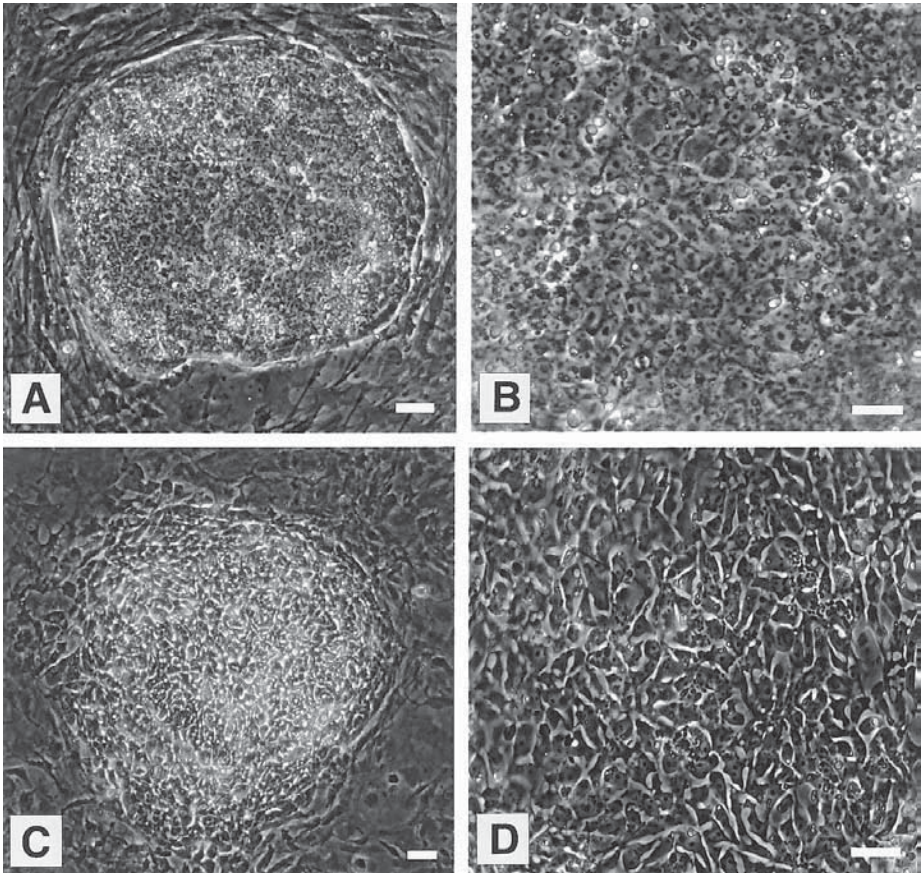

Fig. 2. Primate embryonic stem cells. **(A, B)** Marmoset ES cell colony. **(C, D)** Rhesus ES cell colony. Bar = 50 μm.

ate into cell types and complex structures that may not form if ES cells differentiate in vitro. This provides a way to assess the developmental potential of the ES cells and to study the development of specific cell types or tissues.

1. Culture at least 2×10^6 cells per injection site.
2. Remove cells from culture plate as described in **Subheading 3.3., step. 4a**.
3. Centrifuge gently for 5 min in benchtop centrifuge, resuspend in 0.1 mL culture medium, and place on ice.
4. Load cells into a 1 mL tuberculin syringe.
5. Using a 22 gage needle, inject the cell suspension into the hind leg muscle of a SCID mouse.
6. Observe mouse daily and palpate hind leg weekly. Palpable tumors are usually present within 4 wk.

4. Notes

1. Batches of guinea pig complement may give variable results and must be tested for toxicity.
2. Primate embryonic stem cells require extremely high-quality water for all culture media. We use a Milli-Q filtration system (Millipore), which is sanitized monthly. Batches of water are stored in multiple 2 L bottles and tested in culture medium before use.
3. FBS suitability for culture medium varies among lot numbers and needs to be tested before use. Primate ES cells are particularly sensitive to endotoxin. We test sera of different lot numbers directly on primate ES cells but sera can be tested also by assessing the cloning efficiency of mouse ES cells, grown in the presence of leukemia inhibitory factor (LIF) without feeder layers.
4. We usually perform the first passage within a week of immunosurgery. If this procedure is performed too soon after immunosurgery there will be too few cells, and the culture may be lost. If the first passage is left too long, there is a risk of losing the culture to differentiation.
5. This method is most appropriate when dealing with small numbers of cells, or when it is necessary to select undifferentiated colonies from a partially differentiated culture.
6. Always follow local animal care-and-use protocols.

Acknowledgments

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3

Gene Targeting in ES Cells

Thomas M. DeChiara

1. Introduction

In the ten years since the first gene-targeting experiments were performed in murine embryonic stem (ES) cells ***(1–5)*** and the mutations successfully transmitted through the mouse germline ***(6–11)***, the application of a variety of gene-targeting methodologies has generated a remarkable number of novel and informative mutant mouse strains that have been invaluable not only for the study of gene function in vivo, but also serving as model systems for human disease.

Gene targeting is a term that is used to describe the predetermined mutation of an endogenous gene that results from a homologous recombination event between a mutated version of the gene carried on a targeting vector and the endogenous genetic locus. When performed in ES cells, gene-targeting is a powerful means for introducing specific genetic mutations into the mouse genome. Gene-targeting strategies can employ two different types of vectors, sequence replacement or sequence insertion vectors, that differ not only in their mechanisms of chromosomal integration and gene targeting frequencies, but also in their utility. In their most basic form, replacement vectors ***(1)*** are used to disrupt target gene function by deleting specific sequences and replacing them with heterologous DNA, usually a drug selection gene or a marker gene to analyze target gene expression. Insertion vectors ***(1,2)***, which may offer increased gene-targeting frequencies at a given genetic locus compared to a replacement vector ***(12)***, can also be used to disrupt gene function by inserting heterologous DNA, but also allow for the introduction of more subtle genetic alterations such as point mutations ***(13,14)***. Within the past several years new technologies have been developed that allow for inducible gene expression in transgenic mice that can be combined with more conventional gene-targeting approaches to control the timing and tissue-specificity of the desired mutation.

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Homologous recombination between the targeting vector and the endogenous locus in ES cells is a rare event, but gene-targeting vectors incorporate marker genes for drug selection schemes to enrich for the recovery of homologous recombinant ES cells. In addition, gene-targeting frequencies can be optimized by a number of experimental parameters that affect the composition of the homologous DNA sequences comprising the targeting vector.

As exciting as it is to clone a novel gene and begin to determine its function in vivo by performing a gene-targeting experiment, it is premature to decide on a strategy without having some information about the in vivo expression pattern of the target gene provided by a Northern blot analysis of developmentally staged tissue-derived RNA or from an *in situ* hybridization analysis of developmentally staged mouse embryos. The target gene expression pattern will invariably dictate the type of gene-targeting scenario by incorporating the timing or tissue specificity of the desired mutation. For example, if the gene expression pattern is restricted to the embryonic period, a targeting approach that establishes the desired mutation in the germ-line is more appropriate than if the target gene is transiently expressed during embryogenesis and re-expressed postnatally in a tissue-specific manner. A gene targeting strategy that establishes the mutation in the germ-line may result in embryonic lethality and preclude the postnatal assessment of gene function. In this case, a conditional gene-targeting strategy using an inducible system would better address the study of the mutation in the tissue of interest. Finally, if the gene is expressed postnatally in a specific tissue(s), either a germ-line mutation or a conditional mutation strategy can be employed, with the latter offering more flexibility for establishing the mutation at a desired time in a particular tissue.

This chapter examines the current strategies for generating specific mutations in ES cells by describing the utility of the two general types of gene targeting vectors, describing the systems for inducing conditional mutations, and examining the parameters for designing a gene targeting vector for the experimental application of gene targeting in ES cells.

1.1. Sequence Replacement Vectors

The majority of gene-targeted mutations that have been engineered in the mouse have relied on gene-targeting strategies that employ a replacement vector design ***(1)***. These vectors are useful for disrupting the target gene by deleting specific sequences such as coding exons or transcriptional control regions, and replacing them with a heterologous DNA. As shown in **Fig. 1**, the basic replacement vector is composed of three essential DNA elements arranged in a specific order: (1) a region of 5' target gene homology, (2) a drug resistance marker gene for the positive selection of cells that integrate the targeting vector, and (3) a region of 3' target gene homology. The DNA

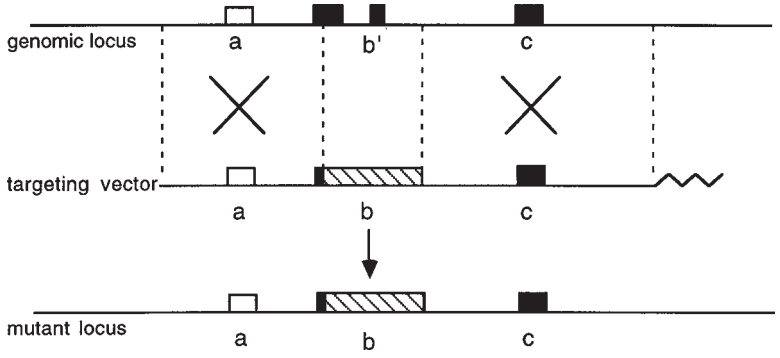

Fig. 1. The basic sequence replacement vector for positive selection in ES cells. The vector is linearized at the 5' end of homology region "a" placing the plasmid sequences at the 3' end of homology region "c." The marker gene cassette (striped box) interrupts the first coding exon and replaces endogenous gene sequences (b'). The host plasmid sequences on the vector are represented by the wavy lines. Target gene noncoding sequences (open box), coding sequences (filled boxes).

sequences resident in regions "a" and "c" on the targeting vector are the substrates for a double reciprocal crossover or gene conversion mechanism that transfers the "b" sequence element into the endogenous locus. The homologous sequences are derived from the cloned genomic portions the endogenous target gene and flank the target gene sequences that are being replaced by "b" sequences to generate the mutant locus. The vector is linearized at either end of the 5' or 3' homology regions so that the DNA ends can serve as a substrate to initiate homologous recombination with the target locus.

1.1.1. Marker Genes for the Positive Selection of ES Cells

Following the introduction of the linearized targeting vector into ES cells, a drug selection is performed to enrich for those ES cells that have integrated the targeting vector and are expressing the drug-resistance gene. Depending on the transcriptional state of target gene in ES cells, the configuration of the selectable marker gene can be modified to further enrich for homologous recombinant ES cell clones. If the target gene is transcriptionally active in ES cells, such an enrichment can be achieved by the use of a selectable marker gene that is lacking transcriptional regulatory DNA sequences, and is expressed only when it acquires the appropriate regulatory sequences of an active transcription unit. In contrast, if the target gene is not expressed in ES cells, the target locus is said to be "nonselectable" and the "b" sequence portion of the targeting vector should be composed of a marker gene expression cassette that includes all of the regulatory sequence elements needed for transcription.

1.1.1.1. Targeting of Nonselectable Genes

When the target gene is not expressed in ES cells, or if its expression is unknown, a targeting vector should include a drug-resistance gene expression cassette to allow for the positive selection of ES cells. The most commonly used selectable marker has been the neomycin phosphotransferase gene (*neo*)***(1)*** which confers resistance to the neomycin analog, G418. The *neo* gene is normally used as part of a cassette driven by the phosphoglycerate kinase gene (*PGK*) promoter or the herpes simplex virus thymidine kinase gene (HSV*tk*) promoter contained on the pMC1*neo* cassette . An alternative choice for a selectable marker is the hygromycin B phosphotransferase gene (*hyg*) ***(15)*** to select for the survival of ES cells in the presence of hygromycin. Both *hyg* and *neo* genes and can be used in independent targeting vectors to simultaneously target both alleles of an endogenous locus in an ES cell by employing a double drug selection with both G418 and hygromycin ***(16)***. An additional selectable marker for mammalian cells, the Zeocin resistance gene (*zeo*) ***(17)***, is seldom incorporated into gene-targeting vectors for the positive selection of ES cells, but is commercially available from Invitrogen (San Diego, CA).

Perhaps the most effective use of the selection marker expression cassette is in combination with reporter genes such as *lacZ* ***(18)*** or green fluorescent protein (GFP) ***(19)***. As shown in **Fig. 2A**, an expression cassette can be configured such that the reporter gene is constructed as an in-frame fusion with coding sequences to disrupt the target gene to serve as a sensitive marker to visualize target gene expression in mice heterozygous or homozygous for the mutant locus. The second element of the cassette is an expression competent marker gene for the positive selection of vector-recipient ES cells.

1.1.1.2. Targeting of Selectable Genes

If the target gene is expressed in ES cells, it is possible to enrich for homologous recombinants by constructing a targeting vector with a selectable marker gene that is lacking transcriptional regulatory elements such as a promoter, and downstream polyadenlyation signals, so that recipient ES cells will survive selection only when the marker gene is expressed when it acquires the proper transcriptional control elements by the integration of the targeting vector into an active transcription unit ***(3,7,20–24)***. In the example shown in **Fig. 2B**, the targeting vector contains the *neo* gene lacking an ATG and polyadenylation sequences fused in-frame with the target gene coding sequences. Following homologous recombination at the target locus, *neo* gene expression will be regulated by the *cis*-acting sequences of the endogenous target gene to produce a *neo* fusion protein that confers resistance to G418.

It has recently become possible to easily monitor target gene expression following homologous recombination by using a reporter cassette in the gene-

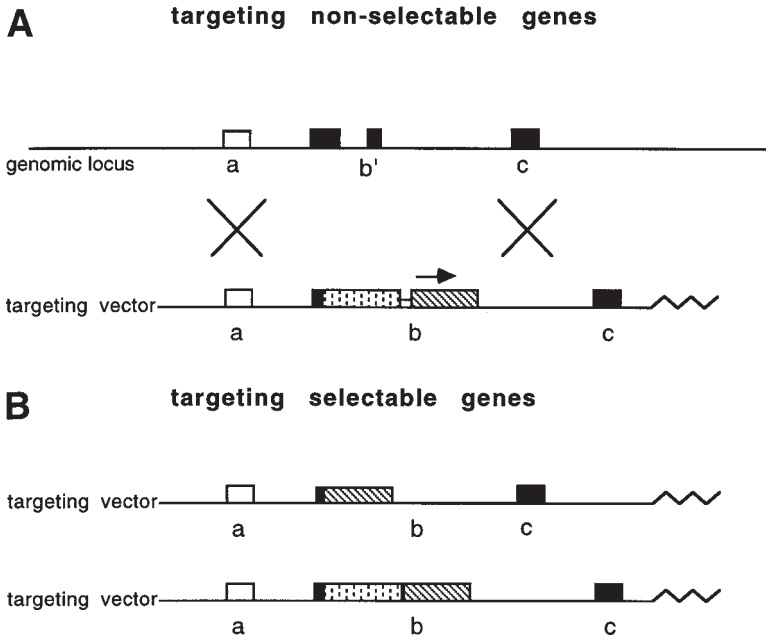

Fig. 2. Targeting "nonselectable" and "selectable" genes. **(A)** The vector contains a lacZ or GFP reporter gene cassette including a polyadenylation signal (stippled box) fused in-frame with target gene coding sequences. A marker gene (*neo* or *hyg*) expression cassette (striped box) is uses for positive selection of ES cells. The arrow indicates that the marker gene cassette is expression competent. **(B)** When the target gene is expressed in ES cells, a marker gene (*neo* or *hyg*) can be fused in-frame with the coding sequences of the target gene. Alternatively, β*geo* and β*gyg* cassettes (stippled and striped box) can serve a dual purpose.

targeting vector which is comprised of a β-galactosidase gene fused with *neo* (β-*geo*) ***(25)*** or similarly with *hyg* (β***gyg***) ***(26)***. These fusion cassettes serve a dual purpose: to perform positive drug selection of vector recipient ES, and to visualize target gene expression patterns in heterozygous and homozygous mice by staining with X-gal.

1.1.2. Positive-Negative Selection Using Replacement Vectors

A widely used method to enrich for homologous recombinant ES cell clones is a gene targeting strategy that employs a replacement vector designed for use in a double drug selection protocol known as positive-negative selection (PNS) ***(4)***. In this scheme, the targeting vector is composed of a positive selection marker used either as an expression cassette **(Subheading 1.1.1.1.)** or as a fusion gene **(Subheading 1.1.1.2.)**, positioned between the 5' and 3' target gene

homologous regions, in addition to a marker gene cassette for negative selection (d) located on either end of the homology regions (*see* **Fig. 3**). The negative selection gene that has been most commonly used is the herpes simplex virus thymidine kinase (HSV*tk*) gene expression cassette, which confers sensitivity to the guanosine analog, gancyclovir (GANC). The HSV*tk* gene phosphorylates GANC, allowing it to be incorporated into replicating DNA to act as an inhibitor of DNA synthesis. As an alternative to using the HSV*tk* gene, the diphtheria toxin A-chain gene has successfully been used as a negative selection marker for gene targeting in ES cells ***(27)***. The PNS targeting vector can be constructed in two configurations as determined by the placement of negative selection gene cassette (d), either upstream of the 5' target gene homology (a), or downstream of the 3' target gene homology (b). However, depending on the final configuration, the vector must be linearized at the end of homology regions "a" or "c."

In a typical gene-targeting experiment, the PNS replacement vector will randomly integrate by its linear ends into the genome of the majority of recipient ES cells, retaining both the positive selection cassette and the *tk* gene in the genome, conferring resistance to positive selection and sensitivity to GANC, respectively. However, in the recipient ES cells where homologous recombination between the targeting vector and the endogenous gene has occurred, the positive selection cassette will be integrated into the target locus while the *tk* gene is lost and these cells and the selection cassette will survive both positive and GANC selection. By employing a PNS scheme, the absolute number of ES cell colonies that survive double drug selection is about 5 to 10-fold lower than the number of colonies that survive positive selection only, greatly reducing the number of colonies to be screened for the gene targeting event. Empirically, not all of the ES clones that survive positive-negative selection are recognized as being homologous recombinants by a screen of the target locus in ES cell clones. These nonhomologous recombinants may have survived negative drug selection because the negative selection marker gene acquires inactivating mutations prior to the random integration of the targeting vector into the ES cell genome ***(28)***.

1.2. Sequence Insertion Vectors

In contrast to the mechanism of replacement vector homologous integration by a double reciprocal cross-over or gene conversion, insertion vectors undergo homologous recombination via a single cross-over, initiated by linear ends within a region of homology ***(1,2)***. As illustrated in **Fig. 4A**, insertion vectors have a basic structure consisting of (1) a contiguous stretch of homologous gene sequences and (2) a positive selection marker cassette that is positioned to disrupt the target gene by interrupting a coding exon as either an expression cassette **(Subheading 1.1.2.)**, or as an in-fame fusion gene **(Subheading**

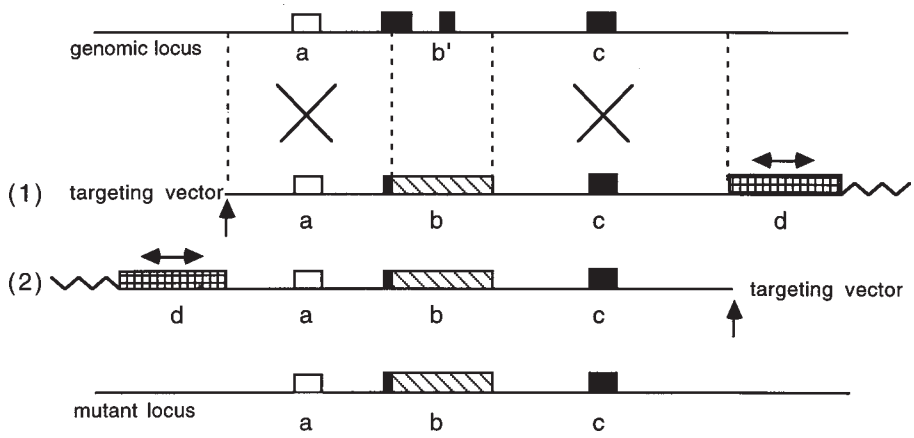

Fig. 3. Sequence replacement vector for positive-negative selection in ES cells. Building on the basic sequence replacement vector, the PNS vector includes an expression cassette of the HSV*tk* gene (d). Vector configurations 1 and 2 differ in the positioning of (d). The vertical arrows indicates the site where the targeting vector can be linearized. The double arrows indicate that (d) can be cloned in either transcriptional orientation relative to the other sequences on the targeting vector. Following homologous recombination the positive drug selection cassette is incorporated into the target locus and the negative selection cassette is lost.

1.1.3.). Insertion vectors are linearized at a point within the homology region and the linear ends become the substrate for chromosomal integration of the vector. Because they integrate via a single cross-over event rather than two, insertion vectors generally target with higher homologous recombination frequencies than replacement vectors ***(12)***, but the entire vector is inserted into the target locus, which results in a duplication of endogenous target gene sequences by those that are resident on the targeting vector. The presence of duplicated gene sequences at the target locus could result in low levels of wild-type protein being generated from the mutant locus as a result of alternative RNA splicing bypassing the mutated exon.

A major benefit of insertion vectors is their use in gene targeting scenarios that are intended to introduce a point mutation in the target gene. This has been called a "hit and run" ***(13)*** or "in-out" ***(14)*** procedure that is performed with successive rounds of drug selection. The insertion vector is composed of the following sequence elements: (1) target gene homologous sequences with a point mutation at a defined position, (2) a positive selection marker gene cassette, and (3) an expression cassette for negative drug selection (*see* **Fig. 4B**). Following the introduction of the targeting vector into ES cells, positive drug selection is applied and the surviving ES cell colonies are screened for

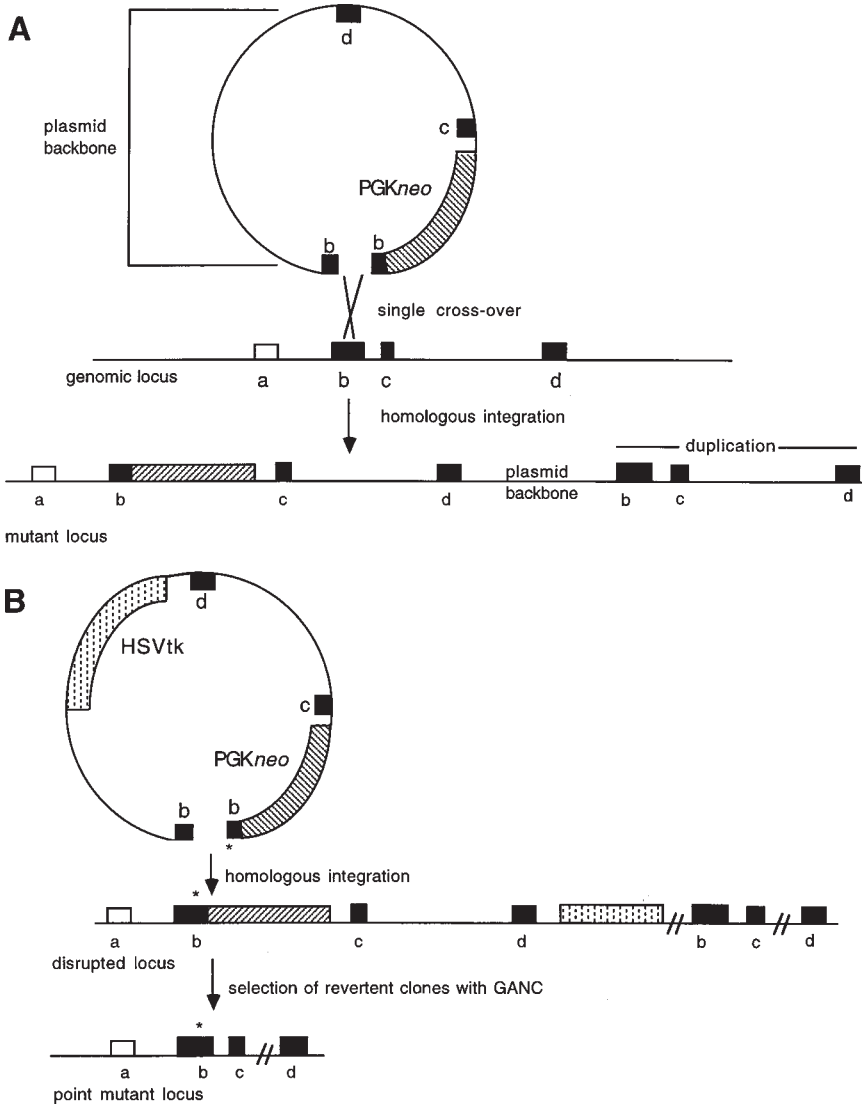

Fig. 4. Sequence insertion vector for homologous recombination in ES cells. **(A)** To disrupt the target gene, the PGK*neo* cassette interrupts exon b. The vector is linearized within exon b to provide the ends for a single crossover into the endogenous locus. **(B)** In a "hit and run" experiment, the vector is engineered with a point mutation (*) at the desired location and includes a cassette for negative selection (HSV*tk*). Following the generation of the disrupted locus by homologous integration, a second selection step with gancyclovir (GANC) can be applied to the targeted ES clone to allow for an intrachromosomal recombination to occur at exon b resulting in the excision of the intervening vector sequences.

homologous recombinants. These ES clones are expanded and then reselected in the medium containing negative selection. Only those cells that undergo an *intra*chromosomal homologous recombination event will survive the selection, excising a portion of the targeting vector that includes all of the nonhomology sequences in the targeting vector, leaving behind the desired point mutation in the target gene.

1.3. Inducible Systems for Gene Targeting

The gene-targeting vectors that have been described thus far have incorporated either a replacement or insertion vector design to generate mutations that are preexisting in the germ-line of chimeric mice and their progeny. A limitation with this approach is that germ-line mutations are not amenable to further genetic manipulation, which is disadvantageous particularly when the mutation results in embryonic lethality, precluding a phenotypic analysis in a developmental or tissue-specific manner where the biological role of the target gene is intended for study. Recently, gene-targeting strategies have been developed that permit the establishment of gene targeted "conditional" mutations that can be induced in a controlled fashion. This section will describe the utility of three inducible systems: (1) Cre/*lox*P, (2) tetracycline inducible, and (3) ecdysone inducible, for controlling gene targeted mutations in mice. The tetracycline- and ecdysone-inducible systems can be designed to incorporate Cre/*lox*P technology to offer an additional level of control for creating the desired mutation by the addition of exogenously added substances.

The use of inducible systems require at least two independently engineered mouse lines that are then interbred to induce the desired genetic mutation. One of the mouse lines is engineered with a gene-targeted conditional mutation, in such a way that it is not inhibitory to normal gene function, but consists of sequence elements that mediate the conversion to the mutated state. A second and third mouse line will harbor the mutation-inducing genes in the form of a transgenes expressed in a developmental or tissue-specific manner and can be controlled by the addition of an exogenous substance. Breeding of the lines will ultimately result in progeny that are hemizygous for the inducing transgenes and homozygous for the conditionally mutant locus, the latter owing to the presence of the inducing transgene. The major difficulty in performing these types of experiments is that they require an adequately sized mouse colony to generate sufficient numbers of homozygous F2 mutants and control mice for phenotypic analysis.

1.3.1. Cre/loxP

In contrast to the long stretches of homology that are required by mammalian cells for the homologous recombination between a gene targeting vector

and the target locus, intrachromosomal recombination is mediated by short homologous regions ***(29)***, such as the joining sequences used for immunoglobulin gene rearrangements. One of the first systems developed to mediate the site-specific intrachromosomal recombination of heterologous DNA integrated into the genome of mammalian cells, makes use of the bacteriophage P1 Cre recombinase which catalyzes a conservative intrachromosomal recombination event between two 34 nucleotide *lox*P recognition sites resulting in the excision of the intervening chromosomal DNA ***(30–32)***. As shown in **Fig. 5**, an inducible gene-targeting strategy can be designed as a binary system wherein one transgenic mouse line expresses the *Cre* gene from a promoter that is temporally regulated ***(33)*** or directs tissue-specific expression ***(31)***. This *Cre*-expressing mouse line is then bred to a second mouse line that is carrying a gene-targeted locus that has been conditionally mutated by a utilizing a PNS replacement vector strategy to position *lox*P sites flanking the endogenous sequences intended to be excised. The targeting vector includes a positive selection expression cassette located between the *lox*P sites so that it is excised along with the coding exons of the target gene. When generating the endogenous locus with the conditional mutation by gene targeting, the presence of *lox*P sites should not interfere with target gene expression at the level of transcription or RNA processing. Crossing of F1 progeny will result in F2 progeny homozygous for the mutant locus and hemizygous for the Cre-expressing transgene as well as the appropriate control progeny.

1.3.2. Tetracycline-Responsive Gene Targeting

The ability to control gene expression, and ultimately gene targeting, in transgenic mice by simply administering an exogenous substance affords an additional level of control over the Cre/*lox*P system for inducing gene mutations in mice. The tetracycline-responsive system ***(34,35)*** is composed of (1) the tetracycline repressor protein (TetR) fused to the herpes simplex virus VP16 transcriptional activation domain, and (2) the tetracycline operator sequence (*tet*O) linked to a minimal promoter element to control the transcription of a downstream gene following *TetR–VP16* binding to *tet*O. The vectors for expression of *TetR–VP16* and for *tet*O-minimal promoter directed gene transcription in mammalian cells are now commercially available from Clontech (Palo Alto, CA).

The tetracycline-responsive system has recently been used in concert with Cre/*lox*P technology to allow for the temporal control of gene targeting in mice ***(36)*** by placing the expression of the Cre gene under the control of the *tet*O-minimal promoter. For more specificity, a tissue-specific promoter can be used to drive the expression of the *TetR–VP16* gene. Moreover, there are Tet-Off and Tet-On variations for controlling tet-responsive gene expression, which depend on the binding of tetracycline to TetR-VP16.

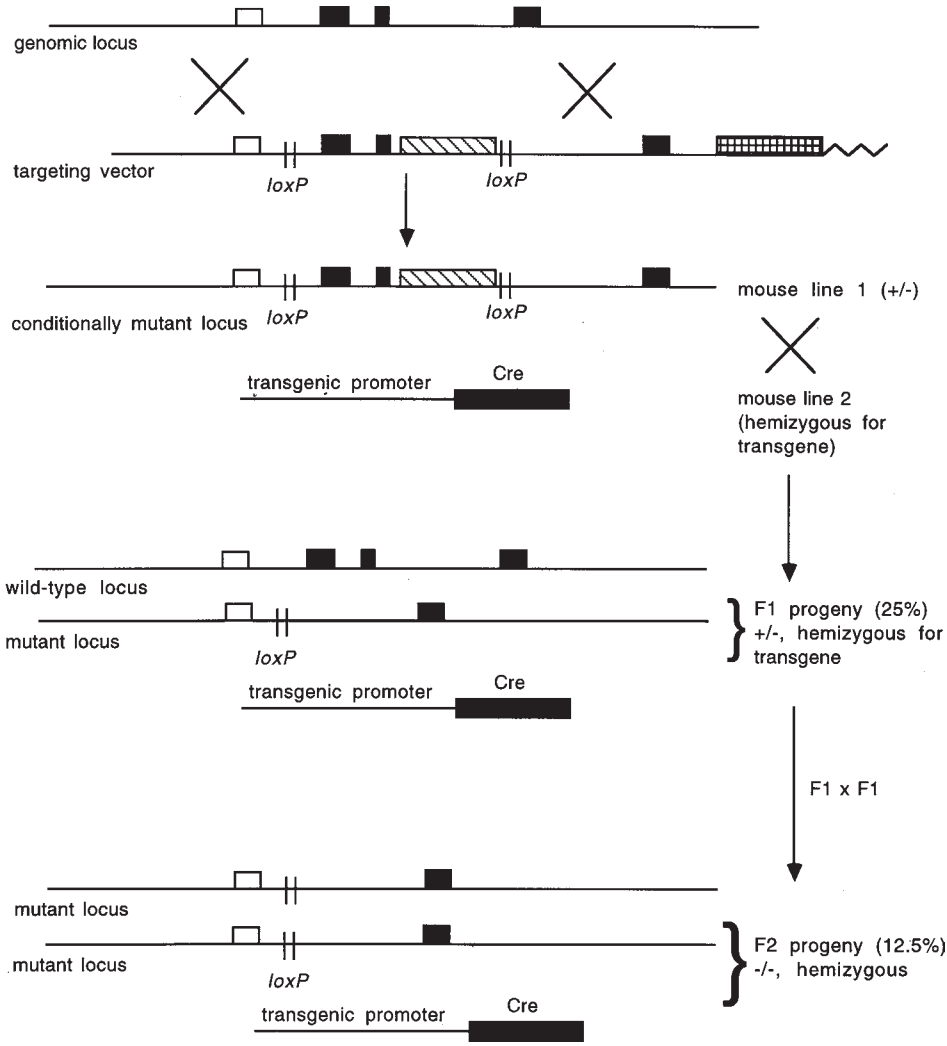

Fig. 5. Inducing gene-targeted mutations with Cre/*lox*P. To generate a conditionally mutant target locus in mouse line 1, a PNS replacement vector strategy can be used to position *lox*P sites (//) flanking the target gene sequences intended to be deleted. The targeting vector contains a positive selection expression cassette (striped box) between the *lox*P sites, and negative selection expression cassette (hatched box). Mouse line 2 carries a transgenic promoter driving the Cre gene expression in a tissue-specific or general manner. Approx 25% of the F1 progeny are heterozygous for the mutant locus derived from *lox*P recombination directed by Cre gene expression. In the next generation, approx 12.5% of the F2 progeny will be homozygous for the mutant locus and hemizygous for the transgene. The wild-type alleles of the endogenous target locus are not shown in the heterozygous progeny.

1.3.2.1. Tet–Off Gene Expression

In the Tet–Off version of this system, the *TetR–VP16* transactivating protein, tTA, will bind to the *tet*O-minimal promoter in the presence of tetracycline to repress gene expression. When tetracycline administration is stopped, tTA no longer binds to the *tet*O-minimal promoter and transcription is induced by as much as several thousandfold in transgenic mouse tissues ***(37)***. As illustrate in **Fig. 6**, it is useful to initially create three independent F0 mouse lines, but because a three-gene cross is involved to combine the genetic components for tetracycline control, it is more practical to generate two F2 mouse lines that are (1) homozygous for the tTA transgene, heterozygous for the conditionally mutant locus and (2) homozygous for the *Cre* expressing transgene, heterozygous for the conditionally mutant locus. In these mice, the conditionally targeted gene can be generated by using a PNS replacement vector strategy as was shown previously in **Fig. 5**. By breeding the two F2 lines, 25% of the F3 progeny will be homozygous for the conditionally mutant locus and hemizygous for both the tTA transgene and the *Cre*-expressing transgene. To maintain the repression of *Cre* gene expression and prevent *lox*P-mediated recombination of the targeted locus during embryogenesis of the F3 progeny, a tetracycline analog, doxycycline, can be supplied in the drinking water (200 μg/mL) given to the F1 females. Upon the removal of doxycycline, *Cre* gene expression is derepressed, resulting in *lox*P-mediated recombination to disrupt the target gene. A drawback to the system has been the reported leakiness of *Cre* gene expression in the repressed state, as measured by a precocious *lox*P-mediated recombination, observed between litters and littermates ***(37)***.

1.3.2.2. Tet–On Gene Expression

Perhaps a more experimentally practical system of tetracycline control is the Tet–On version in which gene expression from the *tet*O-minimal promoter is induced rather than repressed by the administration of doxycycline. This scenario employs a mutant form of *TetR–VP16*, rtTA ***(38)***, which normally represses the *tet*O-minimal promoter. Following the addition of doxycycline, rtTA is released from the *tet*O allowing expression of the *Cre* gene. As with the Tet–Off approach, a drawback is the observable expression from the *tet*O-minimal promoter when it is expected to be repressed by rtTA. The design for a gene-targeting experiment utilizing the Tet–On system is illustrated in **Fig. 7**, showing a breeding scenario that leads to the derepression of the *tet*O-minimal

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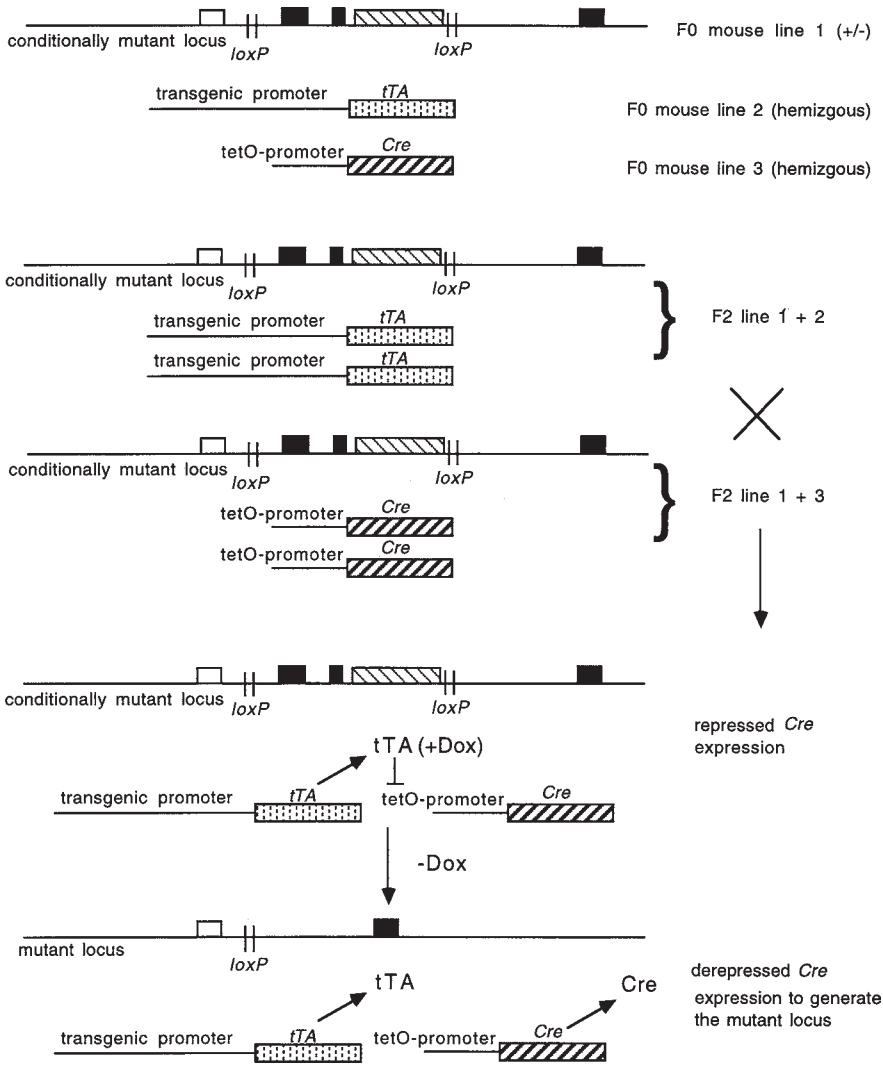
Fig. 6. (*Opposite page*) Tet–Off-inducible gene targeting. Mouse line 1 is heterozygous for a conditionally mutant target locus with *lox*P sites (//) flanking the target gene sequences intended to be deleted, including the positive selection cassette (striped box).



Mouse line 2 is hemizygous for a transgene to express the tetracycline repressor protein (stippled box) in a tissue-specific or general manner. Mouse line 3 is hemizygous for a *Cre*-expressing transgene (heavy striped box) responsive to tetracycline. By crossing lines 1 and 2, and crossing lines 1 and 3, F2 can be obtained that are heterozygous for the conditionally mutant locus and homozygous for the respective transgenes. Crossing the F2 mice will produce progeny that are hemizygous for both transgenes to maintain the repression of the *Cre* gene and will carry the conditionally mutant locus and the wild-type endogenous locus in the expected Mendelian ratios. The removal of doxycycline results in the expression of the *Cre* gene and the creation of mutant target locus. The wild-type alleles of the endogenous target locus are not shown in the heterozygous progeny.

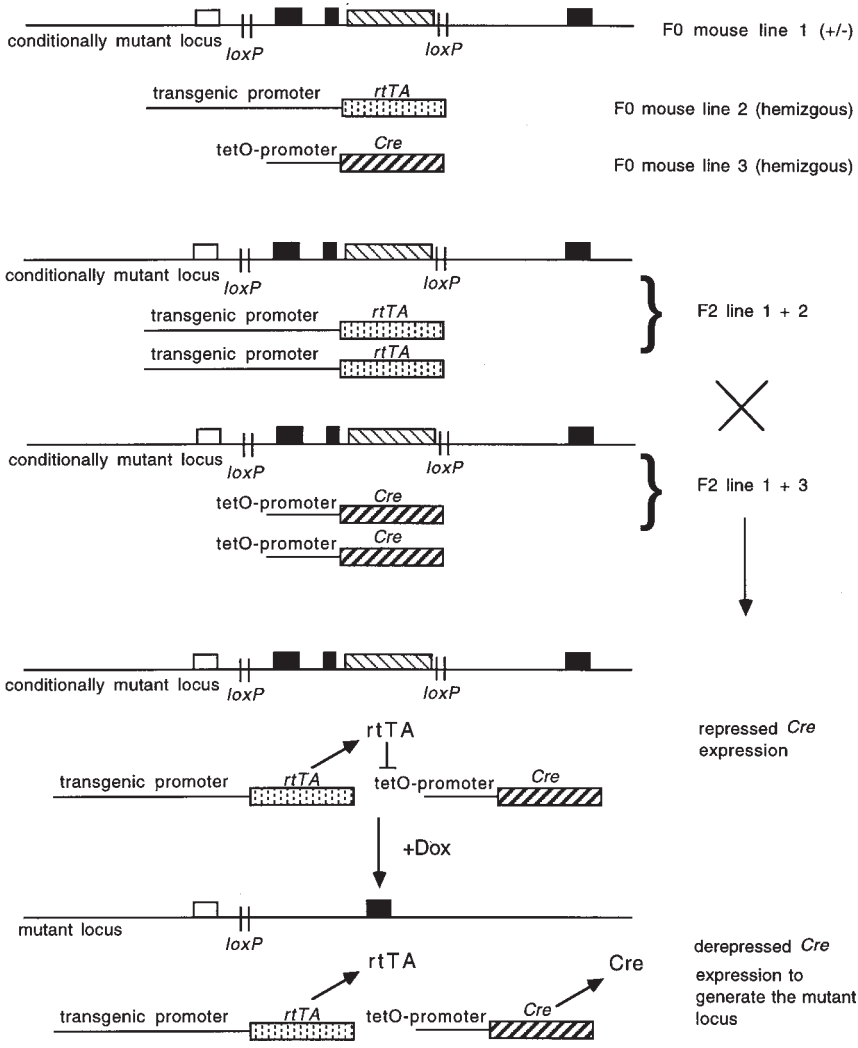


Fig. 7. Tet–On-inducible gene targeting. Mouse line 1 is heterozygous for a conditionally mutant target locus. Mouse line 2 is hemizygous for a transgene to express the mutant tetracycline repressor protein (stippled box) in a tissue-specific or general manner. Mouse line 3 is hemizygous for a *Cre*-expressing transgene (heavy striped box) responsive to tetracycline. F2 can be obtained by breeding that are heterozygous for the conditionally mutant locus and homozygous for the respective transgenes. Crossing the F2 mice will produce progeny that are hemizygous for both transgenes to maintain the repression of the *Cre* gene and will carry the conditionally mutant locus and the wild-type endogenous locus in the expected Mendelian ratios. The addition of doxycycline results in the derepression of the *Cre* gene and the creation of mutant target locus. The wild-type alleles of the endogenous target locus are not shown in the heterozygous progeny.

promoter to achieve *Cre* gene expression to convert the targeted locus from the conditional state to the mutant state.

1.3.3. Ecdysone-Inducible Gene Targeting

In *Drosophila melanogaster*, the molting hormone ecydsone exerts its biological effect by binding to a heterodimeric receptor composed of the ecdysone receptor (EcR) and the ultraspiracle (USP) gene product ***(39,40)***. Following ligand binding, the activated ecdysone receptor can then bind to DNA and induce transcription from a responding promoter that contains ecdysone/glucocorticoid response elements (E/GRE) to achieve highly inducible gene expression in mammalian cells. A vector that encodes the *Drosophila* EcR, and the mammalian homolog of the *USP* gene, RxR, to produce the heterodimeric ecdysone receptor, which binds the ecdysone analog, muristerone A, which is commercially available from Invitrogen Corp. (San Diego, CA). Unlike the low levels of expression observed in the repressed states using the tetracycline-inducible systems, ecdysone-regulated gene expression in mammalian cells exhibits no background levels of gene expression from the minimal promoter in the uninduced state. However, because the ecdysone receptor is a heterodimer, it is more cumbersome in practice from the standpoint of transgene expression, as both the EcR and RxR must be produced in the same tissue. This expression problem can be overcome by incorporating the picornavirus IRES (Internal Ribosome Entry Site) element ***(41,42)***, which can be used to generate a bicistronic mRNA that would allow for the translation of both EcR and RxR proteins from a single mRNA. As shown in **Fig. 8**, a conditional gene targeting strategy would be to induce the expression of the *Cre* gene under the control of exogenously added muristerone A by combining the genetic elements from several independent mouse lines carrying (1) a transgene using a promoter to express an EcR-IRES-RxR gene cassette, (2) a transgene consisting of the muristerone A-responsive promoter to express the *Cre* gene, and (3) a conditionally targeted locus with the *lox*P site flanking the endogenous sequences to be deleted.

1.4. Designing a Gene-Targeting Vector

One of the initial considerations in the design of a gene-targeting experiment is the type and location of the genetic mutation to be introduced, which is often influenced by the nature of the protein encoded by the target locus. For example, if the target gene encodes a multidomain proteins such as a transmembrane receptor tyrosine kinase, very different types of mutations could be planned for studying protein function such the use of a replacement vector strategy to generate a deletion of the tyrosine kinase domain to eliminate the catalytic activity of the receptor, or the use of an insertion vector “hit and run”

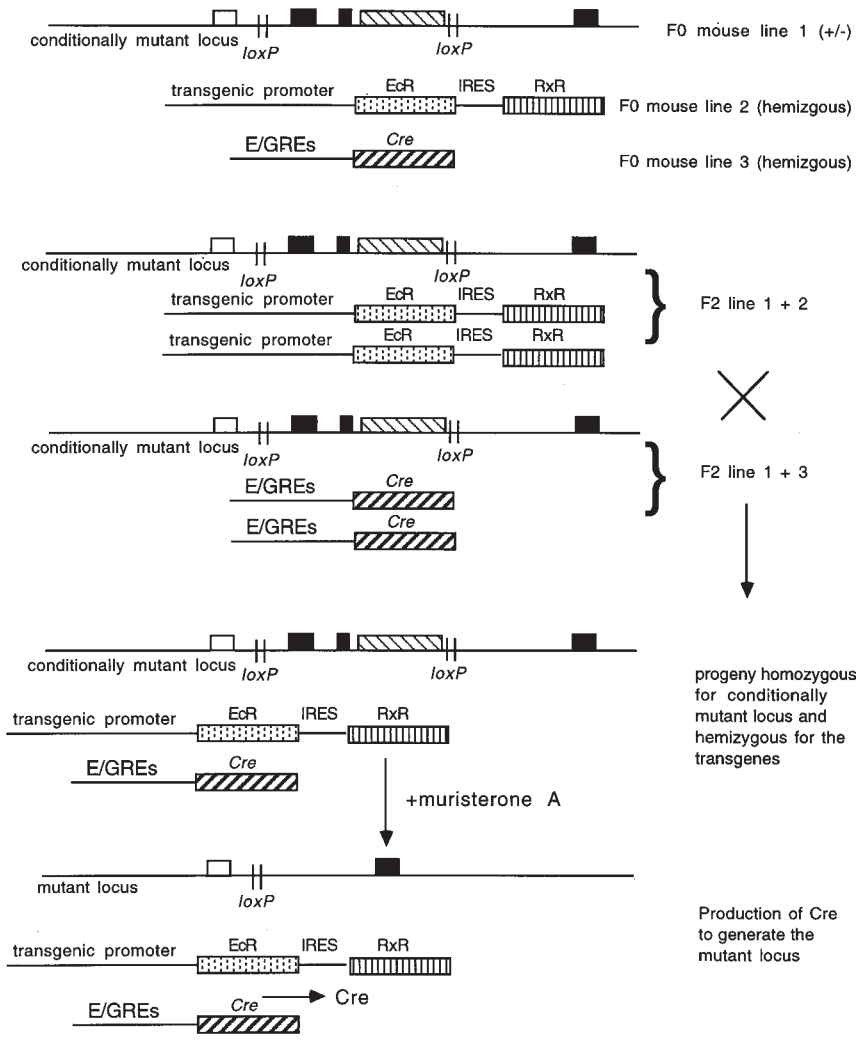


Fig. 8. Mouse line 1 is heterozygous for a conditionally mutant target locus. Mouse line 2 is hemizygous for a transgene to express a bicistronic message encoding the ecdysone receptor (stippled box) and the RxR (vertical stripes). Mouse line 3 is hemizygous for a transgene to express the *Cre* gene (heavy striped box) from a promoter containing ecdysone/glucocorticoid response elements. F2 can be obtained by breeding that are heterozygous for the conditionally mutant locus and homozygous for the respective transgenes. Crossing the F2 mice will produce progeny that are hemizygous for both transgenes and will carry the conditionally mutant locus and the wild-type endogenous loci in the expected Mendelian ratios. The addition of muristerone A results in the expression of the *Cre* gene and the creation of mutant target locus. The wild-type alleles of the endogenous target locus are not shown in the heterozygous progeny.

approach to introduce point mutations to study the role of individual tyrosine residues in the kinase domain. The average gene contains coding exons dispersed throughout about 10 kilobases (kb) of genomic DNA, and thus the location of the mutation will determine the regions of endogenous sequences used to construct the gene-targeting vector. As described in **Subheading 1.5.**, the sequence context of a particular region of genomic DNA may make it a poor substrate for homologous recombination with the target locus.

As a first pass in most gene-targeting experiments, it is usually the intention to inactivate the target gene by deleting some or all of the coding exons. In a replacement vector scenario, it might be sufficient to make a small deletion (0.5–1 kb) to eliminate one or two exons that encode a critical domain required for protein function. However, the genomic structure of some genes may require larger deletions in the range of about 15–20 kb to delete exons spread over many kilobases. Fortunately, deletions of this size can seemingly be generated with targeting frequencies similar to those obtained for small deletions ***(43)***.

Regardless of the gene-targeting strategy that is used, there are a number of important parameters that should be considered to optimize the frequency of obtaining homologous recombinant ES cells. As described **Subheading 1.5.**, these parameters mostly pertain to the DNA sequence composition of the gene-targeting vector, but also include the optimization of ES cell growth conditions.

1.5. Isogenic vs Nonisogenic DNA

The homologous recombination between the gene-targeting vector and the endogenous target locus in ES cells requires extensive stretches of homology to proceed efficiently and with fidelity. Because there are stain-specific sequence polymorphisms can interfere with homologous recombination in mammalian cells ***(44)***, it is advisable to construct a gene-targeting vector using homologous DNA regions obtained from a mouse genomic DNA library that is isogenic with the endogenous locus in the ES cell line. It has been shown that the *Hprt* ***(45)***, *Rb* ***(46)***, and *CKM* ***(47)*** loci in 129 strain ES cells are targeted 5-, 20-, and 25-fold more efficiently, respectively, using targeting vectors that are comprised of isogenic homologous DNA compared with a non-isogenically derived homology regions. Most research institutions now have a centralized transgenic facility that routinely perform ES cell culture and transfection experiments to obtain gene-targeted ES clones, so it is important to know the genetic background of the ES lines in use. The most commonly used ES cell lines are derived from different substrains of the 129 mouse strain, these include CCE (129/Sv/Ev) ***(48)***, E14.1, a derivative of E14 (129/Ola) ***(49)***, D3 (129/Sv) ***(50)***, R1 (129/Ola) ***(51)***, and AB1 (129/Sv/Ev) ***(52)***. Although it is best to prepare a genomic DNA library from the ES cell line-derived DNA, a representative genomic DNA library from 129 strain with insert sizes ranging from

9–23 kb in bacteriophage lambda vector Lambda FIX II is commercially available from Stratagene (La Jolla, CA).

1.6. Homology Length and Targeting Frequency

One of the most important parameters of the gene-targeting vector that affects the targeting frequency at a particular genetic locus is the total length of the homologous DNA regions resident on the vector ***(45,53)***. In a test of replacement vectors to target the Hprt locus using homologous regions derived from isogenic DNA , it has been shown that increasing the length of the total homology from 1.3–6.8 kb dramatically increased the absolute targeting frequency by 250-fold, and that there is an exponential relationship between the total length of homology and the targeting frequency when the homologous DNA ranges from 2 to about 14 kb in length. The use of longer stretches of homology is particularly important when the targeting vector incorporates larger amounts of heterologous DNA, such as when *Bgeo* or *Bgyg* selection cassettes **(Subheading 1.1.1.2.)** are being employed for positive selection and to visualize the expression profile of the target gene.

In the design of a replacement vector, it is usually the case that the homologous DNA will be distributed unsymetrically in the 5' and 3' flanking homology regions of the vector such that one of the arms will be considerably shorter to make it more convenient for performing the initial diagnostic screen of homologous recombinant ES cell DNA either by PCR or Southern blotting (*see* below). The minimum length for the short arm on the vector is about 500 base pairs (bp), but it is important to note that homology arms shorter than about 1 kb can reduce in the fidelity of homologous recombination at the target locus ***(45,54)***. As discussed above, the length of the homologous flanking regions depends on the availability of genomic clones contained in the library and the location of convenient restriction enzyme sites for generating fragments of flanking DNA. For insertion vectors that depend on a single crossover for homologous recombination, it seems that a short arm does not unfavorably affect the targeting frequency because recombination can be achieved by a crossover at a location in the long arm ***(53)***.

2. Materials

1. Restriction enzyme HpaII.
2. Klenow fragment of DNA polymerase.
3. ^{32P}-dCTP.
4. Hybridization solution: 0.5 *M* phosphate buffer, pH 7.4, 7% sodium dodecyl sulfate (SDS,) 1% bovine serum albumin (BSA), 0.01 *M* ethylene diamine tetraacetic acid (EDTA).
5. 2X Standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS).
6. XAR X-ray film (Kodak, Rochester, NY).

7. Intensifying screens (Lightning Plus, Dupont).
8. TE: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA.
9. Trypsin solution.
10. 1X Phosphate-buffered saline (PBS) containing Mg^{2+} and Ca^{2+}.
11. Gene Pulser cuvets : 0.4 cm Electrode, cat. no. 165-2088 (Bio-Rad Laboratories, Hercules, CA).
12. Bio-Rad Gene Pulser.
13. ES culture media.
14. G418 (Gibco).
15. Hygromycin.
16. GANC (gancylovir, Roche).
17. DNA lysis buffer: 0.1 *M* Tris-HCl, pH 7.5, 0.2 *M* NaCl, 0.2% SDS, 1 m*M* EDTA and 25 µg/mL proteinase K.
18. Isopropanol.
19. 70% ethanol.

3. Methods

3.1. Cloning 5' and 3' Target Gene Homology

The cloning of the DNA components of in a basic sequence replacement gene-targeting vector can be a time consuming aspect of any gene-targeting experiment. A rapid cloning strategy can easily be developed by mapping restriction endonuclease sites on the homology regions that can be used to generate fragments for one-step cloning in the proper orientation and juxtaposition to the other components of the vector. A basic strategy is to first construct a plasmid vector backbone that contains the selectable marker genes cassettes and cloning sites that are unique in the vector that would allow for the cloning of the 5' homology region immediately upstream of the positive selection marker gene cassette, and for the cloning of the 3' homology region between the positive and negative selection cassettes if a PNS strategy is planned (*see* **Fig. 9A**).

The next step is to characterize the target gene genomic clones by mapping the location of restriction endonuclease sites relative to the position of intron and exon sequences on the clones. To clone restriction fragments from the homology regions, it is convenient to use truncated exons to specify the deletion boundaries flanking the positive selection marker cassette on the targeting vector because it simplifies the analysis of the 5'–3' orientation of these fragments when cloning, compared with using uncharacterized intron sequences. A simple method for cloning the 5' and 3' homology regions is described in the following list and illustrated in **Fig. 9B**.

1. Determine the complete DNA sequence of the exons that have been selected as the 5' and 3' deletion endpoints, and generate a sequence-derived restriction map of the exon.

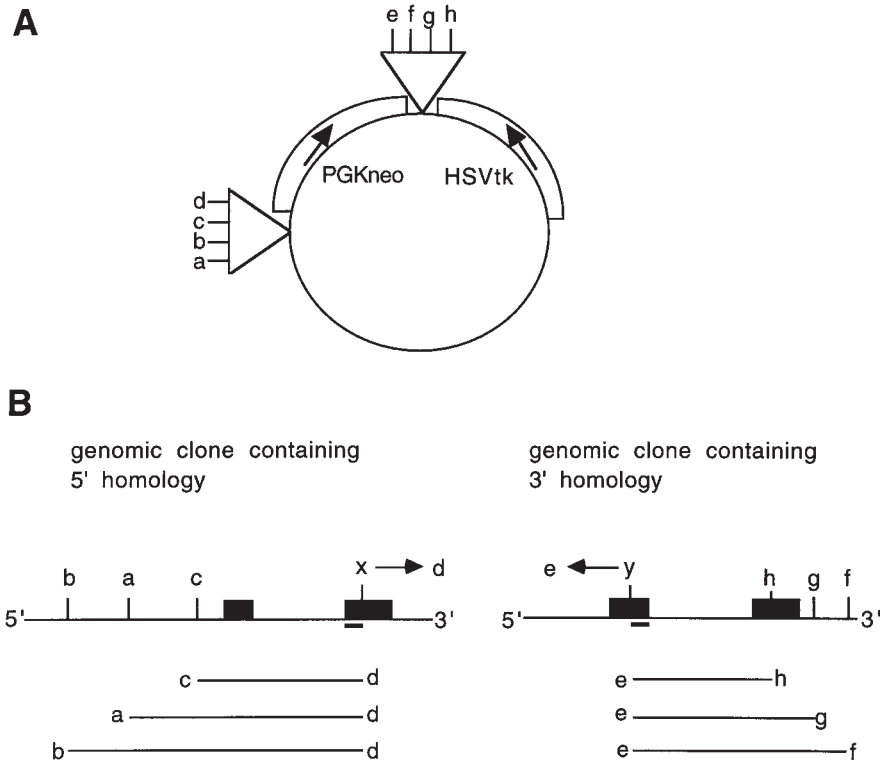


Fig. 9. 5' and 3' Homology region cloning. **(A)** A plasmid vector containing the drug selection cassettes for positive-negative selection and unique restriction endonuclease sites (a–h) to clone DNA fragments from the 5' (a–d) and 3' (e–h) homology regions. **(B)** "x" and "y" are restriction sites that define the deletion endpoints of the 5' and 3' homology regions, respectively, and can be converted to sites "d" and "e" with the use of linkers or adapters. The sites upstream of "x" and downstream of "y" are determined by mapping as described in the text. The resulting fragments can be cloned unidirectionally into the respective multiple cloning sites to complete the gene-targeting vector. In this example, the vector can be linearized at site "a" prior to introduction of the vector into ES cells.

2. Select a restriction enzyme that digests only once within the exon, preferably an infrequent cutter, as the downstream cloning site of the 5' homology region, or the upstream cloning site of the 3' homology region, respectively. If there are no convenient sites residing within the selected exons, use sites in the introns located upstream or downstream of the respective exons.
3. Select a panel of restriction enzymes, compatible with cloning into the unique sites on the plasmid vector (*see* **Note 1**), to restrict the genomic DNA insert individually and in combination with the restriction enzyme chosen in step 2 to truncate the exon.

4. Perform a Southern blotting analysis of both the 5' and 3' candidate homology fragments using as probes only the regions of the individual exons contained within the 5' or 3' fragments, respectively.
5. Based on the length of the restriction fragments from both the 5' and 3' homology regions a determination can be made for cloning into the targeting vector. The length of 5' and 3' homology regions will depend on the configuration of the available genomic clones, the presence of convenient restriction sites for cloning, and the location of repetitive DNA.

3.2. Determining the Location of Repetitive DNA

Gene-targeting vectors require long regions of target gene homology and will often include intron sequences or other untranslated segments of the endogenous gene. Untranslated portions of genes can be problematic for the construction of a targeting vector in the sense that these regions can contain stretches of interspersed repetitive DNA which, if located within several hundred base pairs of the linearized end of the vector, will drive the vector to target homologous repetitive sequences in the genome instead of the endogenous target gene. A method for easily mapping the location of repetitive DNA present on the targeting vector is to digest the homologous DNA regions with previously mapped restriction enzymes, and perform a Southern blot analysis using an end-labeled probe of total genomic DNA (*see* **Note 2**).

1. Based on the restriction map of the homologous DNA regions in the targeting vector, use the appropriate combination of enzymes to generate restriction fragments that average about 300–500 bp in length for Southern blotting.
2. Digest total mouse genomic DNA with HpaII, and end-label 100 ng of the DNA with α-^{32}P-dCTP using the Klenow fragment of DNA polymerase I.
3. Hybridize the Southern blot in with DNA hybridization solution at 65°C for 16 h.
4. Wash the filter in 2X SSC, 0.1% SDS at room temperature for 30 min and then at 65°C for 30 min.
5. Expose the blot to XAR X-ray film (Kodak) for several hours to overnight at –80°C using intensifying screens.
6. Identify the labeled restriction fragment and locate their position on the homology regions to be used in the targeting vector.
7. If repetitive DNA elements are determined to be within several hundred base pairs from the linearized end of the homology region in the targeting vector, they should be eliminated from the vector.

3.3. Electroporation and Drug Selection of ES Cells

There are a number of methods for introducing DNA into ES cells including the nuclear microinjection of naked DNA, calcium phosphate coprecipitation, and electroporation. The most desirable method for the purposes of gene targeting is by electroporation, which is the application of a brief electric current that creates pores in the cell membrane through which the DNA can pass.

1. Linearize the targeting vector by digesting to completion with the appropriate restriction enzyme, phenol-chloroform extracted, ethanol precipitated twice, and resuspended in sterile TE at a concentration of 1 mg/mL.
2. Harvest a 50% confluent 10 cm plate of ES cells growing in the log phase *(55)* by trypsinization and collected by centrifugation.
3. Wash the cells once in 1X PBS containing Mg^{2+} and Ca^{2+}, and resuspend in the same buffer at a concentration of 2×10^7cells/mL.
4. Combine 10–20 μg of the linearized targeting vector DNA and 1×10^7 ES cells in a Gene Pulser cuvet and electroporate the cells at 240 V, 500 μF, and again at 230 V, 500 μF.
5. Allow the cells to recover for 5 min at room temperature.
6. Distribute the contents of one cuvet onto four 10 cm tissue culture dishes containing the appropriate feeder cells. If a PNS selection scheme is being employed, the cells in one of the plates should be selected with only the positive selection drug so that the enrichment with negative selection can be monitored.
7. Forty-eight hours after the electroporation begin positive drug selection using 220 μg/mL G418 (effective concentration), or 300 μg/mL hygromycin.
8. The next day, apply negative selection (if appropriate) by adding 2 μ*M* GANC .
9. Change the culture medium every 2 d and continue the selection for 12–14 d.
10. Harvest ES cell colonies for about 10–14 d following the initiation of the drug selection.

3.4. Identification of Homologous Recombinant ES Cells

ES cells that have undergone homologous recombination will be genetically heterozygous at the target chromosomal locus. Thus, in planning any gene-targeting experiment it is important to have a genotyping strategy that will easily discriminate between the wild-type and mutant loci in the cells that harbor a homologous recombination event. When selecting the target gene homology regions for cloning into the targeting vector, it is important to anticipate the use of a polymerase chain reaction (PCR) or Southern blot analysis by considering oligonucleotide primer pairs to generate diagnostic PCR products or available restriction enzyme sites to generate diagnostic restriction fragments, respectively (*see* **Note 2**). Compared with a PCR screen, Southern blotting analysis of ES cell DNA is a more reliable method for detecting homologous recombinants. The success of this type of analysis is dependent on the selection of DNA probes and the choice of restriction enzymes that will identify restriction fragments that are unique to the mutant and wild-type alleles. An example of such an analysis is illustrated in **Fig. 10**. (*see* also **Note 4**). Regardless of the screening method that is used to identify homologous recombinants, the preparation of the ES cell DNA is identical.

1. Harvest ES cell colonies surviving drug selection individually, trypsinize, and replate in a 24-well culture plate using the conditions (feeders and medium) that is recommend for the particular ES cell line.

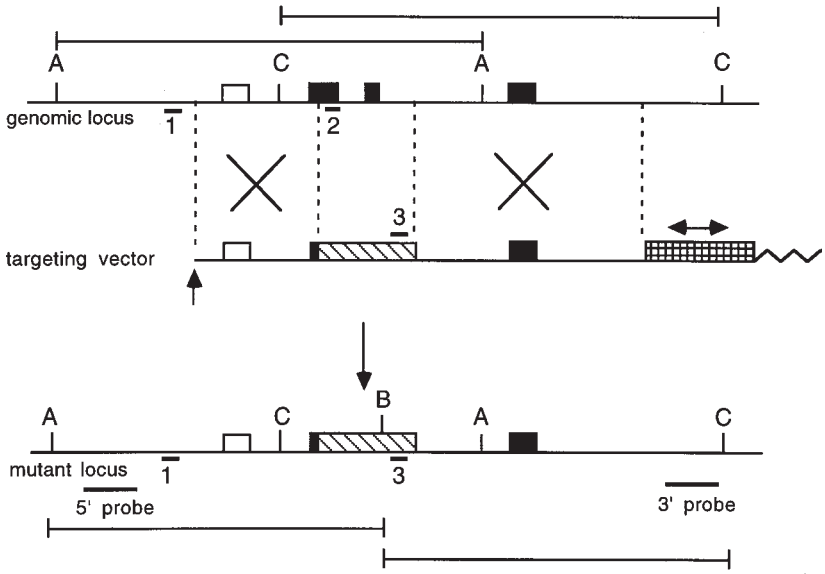


Fig. 10. Screening for gene targeted ES cells by PCR and Southern blotting. A PCR-based strategy to discriminate between the endogenous and mutant loci utilized three synthetic oligonucleotides (1, 2, and 3) to generate PCR products that are specific to the wild-type gene (oligos 1 and 2) and the mutated gene (oligos 1 and 3). The PCR products are size analyzed by gel electrophoresis to identify the homologous recombinant ES clones. A Southern blotting analysis uses diagnostic restriction endonuclease sites to generated restriction fragments specific to either the wild-type or mutated gene. A 5' probe can distinguish between electrophoretically sized fragments generated by digesting ES cell DNA with restriction enzymes "A" and "B", whereas a 3' probe can be used for "B" and "C" generated restriction fragments. PGK*neo* cassette (striped box), HSV*tk* cassette (hatched box) .

2. Change the ES cell medium (without drug selection) daily, and when the clones become 50–75% confluent harvest the cells by trypsinization.
3. Aliquot cells for DNA analysis (2/3) and for freezing down (1/3).
4. For DNA analysis, pellet the ES cells at 12,000*g* for 2 min to remove the trypsin solution.
5. Resuspend the cells in 0.5 mL of at 55°C for 6–16 h.
6. Centrifuge the lysates DNA lysis buffer and incubate for 10 min to remove cellular debris.
7. Precipitate the DNA from the supernatant by adding an equal volume of isopropanol.
8. Remove the precipitated DNA with an inoculation loop, dip in a 70% ethanol rinse, and resuspend in 50–100 μL of TE.
9. Heat the DNA suspension at 65°C for 5–10 min immediately prior to pipetting aliquots for analysis. Typically, a 1–2 μL aliquot of the DNA solution can be used for PCR, and an 8–10 μL aliquot is adequate for Southern blot analysis.

4. Notes

1. Gene targeting vectors end up being quite large, on the order of 15–20 kb, so it is important to use plasmids with replication origins that direct high copy number and those that accept large inserts of DNA, such as pUC or pBluescript.
2. Using an end-labeled probe, only the repetitive sequence population in the genomic DNA will be labeled with high enough specific activity to detect similar repetitive sequences carried on the homologous regions of the targeting vector. In this way the location of repetitive DNA sequences can be mapped.
3. For a PCR-based screen, a minimum of three oligonucleotide are needed; one oligonucleotide will anneal to a flanking genomic sequence that is *not* present on the targeting vector and will prime both the mutant and wild-type alleles when paired with oligonucleotides that are specific for either the wild-type or mutant alleles. The PCR primer pairs should be arranged so that the predicted PCR product sizes from the two alleles can easily be distinguished in a gel electrophoretic analysis. Although a PCR-based analysis is the most rapid method for analyzing the genotype of ES cell clones, a major drawback is the technical inconsistency of the method that may compromise the overall detection of homologous recombinants.
4. When using replacement vectors, it is important to analyze both the 5' and 3' ends of the recombination event to determine that the genomic configuration of the targeted chromosomal locus is correct, as a significant proportion of homologous recombination intermediates are not resolved faithfully, resulting in one arm of the vector integrating in a nonhomologous fashion ***(12,56)***.

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4

Manipulating Mouse Embryonic Stem Cells

Lino Tessarollo

1. Introduction

In recent years, gene targeting technology has been rapidly evolving and has been used by a number of laboratories to systematically alter the mouse genome and to generate mice with specific targeted gene mutations. The improvement in the quality of reagents (embryonic stem (ES) cells and media) available to the scientific community has made this technology accessible to many laboratories.

ES cells are derived from the inner cell mass (ICM) of a 3.5-d postcoitus embryo at the blastula stage of development. ES cells can be cultured, and still retain their ability to contribute to all cell lineages when reintroduced into a host blastocyst. Information has been published describing methods to culture and genetically manipulate ES cells without compromising their ability to contribute to the germline ***(1–4)***. Differences exist in the requirements of specific ES cell lines. However, there are some fundamental steps that must always be followed to preserve ES cell totipotency. For example, basic good tissue culture rules should be followed; ES cells should be passaged as little as possible, as prolonged culture periods will affect their ability to contribute to the mouse germline; plated ES cells should never reach confluency or be exposed to exhausted medium for prolonged periods of time. In most instances, ES cells will show the adverse effects of stress due to poor culture conditions only at the time their totipotency is evaluated by their ability to generate "germline chimeras" (several months after the beginning of the experiments!). This chapter discloses some of the basic principles to establish ES cell technology in a laboratory. The protocols described here are relatively simple and will be very effective if followed carefully. In my laboratory, these protocols have worked very well with several ES cell lines of the 129SV mouse strain, and in particular with the CJ7 ES cell line ***(5)***. It is, however, very important to follow additional specifications given by the source of the specific ES cell line in use.

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2. Materials

2.1. Equipment

The following equipment can be found in almost every tissue culture area. However, special care should be taken to assure that every piece is decontaminated before use.

1. Tissue culture incubators: Set at 5% CO_2, 20% O_2, with a saturated aqueous atmosphere. It is extremely important to assure the proper function of this piece of equipment. In particular, special care should be taken in monitoring precise CO_2 levels every week and assure that the recovery time after openings is not too extended. If possible, reserve one incubator for ES cell work exclusively, keeping track of its usage and users.
2. Inverted microscope with 5× and 10× objectives.
3. Electroporator: Capable of reaching the capacitance of 500 μF. (i.e., Bio-rad Gene Pulser).
4. Tissue culture hood with UV light: Before use, make sure that it has been properly decontaminated and the air flow is working properly. If possible keep it separate from other users. Hood should be wiped down with 70% ethanol before and after use. Also, turn on the UV light when not in use.
5. Tabletop centrifuge.
6. Water bath: Set at 37°C for regular use but can also be used at 56°C to heat inactivate serum.

Once a tissue culture area is set up for ES cell use, it is extremely important to periodically check that the instruments are functioning properly.

2.2. Tissue Culture Reagents and Solutions

Many of the following reagents can be prepared from powder. However, it is recommended to purchase premade or stock solutions to avoid variability in quality because of in-house preparation.

1. Dulbecco's Modified Eagle Medium (DMEM): With high glucose (4500 mg/L) without sodium pyruvate (however, some ES cell lines may require sodium pyruvate) (e.g., Gibco-BRL cat. no. 11960-044). The following components should be added to fresh DMEM.
2. Fetal calf serum (FCS):- FCS can be purchased from several companies that provide ES cell-tested batches. However, an in-house test should be performed for every new lot of FCS purchased to ensure that it is optimal for the ES cell line in use in the laboratory (*see* **Note 1**).
3. 100X Penicillin-streptomycin (pen/strep): 50 Units/mL each of penicillin and streptomycin. 100X solutions are commercially available (e.g., Gibco-BRL, BioWhittaker).
4. 100X L-Glutamine: 200 m*M* (e.g., Gibco-BRL, cat. no. 25030-081).
5. MEF media: To make 100 mL combine 83 mL DMEM, 15 mL fetal bovine serum (FBS), 1 mL 100X pen/strep, 1 mL 100X L-glutamine.

6. 100X β-mercaptoethanol: 7 μL β-Mercaptoethanol (β-ME) (Sigma cat. no. M-7522) in 10 mL of phosphate-buffered saline (PBS). Store at 4°C and replace weekly.
7. 100X MEM nonessential amino acids: Gibco-BRL, cat. no. 11140-050.
8. Leukemia inhibitory factor (LIF): Available from Chemicon International at 10^7 Units/mL (cat. no. ESGI107).
9. ES cell media: To make 100 mL combine 81 mL DMEM, 15 mL FBS, 1 mL 100X pen/strep, 1 mL 100X L-glutamine, 1 mL 100X sol β-ME, 1 mL 100X MEM nonessential amino acids and 10 μL LIF. ES cell media should be prewarmed only before use, and it should be kept protected from light. This media should be stored for not more than 2–3 wk if used with ES cells. Older media can be stored and used for expanding cloned ES cells used for DNA analysis.
10. Freezing medium: 60% FCS, 20% D-MEM, 20% dimethylsulfoxide (DMSO).
11. PBS: Gibco-BRL cat. no. 14040-133.
12. PBS without Ca^{2+} and Mg^{2+} (PBS w/o): Gibco-BRL, cat. no. 14190-045.
13. 0.1% Gelatin. Dissolve 1 g of gelatin (porcine skin gelatin, cell culture tested; Sigma, cat. no. G 1890) in 1 L of distilled water, aliquot and autoclave; store at 4°C.
14. Dimetil sulfoxide (DMSO): Sigma cat. no. D 2650.
15. Trypsin-ethylene diamine tetraacetic acid (EDTA): Gibco-BRL, cat. no. 25300-054.
16. DNase I solution: 100 mg (2000 U/mg) in 7.0 mL, 0.154 *M* NaCl, 50% glycerol (e.g., Boehringer Mannheim, cat. no. 104159).
17. Collagenase H solution: 100 mg (>0.5 U/mg) in 7 mL PBS, 50% glycerol (e.g., Boehringer Mannheim, cat. no. 1074032).
18. Hyaluronidase: 100 mg (approx 800 U/mg) in 7 mL PBS, 50% glycerol. (e.g., Sigma, cat. no. H-3884).
19. MEF digestion media: 50 mL DMEM supplemented with 500 μL DNase I, 500 μL collagenase H, and 500 μL hyaluronidase solutions.
20. Mitomicyn C (MC). 100X stock: Resuspend 2 mg of MC in 2 mL PBS (e.g., Boerhinger Mannheim, cat. no. 107-409). Filter through a 0.22-μm syringe filter. Aliquot and store at –20°C for up to a month.
21. Geneticin (G418): Gibco-BRL, cat. no. 11811-023.
22. Gancyclovir: Use Cytovene (Syntex) at 2 μ*M* or FIAU (Oclassen Pharmaceuticals) at 0.5 μ*M*. Different concentrations of gancyclovir or FIAU should be tested considered the sensitivity of ES cells to these compounds.

2.3. Plasticware and Other Materials

It is recommended that disposable plastic materials are used for all tissue culture work.

1. Culture plates:
   a. 10 cm Polystyrene dishes (use 10 mL media).
   b. 6 cm Polystyrene dishes (use 4 mL media).
   c. 12-Well cluster plates, tissue culture treated (use 2–2.5 mL media).
   d. 24-Well cluster plates tissue culture treated (use 1.5-2 mL media).
   e. 96-Well cluster plates, round bottom, tissue culture treated.

2. 50 mL and 15 mL tubes.
3. 1 mL freezing vials.
4. 1 mL Eppendorf tubes.
5. 25 mL, 10 mL, 5 mL, 1 mL plastic pipets.
6. 200 µL Aerosol barrier tips.
7. 12-Well multichannel pipettor.
8. Repeat pipetman.
9. 0.4 cm Electrode gap electroporation cuvettes (e.g., Bio-Rad Gene Pulser cuvets, cat. no. 165-2088)
10. Equipment for dissection: razor blades, scissors, microdissecting scissors, straight and curved forceps, tweezers.

2.3. Molecular Biology Reagents

For additional information about molecular biology procedures and reagents *see* **ref. *5***.

1. Lysis buffer: 100 m*M* Tris-HCl, pH 8.5, 5 m*M* EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 m*M* NaCl, 0.2 mg/mL proteinase K. Proteinase K is prepared as a stock solution of 10 mg/mL in water and stored at –20°C. Note: Proteinase K should be added fresh to the other components of the lysis buffer just before lysing the cells.
2. 0.1 *M* Spermidine trihydrochloride: Sigma, cat. no. S2501.
3. 10X TPE: 1 *M* Tris-phosphate, 20 m*M* EDTA. For 1 L use 108 g Tris base, 15.5 mL 85% phosphoric acid (density 1.679 g/mL), 40 mL 0.5 *M* EDTA, pH8.0, and adjust the volume to 1 L.
4. Alkali solution: 1.5 *M* NaCl, 0.5 *M* NaOH.
5. 20X standard saline citrate (SSC): For 1 L dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 mL water, adjust to pH 7.0 with a few drops of 10 *M* NaOH and adjust the volume to 1 L.
6. 20X SSCP: For 1 L dissolve 140.2 g NaCl, 88.2 g sodium citrate, 43.7 g Na_2HPO_4, 12.7 g NaH_2PO_4 and adjust the volume to 1 L.
7. Prehybridization solution: 4X SSCP, 1X Denhardt's solution, 1% SDS, 80 mg/mL boiled sonicated salmon sperm DNA (e.g., Digene, cat. no. 3100-1024). Store at 4°C.
8. Hybridization solution: 4X SSCP, 1X Denhardt's solution (5), 1% SDS, 100 mg/mL boiled sonicated salmon sperm DNA, 10% dextran sulfate. Store at 4°C.
9. Loading buffer (6X): 0.25% bromophenol blue, 0.25% xylene cyanol; 15% Ficoll type 400.
10. Hybond N(+): Amersham (cat. no. RPN 203 B).

3. Methods

3.1. Preparation of Feeder Cells

ES cells are usually grown on mitotically inactive primary mouse embryo fibroblasts (MEFs) or SIM mouse embryo (STO) fibroblasts ***(1)***. These mitotically inactive feeder cells provide support to the ES cells and produce growth factors, including LIF, that allow the ES cells to maintain their totipotency (*see* **Note 2**).

3.1.1. Isolation of MEFs

MEFs are isolated from 12–14 d postcoitus (dpc) embryos derived from a mouse strain transgenic for a gene that confers resistance to the antibiotic (e.g., neomycin) used for the selection of ES cell clones subsequent to the electroporation process.

1. Kill a 12–14 d pregnant mouse by CO_2 asphyxiation.
2. Aseptically dissect out the uterine horns. With large scissors and forceps cut the skin and then with sterile smaller scissors and tweezers cut the peritoneum. Remove the uterine horns by holding the cervix with sterile tweezers and cutting the connective vaginal side.
3. Rinse uterine horns twice in PBS.
4. Place uterine horns into a 10-cm tissue culture dish with PBS (in tissue culture hood) and dissect out embryos.
5. Place embryos in a fresh dish with 10 mL PBS. Decapitate and eviscerate the embryos.
6. Place carcasses into a clean 10 cm dish and mince them with a sterile razor blade until a gelatinous mass is created.
7. Add 5 mL of DMEM and transfer the mix into a 200-mL flask containing a stirbar.
8. Add 50 mL of MEF digestion media.
9. Stir at ≤ 37°C on a warm plate for 30–40 min.
10. Pipet off supernatant and save in a 50-mL tube.
11. Add 50-mL of fresh MEF digestion media to remaining chunks in flask and stir again at ≤37°C for 30–40 min.
12. Transfer supernatant to a new 50 mL tube and set aside.
13. Add 20 mL of trypsin-EDTA to remaining particles and stir at 37°C for 15–20 min.
14. Neutralize trypsin with approx 30 mL of MEF medium and transfer the supernatant to a 50 mL tube.
15. Spin all three tubes in a centrifuge at 300*g* for 5 min.
16. Pipet off supernatants.
17. Use 5 mL of MEF media per tube to resuspend pellets and pull the three fractions.
18. Bring volume to 10 mL of MEF media per dissected embryo and aliquot 10 mL per 10 cm tissue culture dishes (e.g., for 10 embryos, resuspend in 100 mL of media and plate into 10 plates).
19. Grow the cells until 80% confluency, at which time you may freeze them down (4 vials/plate) or trypsinize and replate for feeder production.

3.1.2. Preparation of Mouse Embryo Fibroblast Feeder Cell Layer

1. Thaw 1 vial of primary MEFs into a 10-cm plate with MEF media.
2. Grow cells to confluency and split 1:4. When these 4 plates are again confluent, split one more time 1:5 (total 20 plates).
3. When MEFs have almost reached confluency, pipet off the medium and add 5 μL of MEF media with MC (50 μL of 100X solution) to each plate.
4. Incubate for 3–3.5 h at 37°C.

5. After incubation, remove media and wash 3X with PBS.
6. Add 1.5 mL trypsin-EDTA to each plate and incubate 2–3 min (the cells start coming off the plate very quickly) at 37°C.
7. Stop the trypsin reaction by adding 1.5 mL of MEF media. Pipet to dissociate the cells. Collect all cells by rinsing the plates with media.
8. Determine the total number of cells.
9. Spin the cells down and resuspend them in an adequate volume of MEF media for immediate plating or for freezing. MEFs can be frozen down at a density of about 7×10^6 per vial for later use. Mitotically inactive MEFs (feeder cells) are sensitive. Therefore, they should be seeded onto gelatin coated plates to facilitate cell adesion.
10. Prepare gelatin-coated plates by covering the dish surface with 0.1 % gelatin. After incubating for at least 30 min (overnight is also good), remove the liquid. The plate is ready for seeding the feeder cells. Plates can also be dried for later use.
11. Plate MEFs at the following density onto gelatin coated dishes in order to obtain a monolayer **(Fig. 1A)**:

Plate size	MEFs
10 cm	3.5×10^6 cells per plate
6 cm	1×10^6 cells per plate
12-well plate	5×10^5 cells per well
24-well plate	2×10^5 cells per well

12. Change the medium every other day. Use the cells within 1 wk.

3.2. Culturing ES Cells

ES cells (*see* **Notes 3** and **4**) are totipotent cells with a doubling time of approx 12 h. They should be monitored twice a day to prevent overgrowth. However, they should be seeded at a certain density (about 10%), as they like to grow in clusters **(Fig. 1B)**. To avoid temperature shock, ES cell media should always be warmed to 37°C before adding to the culture.

1. Thaw 1 vial of ES cells (ES cells should be frozen at a concentration of about 2×10^6 cells/vial) by warming quickly at 37°C. Transfer the cells to a tube with 5–6 mL of ES cell medium.
2. Spin down for 10 min at 300*g*, remove the supernatant, and resuspend the cells in 10 mL of fresh ES media.
3. Plate onto a 10-cm feeder dish.
4. Change medium every day.
5. After about 3 d the cells should be ready for splitting **(Fig. 1C)** (*see* **Note 5**).
6. Feed the cells 2 h before splitting.
7. Remove the medium and rinse briefly with 5 mL of trypsin-EDTA (37°C).
8. Add 1 mL of fresh trypsin-EDTA and incubate 3–5 min at 37°C.
9 Check under the microscope to see if the colonies look like grapes **(Fig. 1D)**.

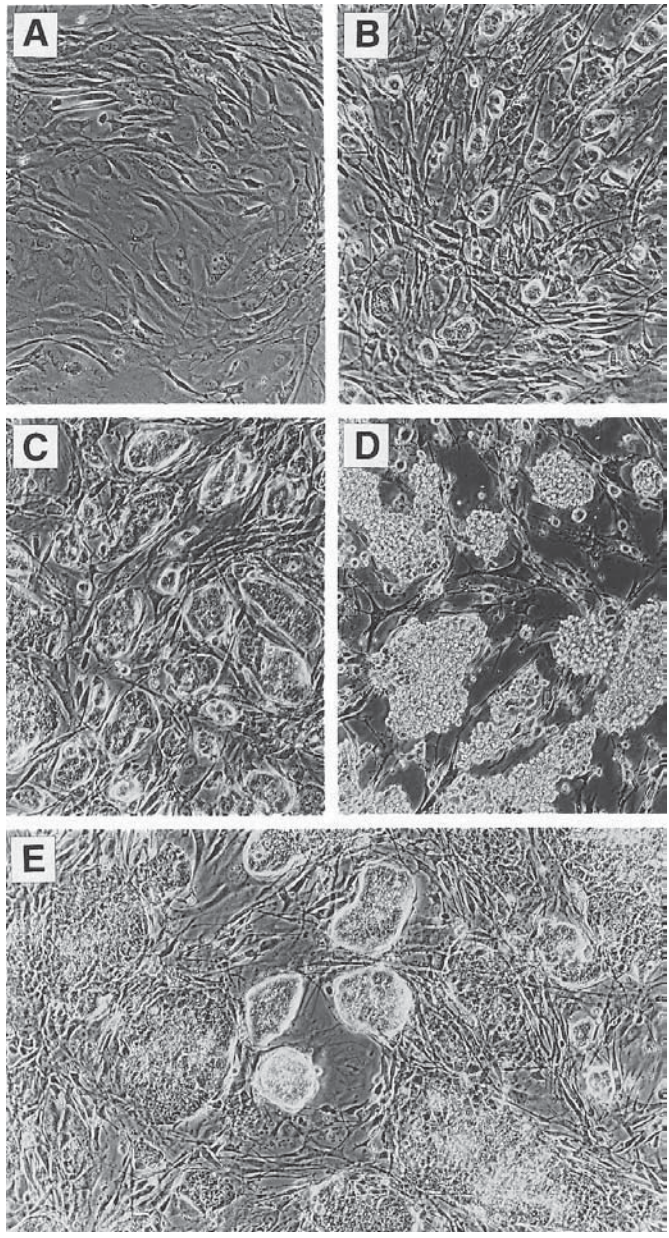

Fig. 1. Culturing ES cells. **(A)** Monolayer of feeder cells, **(B)** Density at which ES cells should be plated after thawing, **(C)** Semiconfluent ES cell culture, **(D)** Incubation of ES cell cultures with trypsin for 3–5 min makes ES cell clusters look like grapes, **(E)** Highly differentiated ES cell culture (note the high degree of differentiation toward the epithelial lineage). 100× magnification.

10. Neutralize the trypsin by adding 1 mL of MEF medium (during the splitting it is not required to use ES medium) and dissociate the colonies into a single cell suspension by pipetting at least 15–20 times with a sterile plugged pasteur pipet (the tip of the pasteur pipette should be flame polished to create a slightly smaller opening).
11. Collect the cells in 5–7 mL MEF medium.
12. Spin down and resuspend in ES cell medium for plating. Usually one subconfluent plate of ES cells is split 1:6 or frozen 1:5.
13. To freeze one plate of ES cells, resuspend the cells in 2.5 mL of DMEM and slowly add 2.5 mL of freezing medium.
14. Mix gently, aliquot into five cryopreservation vials, and store overnight at –80°C in a partially insulated box so that the cells freeze slowly. The next day transfer to liquid N_2.

3.3. Genetic Manipulation of ES Cells

3.3.1 Electroporation

Day 0

1. Split one plate of subconfluent ES cells into four dishes such that the next day the cells are ready to be split again **(Fig. 2A)** (*see* **Note 6**).

Day 1

2. Change the media in the morning, as these cells are plated more confluently than usual **(Fig. 2B)**.
3. In the afternoon, trypsinize plates as previously described and collect the cells.
4. Spin down cells for 10 min at 300*g* and resuspend in 50 mL of room temperature PBS w/o. Count cells. There should be 5–8 × 10^7 cells.
5. Spin down and resuspend cells to a final concentration of 1.5–2 × 10^7 cells/0.8 mL PBS w/o (e.g., if you have 7 × 10^7 cells total, resuspend the pellet in 3.2 mL PBS w/o).
6. Transfer 0.8 mL of cell suspension to a cuvette with 20 µL of DNA (1 µg/µL of linearized and purified DNA vector in water). Mix the cells with the DNA by pipetting with a 1 mL plastic pipette.
7. Quickly electroporate the cells. The electroporator is set at 250 V with a capacitance of 500 µF. (During the process many cells die, making the cell suspension appear somewhat viscous. The viscosity is caused by the DNA released by lysed cells.)
8. Transfer the cells from the electroporation cuvette to 30 mL of ES cell medium and plate onto three 10-cm dishes with feeders.
9. Return electroporated ES cells to the incubator.

3.3.2. Selection

Day 2

10. Start selection 18–24 h after the electroporation with 250 µg/mL G418 and 0.5 µ*M* FIAU (or 2 µ*M* gancyclovir) for 3–4 d **(Fig. 2C)**.

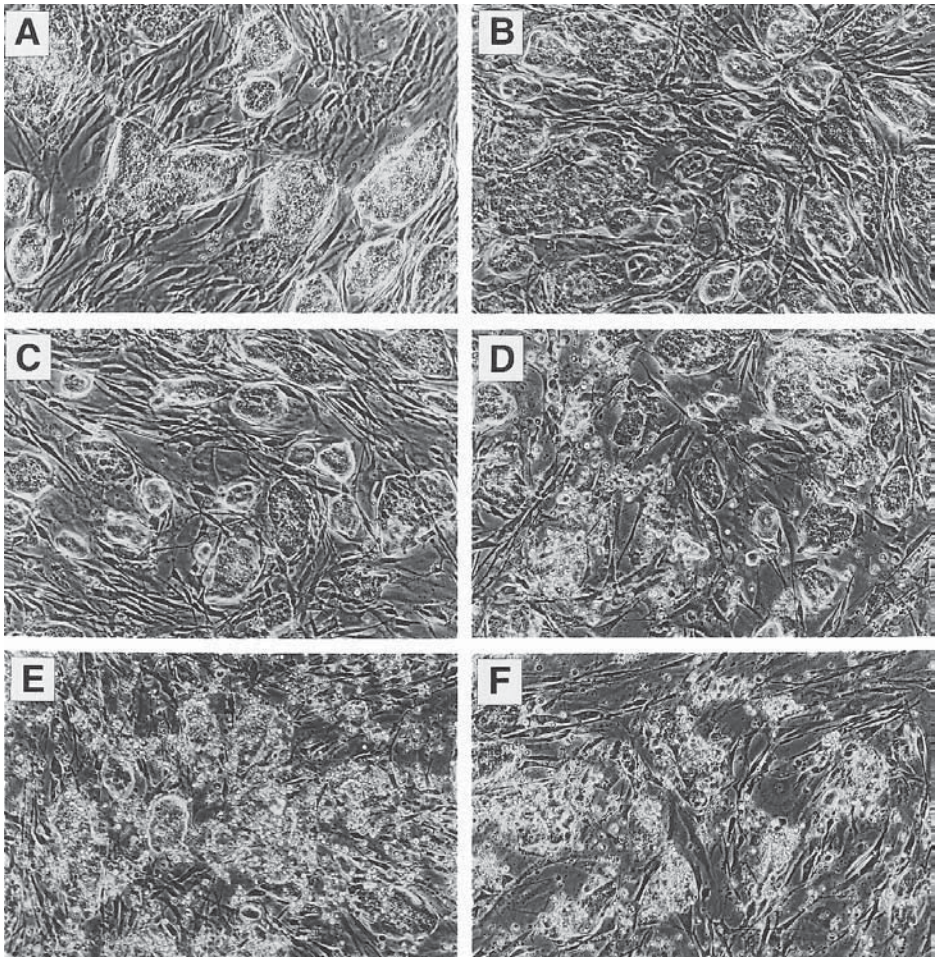

Fig. 2. Electroporation and selection. **(A)** Subconfluent ES cell plate (day 0). **(B)** appearance of ES cell plate before being used for electroporation (day 1). **(C)** Appearance of cells 24 h after the electroporation (day 2). **(D)** ES cell culture after the first day of selection (day 3). **(E)** Massive cell death is seen after 48 h of selection (day 4). **(F)** Almost all ES cells are dead on the third day of selection; debris is still present (day 5). 100× magnification.

Day 3

11. Change media (ES cell medium + G418 + FIAU). Now the cells have stopped growing. Some cells look vacuolated and debris from cell death appears on the plate **(Fig. 2D)**.

Day 4

12. Massive cells death appears on the plate **(Fig. 2E)**. Rinse off debris with PBS and change the media (ES cell medium + G418 + FIAU).

Day 5

13. Cell death still extensive. Rinse plate with PBS. This is the end of double selection. Change to G418 media (ES cell medium + 250 μg/mL G418). Few ES cells should be present on the plate among the feeders **(Fig. 2F)**.

Day 6/ Day 7

14. Colonies appear on the plate. Rinse plate with PBS if debris is still present and change G418 media every day. Prepare 24-well feeder plates for the colonies to be transferred
15. Resuspend feeder cells to 2×10^5 cells/mL and plate 1 mL/gelatin coated well.

3.3.3. Isolating ES Cell Clones

Day 8–9

1. Pipet 100 μL/well of trypsin-EDTA to alternate wells of a 96-well plate **(Fig. 3)**. **Do not** pipet more than 12–24 wells at a time.
2. Replace the medium in the dish with the colonies with 5–8 mL PBS. Pipet gently along the side of the plate to avoid detaching the colonies.
3. Place the open dish under the microscope and score for good-quality colonies **(Fig. 4A)**. Colonies are chosen based on their morphology and size. Only colonies with irregular tridimensional growth should be picked **(Fig. 5A, C, E)**. Colonies that show signs of differentiation should not be picked **(Fig. 5B, D, F)**.
4. Pick a colony using a P20 tip mounted on micropipetman set at 10 μL and transfer to well with trypsin **(Fig. 4B)**.
5. After picking 12–24 colonies (do not leave colonies for more than 10–15 min in trypsin), incubate the plate for 3–4 min at 37°C.
6. Dissociate colonies by pipetting 10–15 times with a multichannel P200 pipettor set at 100 μL. Aerosol barrier tips of 200 μL should be alternated in the multichannel pipettor so that 6 colonies at a time can be dissociated and transferred to a 24-well plate **(Fig. 3)**. This method allows quick colony processing, therefore reducing the stress of trypsinization.
7. Transfer the cells to a 24-well feeder plate with G418 medium **(Fig. 3)**. Use 2 mL/well. This medium does not need to be changed after colony plating.
8. After picking the colonies, replace the PBS on the 10-cm plate with fresh G418 medium and store for more picking on the following day.

3.3.4. Freezing ES Cell Clones (see **Note 7**)

Day 13–14

1. Remove media from clones in 24-well plates.
2. Rinse each well with 0.5 mL of trypsin.
3. Add 150 μL of fresh trypsin and incubate the plate at 37°C for 3–5 min (use repeater pipetman for speed).

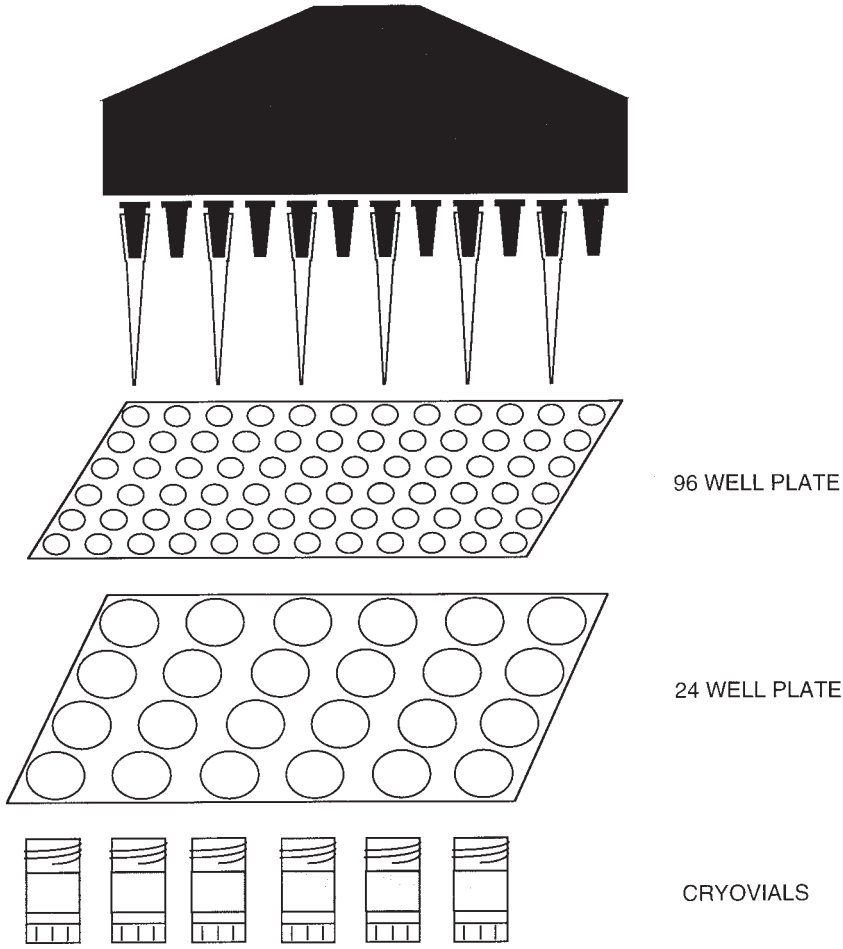

Fig. 3. Schematic illustrating how to arrange the tips in the multichannel pipettor in order to perform dissociation **(Subheading 3.3.3.)** and freezing **(Subheading 3.3.4.)** of several ES cell clones simultaneously. After colonies are placed in individual wells of a 96-well plate, cells can be handled with a multichannel pipettor for the dissociation and transfer to a 24 well plate. At the time of freezing, the same tip arrangement can be used for dissociation of ES cells and transfer to tubes for cryo-preservation.

4. Neutralize trypsin with 200 μL of MEF media.
5. Dissociate cells from 6 wells at a time by pipetting 10–15 times with a multichannel pipettor set at 200 μL with tips arranged as in **Fig. 3**.
6. Transfer 200 μL of cells to cryovials containing 200 μL of freezing media.
7. Mix by pipetting twice with the multichannel pipettor, close the tubes, and store in a styrofoam box on top of dry ice (in this way the freezing process will occur slowly).

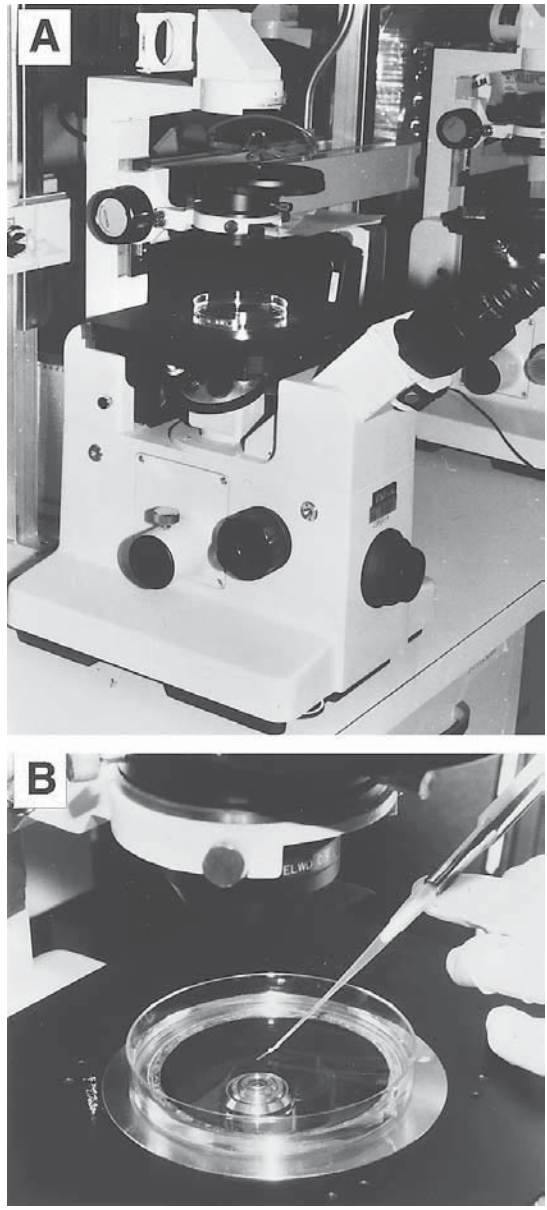

Fig. 4. Picking of ES cell clones. **(A)** Typical tissue culture microscope used for viewing and picking of ES cell clones. **(B)** Picking of ES cell clone.

8. After 2 h transfer the tubes to a –80°C freezer. The clones can be stored until results are obtained from the DNA screening.

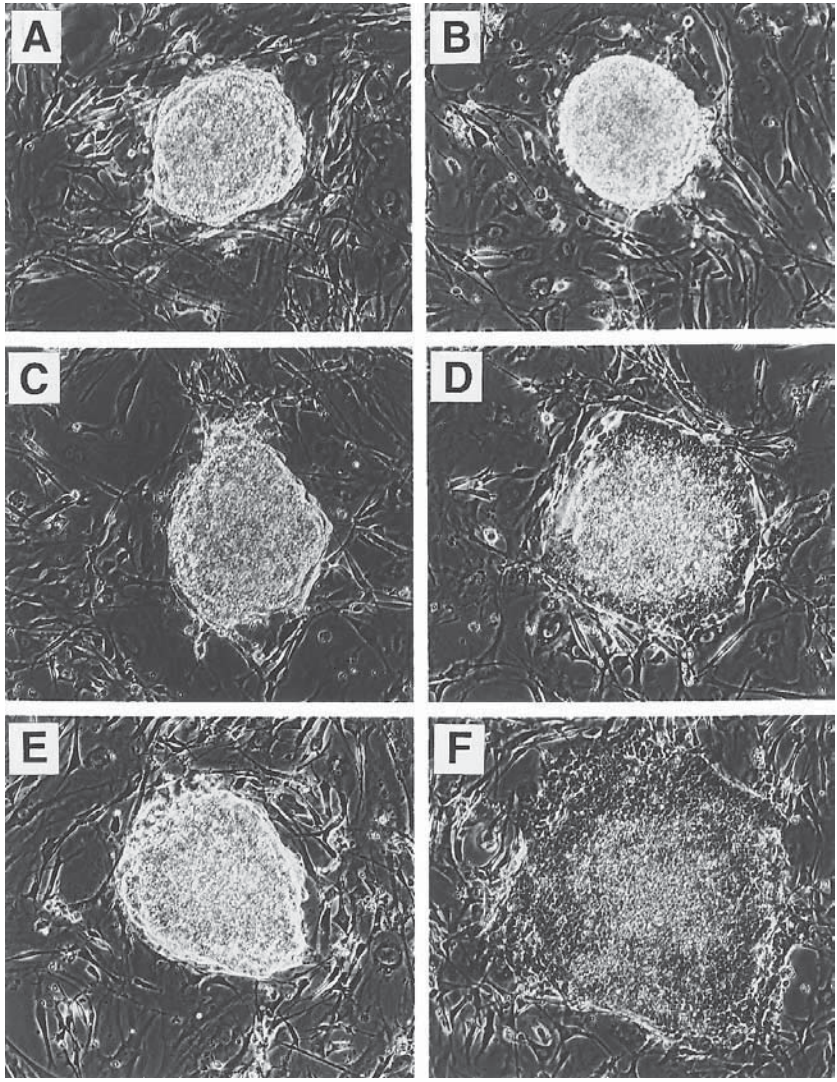

Fig. 5. Morphology of undifferentiated **(A, C, E)** and differentiated **(B, D, F)** ES cell colonies. Undifferentiated colonies display slightly irregular threedimensional ES cell growth **(A, C, E)**. Colonies which grow three dimensionally but appear rounded are differentiating as simple embryoid bodies (**B**; **ref. *1***) whereas flat colonies **(D, F)** are differentiating as epithelial cells. 100× magnification.

9. The remaining cell suspension from the trypsinized plate can be transferred to a new gelatin-coated 24-well plate with G418 media. These cells will be grown and used for DNA analysis.

3.4. Analysis of DNA from ES Cell Clones

3.4.1. Lysis of ES Cell Clones

A key issue at this step is to maximize DNA recovery from these ES cell clones. Therefore, cells can be overgrown and should be harvested at maximum confluency.

Day 19–21

1. Remove medium from well.
2. Rinse with 0.5 mL of PBS.
3. Remove PBS and add 500 μL of lysis buffer.
4. Let the cells sit for few minutes until lysis is complete and harvest the lysates to a 1.5-mL Eppendorf tube.
5. Incubate overnight in a shaking incubator at 55°C.

3.4.2. DNA Extraction

The following protocol is a very simple and crude method for DNA extraction (modified from **ref. *7***) (*see* **Note 8**).

1. Add 1.0 mL of 100% ethanol to the 0.5 mL of cell lysate.
2. Mix thoroughly by hand until the DNA is completely precipitated.
3. Centrifuge for 15 min at high speed in a microfuge.
4. Remove supernatant without dislodging the pellet.
5. Rinse the pellet by adding 0.5 mL of 70% ethanol and vortex.
6. Spin 10 min and remove as much of the supernatant as possible.
7. Allow to air dry 5–10 min.
8. Resuspend in 100 μL of sterile water.

3.4.3. Southern Blot Analysis (see **Note 9**)

DNA recovered from one ES cell clone expanded in a 24-well plate is sufficient for at least three restriction digest analyses. The digestions can be performed in a 96-well plate to reduce manipulations and preserve the sequence of the samples more easily.

1. Check the DNA concentration of several clones by spectrophotometric analysis to determine more precisely the average amount of DNA ***(6)***. About 10–30 μL contains, on average, 10 μg of DNA.
2. Prepare a master mix containing 5 μL 10X enzyme buffer, 2 μL 0.1 *M* spermidine trihydrochloride, 30–50 U of restriction enzyme and water to make a total of 55 μL after addition of 10–20 μL of DNA.
3. Mix the reaction by pipetting in the DNA sample.
4. Incubate for 8–20 h at 37°C.
5. Stop the digestion with 10 μL of loading buffer.
6. Load the samples on a 0.8% agarose gel in 1X TPE buffer and subject to electrophoresis over night at approx 30 V.

7. Nick the DNA with 245 nm reflected UV light for 5 min or depurinate with 0.25 *M* HCl for 15 min (with shaking).
8. Soak gel in alkali solution for 45 min (with shaking).
9. Set up transfer and blot onto nylon membrane (e.g., Hybond N+) over night in alkali buffer.
10. Take apart the Southern blot and soak the membrane in 2X SSC for approx 30 s.
11. UV crosslink for a few seconds or bake for 1 h.
12. Prehybridize for at least 2 h at 65°C.
13. Hybridize overnight (at least 8 h) at 65°C. Several commercial kits (e.g., a DNA labeling kit from Boehringer Mannheim or Stratagene) allow the labeling of probes with radioactively modified deoxyribonucleoside triphosphates. Probes with a specific activity of 1.5–2.0 × 10^9 dpm (disintegration per minute)/μg should be used to obtain relatively quick results (i.e., with an O/N exposure).
14. Wash blots two times with 0.2X SSCP and 0.1% SDS for 20–30 min at 65°C.
15. If necessary, wash once with 0.1X SSCP and 0.1% SDS for 20–30 min 65°C.
16. Wrap the filter in Saran Wrap and apply O/N to X-ray film to obtain an autoradiographic image.

3.5. Preparation of ES Cell Clones for Expansion and Injection

1. Quickly thaw the selected clones at 37°C and transfer to 5 mL of MEF media.
2. Spin down for 10 min at 300*g*.
3. Remove the supernatant without dislodging the small pellet.
4. Resuspend in ES media and plate 1 clone/well on a 12-well feeder plate (from now on the cells should get only ES media).
5. Expand the clone and split into one or two 6-cm feeder plates after 4–5 d.
6. When these cells are ready for splitting, freeze down a few vials and split some more cells for a second DNA analysis. With this analysis you want to ensure the identity and the clonality of the positive clones (*see* **Note 10**).
7. Perform injections with cells trypsinized and resuspended in ES medium (*see* Chapter 9 this volume).

4. Notes

1. The two parameters to be tested are (a) plating efficiency and (b) toxicity. Briefly, 10^3 cells are plated onto a 6-cm plate with feeder cells. Media is prepared with 10% or 30% of the new batch of heat inactivated (30 minutes at 56°C) FCS. Plate cells in triplicate for every batch of FCS to be tested. Colonies are counted after 5–7 d. The number of colonies is compared between different lots. The presence of more colonies in the 10% FCS media compared with the 30% is an indicator of serum toxicity. These lots should be avoided (also, *see* **ref. *1***). Serum can be stored between –20 and –80°C for up to 3 yr.
2. The production of good-quality feeder cells, in our experience, is a critical step in obtaining genetically manipulated ES cells that retain their ability to form a

functional mouse germline. It is important to note that there is a certain degree of variability between batches of feeder cells produced. However, the growth rate of the primary fibroblast, from which feeder cells will be derived, can be used as a quality indicator; that is, fast growing MEFs will generally produce good feeders.

3. Upon receiving an early passage of ES cells it is important to expand them to create a stock of frozen cells to be used for electroporation experiments. A few ES cells from the stock should be tested for the presence of mycoplasma, as contamination may greatly reduce ES cells efficacy. Finally, a sample of the expanded cells should be tested for their ability to contribute to the mouse germline on blastocyst injection.
4. ES cells should always be grown with ES cell medium on feeder cells. Therefore, special care should be taken in assuring that enough feeder plates are available. When grown on feeders, ES cells may not require addition of LIF to the media. However, we found that it is a good practice to grow them with this additive to buffer possible feeder deficiency.
5. It is very important to split ES cells before they reach confluency. A confluent culture may result in high degree of cell differentiation **(Fig. 1E)**. It is also very important to culture ES cells only when needed, as these cells tend to lose their totipotency after multiple tissue culture passages.
6. It is important to split cells 24 hrs before the electroporation to have a culture where the majority of cells are actively dividing. In fact, it is believed that recombination with the foreign DNA vector occurs during the S phase of the cell cycle.
7. After plating, individual cells from dissociated colonies will take 4–5 d to produce new clusters of cells. It is important not to let these cells overgrow and differentiate. After a maximum of 6 d every clone should be frozen. For planning purposes, it is important to note that almost all clones picked on a certain day are going to be ready simultaneously. Therefore, materials should be prepared prior to starting (i.e., gelatin-coated 24-well plates, label cryovials, prepare freezing media, arrange tips for multichannel pipettor).
8. The protocol described is extremely fast and has been proven very effective with DNA restriction endonucleases that require high-salt buffer conditions (e.g., *Bam*HI, *Bgl*I, *Bgl*II, *Hind*III, *Eco*RI, *Eco*RV, *Pst*I, *Not*I, *Xba*I, and the like). However, before proceeding with the extraction of all clones, a test should be performed to ensure that the DNA samples extracted with this method are suitable for analysis with the endonucleases required for the screening for homologous recombination. Use the phenol/chloroform method for extraction ***(6)*** if cleaner DNA is needed.
9. For more complete and detailed protocols for Southern analysis, *see* **ref. *6*** or follow the recommendations of the manufacturer of the membrane used for Southern analysis.
10. If a clone represents a mixed population of normal ES cells and G418-resistant cells, it can be grown in G418 media until clonality is achieved. However, before a clone is injected it should be grown for at least 1 wk in ES media without G418.

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5

Gene Targeting in a Centralized Facility

Louise D. Barnett and Frank Köntgen

1. Introduction

Gene targeting in mice has become a commonly used technique to elucidate gene function. The principles for the generation of such gene knockout mice are relatively straightforward and many of the experimental procedures are standardised ***(1–3)***. A further step toward increasing the efficiency in the generation of knockout mice lies in the pooling of resources and knowledge by establishing a centralized gene-targeting facility.

The methodology used in the Genetically Modified Mouse Laboratory (GMML) at the Walter and Eliza Hall Institute of Medical Research at the Royal Melbourne Hospital has generated 17 mutant mice strains ***(4–8)*** over the last few years and also has been successfully adopted by other institutes. This method utilizes a Southern blot-based screening approach to routinely screen 720 embryonic stem (ES) cell clones from which two independent heterozygous mouse strains are established. The method has also been used successfully to generate double-targeted germline chimeras ***(6)***.

A protocol based on the everyday operation of the gene-targeting facility is described.

2. Materials

2.1. Buffers and Solutions

1. Genomic DNA lysis buffer: 100 m*M* Tris-HCl, pH 8.5, 5 m*M* ethylene diamine tetraacetic acid (EDTA) pH 8.5, 0.2% sodium dodecyl sulfate (SDS), 200 m*M* NaCl ***(9)***, store at room temperature (RT). Make 20 mg/mL stock solution of Proteinase K in H_2O, store aliquots at –20°C. Add 100 μg/mL Proteinase K for

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ES cell lysis. For mouse tail lysis, increase SDS concentration to 0.5% and add 500 μg/mL Proteinase K.

2. Southern blot hybridisation solution: To make 1 L, add components sequentially and allow to dissolve completely after each addition: 50 mL 20% SDS, 50 mL 1 *M* Tris-HCl, pH 7.5, 2 g bovine serum albumin (BSA), 1 g tetra-sodium pyrophosphate, 2 g polyvinyl-pyrrolidone MW 40,000, 2 g Ficoll MW 400,000, 500 mL formamide (molecular grade), 200 mL 50% dextran sulphate, 200 mL 5 *M* NaCl. Mix well and store at –20°C.

2.2. ES Cell Culture Media and Solutions

1. Dulbecco's modified Eagle's medium (DMEM): Containing high glucose and low bicarbonate (17 g $NaHCO_3$ per 5L).(Gibco-BRL, cat. no. 074-02200P).
2. PMEF-DMEM: Add 10% fetal calf serum (FCS) (heat-inactivated, ES tested) to DMEM and filter sterilize through 0.22 μm filter. Store at –20°C and use within 3 wk of thawing.
3. ES-DMEM: Add 15% FCS to DMEM and handle as in **step 2**. On thawing, add LIF (AMRAD/ Pharmacia, cat. no. 90001106) to a final concentration of 1000 U/mL.
4. Gelatin solution 0.1% (w/v): Dissolve 1 g porcine skin gelatin type A (Sigma, cat. no. G-1890) in 1 L mouse tonicity phosphate-buffered saline (MTPBS) and autoclave to dissolve and sterilize.
5. Trypsin-versene/ 1% (v/v) chicken serum (CS): Chicken serum (Pacific Shoji, cat. no. 110-100) is heat inactivated, filtered, and stored in aliquots at –20°C. Add 1 mL of CS to 100 mL trypsin-versene (Commonwealth Serum Laboratories, cat. no. 9601901) and keep at 4°C for use within 2 wk or store in aliquots at –20°C (*see* **Note 1**).

2.3. Sources of Cells

1. ES cell line "W9.5". 129/Sv.C3-+^{c}+^{p} inbred strain ***(10)***. This line was isolated on PMEFs.
2. ES cell line "Bruce4". C57Bl/6 Thy 1.1 congenic strain ***(11)*** isolated on PMEFs.
3. Primary mouse embryonic fibroblasts (PMEF): ICR-M-TK*neo* 2 embryos ***(12)***, as a source of *neo*^{r} fibroblasts are prepared according to Köntgen and Stewart ***(2)***. Batches of PMEFs are passaged 1:4 on d 4, 1:4 on d 7, 1:3 on d 10, then harvested on d 14, pooled, and irradiated in a cesium source with 3000 rads at RT. PMEFs are pelleted at 1000 rpm/5 min, aliquoted, and frozen at –70°C in 10% (v/v) DMSO/ PMEF-DMEM. PMEFs from a 175-cm^2 flask are frozen as "150 cm^2" surface area or 3 × "50 cm^2" to account for any losses due to freezing and/or thawing. PMEFs are thawed in a 37°C waterbath, washed in PMEF-DMEM, and plated.
4. Slow-twitching oxidative (STO) cells: A *neo*^{r} and *hygro*^{r} fibroblast cell line ***(13)*.** STOs are growth arrested with 4000 rads irradiation in a cesium source at RT.

3. Methods

3.1. Targeting Vectors

3.1.1. Preparation of Targeting Vector DNA Suitable for Electroporation

1. Prepare targeting vector DNA by either alkaline lysis method (Qiagen, cat. no. 12163) or by cesium chloride gradient and has not been in contact with phenol.
2. Linearize vector with an appropriate restriction enzyme. The 10–25 μg vector DNA is electroporated for gene targetings utilizing a promoter-driven antibiotic resistance cassette and 25–150 μg vector DNA is used for promotor–trap constructs (refer to **Note 2**). There is no requirement for the insert to be purified away from the vector backbone—linearization is sufficient and the recommended practice. Supercoiled DNA has been electroporated with only a two- to five-fold reduction in targeting efficiency.
3. Following digestion, precipitate the targeting constructs with ethanol, washed with 70% ethanol and resuspended at 100 μg/mL, in sterile MTPBS.
4. Run 1 μL DNA on an agarose gel to check completeness of digestion and recovery. Make the DNA up to 750 μL with sterile MTPBS for electroporation.

3.1.2. DNA Probe Specifications: Size, Purification, and Amounts Required

The "external" (nontargeting vector-derived) DNA probe used to detect targeted ES cell clones by Southern blot analysis is 0.2–2 kb in size. It is recommended to have a second "external" probe located at the opposite end relative to the first probe to confirm the homologous recombination before targeted ES cell clones are chosen for expansion and blastocyst injection. One can also screen for homologous recombinants using a probe derived from the selectable marker, however, clones with a random integration will make it difficult to interpret the results. The latter approach is not recommended, but possible.

1. Run the DNA probes on an agarose gel until the insert is away from the vector.
2. Excise the insert band, place it on a gel tray, and pour a lower percentage gel around the slice and electrophorese.
3. Excise band, purify the DNA using a suitable method (e.g., Qiagen, cat. no. 28704).
4. Test the probes for their sensitivity and purity on genomic Southern blots of ES cell DNA digested with a variety of enzymes. Purified DNA probe of 250 ng (i.e., 10 labelings) per project is requested from facility users.

3.1.3. Screening Strategy

Southern blot analysis is the method of choice to screen for targeted ES cells within a service facility. This method is more robust and reliable compared to PCR but is not necessarily more labor or time intensive. Negative results, e.g., in a PCR-based screening assay are potentially indistinguishable from techni-

cal error, whereas Southern blot results provide evidence of experimental success via a visible endogenous band.

In order to achieve reproducible results, it is important to choose restriction enzymes that are reliable cutters of genomic DNA—such enzymes were found to be *Bam*HI, *Bg*lII, *Eco*RI, *Eco*RV, *Hind*III, *Sac*I, and *Xba*I. Problems with incomplete digestion of DNA, especially from mouse tails, have been rectified by changing the brand of enzyme. It should also be noted that the foregoing enzymes are chosen because they are relatively inexpensive.

If no homologous recombinants are found among 720 ES clones screened, redesign of the targeting construct is recommended before a new experiment is started.

3.2. Cellular Techniques

3.2.1. ES Cell Culture

The methodology presented hereafter represents an adaptation of previously published procedures (***2***,***3***, and ***14***) geared toward multiple parallel experiments as performed within a gene-targeting facility. All ES cell culture follow previously documented conditions ***(2)***.

1. Passage ES cells when they begin to form three-dimensional, phase-refractive colonies at 70–80% confluency.
2. Swirl dishes to dislodge any nonadherent dead cells and/or debris, aspirate the media, and wash twice in MTPBS.
3. Incubate at 37°C in trypsin-versene/1% CS for 3–5 min. Perform microscopic assessment of trypsinization after dislodging the cells by shaking. Clusters of cells may be visible, but no cells should be adherent.
4. Add ES-DMEM and disperse clumps by gentle pipetting (*see* **Note 3**).
5. Plate ES cells dropwise onto the middle of the culture dish, ensuring that the PMEF layer remains intact and the dispersal of ES cells is uniform.
6. Carry the dishes to the incubator held on a slight tilt to allow even cell coverage. If the dishes are carried flat the cells move into the media swirl and settle toward the edges and center of the dish.

3.2.2. Testing Fetal Calf Serum for ES Cell Cultures

FCS used for culturing ES cells must not contain any components that promote differentiation and/or inhibit proliferation, enabling ES cells to retain their totipotent state to generate germline chimeras. FCS within our facility is tested through three rounds of screening and scored by observers blinded to the identity of the batches.

1. Initially check all FCS samples for their influence on the media's pH after overnight incubation and for plating efficiency of PMEFs and ES cells. Perform the tests in six-well plates using the current FCS batch as a standard.

2. Score the first screen by a number of experienced personnel. FCS samples are eliminated due to acidic or basic pH, decreased plating efficiencies of PMEFs and/or ES cells, as well as phenotypic changes in cell morphology as compared to the standard.
3. For the next round of screening that tests the FCS toxicity, plate known numbers of ES cells in 15% and 30% FCS.
4. Score the second screen. No difference should be observed in plating efficiency and morphology between the two concentrations.
5. For the final screening, two individuals perform parallel experiments with duplicate platings of known numbers of ES cells on PMEFs and gelatin alone.
6. In the absence of PMEFs, FCS-induced differentiation or the lack of being able to support undifferentiated growth becomes more obvious.
7. Rank the remaining FCS batches. Choose the the best performing batch in all categories. The ultimate test of a chosen FCS batch would be its ability to promote germline transmission of ES cells. This, however, can rarely be tested, as companies do not reserve batches for the required period of time.

3.2.3. Transfection of ES Cells

3.2.3.1. Day 4

1. Gelatinize a 60-mm dish.
2. Thaw 21 cm^2 PMEFs in a 37°C waterbath, resuspend in 5 mL PMEF-DMEM, and plate dropwise onto the center of the dish using a pipetman (Medos, cat. no. TEC 155016) set on the slowest dispensing setting.

3.2.3.2. Day 3

1. Swirl the PMEF culture to dislodge any nonadherent dead cells, tilt the dish, and aspirate the supernatant close to, but not touching, the PMEF monolayer.
2. Plate approximately 1–5 × 10^6 thawed ES cells dropwise onto the PMEF layer in ES-DMEM.

3.2.3.3. Day 1

1. Gelatinize 10 × 60 mm dishes.
2. Thaw and plate approx 210 cm^2 of PMEFs. This number is scaled according to the number of constructs being electroporated.
3. Color-code and label the side of each dish so that both lids and bases of the dishes are identifiable. This minimizes confusion with multiple sets of dishes in the incubator and possibly different selection agents (*see* **Note 4**).
4. Refeed ES cultures if required.

3.2.3.4. Day 0

1. Assess colony growth of ES cells, for this is what dictates when to perform the electroporation. A "healthy" culture will be growing rapidly (doubling time of approx 10 h) with ES cell colonies beginning to become three dimensional,

having discrete colony boundaries, lacking distinct cell borders, and cover 70–80% of the surface area.
2. Change media in the morning (should be yellow in color) before electroporating in late afternoon.
3. Assess the plating efficiency of the PMEFs from day 1: a suboptimal confluency can be rectified by plating more PMEFs with the ES cells after electroporation.

3.2.3.5. Electroporation

1. Chill the DNA solution and electroporation cuvet (Bio-Rad, 0.4 cm electrode gap, cat .no. 165-2088) on ice before use.
2. Trypsinize ES cells using 0.5 mL trypsin solution to a single-cell suspension, add 9.5 mL PMEF-DMEM, and pellet cells at 1000 rpm for 5 min at 4°C. During this centrifugation, set up a 50-mL tube containing 40 mL ES-DMEM for recovery of ES cells following electroporation.
3. Aspirate all traces of media away from the cell pellet by tipping the tube toward the pipet rather than sucking too close to the cells.
4. Resuspend cell pellet in 750 μL DNA solution and transfer to the electroporation cuvet without creating air bubbles. Replace on ice.
5. Wipe the outside of the cuvet to remove water and/or ice before electroporating at 250 V/500 μF (Bio-Rad Gene Pulser, cat. no. 165-2078). The time constant should fall between 7 and 11 ms. Times outside this range have still been found to produce colonies, however, the efficiency is likely to be diminished (*see* **Note 5**).
6. Return cells to recovery media and rinse the cuvet once with ES-DMEM. A flocculant precipitate of dead cells/ DNA should be visible.
7. Aspirate media from PMEF cultures and plate 4 mL ES cells dropwise after thorough resuspension.

3.2.4. Selection of ES Cells

3.2.4.1. Day 2

1. Add G418 or Hygromycin β to ES-DMEM at 200 μg/mL final concentration. Electroporated cultures should look no different from sparsely plated ES cells beginning to divide. Dead cells are indicative of the electroporation efficiency, and there should be no more than 10% present.
2. Swirl cultures to dislodge these dead cells and aspirate close to the PMEF layer without touching the surface. This procedure is crucial to the success of the picking process later on, for damage to the monolayer can result in it rolling back on itself and reducing the ability to pick clonies (*see* **Note 6**).
3. Restack the dishes to minimize the time any culture is without media; the first aspirated is the first to receive fresh media. Perform all media changes with dropwise dispensing, as fast spurts cause damage to the PMEF monolayer.

3.2.4.2. Days 3–9

1. Check the cultures daily. In the first few days under selection, the cultures will still grow rapidly and might require frequent media changes (*see* **Table 1**).

Although the selection agents are stable in the ES-DMEM, frequent media changes are necessary during selection, as dead cells should be removed. G418 kills less efficiently than Hygromycin β, so additional media changes minimize background debris. Cell death should begin at d 4 and last until d 7 but could linger longer in G418-selected cultures. Colonies are usually apparent by d 7 and visible to the naked eye by d 9. This is easy to assess by simply looking from underneath the dish or after aspiration during media changes.

3.2.5. Picking and Expansion of ES Cell Colonies After Selection

3.2.5.1. Preparation for picking (Day 9)

Our facility picks 720 individual ES cell colonies (15 × 48-well plates) for a standard targeting experiment in which the targeting frequency is unknown.

1. Based on the estimated numbers of colonies, prepare 48-well culture plates with PMEFs. Prepare an appropriate number of individual glass capillaries.
2. Coat the wells of each plate with 250 μL gelatin using a multistep dipenser with the tip aimed vertically into the well.
3. Incubate for 30 min at RT before aspirating gelatin with a Pasteur pipet. Tip plate forward at an angle to assess that *all* the gelatin has been removed.
4. Label plates on both lid and base with name of construct, date, number of plate and "3°" (tertiary plate for DNA, *see* below). With the labelling facing forward, color the top right-hand corner as a visual signal to aid in orientation of plates. This becomes crucial at later stages of the procedure.
5. Thaw 750 cm^2 PMEFs (15 × 50 cm^2), wash to remove freeze mix, and resuspend in 187.5 mL ES-DMEM G418 or Hygromycin β (12.5 mL per plate) (*see* **Note 7**). Forty-eight-well plates are assumed to be 50cm^2 in surface area.
6. Add 250 μL PMEFs to each well, vertically and off center. If PMEFs are plated in the center, the force of expulsion is likely to distribute them to the periphery or the center of the well, leading to uneven coverage. The same is true for dispensing on the side of the well.
7. Prepare drawn-out, thin-wall, hard-glass capillaries (BDH Lab Supplies, cat. no. 321242C) for picking individual colonies. The method for pulling capillaries has been described ***(14)***. The investigator needs practice to master this technique . If the capillary is breaking off center then the pulling tension between hands is uneven. The capillary tip should have a smaller diameter than the size of the ES cell colonies so that single-cell suspensions are generated during picking. Pipet-holders are made with a 1.5-mm drill boring holes around the edge of a Perspex block to sit the nonextruded capillary end in. Pull capillaries on the lab bench and transfer to a culture hood for sterile storage overnight.

3.2.5.2. Picking ES Cell Colonies (Day 10)

Day 10 is chosen for picking, as the majority of colonies are still in exponential growth. This also falls on a weekday according to the timetable used here **(Table 1)**. If desired, picking may also be done at any time until d 13. By

Table 1
Gene Targeting Time Table

Day		Experimental Procedures
–3	Sat	Thaw ES cells
0	Tues	Electroporate ES cells and plate on 10 × 60 mm dishes
1	Wed	Begin selection with Geneticin or Hygromycin B
10	Fri	Pick 720 individual colonies into 48-well plates
13	Mon	Split each plate three ways: injection, backup, and DNA
16	Thurs	Freeze injection and backup plates at –70°C
17	Fri	Lyse cultures on DNA plates O/N
18	Sat	Precipitate DNA in 48-well plates
19	Sun	Wash and dry DNA on 48-well plates Digest DNA of first two 48-well plates O/N
20	Mon	Electrophorese 35 mL of digested DNA O/N
21	Tues	Vacuum blot ≥4 h. Label probe. Hybridize O/N 42°C
22[a]	Wed	Stringent wash ≥1 h 68°C. PI exposure ≥4h
23–30	Thurs–Fri	Screen remaining 13 × 48-well plates
33–35	Mon-–Wed	Re-electrophorese positive clones to confirm clone position
36–37	Thurs–Fri	Strip filter, re-probe with selective marker (neo/hygro) to check number of integrations
40	Mon	Thaw positive, single integration clones onto 12-well plates
45	Sat	Expand 12-well culture onto 60 mm dish
48	Tues	Expand 60 mm culture onto 100 mm dish
51	Fri	Freeze 6X vials from 100 mm culture at –70°C
52	Sat	Thaw 1 vial onto 60 mm dish
55	Tues	Assess recovery from freeze. Plate onto 48-well plate for DNA
57–62	Wed–Mon	Confirm clone with multiple digests and/or alternative probe

Once confirmed, schedule blastocyst injection.

Two gene-targeting projects can be handled at any one time per person. ES cell transfections are spaced 1 wk apart to stretch the tissue culture work over a longer period of time. The molecular experiments are done during the electroporation/selection phase when the tissue culture workload is relatively low.

[a] Proceed with remaining screening at own pace from this point if necessary

d 13, some of the colonies may have begun to overgrow, having darkened centers and/or flat edges around the periphery of the colony. The ES cell colonies are picked in a laminar flow cabinet.

1. Prepare the ES colonies for picking by aspiration of the media, gently wash twice with MTPBS and adding 2 mL trypsin/1% CS solution per 60 mm dish (*see* **Note 1**).
2. Immediately transfer the culture to the stage of a zoom-focus stereo microscope under dark-field optics. Following the addition of trypsin, allow it to sit for 3–7 min to trypsinize. Do not move the dish excessively.

3. Pick colonies using a "homemade" mouth pipet that has been described by Abbondanzo, Gadi, and Stewart ***(14)***. Non-differentiated ES colonies resemble small mounds under dark-field and look refractile. Differentiated colonies have flattened halos and should be avoided. Colonies of a similar size are chosen to standardise the growth rates of the clones.
4. Insert the capillary in the mouth pipet and allow it to break the surface tension away from any colonies as some trypsin will enter the capillary by capillary action.
5. Guide the capillary to the edge of a colony and apply suction to withdraw the colony as a single cell suspension into the capillary for transfer into a prepared 48-well plate. Choose well-spaced colonies to avoid cross-contamination of clones.
6. Expel the ES cells into the wells together with some air bubbles, which mark a "filled" well. Discard capillaries are after a single use. The surface of the dish may be scored by the capillary after picking as a reminder of territory covered. Two 48-well plates are safely picked per dish. Picking time for a beginner is estimated at 20–30 min per 48-well plate, decreasing to 10–15 min for a more experienced worker.
7. Prepare an additional set of 15 × 48-well plates with PMEFs in ES-DMEM to split into at d 13. This set is used as the injection stock and labelled 1° (primary plate).

3.2.5.3. Days 11 and 12

1. Examine plates. Nothing remarkable should be seen seen. Any overt contamination can be treated by addition of two pellets of NaOH per well, allowed 5 min to kill, and then thoroughly rinsed away with MTPBS and left to dry.

3.2.5.4. Day 13

1. Examine plates. ES cells will be visible and beginning to form colonies. The majority of the ES cells may have dispersed to the outer edges of the well with the expulsion of bubbles while picking. The media will begin to change color. The decision to split cultures is based on growth of the majority of the ES cell clones in a plate.
2. Gelatinize a further set of 15 × 48-well plates, label 2° (secondary plate), and add ES-DMEM. This culture set is used as a back-up for the screening assay, however, it can also be used for the preparation of injection stock should the original culture be lost due to contamination or similar.
3. To split cultures into triplicates, aspirate the media from the tertiary ES cell plate close to but not touching, the PMEF layer holding the plate at a forward-facing angle to see each well.
4. Wash with 250 μL MTPBS added to the side of each well with a multistep disenser held horizontally so that cells are not dislodged and lost during aspiration. It is important to aspirate all MTPBS from wells so that the volume of the trypsin is not diluted and increased. To avoid cells drying out, wash and trypsinize only five plates at a time and restack them so that the first culture aspirated becomes the first to receive MTPBS or trypsin.

5. Add 50 μL of trypsin solution vertically, as the cell layer will be disrupted anyway. This volume is the minimum needed to cover the well surface, however, it does gravitate to the edges of the well. Tap the plates on all sides to redistribute the solution before and again after approx 3 min of incubation. Total incubation time is 5–7 min or until all cells are detached.
6. Following incubation hit all sides of a plate approx 10 times against the side of a bench to disrupt the large colonies. It is not critical to get single cells at this stage as the cells will be pipetted during the three-way split.
7. To each well, add 250 μL of ES-DMEM and return the four plates that are not in use to the incubator. Line up the corresponding primary and secondary plates to the tertiary plate all labels facing you. A quick confirmation is the visual cue of colored corners facing the same direction.
8. Double check that all plates are labeled with the same plate number. Place the tertiary plate to the front and the primary furthest away.
9. Use a 12-multichannel pipettor for the splitting with tips placed on channels 1,3,4,6,7,9,10, and 12 which fits the spacing of the 8 wells in a row of a 48-well plate; prepare tip boxes beforehand in the same pattern for convenience.
10. Tilt the tertiary plate sideways and use the multichannel pipet to mix the ES cell suspension with repeated pipetting and flushing of the raised surface of the wells.
11. Transfer 100 μL of ES cells into the primary plate with complete expulsion to introduce a few bubbles identifying "filled" rows. Repeat for the secondary plate. Change tips after each row of clones has been transferred.
12. When all six rows have been plated, add 250 μL ES-DMEM per well to the tertiary cultures and incubate triplicates for a further 2–3 d at 37°C.

3.2.6. Freezing ES Cell Clones (Primary and Secondary Plates) in 48-Well Plates

Freeze plates when the majority of ES cell cultures are approx 75% confluent with rapidly dividing colonies becoming three dimensional. The media should be changing pH in that which are growing well.

3.2.6.1. Days 16 or 17

1. Prepare freezing medium by adding DMSO to ES-DMEM at a final concentration of 10%. ahead of time and chill, as addition of DMSO promotes an exothermic reaction. Each clone is frozen in a 250-μL volume The freezing mix is made up to accomodate the 50 μL of trypsin already in the well, therefore 200 μL of freezing mix is added to each well. For the 30 plates to be frozen (15 primary and 15 secondary), add 37.5 mL DMSO to 262.5 mL of ES-DMEM.
2. Wash and and trypsinize the ES cells as is described for the splitting—*see* **Subheading 3.2.5.4.**, d 13. This time, however, it is crucial to obtain a single-cell suspension from the trypsinization when "hitting" the plate, as there is no further pipetting involved.
3. Add 200 μL freeze mix off centre to each well and seal the plate with waterproof plastic tape (3M Scotch, cat. no. 471) and store at –70°C (*see* **Note 8**).

4. Leave the tertiary cultures growing a further day to approach ≥100% confluency in order to maximize DNA yields. There is no need to change the media, as some overgrowth/differentiation will not affect the Southern blot analysis.

3.2.7. Direct Preparation of DNA in 48-Well Plates

The following is an adaptation of the method described for screening 96-well plates by Ramírez-Solis, Davis, and Bradley ***(1)*.**

3.2.7.1. Days 17 and 18

1. The tertiary ES cell cultures should now have bright yellow media color.
2. Pre-warm an oven to 55°C. Add 250 μL of 20 mg/mL Proteinase K frozen stock per 50 mL of lysis buffer. Lysis buffer of 12.5 mL is required for each plate (187.5 mL for 15 × 48-well plates).
3. Aspirate media from the plates, being careful not to touch the bottom of the well, as this could cause the cell layer to lift off during subsequent steps.
4. Without disturbing the monolayer, add 250 μL lysis buffer to the side of the well.
5. Tape each plate with waterproof plastic tape to prevent evaporation and as a further precaution, place in a ''wet box'' (a closed container with water-saturated tissues) inside the 55°C oven. Incubate stationary O/N. Minimize movement of the plates, as adherence of DNA depends on maintaining contact between the cellular matrix and the well surface.
6. Next day, carefully add 500 μL absolute ethanol dropwise to the side of the well with a multichannel pipettor and leave O/N at RT wrapped in plastic to prevent evaporation.
7. The following day, the precipitated nucleic acid "cobwebs" are best viewed against a black background. Note the wells without DNA.
8. Carefully invert the plate to discard the ethanol, leaving the DNA attached. Blot residual ethanol on paper towelling.
9. Wash the DNA twice with 250 μL 70% ethanol, again blotting the excess. At the final inversion, leave the plate upside down to dry on its lid at 37°C. Dry completely (≥ 1 h) and store either at RT or –20°C.

3.2.8. Thawing ES Cell Clones from 48-Well Plates

1. Plate 4cm^2 PMEFs in 1 mL PMEF-DMEM onto gelatinized 12-well plates the day before the proposed thawing.
2. Individually remove primary plates from –70°C and place on a styrofoam block in a culture hood.
3. Thaw only targeted clones by pipetting prewarmed 37°C ES media into the wells. After media has cooled and thawing slowed, transfer the cell suspension onto the aspirated 12 well PMEF culture.
4. Repeat until the entire contents of well is thawed.
5. Finally, rinse well around the edges, and top up the 12-well culture to 4 mL to dilute DMSO and leave O/N at 37°C. Return the frozen 48-well plate to the –70°C freezer.

6. Next day, refeed the plate with 1 mL ES-DMEM to remove all DMSO.
7. Treat each targeted ES cell clone individually during expansion to allow for optimum growth and recovery. If there are only few colonies or clumps of ES cells, you may need to trypsinize cultures within the 12-well plate.
8. Expand the ES cell clones by transfer from a 12-well plate to a 60-mm dish, then to a 100-mm dish, from which six 1 mL aliquots are frozen. This equates to approx 8 cm^2 or 1 – 5 × 10^6 ES cells (*see* **Note 9**).
9. To freeze ES cells, wash the culture twice in MTPBS and trypsinize with 1 mL, add 9 mL PMEF media, pellet cells at 1000 rpm for 5 min at 4°C.
10. Resuspend in premade freeze mix (10% DMSO in ES-DMEM). Freeze cells in 1 mL cryovials at –70°C for at least 12 h before transferring to liquid nitrogen for long-term storage.

3.2.9. Reconfirmation of Homologous Recombination in ES Clones before Chimera Production

1. Thaw a vial of the frozen stocks onto a 60-mm dish for reconfirming of homologous recombination and to establish the growth rate and recovery from freeze.
2. After 2–3 d, passage cultures onto a gelatinized 48-well plate without PMEFs. The number of rows plated depends on the number of different restriction enzyme digests that one wants to perform for additional mapping studies of the targeted locus (*see* **Subheading 3.2.7.**).

3.2.10. Preparing ES Cell Clones for Blastocyst Injection

1. Thaw targeted clone onto 60-mm dish timed according to the growth rate previously observed, usually 2–3 d in culture.
2. For each injection, gelatinise a 100-mm dish. The ES culture should look like a healthy culture ready to be electroporated.
3. Wash the culture twice with MTPBS and add 0.5 mL trypsin/CS, which has been stored frozen in aliquots at –20°C (*see* **Note 1**).
4. Incubate for 3–5 min at 37°C or until the cells lift off the surface upon shaking.
5. While trypsinizing label three tubes—one for media (to dilute the cells as required by the injectionist), one with the clone name (gene, plate location, and antibiotic resistance) and its genotype, and the third having the clone details with additional information that the preparation has been adherence depleted of PMEFs (i.e., ES cell enriched).
6. To achieve a single ES cell suspension, carefully pipet cells in the trypsin solution using a Gilson P200 pipet or equivalent. Add 9.5 mL of PMEF-DMEM to the dish and pipet up and down without introducing air bubbles.
7. Transfer the cell suspension to the gelatinized 100 mm dish and incubate at 37°C for 1h to allow the PMEFs to adhere to the surface (*see* **Note 10**).
8. Collect remaining ES cells from original dish in 200 μL media, spin at 1000 rpm for 5 min at 4°C and use for initial injections.
9. Resuspended ES cells for injection in PMEF-DMEM/20 m*M* HEPES (Gibco-BRL/Life Technologies, cat. no. 15630-31). Keep cells on ice or at 4°C.

11. Remove the supernatant from the 100-mm dish after swirling to dislodge any cells just settled on the surface and flush a small area of the plate a few times with 200 μL of media to give a single-cell preparation. Spin as above. The injectionist chooses the best preparation to inject.
11. Add 10 mL of ES media to the 100-mm dish and return it to the incubator for a further 2 d of growth before the second injection day.

3.3. Molecular Techniques

3.3.1. Southern Blot Analysis

3.3.1.1. Restriction Enzyme Digestion

Two 48-well plates from a screen are processed in a pilot experiment to test enzymatic digestion, adequate electrophoretic separation of endogenous from targeted alleles, and probe specificity. Several targeted clones frequently can be found among the first 96 clones saving cost and labor compared with performing a full screen immediately. As mentioned in **Subheading 3.1.3.**, the choice of restriction enzyme used is paramount (*see* **Note 11**). Excess enzyme (20 U per well) is used to ensure that genomic DNA is digested to completion. The cost of a labor-intensive technique like Southern blotting is usually justified if the procedure only has to be performed once.

1. To each well containing dried DNA, add 20 U of enzyme in 100 μL of 1X restriction enxyme buffer containing 100 μg/mL RNase A (Boehringer Mannheim, cat. no. 109-169) (DNA in the wells is resuspended during the enzymatic digestion).
2. Gently tap all edges of the plate to disperse the mix evenly over the surface and incubate O/N in a 37°C humidified tissue culture incubator to prevent evaporation.
3. After an hour, tap the plate again to aid the resuspension and digestion.
4. Next day, remove the plates and determine the extent of digestion by examining the viscosity of the solution. Freeze digests in plates at –20°C until electrophoretic separation (*see* **Note 12**).

3.3.1.2. Gel Electrophoresis

1. Add 10 μL of 10X concentrated glycerol loading dye (14) per well and load 35 μL of digest into an appropriate percentage gel for adequate separation of endogenous and targeted alleles.
2. Refreeze the remaining digest at –20°C for later use in reconfirmation of clones (*see* **Subheading 3.3.2.**). We use 20.5 × 24 cm size gels (IBI, cat. no. HRH 55000) with modified gel trays to enable three rows of samples to be run per gel (*see* **Note 13**).
3. Run 1X Tris-acetate/EDTA (TAE) gels either for 4–5 h at 100 V or O/N at 20–30 V (*see* **Note 14**).

3.3.1.3. Southern Blotting

1. Vacuum blot DNA onto nylon membrane. In the system described, the best membrane for Southern blotting of genomic DNA with the least background is

Zeta-probe Genomic Tested™ nylon membrane from Bio-Rad (cat. no. 162-0194). Vacuum blotting (Pharmacia Biotech, cat. no. 80-1266-24 AG) has been found to expedite the Southern blotting process and has been shown to transfer routinely greater than 90% of the DNA in less than 4 hours with (*see* **Note 15**).
2. If depurination is required, perform it on the apparatus whilst the transfer is taking place with 0.25 *M* HCl and stop when the dyes in the loading buffer have changed color (approx 20 min). The gel should never be permitted to dry. Gently pour the acid off and flood the entire chamber with 0.4 *M* NaOH.
3. Vacuum transfer in 0.4 *M* NaOH for for 4 h in a 55-m bar vacuum.
4. Remove membrane and briefly rinse in 2X standard saline citrate (SSC) and either air dry at RT or place the membrane in an 80°C oven.

3.3.1.4. Radioactively Labelling DNA Probes

1. Make up 25 ng of gel-purified probe to 6 µL with ddH_2O in a screw-capped tube for random prime labelling (Bresatec Gigaprime DNA labelling kit cat. no. GPK-1).
2. Add 6 µL of decanucleotides, mix, boil for 5 min and spin briefly to collect condensation from the lid. Snap cooling on ice is unnecessary.
3. Add 6 µL of nucleotide/buffer cocktail A, 1 µL of the enzyme and 5 µL of α–^{32}P-dATP, mix well and incubate ≥1h at 37°C.
4. Purify probe away from unincorporated nucleotides with Nuctrap™ probe purification column (Stratagene, cat. no. 400702) or similar.
5. Determine the activity of the probe. Each probe should be at least 2×10^7dpm/25 ng of DNA labeled. Probes are not used if they fall below this threshold.

3.3.1.5. Hybridization and Washing

Hybridization and washing is carried out in roller bottles in a hybridization oven at 42°C.

1. Swirl the hybridization solution to mix precipitates, and warm to 42°C.
2. Roll the membrane DNA side in and prewet with 2X SSC before adding to the hybridization bottle. Prehybridize for ≥15 min at 42°C.
3. Boil purified probe for 5 min in the presence of 500 µL 10 mg/mL sheared herring sperm DNA (Boehringer Mannheim, cat. no. 223-646) and add to the hybridization solution. Return to 42°C oven and hybridize O/N.
4. Next day, wash membranes under maximum stringency in 0.1% SSC/0.1% sodium dodecyl sulfate (SDS) at 68°C with agitation for 3X 20 min. A final 20-min wash with 0.1X SSC/0.5% SDS will further reduce any background.
5. Expose filters on a PhosphoImager for ≥4 h. This is usually sufficient to obtain an interpretable result.
6. Strip the membranes in several changes of 0.4 *M* NaOH at RT, neutralized in 2X SSC and store in a wet bag at RT (*see* **Note 16**). Membranes are reprobed with a probe derived from the selectable marker to establish the number of integrations in each targeted clone.

3.3.2. Verification of Targeted ES Cell Clones

Targeting events in ES cell clones are reconfirmed with a further Southern blot to ensure that no mixup occurred during handling. From a targeted clone a further 35 μL digest of the frozen 48-well plate is run on a gel. Targeted clones verified in this second round of screening with single integrations are chosen for expansion and injection (*see* **Subheading 3.2.8.**). Refer to **Note 17**.

3.3.3. Genotyping the Germline Offspring

1. Take 0.5 cm of the tails from 3-wk-old coat-color germline offspring at weaning into a safe-lock tube (Eppendorf, cat. no. 0030 120.086) or screw-cap tubes and place on dry ice to prevent degradation.
2. Add 0.5 mL of lysis buffer/ Proteinase K per tail and digest rotating O/N at 55°C.
3. Next day, spin at 13,000 rpm for 10 min to pellet undigested material such as hair and bone.
4. Transfer to a new tube, add 0.5 mL isopropanol with a multidispenser, mix by inversion, and either leave the DNA to settle or spin briefly to pellet. Tip off the supernatant carefully.
5. Wash the pellet with 1 mL 70% ethanol, mix by inversion, and pellet the DNA as before.
6. Leave tubes with DNA inverted to air dry.
7. Add 100 μL of TE pH7.5 per tail and resuspend the DNA by flicking the end of the tube several times, incubate at 37°C. Do not vortex or pipet the DNA for resuspension ***(18)***
8. Digest 25 μL of DNA in a total volume of 50 μL with 100 μg/RNase A. Incubate O/N 37°C. Perform Southern blots as in **Subheading 3.3.1.**

3.4. Mouse Techniques

The mouse techniques involved in the generation of genetically modified mouse strains are beyond the scope of this chapter and can be found in Chapter 9 in this volume.

4. Notes

1. Trypsin/CS is aliquoted and frozen, as it has been found to diminish in potency on storage at 4°C. Chicken serum does not contain a trypsin inhibitor and prevents ES cells from sticking to each other.
2. Promoter-trap constructs result in fewer colonies and theoretically a higher targeting frequency. We have found that ES cells can exhibit a slower growth rate that increases the culture time to approx 14 d before picking.
3. If the original growth of the culture is not uniform (i.e., there are a few larger clumps of ES cells among many smaller ones) as seen with an inexperienced worker, then the clumps may be dispersed via pipetting of the ES cells in the trypsin solution before media is added to the cell suspension.

4. The electroporated ES cells can also be plated onto slow-twitching oxidative (STO) cells. STO cells do not exhibit as tight cellular contact in a monolayer as PMEFs and therefore show less tendency to "roll up" upon trypsinization. Generally it is easier to pick colonies from a STO than from a PMEF feeder layer.
5. Shorter time constants are indicative of a high salt concentration or higher temperature than recommended.
6. It is good practice to learn to hold the dish in one hand and use index and middle fingers to pry the lid and plate apart, with the thumb acting as a hinge, to allow the aspiration of media by introduction of a Pasteur pipet through the gap when the plate is tilted at an angle away from you. This ensures that the culture remains sterile, no juggling of the lids is required, and the distance from the monolayer's surface and the pipet can be easily judged.
7. Colonies are picked into selection media to ensure that nontransfected ES cell survivors will now be selected upon trypsinization into this media, ensuring monoclonality of the expanded ES cell clones.
8. The boxes in which the cryovials (Nunc, cat. no. 377224) are packaged fit 16 × 48-well plates and are useful to package projects together.
9. With inexperienced workers where the chance of contamination is increased, one is able to leave a few cells behind during the expansion to ensure the clone is not lost in subsequent steps. These cells can also be used for differentiation studies or to make DNA.
10. Attachment of the PMEFs will occur faster in 10% than in 15% FCS. Viable ES cells will be those that loosely attach to the surface in 1 h, whereas any dead cells will be nonadherent.
11. The screening procedure outlined here requires 15,000 units (U) of enzyme to screen 720 ES cell clones and can be extremely expensive depending on the enzyme used (>A$5000). High-concentration enzyme is chosen preferentially to limit the effects of glycerol on the digestion (star activity).
12. The procedure outlined never involves the pipetting of the genomic DNA, thus no shearing of the high molecular weight species occurs. It is routinely used to identify endogenous DNA fragments in excess of 23 kilobases (kb) in size.
13. This modification enables 96 clones to be analysed at once (2 × 48-well plates) and reduces membrane and agarose costs as well as time. Combs were specially made with 2 mm-wide-teeth in thickness and 34 wells across. The middle 32 wells have samples loaded and the outer two are reserved for the molecular weight markers (DRIgest III from Pharmacia Biotech, cat. no. 27-4060-01). Sides for the markers are alternated to ensure that they do not run into each other.
14. Well-digested DNA, using a six-cutter, should have a clearing just below the wells and the majority of the DNA should be in the upper two-thirds of the gel if it appears lower it is likely that some degradation has occurred. Satellite bands are seen with many enzymes and are usually indicative of adequate digestion.
15. The system has been further modified to ensure robust, repetitive results by replacing the scintered glass support with stainless steel mesh, as the support slowly becomes clogged with continual use under alkaline blotting conditions,

and replacing the supplied masks with a thicker plastic that is less prone to crease and buckle, preventing vacuum leakage.

16. Membranes have been successfully probed and stripped at least 10 times. This is especially true for the panel filters derived from ES cell DNA digested with several different restriction enzymes that are used to assess the purity of probes at the beginning a project.
17. It may be possible to breed out extra integrations if no single integrants are found.
18. The quality of DNA from a tail is never as good as the DNA obtained from the ES cells, and often a different strategy might need to be employed to screen when DNA fragments are larger than 15 kb in size.

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6

The *LoxP*/CRE System and Genome Modification

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1. Introduction

Conventional gene knockout technology by homologous recombination can provide important information toward elucidating the function of some genes; however, the role of many genes cannot be investigated due to early embryonic lethality. Alternatively, the role of a particular gene in adult tissues may be masked by developmental abnormalities in any knockout animals generated. The implementation of site specific recombinases, such as the bacteriophage P1 *LoxP*/CRE system, enables the development of conditional knockouts that lack a particular gene only in a specific tissue or after a specific stage of development. This system can also be used to facilitate transgene activation/inactivation in vivo, deletions of large stretches of genomic DNA, chromosomal translocation and subtle alterations to genes and/or their regulatory sequences in vivo.

The CRE enzyme is a 35-kDa protein isolated from bacteriophage P1, which acts as a site specific DNA recombinase ***(1,2)***. The sequence recombined by CRE, termed *LoxP*, consists of two 13-base pair (bp) inverted repeats (CRE recognition sites) separated by an 8-bp spacer **(Fig. 1A)**. The 8-bp spacer region is asymmetrical and is responsible for the directional nature of the *LoxP* site. Two *LoxP* sequences in the direct orientation will result in excision of the intervening DNA by CRE leaving one intact *LoxP* site **(Fig. 1B)**. In contrast, where two *LoxP* sites are in the opposite orientation, CRE will invert the intervening piece of DNA **(Fig. 1C)**. The specificity and efficiency if this system in vitro and in vivo has made it ideal for applications where genome modifications are required. It can be adapted to translocate, delete, or invert large pieces of DNA or to modify the genome only where and when required by the investigator. The latter is limited only by the availability of suitable regulators of the CRE recombinase.

The CRE recombinase will tolerate some alterations in the *LoxP* sequences. These include base changes in the "spacer" region, which allows the use of

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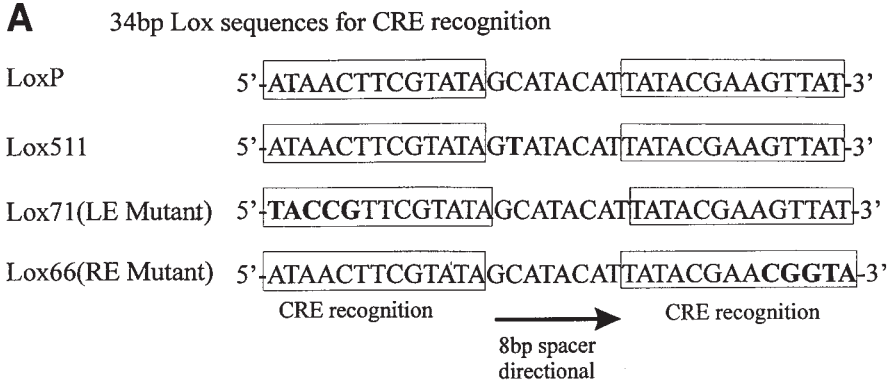

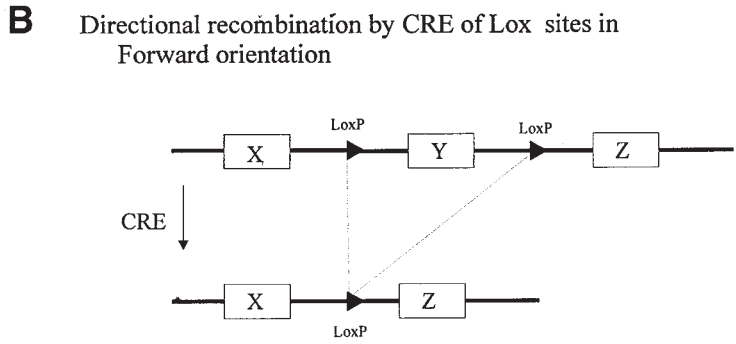

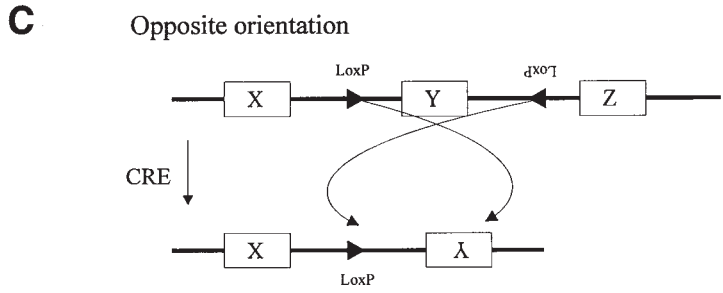

Fig. 1. Recombination of specific Lox sequences recognized by CRE (**A**) 34-bp sequences recognized and recombined by CRE recombinase. *LoxP* is the wild-type sequence containing two 13-bp-inverted CRE recognition sequences separated by an 8-bp directional spacer. Lox511 is a mutant site differing by a single base in the spacer region. It is recognized and recombined by CRE, but is not recombined to wild-type LoxP sequences. Lox66 and Lox71 each contain one mutant CRE recognition site, but are still recognized and recombined by CRE. The product of Lox66 and Lox71 recombination containing both mutant CRE recognition sites can no longer be recognized by CRE. (**B**) and (**C**) Directional nature of Lox sites and CRE recombination ► indicates Lox sites and orientation). Where sites are in the opposite orientation the DNA is inverted during the recombination event.

Lox sites that cannot recombine with *LoxP* sites (e.g., *Lox511 (3)*; see **Fig. 1A**). Furthermore, CRE will also recognize and recombine Lox sites that have alterations in one of the CRE recognition sequences (e.g., *Lox71* and *Lox66*; *[4]*). This can result in the production of Lox sites which, when recombined by CRE, generate a sequence which CRE can no longer recognize/recombine. For example, the *Lox71* site has a mutation in the left CRE recognition element, whereas the *Lox66* site has a similar mutation in the right element. When these two sites get recombined by CRE, *Lox* sites containing either the *LoxP* sequence or both mutant elements are generated. CRE can no longer recognize the site containing both mutant elements. These features can be utilized to generate preferential recombination events.

2. Construct Design for Conditional Gene Knockout Targeting

2.1. Concepts for Conventional Gene Knockout Constructs

Targeting constructs for the insertion of *LoxP* sites into genomic DNA are very similar to those used for the generation of conventional gene-knockout embryonic stem cells (ES) *see* **Fig. 2** and their subsequent progeny. The design and use of these conventional constructs is described elsewhere in this volume, together with strategies for transfection, selection, and screening of ES cells and thus is not discussed here. However, many concepts to improve targeting and screening efficiency are common to both types of constructs including the following:

1. Genomic DNA isogenic to that of the embryonic stem cells to be targeted should be used for the development of targeting constructs
2. The specificity and length of DNA sequences flanking the targeted region should be maximized for efficient homologous recombination, i.e., repetitive sequences should be avoided. The specificity of flanking sequences can easily be verified by using them as probes on Southern blots
3. Neomycin/hygromycin or similar resistance genes are used to select for integration.
4. The thymidine kinase enzyme is included immediately adjacent to the flanking sequences to allow for selection against random integration (i.e., total insertion), of the targeting construct
5. Specific genomic probes outside the targeting construct (5' and 3') should be isolated and tested for efficient detection of correctly targeted clones
6. Appropriate restriction enzyme-cutting sites should be included within the construct for an obvious band shift in the targeted clones when using these probes in Southern blot.

2.2. Additional Considerations for Conditional Gene-Knockout Constructs

In addition to these aspects, the design of conditional gene knockout constructs requires a number of important additional considerations:

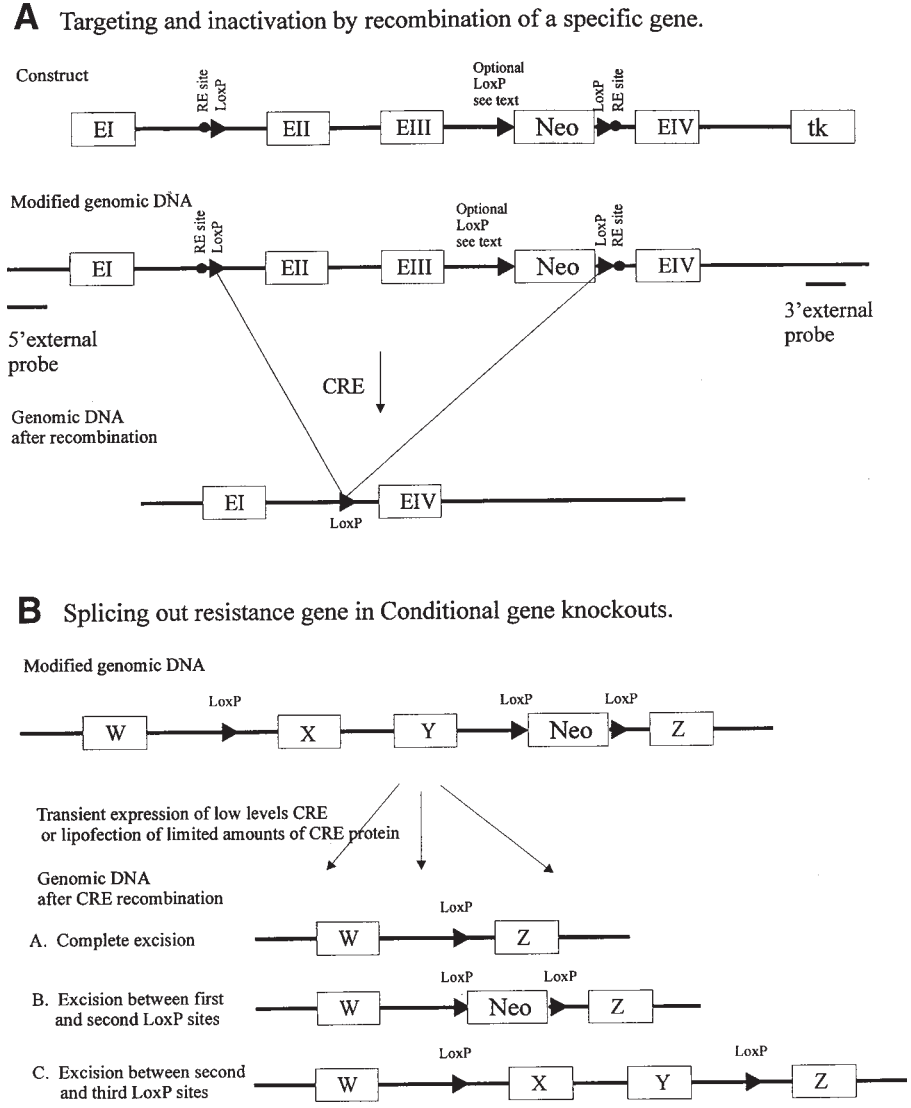

Fig. 2.Conditional gene knockouts using *LoxP*/CRE. **(A)** Drawing of a potential conditional targeting construct showing *LoxP* sites flanking neomycin and exons II and III. After recombination by CRE these exons and the neomycin resistance gene are removed. **(B)** Three possible recombination events after exposure to CRE when a floxed neomycin cassette is included. NB. B and C are intermediates that will be recombined to form A in the presence of CRE. Limiting amounts of CRE can increase the number of clones with B or C intermediates. C is the form where neomycin has been removed, but the gene not yet deleted. This is the ideal form for introduction into the germline. (▶ indicates Lox site. ● indicates restriction-enzyme site.)

1. Conventional knockouts are usually designed so that the neomycin gene (or other resistance gene) disrupts the transcription/translation of the endogenous gene. In conditional gene knockouts, the endogenous gene must function normally until recombined by CRE and thus any modifications must be outside coding regions or intronic and not interfere with regulatory regions.
2. The *LoxP* sites must be inserted in a manner where splicing will result in excision of sufficient DNA to render the gene inactive. This can be particularly difficult for genes with large introns where only relatively small amounts of coding sequence can be removed (unless a multiple targeting strategy is used, *see* **Subheading 4.**). In this case, it is important to have the complete genomic structure defined, including any alternative splicing that may occur. Then the *LoxP* sites can be positioned to remove exons that encode an important functional domain and/or removal of exons would result in a frameshift if alternative splicing occurs after recombination. Similarly, insertion of *LoxP* sites into intronless genes can be difficult, as care should be taken to avoid regulatory sequences in 5' and 3' untranslated regions (UTRs. It is also important to note that the distance between the *LoxP* sites and the resistance gene could influence the efficiency of generating clones containing all elements of the targeting vector. An alternate strategy is to use a reversible-knockout approach. In this case, disruption of the gene occurs until specific CRE recombination normalizes gene function.
3. Restriction enzyme-cutting sites should be included at each site of modified DNA integration. Screening strategies for the detection of correctly targeted embryonic stem cell clones commonly utilize restriction enzyme sites within neomycin or similar resistance genes, which are inserted at a single site in the gene of interest. For conditional gene knockouts, there is insertion of foreign/modified DNA into multiple sites within the genomic DNA, only one of which normally would include a resistance gene with appropriate restriction sites. We have found that the presence of correctly integrated *LoxP* sites are easily confirmed by Southern blot, utilizing restriction enzyme sites included in the targeting construct proximal to each *LoxP* sequence.
4. Screening strategies should be designed to include the ability to easily distinguish between at least three versions of the gene of interest: wildtype, targeted but not recombined and recombined. With the conventional gene-knockout approach, only two versions need to be distinguished: wild-type and knockout.

2.3. Should the Resistance Gene Be Flanked by LoxP Sites?

As mentioned previously, conditional gene-knockout strategy requires the gene to function normally until recombination occurs. Resistance genes included for the selection of correctly targeted clones usually include ≥1 kilobase (kb) of foreign DNA, which has the potential to influence the expression of the targeted gene prior to recombination. This is especially true of resistance genes inserted 5' of transcription and/or translation start sites or into the first intron. There is also some evidence that resistance genes located in

introns can be spliced to upstream exons in some transcriptions, effectively generating a truncated or mutant protein. To minimize this potential influence on gene expression or transcript, it is possible to splice out the resistance gene from the correctly targeted embryonic stem cell clone in vitro, prior to blastocyst microinjection or morulae aggregation ***(5)***. To remove the resistance gene, an additional *LoxP* site (or a pair of sequences specific for another recombinase, e.g., FRT/flp) ***(6)*** must be included to flank the resistance gene (*see* **Fig. 2B**). The modified genomic DNA would thus include three *LoxP* sites, two positioned flanking the resistance gene, and the third located to cause the desired deletion when recombination occurs. Transfection of the correctly targeted embryonic stem cell clone with limiting amounts of Cre construct or protein results in inefficient recombination, producing embryonic stem cell clones with three alternative genome configurations (*see* **Fig. 2B A–C**) in addition to nonrecombined clones. The amount of *Cre* required in vitro to give a sufficient number of embryonic stem cells with intermediate configurations will depend on (1) transfection efficiency, (2) the *Cre* promoter, (3) whether a nuclear localization signal is included, and (4) the activity of the enzyme. The presence of intermediates can be confirmed by test transfections and screening of ES cell pools. Selection of clones where the resistance gene has been deleted and the genomic DNA, with its appropriate modifications, has been left intact **(Fig. 2B,C)** can be made by PCR or Southern-blot screening.

2.4. Generation of the Conditional Knockout Construct

With these considerations in mind, the design and manufacture of the conditional gene-knockout construct by an experienced molecular biologist is fairly straightforward. We have used a combination of polymerase chain reaction (PCR) (to insert *LoxP* and restriction enzyme sites) and cloning (to insert selection gene with/without *LoxP* sites) to produce our targeting constructs. As the final construct is often quite large (>16 kb), we have produced the construct initially in portions, using a highly efficient cloning step to produce the final construct after initial portions have been verified by restriction analysis, PCR, and sequencing. The final construct is also verified by similar methods and recombination by CRE in vitro (*see* **Subheading 6**).

3. Transgenes that Use *LoxP*/CRE for Activation/Inactivation

The use of *LoxP*/CRE in modifying the genome of mammals is not limited to knocking out genes (*see* **Fig. 3**). Overexpression transgenic animals can be developed such that expression of a transgene can be switched on/off by the subsequent induction of CRE in a tissue specific or nonspecific manner. For example, a transgene that may be toxic or have other unwanted effects during development can be inserted into the genome as a ‘‘silent’’ gene (*see* **Fig. 3A**)

until CRE recombinase activates it. This activation could take place via an inducible and/or tissue specific promoter. This system has at least three advantages over using inducible promoters to express the transgene directly:

1. Most inducible promoters have a low basal level, which may be undesirable for many transgenes. It is unlikely that these ''leaky'' levels would be sufficient to produce enough CRE to cause recombination.
2. It is unnecessary to maintain animals on the agent required to induce the promoter, which can be expensive or potentially have other biological effects, as activation by recombination is permanent for both recombined cells and their progeny.
3. Phenotypes induced cannot be due to transgene positional effects, as the location of the transgene in the genome is not altered by CRE recombination.

This concept is illustrated by Lakso and colleagues *(7)*, where lens tumors developed in mice after a 1.3-kb STOP fragment inhibiting the lens-specific aA-crystallin promoter transcribing the SV40 large T antigen was removed by CRE. Similarly Akagi and co-workers *(8)* described a mouse where CRE removed a CAT sequence, allowing transcription of a *LacZ* reporter gene in vivo.

In contrast, transgenes can also be inactivated by CRE mediated deletion **(Fig. 3B)**. This concept has been utilized to develop control mice for *Cre* constructs where a *LacZ* reporter gene was inactivated by CRE in vivo.

4. *LoxP*/CRE-Induced Chromosomal Translocation

Chromosomal translocations (*see* **Fig. 4**) are the cause of many human diseases, particularly carcinogenesis. These diseases are often cause by alterations in gene regulation, formation of mutant or truncated proteins, or by the actions of any fusion proteins generated. The *LoxP*/CRE system provides an exciting new approach to the analysis of these diseases with the potential to mimic these translocations in mice. These animals can be generated by first targeting *LoxP* sites to the specific chromosomes by homologous recombination (*see* **Fig. 4**). These are done consecutively using different antibiotic resistance genes (A similar double targeting of *LoxP* sites strategy could also be used to delete large pieces of DNA on the same chromosome). A clone with correctly inserted *LoxP* sites is then transfected with CRE similar to that described for the removal of resistance genes in **Subheading 2**. The CRE will cause recombination between the *LoxP* sites on the distinct chromosomes. Although the desired translocation can be detected directly (by PCR or Southern blot), because of to the large distance between these *LoxP* sites the efficiency of CRE recombination is much lower than for that described above. Furthermore, *LoxP* sites are regenerated on translocated chromosomes after recombination and thus are equally likely to undergo further recombination back to the original configuration, unless mutant *Lox* sites such as those

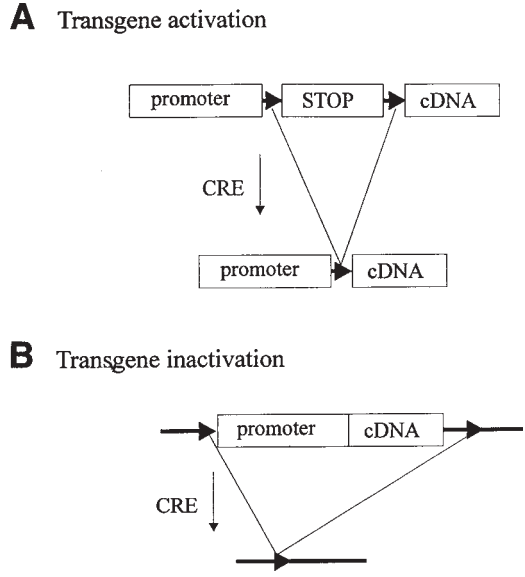

Fig. 3. Constructs for activation and inactivation of transgenes by CRE. **(A)** Transgene that is activated following CRE-mediated excision of a "STOP" sequence. **(B)** Removal of a floxed transgene by CRE. ► indicates Lox site.

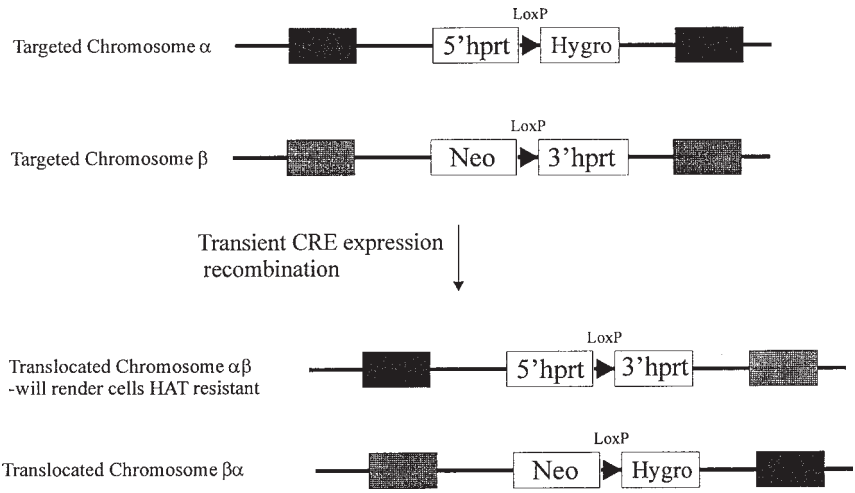

Fig. 4. Strategy for CRE-induced chromosome translocation. Chromosomes are sequentially targeted with Lox sites using different antibiotic resistance genes. Transient CRE expression then induces chromosome translocation and selected using HAT media. Note:- use of mutant Lox sites will improved the efficiency of this recombination event. ► indicates Lox site.

mentioned in ***(1)*** are used (eg. *Lox71* and *Lox66*). It can, therefore, be convenient to use an additional selection criteria (**Fig. 4**; e.g., Hprt ***[9]***), to select the desired recombinants. This can be facilitated by inserting segments of the selection gene into the targeting constructs so that the gene will be complete, in frame, and functional only after specific recombination.

5. Subtle Genome Modification

One of the other uses of homologous recombination technology is the introduction of point mutations, or other modification of genes, to examine functions of gene regulatory regions in vivo, mutate functional domains of proteins, or to mimic mutations observed in human disease (*see* **Fig. 5**). One of the major problems with such an approach is that the antibiotic resistance genes required to select correctly targeted cells have the potential to influence the gene function greatly, perhaps even more than the mutation itself. The use of *LoxP*/CRE in the experimental approach to these issues provides a mechanism where modifications can be made with minimal alteration to the surrounding genome. The mutation construct would contain the antibiotic resistance gene far enough 5' or 3' to avoid complications (e.g., away from vital elements in promoters or outside exons for protein mutations) and flanked by *LoxP* sites. Thus, the correctly modified clone could be transfected with CRE to excise the resistance gene. It is less likely that the 34-bp *LoxP* site that remains in the modified genomic DNA will effect gene expression/function in comparison with an entire resistance gene, including promoter and coding regions. This approach has been used successfully to alter the IgH switch control element in vivo ***(10)***.

6. Recombination by CRE

The foregoing sections indicate the potential use of *LoxP*/CRE in various approaches for modification of the genome and the variety of ways *LoxP* sites can be inserted to facilitate these modifications. The feasibility and success of these approaches is dependent on the mechanism by which CRE is regulated and the efficiency of the recombination event in vitro and/or in vivo.

6.1. Recombination In Vitro

Recombination in vitro is required for many of the processes outlined including verification of constructs prior to transfection, removal of resistance genes, and chromosomal translocation.

1. The ultimate test of a targeting construct containing *LoxP* sites is the ability of that construct to undergo recombination. Competent cells can be easily generated from bacterial cells that express CRE and these can be transfected with the construct ***(11)***. Miniprep DNA can be digested with appropriate restriction enzymes to confirm recombination, and thus functional *LoxP* sites. The original DNA can then be introduced into cells for homologous recombination.

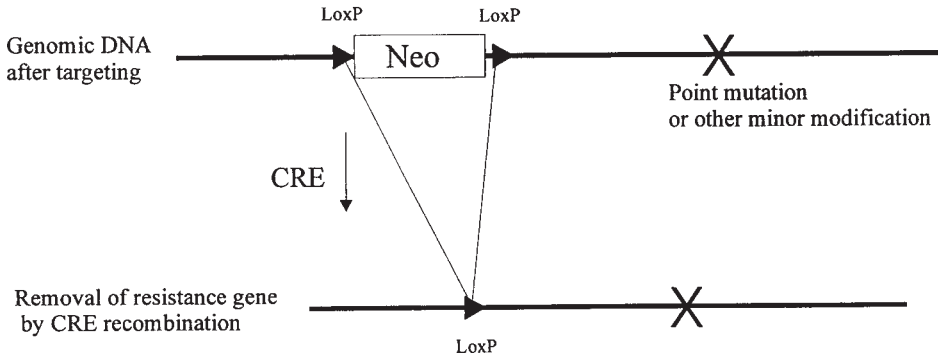

Fig. 5. Subtle genome modification. A minor modification of genomic DNA is targeted into cells which are selected by a nearby neomycin cassette. This neomycin cassette is flanked by Lox sites and thus is removed by CRE-mediated recombination, thus minimizing the alterations to the genomic sequences. ► indicates Lox site.

2. After transfection, selection, and screening, ES cell clones shown to have the modifications correctly integrated can have their resistance gene removed to avoid the influence of such sequences on the gene of interest (or even neighboring genes). To remove resistance genes, clones are transiently transfected with a construct where *Cre* is under the control of a promoter that expresses in ES cells in vitro. Promoters such as CMV, EF1α, β-actin, and EIIa have been shown to be successful. Alternatively, purified CRE protein can be transfected directly using such techniques as lipofection ***(12)***. Where only two *LoxP* sites are present and removal of the resistance gene is the only expected CRE-induced recombination (as in subtle genome modification), clones are readily isolated where recombination can be confirmed by PCR or Southern blot. Where three or more *LoxP* sites are present (e.g., for the removal of resistance genes from conditional knockout constructs), the procedure is more difficult. The amount of *Cre* vector or protein to be transfected must be titrated to ensure that clones with intermediate recombination event occur (*see* **Subheading 2.3.** and **Fig. 2B**). As mentioned earlier, the presence of appropriately recombined ES cells can be confirmed by PCR screening of pools prior to cloning of ES cells and selection of correctly recombined clones.
3. In vitro CRE recombination is generally quite efficient as optimized transfection rates and strong promoters (or pure enzyme) give effective recombination. For chromosome translocation studies, it may be necessary to use some form of selectable marker, as recombined chromosomes can readily recombine back to their wild-type configuration in the presence of CRE and/or the CRE enzyme may be less effective over the vast distances of the mammalian genome (*see* **Subheading 4.** ***[9,13]***). Screening resistant clones is still necessary to demonstrate the correct translocation prior to introduction into the germline.

6.2. Recombination In Vivo

The majority of the current usage of the *LoxP*/CRE system has been in the development of conditional gene knockouts. The array of CRE transgenic animals is increasing daily and the development of a CRE recombinase transgenic database provides an important resource as to the lines that have or are being developed. This database is located at http://www.mshri.on.ca/nagy/Cre.htm and is discussed in more detail by Nagy and Mar (*see* Chapter 7). One significant advantage of conditional gene knockouts is that the same gene (once targeted) can be specifically deleted in a different tissue or cell type (or more precise subpopulations of cells) as the CRE transgenics become available simply by mating mice. Furthermore, with the development of more sophisticated inducible expression systems that enable tight regulation of genes in a tissue-specific manner, more refined biological questions can be addressed. It is not the purpose of this chapter to review these systems, but rather to indicate that the full potential of the *LoxP*/CRE system is yet to be realized and is limited only by where and when Cre can be expressed (*see* Chapter 7).

There are several important considerations in the choice of Cre expression system and in the consequent interpretation of results from these matings:

1. CRE needs to be expressed at a sufficiently high level to get close to 100% recombination. The rate of CRE recombination has been improved by altering the *Cre* cDNA to include a better KOZAK sequence and more mammalian codon usage which will both improve translation efficiency, and a nuclear localization signal has been added to increase nuclear CRE concentration and thus function. Despite these modifications it is important to use a promoter with a reasonably high level of transcriptional activity to ensure total recombination. Results should always be interpreted with the caveat that some nonrecombined cells may remain.
2. All required regulatory elements of a gene promoter should be present. It is very clear from much promoter research that although a few elements may display some cell specificity in vitro, large segments of genomic DNA including 5', intronic and 3' sequences may be required to give appropriate expression in vivo. Where possible promoters should be tested in vivo prior to deleting the gene of interest to verify tissue specificity.
3. The CRE-induced genomic DNA deletion will be present in all daughter cells of a recombined cell. This means that if CRE is expressed in an earlier progenitor cell, many subsequent cell lineages could have the CRE induced deletion, even though CRE may not be expressed.

Although mating with transgenic mice expressing Cre has been the usual mechanism to induce recombination in *LoxP*-targeted mice, an alternative is the use of viruses. Adenoviruses carrying the Cre recombinase gene have been used to induce recombination in vivo ***(14)***. Refinement of the viral targeting specificity could make viral introduction a fast alternative to the generation of *Cre* transgenic mice.

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7

Creation and Use of a Cre Recombinase Transgenic Database

Andras Nagy and Lynn Mar

1. Introduction

Embryonic stem (ES) cell-mediated transgenic approaches have revolutionized mammalian genetics in the last decade. Close to 4000 genes have been targeted to date. Analysis of these genetic alterations has provided an unprecedented understanding of critical gene functions that underlie normal developmental and disease mechanisms in mammals. We have also learned, however, that mammalian genetic determination is complex: genes have multiple functions during the life of an individual. In addition, mammalian genetic determination often utilizes gene families, in which the members have similar structure and overlapping expression and functions. These two phenomena pointed out some limitations of the gene-targeting approach. In many cases a gene "knockout" resulted in early embryonic lethality, which obscured the study of potential later functions. In other cases the "knockout" did not have any phenotype due to the compensation of the gene deficiency by other family members. These limitations have called for further development of the powerful gene-targeting technology ***(1–3)***.

One of the critical tools now being efficiently combined with gene-targeting is site-specific recombination ***(4)***. In fact, to date, two site specific recombinases have been shown to work efficiently in mammalian systems: the yeast-derived Flip (FLP) recombinase ***(5)*** and the P1 phage-derived Cre recombinase ***(6)***. Each recognizes a unique 34-base pair (bp) consensus sequence, designated FRT for the FLP, and *loxP* for the Cre recombinase. These enzymes catalyze a recombination event between two of their recognition sites ***(7)***. Since the 34-bp consensus sequences are asymmetrical, their orientation is an important determining factor for the outcome of the recombination, so that

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recombination between similarly oriented recognition sites in the same DNA strand results in an excision of the intervening sequence **(Fig. 1)**. The Cre/*loxP* system appears to be more efficient in mammalian systems, including ES cells. Therefore it has been much more extensively applied than the FLP/FRT system.

2. Cre Recombinase Action

Cre recombinase is an essential enzyme required for the reproductive cycle of the phage P1 ***(7,8)***. During the lysogenic phase, multiple copies of the phage genome are produced in the form of circular concatamers. Cre resolves these concatamers into multiple copies of single-phage genomes by restricting and covalently ligating two adjacent *loxP* sites, which flank a single genome equivalent.

The *loxP* site is unlikely to be present in mammalian genomes; therefore it has to be introduced in order to provide a substrate to the enzyme. An important and intensive characterization of the enzyme action in mammalian tissue culture systems ***(6–9,10)*** preceded its in vivo applications. The first report illustrating that the recombinase, produced from a transgene, can mediate a very specific alteration in vivo in the mouse was published in 1992 ***(11)***. Shortly after, we learned that it also works in ES cells ***(12)***. These experiments ignited a burst of ideas and applications using the combination of gene-targeting and site-specific recombination (for review, *see* ***[3]***). In this chapter we introduce the major trends through a few examples.

3. Combination with Transgenesis and Homologous Recombination

3.1. Conditional Genome Alterations

3.1.1. Conditional Transgenics

Transgenesis of mammals started with the pioneering work of Gordon and co-workers ***(13)***, who demonstrated that DNA when injected into the pronucleus of a fertilized egg can integrate into the genome, and can even express a delivered gene ***(14,15)*** if it was designed to do so. During the last nearly 20 years this technology has brought us a broad spectrum of information about gene functions, regulation, and the consequence of mutations.

In the past, expression of mutant proteins has been particularly fruitful in determining the function of a normal gene ***(16)***. However, mutations in many genes cause early developmental arrest, and, consequently, cannot adequately address any later roles. Present and future objectives will include expressing mutant proteins only in specific cell lineages and during developmental periods of interest. For instance, to better understand the role of oncogenes in certain organs, or to study the effects of somatic mutations.

One potential strategy takes advantage of the Cre/*loxP* system. Here, a tissue or temporal specific promoter is placed in front of two consecutive genes in

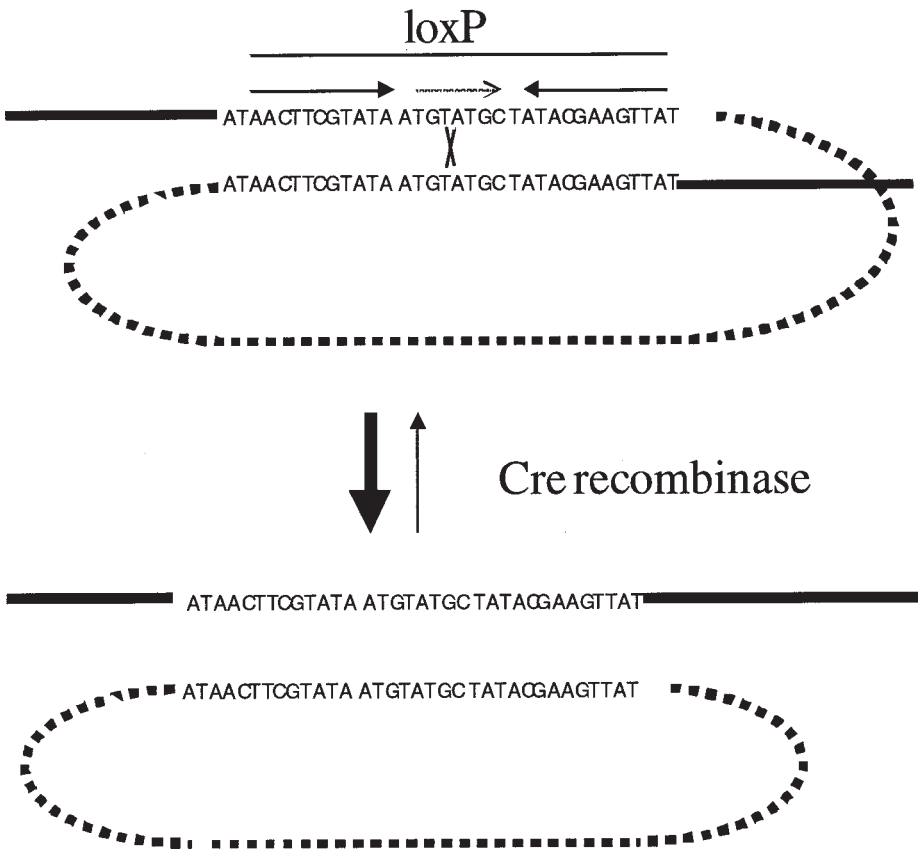

Fig. 1

such a way that it initially directs only the expression of the upstream (5' most) gene **(Fig. 2A)**. This gene is flanked by a pair of *loxP* sites, so that it can be removed on Cre-mediated excision. Consequently, leading to the expression of the downstream gene in place of the first in an identical fashion. If the *lacZ* reporter gene is placed in the upstream position, it facilitates the initial high-resolution characterization of the spatiotemporal expression pattern of a specific promoter. Another variation for this strategy is an arrangement shown in **Fig. 2B**. First, transgenic animals with a conditional transgene similar to the above, but under the control of a ubiquitous promoter are generated. They are then crossed to Cre transgenic lines each displaying different expression patterns. The advantage here is that only the conditional transgenic lines need be established.

An important issue arising when applying such a conditional transgenic strategy is the concern about multiple copy and/or multiple site of integration of the transgene. In this frequently occurring event, several copies of the *loxP*-contain-

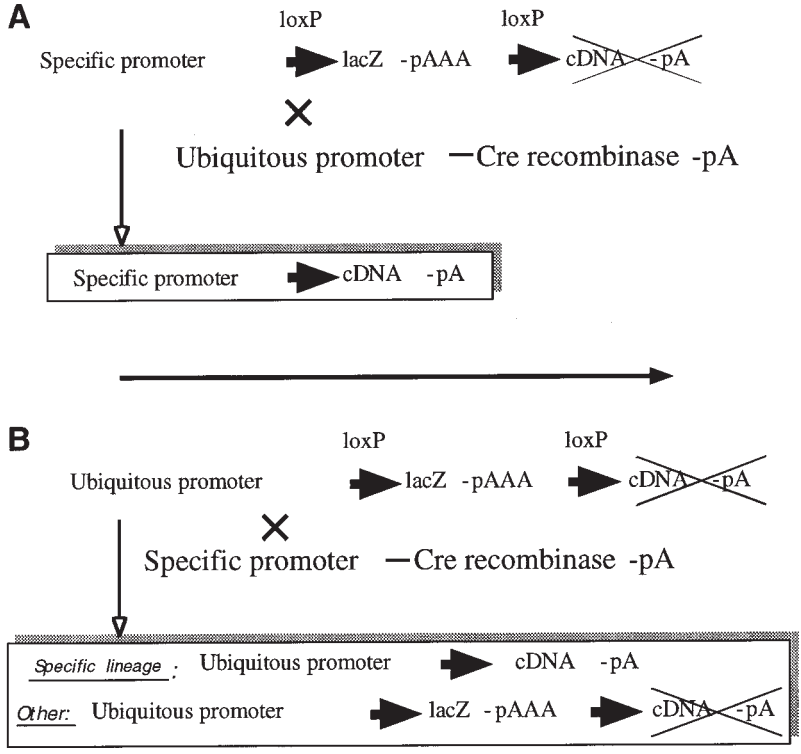

Fig. 2

ing plasmid integrate into the same and/or different regions of the genome with random orientations. In such an event, inverted *loxP* sites are easily created; these can cause severe chromosomal instability during Cre excision ***[17]***), leading to aneuploidity and a potential phenotype that is unrelated to the transgene.

3.1.2. Conditional Knockouts

There are several solutions for conditional gene knockouts ***(3)***. One of them combines gene-targeting and site-specific recombination. The general strategy here is to introduce *loxP* or FRT sites flanking an essential exon of the gene of interest (**Fig. 3**). These intron-inserted sites should not disturb the coding regions, regulatory elements or proper splicing and therefore functioning of the gene. There is a concern, however, about the positive selectable marker that is also introduced to the modified locus. As a consequence, strategies have been developed for the removal of the marker after the targeted allele has been identified. In this respect, the FLP/FRT recombinase system was successfully applied in the FGF8 conditional knockout ***(18)***, and a three *loxP* system based on partial Cre excision in a study of DNA polymerase β gene ***(19)*** has also

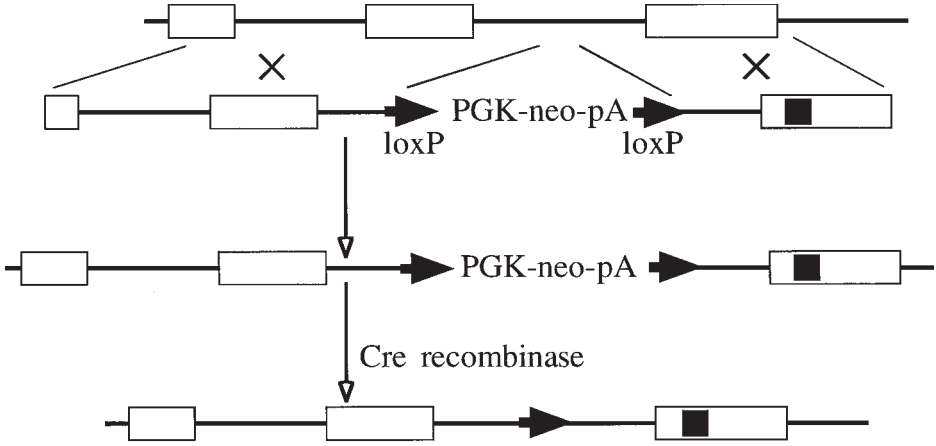

Fig. 3

been used. After the desired insertion is achieved, the selectable marker is removed with the gene being functionally equivalent to wild type so that the homozygous animals should be normal. If, however, Cre recombinase is expressed in any of the cells, it specifically removes the essential exon from the modified alleles and creates a Cre-excision-dependent knockout. If a cell type-specific Cre transgene is utilized, only the lineage expressing Cre will be deficient for the gene ***(20)***.

3.1.3. Conditional Gene Repair

The idea of conditional gene repair is similar to that of the conditional knockout. However, the logic here is complementary. The aim is to create a gene knockout with an insertion into a gene of interest. However, the insertion created, is special in three respects: (1) it interferes with the gene's expression; (2) it is flanked by *loxP* or FRT sites; and (3) after Cre-mediated excision, normal gene function is restored **(Fig. 4)**. When such a gene knockout is introduced into the germline, the consequence of the gene deficiency should be characterized, and the primary lineage affected determined. If there is an embryonic lethality related to the primary defect, a Cre transgenic line with specific expression in this particular lineage is crossed over the knockout. Due to the excision of the interfering sequence, the gene is repaired *in situ* in the primarily affected lineage where the Cre is expressed, resulting in rescue of the primary phenotypic defect. The embryo can thus survive longer, until the chronologically second phenotype is manifest. In theory, this methodology should provide an automatic guide through the multiple functions of a gene. Such an *in situ* gene repair has recently been demonstrated for an *N-myc* hypomorphic allele ***(21)***.

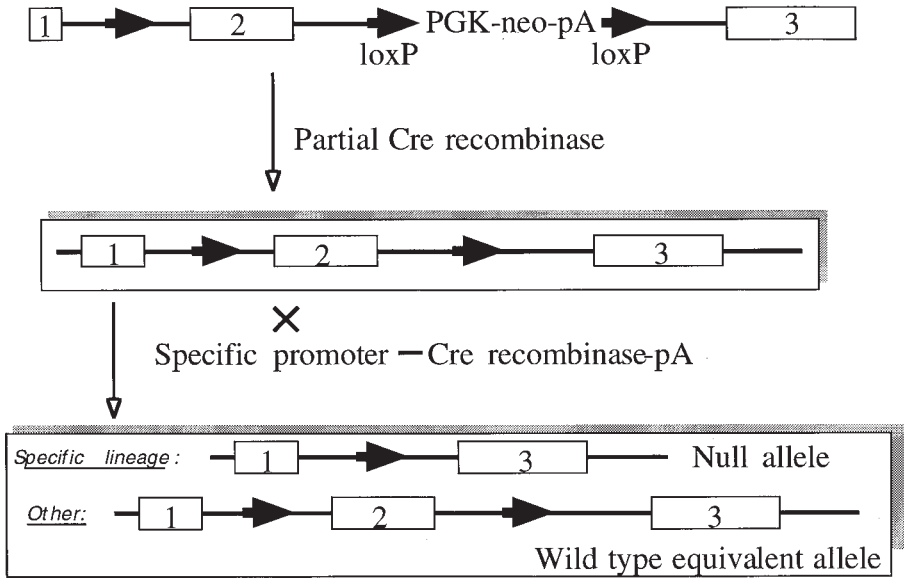

Fig. 4

An important difference between the conditional gene knockout and repair is their different requirement for the fidelity of the Cre-mediated excision. If, for example, a Cre recombinase transgene excises only in 70% of the cells of the desired lineage, the remaining wild-type equivalent 30% could make analysis of the phenotype extremely complex. Even a weakly chimeric lineage (with respect to wild type) could even be sufficient to rescue any phenotypic anomaly. On the other hand, if 70% of cells are repaired in a lineage, it will almost certainly be enough to rescue the phenotype. We expect that numerous Cre transgenic lines will be produced with incomplete Cre-mediated excision in the target lineage. Therefore, many of the lines may not be suitable for most conditional knockout studies, but can be useful for conditional repairs.

There are several ways of creating repairable gene knockouts or alterations. One possible way is to insert a *loxP* flanked positive selectable marker, such as neo, into the 5' untranslated region (5'UTR) of a gene. In this case, transcription of the targeted gene is disrupted, until the point when Cre excision of the *loxP*-flanked marker allows for transcriptional and/or translational read-through. Alternatively, the *loxP*-flanked sequence (including the selectable marker) can be inserted into an intron. In this case, the sequence must contain a strong splice acceptor (SA) site, and a transcriptional termination signal in order to truncate the transcript. Interestingly, a strong disturbance in the transcription of the targeted gene can occur when inserting an inverted neo gene

into an intron ***(22,23)***, because it contains a strong SA site. The frequently used phosphoglycerate kinase (PGK) promoter-neo cassette can also compromise transcription when it is in the same orientation as the modified gene. The compromise, however, is milder: an approximately 60% drop in the mRNA level is expected in this case ***(21–24)***.

3.2. Inducible Genome Alterations

The real power of Cre-mediated genome alteration will be realized when temporal regulation of the recombinase becomes possible. Efforts are now moving toward this direction. Both the tetracycline-inducible ***(25)***, and the mutant estrogen receptor, tamoxifen-inducible systems ***(26,27)*** have been combined with Cre-mediated, site-specific recombination. These experiments have already demonstrated the possibility of inducible Cre recombinase activity and, as a consequence, inducible Cre-mediated excision. The goal for general applicability is to reach minimal or preferably no excision in the noninduced state and the possibility of complete excision in the induced state.

3.3. Introduction of Subtle Changes

Introduction of a subtle change, such as a point mutation, into a gene by homologous recombination is possible by embedding the particular mutation into the target vector. In this case, however, removal of the positive selectable marker is required **(Fig. 5)**. The marker can be removed either by transient expression of Cre in the targeted ES cells or after germline transmission of the marker-containing allele. In the latter, crossing the germline chimera to Cre transgenic animals is less labor intensive***(21–28)***. The Cre transgene should subsequently be segregated from the targeted allele to maintain the clarity of the experiment. A third way to introduce Cre recombinase is to inject its expression vector as a circular plasmid into a heterozygous F1 zygote. This was reported as a very efficient way of removing *loxP*-flanked segments in the few kilobase range ***(29)***. The advantage of this latter method is that the Cre is only transiently expressed during preimplantation stages and does not leave a transgene behind.

3.4. Large Deletions/Duplications and Chromosomal Translocations

The Cre recombinase can also work on two *loxP* sites located at a long range from each other. If two sites with the same orientation are placed on the same chromosome but a few megabases apart, and Cre recombinase is expressed in the cell, excision occurs. It occurs at a low frequency but high enough to identify such an event with a properly designed selection system. The most commonly used selectable marker for this technology is a split HPRT minigene. Neither of the two halves of the hypoxanthine phosphoribosyltransferase (HPRT) selectable marker renders cells HPRT positive. The 5' part of HPRT is placed at

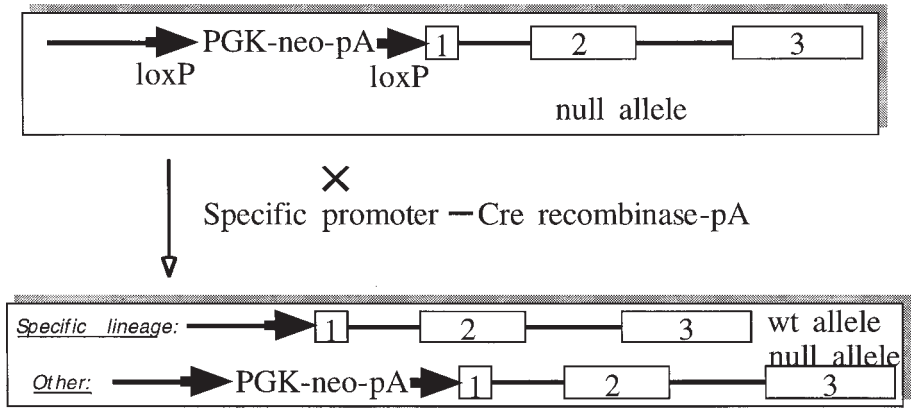

Fig. 5

one end of the planned deletion whereas the 3' part is at the other end of the deletion on the same chromosome in an HPRT-negative cell line. The deletion brings the two HPRT pieces ***(30)*** together and allows the cells to survive in hypoxanthine, aminopterin, and thymidine (HAT) medium **(Fig. 6A)**. If the two *loxP* sites happen to be on homologous chromosomes, the recombination results in a duplication in one chromosome and a deletion on the other **(Fig. 6B)**.

If two *loxP* sites are placed on different and nonhomologous chromosomes, and their orientation is the same relative to their centromere, a reciprocal translocation occurs between the two chromosomes **(Fig. 6C)**. Again, the HPRT selection system mentioned can identify this rare event ***(31)***.

3.5. A Database of Cre Recombinase Transgenic Mouse Lines

As this site-specific recombinase technology further developed, it became evident that this tool was going to have a significant impact on the power of mammalian genetics. At that point one of us (A.N.) sent out a letter to many of our colleagues in the field, calling for a collective effort in order to produce a panel of Cre transgenic mouse lines, each expressing the recombinase with different specificities. The original idea was to coordinate this effort and arrive at an optimized panel of expression patterns, which would cover the mouse as though it were a four-dimensional jigsaw puzzle. We have received many responses welcoming a collective effort. However, it was immediately apparent that the proposed optimization was unlikely to work. As even though almost everyone's reaction was favorable, individuals were more likely to be willing to create specific lines that immediately appealed to work they were pursuing in their own laboratories, thereby curtailing an optimized effort.

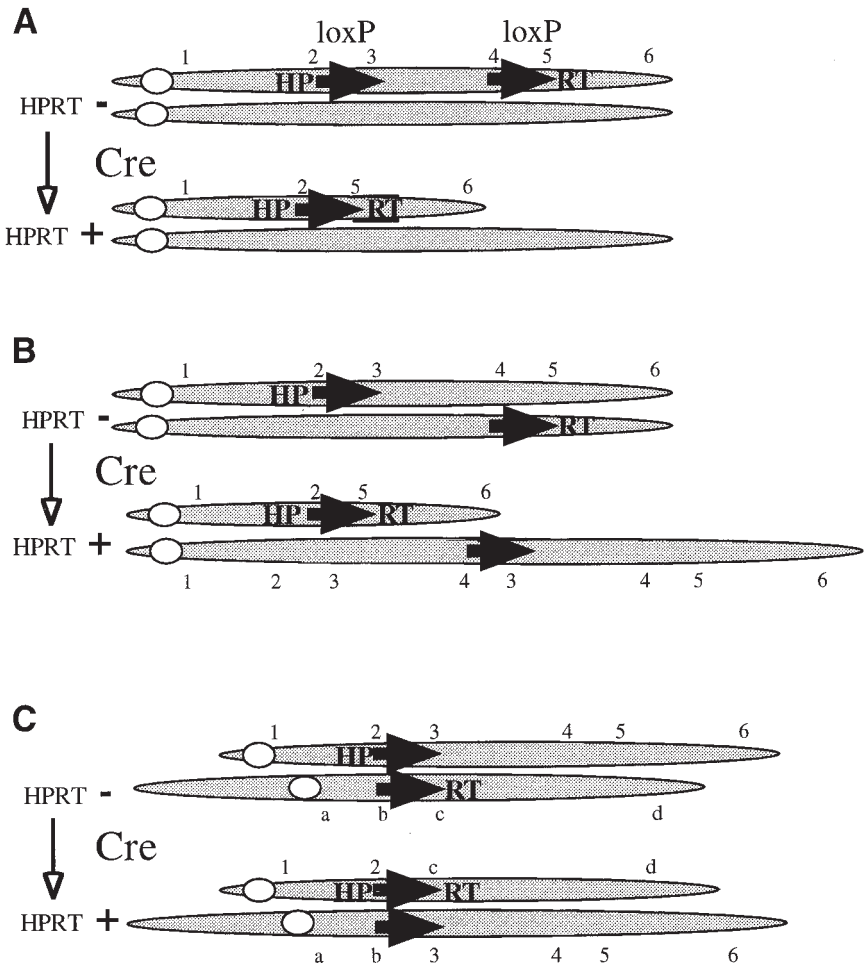

Fig. 6

As a consequence, the goal is now to create a database, listing all the Cre transgenic lines that people have suggested or indicated that they intend to make. During the last three years the database has evolved, and now the current status of the transgenic lines is also listed. Status falls into five categories: (1) suggested, (2) Being made, (3) made and being tested, (4) made and not working according to the expectation, and (5) made and the Cre recombinase properly excises with the expected specificity. The database is published on the World Wide Web: <http://www.mshri.on.ca/nagy/> and is regularly updated. It is formatted as shown in **Table 1**.

Table 1
The Format of the Cre Transgenic Database as Published on the World Wide Web (The underlined words are buttons)

Promoter	Specificity	Status	Last Updated
bHLH-EC2	Mesoderm at 7.5 d. Later restricted to precursor cells in dermamyotome and blood islands	Suggested	Apr 10/97
Hoxa-1	Neuroectoderm and mesoderm	Being made	Apr 10/97
HNF3-alpha	D 7.5 embryonic endoderm; d 8.5 foregut & hindgut endoderm; weak in notochord. After d 8.75: notochord, endoderm-derived tissues; ventral parts of midbrain	Made, not tested yet.	Oct 28/97
Hoxa-1 enhancer III	Floorplate, notochord, for gut epithelium	Made, tested positive expression and recombinase activity	Apr 10/97
—	—	—	—

Submit your Cre transgenic line to the database
Status: *not made * made, did not work * made, not characterized * made, tested positive*

The promoters are buttons, they show the name of the submitter, contact information, or references and Medline listing of the promoter or Cre transgenic line. If the line is already published the Status field contains a <Published> button, which connects to the corresponding MEDLINE listing. An electronic submission sheet is attached that allows a convenient means of information submission and update. Successful production of Cre transgenic lines that have not been submitted to the database is also listed, as the corresponding reports become available.

4. Prologue

There are an increasing number of publications reporting the successful combination of site-specific recombinase and ES cell-mediated genetics, clearly demonstrating that our initial enthusiasm was well founded. Almost any imaginable genomic alteration can now be produced in mice. Certainly, we can create any phenocopies of defects associated with human diseases; from chromosomal aberrations to missense mutations.

Here we have described the creation of a rather unusual database, a database before the data has been produced. The fact that this was possible reflects two important things: the early recognition of the significance of creating such a resource in order to avoid duplicating or multiplying the same labor-intensive effort; and the willingness for a wide range of collaborations where we can share the same tools to address different biological questions. Tools do not have values on their own, but they can be applied to create a variety of new, valuable things. Thus they should be available as shared resource for a wide range of applications that will hopefully provide far reaching insightful information.

Acknowledgments

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8

Choice of Mouse Strains for Gene Targeting

Mireille H. Lahoud, Trevor J. Wilson, and Paul J. Hertzog

1. Introduction

In designing and planning the generation of a gene-targeted mouse, there are several stages at which the choice of mouse strain is critical. Consideration must be given to the genetic strain from which (1) the genomic DNA library, (2) the embryonic stem cells, and (3) the recipient embryos are derived, in addition to (4) the strain with which the chimaeric mice are mated. Ultimately, only the strain of genomic DNA, the (ES) cell strain, and the strain of mice for mating contribute to the genetic composition of the targeted mouse, whereas the strain of host embryos is only relevant in that it must be compatible with the choice of ES cells, otherwise the ability of the chimeric mice to provide germline transmission of the ES cell genome may be compromised.

The genetic background of the targeted mutation has been shown to be an important determinant for the mutant phenotype. Strain variation in the phenotypes of spontaneous mutations was described as early as 1955 by King ***(1)***, who reported a spontaneous mutation, pygmy, later identified to be a mutation of the DNA binding molecule, Hmgi-c ***(2)***. The pygmy mutation resulted in prenatal and postnatal growth retardation in both "small" and "large" strains of mice. However, although the majority of male mice on a "large" background appeared fertile, the mice on a "small" background were infertile and more than half the males were cryptorchid ***(1)***.

In this chapter, we discuss the use of isogenic DNA and choices of strains for ES cells and for breeding. The compatible host embryo strains reported for the generation of chimeras is mentioned briefly. As the influence of genetic background on some knockout mouse phenotypes is reviewed in Chapter 14, only a few examples of the effects of genetic background is discussed where relevant to illustrate the advantages of particular strains or ES cell lines and to act as a guide in choosing strains for the generation of future mutant mouse lines.

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2. Use of Isogenic DNA for Gene Targeting Constructs

Although few direct comparisons have been performed, the use of isogenic DNA has been reported to improve the gene targeting frequencies in some homologous recombination studies. For example, gene targeting of the retinoblastoma susceptibility gene *(Rb)* in 129-derived ES cells was found to be 20-fold more efficient using an isogenic 129-derived targeting construct than a Balb/c-derived construct ***(3)***. The constructs were generated using 17 kilobases (kb) of genomic DNA and were identical, except for sequence divergencies between 129 and Balb/c DNA ***(3)***. Similarly, gene targeting of the *creatine kinase M* gene in 129-derived ES cells was found to be 25-fold more efficient using a 129-derived targeting construct than *a Balb/c d*erived construct ***(4)***. Targeting of the *Hprt* locus using isogenic DNA was reported to be 4 to 5-fold more efficient than targeting using nonisogenic DNA ***(5)***. By contrast, a 129-derived construct for targeting the 5-lipoxygenase activating protein in dibenzanthracene (DBA)/1 ES cells was reported to have a similar targeting frequency to isogenic DNA ***(6)***. The discrepancy between the levels of increased efficiency associated with the use of isogenic DNA reported in different studies probably reflects the extent of polymorphisms between any two strains. Although only a few direct comparisons have been performed, use of isogenic DNA is advisable where possible, as it is likely to improve targeting frequencies. Furthermore, genomic DNA libraries from most commonly used inbred strains such as *129/SvJ*, *C57BL/6*, *Balb/c*, and *DBA/2J* are commercially available from sources such as Stratagene or Clontech, and custom-made libraries can also be ordered. If, however, isogenic libraries are not available, it may be possible to compensate for the polymorphisms by using larger constructs to increase the regions of homology and hence the overall chances of homologous recombination.

3. Embryonic Stem Cells

3.1. 129-Derived ES Cells

The majority of ES cell lines that are available for use in gene targeting have been derived from substrains of the 129 mice. This is reported to be due to the capacity of this strain to generate ES cell lines that have the ability to contribute to the mouse germline after extensive manipulation in culture. Over time, the 129 line has been extensively distributed and a number of breeding colonies set up, resulting in the generation of 129 substrains from which a number of ES lines were generated (reviewed in ***7***). These include the ES cell lines E14TG2a ***(8)***, HM1 ***(9)***, mEMS32, RW-4 ***(10,11)***, D3 ***(12)***, EK.CCE ***(13,14)***, AB1 ***(15)***, AB2.1 ***(16)***, J1 ***(17)***, and H3 ***(18)***, in addition to a cell line derived from deliberate outcrossing, R1: *129/Sv* × *129/SvJ* strains ***(19)***. Accidental and deliberate

outcrossing of the mouse 129 substrains has resulted in discrepancies between the genetic background of ES cell lines and the proposed parental mouse 129 substrains ***(7,20)***; for an analysis, *see* **ref.** ***(7)***. This has placed some limitations on the studies that can be performed, especially in fields such as transplantation and graft rejection where knockout mouse lines were found to be nonhistocompatible with their parental inbred strain ***(21)***.

The extensive efforts of Simpson et al. (1997) to clarify the origin and genetic background of the 129-derived ES cell lines enables users of these cell lines to improve the targeting frequency by using the appropriate genomic DNA library, in addition to an isogenic strain of mouse to generate knockout mice on a pure background.

3.2. ES Cells from Other Mouse Strains

There are instances where the aim of the targeting studies is to determine the role of a gene in a particular response or a model of disease by using a mouse strain that is susceptible to that disease. The Balb/c and C57BL/6 mouse strains have been widely used and are well characterized. They differ in a number of characteristics, including immune responses and behavior. The availability of C57BL/6-derived and Balb/c-derived ES cell lines will facilitate gene-targeting experiments on these backgrounds.

In order to generate gene-targeted mice on a specific genetic background, one option is to generate gene-targeted mice on a mixed background and then to backcross it to the required strain. However, this procedure is time and resource consuming: eight backcrosses will involve approximately 1.5 yr of breeding (*see* **Subheading 5**). If an ES cell line is available from the desired strain, then chimeric mice can be mated directly with the strain of interest to produce not only the background of interest, but also a homogeneous background.

Two *C57BL/6*-derived ES cell lines have been described, the *BL/6-III* ***(22)*** and Bruce-4 ***(23)***. These have been successfully used for the gene targeting of a number of genes including *lymphotoxin*-α, *TNF* and *MHC class II Aa* ***(23,24)***. Balb/cJ-derived ES cells have also been derived that have been reported to produce germline chimeras ***(25)*** one of which has been used for the successful targeting of the *IL4* gene ***(25)***.

ES cells have also been generated from other mouse strains that are highly susceptible to autoimmune disease: DBA/1 mice enable the study of collagen induced arthritis, an experimental model of rheumatoid arthritis ***(26)***. The DBA-1-derived ES cells were used to examine the role of *5-lipoxygenase* (*FLAP*) in the pathogenesis of collagen-induced arthritis. FLAP is an integral membrane protein required for the synthesis of leukotrienes, a class of biologically active lipids with proinflammatory effects ***(26)***. *FLAP* knockout mice were reported to develop and grow normally but had a reduced inflammatory

response to zymosan and a reduced severity of collagen induced arthritis when compared with wild-type or heterozygous animals, demonstrating the essential role of leukotrienes in acute and chronic inflammatory responses ***(26)***. ES cells have also been derived from MRL mice, a strain that spontaneously develops a generalized autoimmune disease with features similar to systemic lupus erythematosus ***(27)***. These cells have been used for gene targeting of the EP_2 prostaglandin receptor and for generating mice heterozygous for *EP2* ***(27)***.

4. Recipient Embryos

Once a gene-targeted ES cell line is generated, the mutation is introduced into the germline. This is usually achieved by either microinjection of ES cells into suitable host blastocysts or by aggregation with earlier stage embryos such as morulae. In either case, the manipulated embryos are transferred into the uterine horns of pseudopregnant foster mothers to generate chimeric mice. A number of factors have been reported to influence the degree of somatic cell and germline contribution of the ES cells and these include the choice of the ES cell line, the passage number of the cells, and how extensively they have been manipulated in culture.

Although the host embryo does not contribute to the genetic background of the final knockout mouse, the combination of the strain of mouse from which the ES cells and the strain of mouse from which the host embryos are derived is critical for the ability of ES cells to generate germline chimeras. This is postulated to be because of to the relative growth properties of the ES cells and the host blastocyst inner cell mass ***(22)***. For example, Balb/c and C57Bl/6 strains appear to have similar growth properties as evidenced from studies of Zinkernagel and co-workers who demonstrated that viable chimeras were formed from the aggregation of reciprocal combinations of morulae-stage Balb/c and C57Bl/6 embryos ***(28)***. By contrast, it has been hypothesised that the differential growth of 129/Sv and C57BL/6 ES cells results in the formation of reduced frequencies of chimeras using C57BL/6-derived ES cells with 129/Sv-derived blastocysts, whereas higher frequencies of germline chimeras are formed using the inverse combination, i.e., 129/Sv-derived ES cells with C57BL/6-derived blastocysts ***(22)***.

4.1. Recipient Embryos for 129-Derived Embryonic Stem Cells

Gene-targeted 129-derived CCE ES cells were tested for their ability to colonize host blastocysts from two albino outbred strains, CD1 and MF1, and one inbred black strain, C57BL/6, during the generation of *c-abl*-targeted mutants ***(14)***. The overall frequency of chimaera formation was approximately equal in all backgrounds, however, the degree of ES cell contribution to the coat and the rate of germline transmission was dependent on the genetic

background of the host blastocyst. On the basis of coat chimerism, the 129-derived ES cells contributed extensively to C57BL/6 blastocysts, at intermediate levels to MF1 blastocysts and poorly to CD1 blastocysts. However, germline transmission was only obtained from chimeras derived from C57BL/6 host blastocysts ***(14)***. This is in contrast with morulae aggregation techniques where aggregation of CD1 morulae with 129-derived ES cells gave a similar rate of germline transmission as the microinjection of these cells into C57BL/6 blastocysts ***(29)***. These data may reflect a difference in the ability of ES cells to infiltrate the inner cell mass of blastocysts from the respective strains.

The 129-derived J1 cells have previously been shown to be compatible with Balb/c and C57BL/6J cells ***(17)***. A comparison of the compatibility of gene-targeted J1 cells with host blastocysts of Balb/c, CF1, and C57BL/6 background has been performed in our laboratory **(Table 1)**. The data were obtained from 13 targeted J1 clones derived in our laboratory (from the electroporation of 9 targeting constructs) that were demonstrated to have germline transmission. The rate of chimera formation was slightly elevated using C57BL/6 host blastocysts: 61% of the mice born after blastocyst transfer were chimeric ($n = 61$), as compared with 48% on a Balb/c background ($n = 88$) and 50% on a CF1 background ($n = 449$). The levels of chimerism were estimated from the proportion of agouti pigmentation in the coats of the chimeric mice. Male mice derived from ES cell transfer into C57BL/6 mice had higher levels of coat chimerism: 86% of C57BL/6-derived mice had 80% or greater agouti pigmentation in their coat, as compared with 33% of Balb/c-derived mice and 23% of CF1-derived mice. This difference in coat chimerism is relevant to the production of gene-targeted animals, as we also observed that the proportion of coat chimerism correlated with germline transmission. Pooled data across the three strains of host blastocysts indicated that 74% of male chimeras that had 80% or greater coat chimerism ($n = 43$) were able to transmit the ES cell genome to their offspring, as compared with 56% of chimeras with 50–79% coat chimaerism ($n = 25$) and 43% of chimeras with 20–49% coat chimaerism ($n = 21$). The proportion of coat chimerism also correlated with the rate at which the ES cell genome was transmitted. Chimeras with a higher level of coat chimerism had higher proportions of ES-cell-derived agouti pups in their litters than chimeras with lower levels of coat chimerism **(Table 2)**. In summary, all three strains of host blastocyst were compatible with J1-targeted clones. The increased levels of high proportion chimeras obtained with C57BL/6 blastocysts and the correlation of the rates of germline transmission with the proportion of coat chimerism promotes the use of C57BL/6-derived blastocysts in order to increase the chances of success in the generation of gene-targeted animals.

Table 1
Formation of Chimeric Mice from the Injection of Targeted J1 Clones into Balb/c, CF1, or C57BL/6 Blastocysts

Host blastocyst strain		Coat chimerism (percentage of agouti pigmentation)			
		<20%	20–49%	50–79%	≥80%
Balb/c	(*n* = 27)	15%	26%	26%	33%
CF1	(*n* = 130)	22%	30%	25%	23%
C57BL/6	(*n* = 14)	—	14%	—	86%

The degree of ES cell contribution was estimated from the agouti pigmentation in the coat.

Table 2
Germline Transmission of ES Cell-Derived Pups from Male Chimaeric Mice

Coat Chimerism		Rate of Germline Transmission (percentage of ES cell-derived pups)		
		<10%	10–60%	≥60%
≥80%	(*n* = 32)	6%	13%	81%
50-79%	(*n* = 14)	29%	21%	50%
20-49%	(*n* = 9)	22	33%	44%

Chimaeras of different levels (>80%, 50–79%, or 20–49% coat chimaerism) were mated to wild-type females and their rate of germline transmission identified as a proportion of ES cell-derived pups (agouti) from the total number of pups born.

4.2. Recipient C57BL/6 Embryos

C57BL/6 blastocysts have also been shown to be effective hosts for the generation of germline transmitting chimaeras from a number of ES cell lines, including the 129-derived ES cells RW4, AB2.1, and AB.1 ***(11,15,16,30)*** and the Balb/c-, MRL-, and DBA-1-derived ES cells ***(25–27)***. However, unlike 129-derived ES cells where the degree of coat color chimerism is reported to be a reliable indicator of predicting the probability of germline transmission (data reported above; ***14,31***), coat color chimerism did not correlate with germline transmission in two reports using MRL- or DBA-derived ES cells ***(6,27)***.

4.3. Recipient Balb/c Embryos

Balb/c blastocysts have been reported to be compatible with both 129-derived D3 ES cells ***(32,33)*** and C57BL/6-derived ES cells; germline chimeras were not obtained using 129-Sv blastocysts ***(22)***. It is well recognized that Balb/c mice show delayed embryonic development and a low yield of embryos per mouse obtained from natural matings compared with other mouse strains. However,

recent studies by Lemckert and colleagues ***(34)*** have described in vitro maturation methods for the optimisation of the quantity and quality of Balb/c blastocysts, to enable a success rate similar to that of 129-derived ES cells. The FvB/N blastocysts have shown mixed results in limited studies: 129-derived H3 cells demonstrated germline competence in the FvB/N background, whereas J1 cells that are derived from the same 129-substrain as the H3 cells, did not demonstrate germline competence ***(18)***.

5. Breeding Strain

Once chimeras are generated, they are usually initially bred to a strain where germline transmission of the ES cell genome can be identified on the basis of the coat color of the pups. Heterozygous pups are generated from this breeding that carry a targeted allele derived from the ES cell line. Heterozygous matings can then be set up to generate wild-type, heterozygous and homozygous gene-targeted mice. However, these pups are of a mixed genetic background, with genetic contributions from the ES cell strain and from the breeding strain. An alternative strategy is to breed germline chimeras (once identified) to the strain from which the ES cell line is derived. This avoids the dilution of the ES cell genome as the host embryo does not contribute to the genetic component of the progeny. If outbred progeny are used for breeding, the relative contributions may vary between littermates and result in variability in the phenotype of the targeted mutants (*see* Chapter 14, this volume). One advantage of analysing mice on a mixed background is that the phenotype observed is more likely to represent the common phenotype across strains, although the penetrance of the phenotype may be variable.

Alternatively, mice can be bred to a specific background to reduce any observed variability. Targeted animals carrying the mutation may be backcrossed for eight generations or more to generate mice on a different genetic background. This increases the representation of genes derived from the breeding strain, reducing the genetic material (other than the targeted allele) derived from the ES cell strain. For example, homozygous gene-targeted mice that were generated with a hypomorphic mutation in CD18 on the 129/Sv or the C57BL/6 strain backgrounds were shown to display mild granulocytosis, an impaired response to chemically induced peritonitis, and delays in transplantation rejection ***(35)***. However, backcrossing of CD18-deficient 129/Sv mice onto the PL/J strain for eight generations resulted in 100% of the homozygotes developing a chronic inflammatory skin disease from about 11 wk after birth, characterized by erythema, hair loss, and the development of scales and crusts, similar to human psoriasis ***(36)***.

However, concerns have been raised by Gerlai ***(37)*** about the background genotype of targeted animals generated by backcrossing. He suggested that where the gene of interest has been targeted in 129-derived ES cells, the chromosome with the targeted locus will carry 129-derived gene alleles.

Following breeding of the heterozygous mice, the 129-derived genes close to the locus of the targeted gene will segregate with the targeted gene, whereas mating-strain-derived genes that are closely linked will segregate with the wild-type allele. Furthermore, even after 12 backcrosses, approximately 16 cM of the genetic material surrounding the targeted locus will be derived from the ES cell strain rather than the required breeding strain. A region of this size has been estimated to contain on average 300 genes (reviewed in ***37***). These ES-cell-derived linked genes may interact with the targeted mutation to modify the phenotype of the mutant animal. For this reason, it may be difficult to determine whether phenotypic differences observed between gene-targeted animals and their wild-type littermates are the result of the gene-targeting event or due to the effect of the other genes closely linked to the targeted locus modulating or being modulated by the background strain genes.

One recently described strategy to aid the generation of congenic strains is that of "speed congenics" ***(38)***. This method uses polymorphic genomic markers to screen the progeny of each backcross generation for the "best male" (i.e., with a genomic content most like the recipient strain) for subsequent mating. It is statistically predicted that, by the use of an effective marker density of 10-cM intervals and screening 20 males/generation, congenic mice closer to the recipient genome are achieved after five generations (approx 100% recipient genome) compared with after 10 generations by conventional methods (approx 99.9% recipient genome). Furthermore, markers located close to the desired conserved allele can selectively identify appropriate recombination events for maximal recipient genome contribution.

Where animals are required on a specific genetic background, it is thus preferable to directly target the gene of interest in ES cells derived from the required background and mate the chimeric animals back to the same strain to generate animals that are isogenic and only differ at the locus of the targeted gene. This is even more critical when closely linked loci are known to influence phenotype. For example, C57BL/6 mice are susceptible to an inflammatory, demyelinating form of experimental autoimmune encephalitis (EAE) induced by immunization with a 35–55 peptide of myelin oligodendrocyte glycoprotein. Exposure to the peptide results in the generation of specific autoreactive TH1 $CD4^+$ T cells that recognise the myelin antigen in the central nervous system, resulting in autoimmune encephalitis (reviewed in **ref. *39***). To examine the role of TNF in EAE, it was necessary to generate gene targeted mice for TNF on a C57BL/6 background, however, it was necessary to directly target a strain in which EAE was inducible, such as C57BL/6, "as the location of the *TNF* gene renders segregation of the mutated allele from the original major histocompatibility complex by backcrossing virtually impossible." Targeting of *TNF* on the C57BL/6 background showed that TNF was required to initiate the

neurological deficit as demonstrated by a significant delay in disease onset and duration relative to wild-type mice ***(39)***. Interestingly, another group that targeted TNF in another strain (129) and backcrossed into C57BL/6 found TNF –/– mice had an increased severity of EAE ***(40)***. These results may indicate the presence of other influencing genes that do not easily segregate upon backcrossing.

5.1. Choice of Mouse Strains in Developmental Biology

The choice of genetic background is important for studies of developmental biology, immunology, and neurobiology. For example, targeting of the epidermal growth factor receptor gene (*EGFR*), which encodes a tyrosine kinase receptor that binds ligands such as EGF and TGFα, indicated that EGFR was critical for normal growth, but the severity of the phenotype was dependent on the genetic background of the targeted mice ***(41,42)***. The phenotype ranged from death at peri-implantation in a CF-1 background ***(42)***, death at midgestation in a 129/Sv background ***(41)*** to survival until birth or postnatal day 20 in mixed or outbred backgrounds. A vast range of phenotypic abnormalities were observed in live-born mutant mice and these were associated with reduced growth and progressive wasting, depending on their genetic background ***(41,42)***. Phenotypic differences between genetic strains have also been observed in penetrance of lethality and severity of phenotype in targeted mutations of the insulin-like growth factor I gene (*Igf1*) ***(43)***, the mouse beta (β)1-adrenergic receptor gene ***(44)***, and the homeobox gene *Hoxb-4* ***(30)***.

5.2. Choice of Mouse Strains in Immunology

The immune system is another example where strain difference has become a critical issue in terms of experimental planning and design. The inbred strains of mice vary in their susceptibility to infection and disease, depending on the type of immune response they mount, such as the polarization of the T helper (Th) response. Th1 responses are characterized by production of IFNγ,IL3, GM-CSF, lymphotoxin, and IL-2, thought to be important for cell-mediated immune responses and are observed in C57BL/6, 129/Sv, C3H/HeJ, or DBA/2 mice, whereas Th2 responses, which are characterized by high levels of IL-4, IL-5, IL-6, IL-10, and strong antibody production are observed in Balb/c mice ***(45–49)***. Th1 type responses are critical in the protection of infections such as *Leishmania major* ***(47)*** and *Coccidiodes immitis* ***(46)***, whereas Th2 responses are required for protection against other infections such as *Borrelia burgdorferi* ***(48,50)***. Due to the polygenic nature of immune responses and the variations between inbred strains, a pure genetic background is preferred for immunological studies.

5.3. Choice of Mouse Strains in Neurobiology

With regard to neurobiology, observed differences between inbred strains have prompted a number of studies to compare behavioral differences between strains

(51) including learning behaviours in different situations *(52,53)*, drug addiction and responses *(54–56)*, aging and neurodegeneration *(57)*. Furthermore, information about the characteristics of a large number of mouse strains can be obtained from the mouse genome informatics site at The Jackson Laboratory, Bar Harbor, ME (http://www.informatics.jax.org/). These studies detailing the baseline behaviors of mice from different strains can be used as a guide to determine the most appropriate strain for targeting specific genes, as reviewed in *(51)*.

In conclusion, the appropriate mouse strain for generating a gene-targeted mutant should be determined by identification of a genetic strain that would provide the best model for the analysis of the knockout mouse, and consideration of the reasons for or against backcrossing the mice, such as genetically linked loci, or availability of ES cells of appropriate strain background. The derivation of ES cell lines from a range of inbred strains known to be susceptible to infections and immune diseases of interest should facilitate the study of genes in the required context. Once an appropriate ES cell strain has been chosen, an isogenic DNA library and a breeding strain would then be employed (where possible) and a compatible host blastocyst strain identified. The generation of targeted mice, initially on a mixed background to identify possible phenotypes, and then on an inbred strain background, should then facilitate analysis of phenotype, reduce phenotypic variability between targeted animals, and allow for improved experimental design.

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9

Isolation, Microinjection, and Transfer of Mouse Blastocysts

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1. Introduction

Over the last half century, the efforts of several pioneering developmental biologists have established the conditions to culture, manipulate, and reintroduce mouse embryos that will successfully develop to term. The isolation of teratocarcinoma cell lines, first, and later of blastocyst-derived embryonic stem (ES) cell lines that can contribute to the germ line of recipient mouse embryos has provided the basis for a system to introduce targeted mutations into the mouse genome (for a historical review *see* **ref.** ***1***. Today, the technology to produce genetically manipulated mice has been made more accessible to investigators by the emergence of a biotechnology industry that provides high-quality instrumentation, materials, and reagents. However, specific technical skills still need to be acquired by the investigator in order to employ successfully this technology for the production of genetically engineered mouse models.

Here, we describe a relatively straightforward method to isolate mouse blastocysts, manipulate them by injecting totipotent ES cells and to generate chimeric mice with an ES cell-derived contribution to the germline. Upon appropriate manipulation of the ES cells in vitro, this system will enable the transmission of new genetic traits to the chimera's offspring.

2. Materials

2.1. Mice

The choice of the appropriate mouse strain as a source of embryos used for injection, and as recipient females to reintroduce the manipulated blastocysts is critical to the success of the outlined procedure. *See* **Notes 1–3**.

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2.2. Media and Supplies

1. Isolation and injection medium: The medium used for isolating and injecting blastocysts is similar to that used for culturing ES cells (for details *see* Manipulating mouse embryonic stem cells, Chapter 4, this volume) but contains the further addition of HEPES buffer: Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4500 mg/L, Gibco-BRL cat. no. 11960-044) supplemented with:
   a. 15% Fetal calf serum (FCS; e.g., Hyclone Laboratories and Gibco-BRL)
   b. 2 m*M* L-Glutamine (e.g., 100X solutioning Gibco-BRL cat. no. 25030-081)
   c. 0.1 m*M* β-mercaptoethanol, tissue culture grade (Sigma cat. no. M-7522)
   d. 0.1 m*M* MEM nonessential amino acids (e.g., 100X solution: Gibco-BRL cat. no. 11140-050)
   e. 500–1000 U/mL of leukemia inhibitory factor (LIF; available from Chemicon International at 10^7 U/mL cat. no. ESG 1107)
   f. 25 m*M* HEPES buffer solution (e.g., Gibco-BRL, cat. no. 15630-080).
2. Embryo-tested mineral oil: Light mineral oil ES cell qualified (Specialty Media, cat. no. ES-005-C).
3. Tribromoethanol (Avertin) anesthesia: Dissolve 25 g of 2,2,2-tribromoethanol (Aldrich, cat. no. T4840-2) in 15.5 mL of ter-amyl alcohol (Aldrich, cat. no. 24048-6) at 37°C. This solution is stable for up to a year if stored at 4°C in dark bottles wrapped in aluminum foil. Keep bottles well sealed and handle quickly to avoid evaporation of ter-amyl alcohol and consequent change in tribromoethanol concentration. Dilute 0.5 mL of the stock solution with 40 mL of 0.9% NaCl solution (this solution is stable for up to 4–5 mo when stored at 4°C in dark bottles wrapped in aluminum foil). Inject intraperitoneal 0.25 mL/10 g body weight.
4. 1 mm Capillaries: For both needle and holding pipets use 30 or 60 μL microcaps (depending on the length required), (Drummond, cat. no. 7695D43); for holding pipets different types of glass can also be used such as Ziptrol tubes (Drummond, cat. no. 7690H12).
5. Pasteur pipets: these are used to make mouth-controlled transfer pipets. Pull Pasteur pipet on a Bunsen burner to make a capillary. Snap the tip to obtain a blunt edge. Connect the pipet to plastic tubing with a filter to prevent contamination during mouth pipetting.
6. Miscellanea: G30 and G25 needles with 1 and 5 mL syringes used to administer the anaesthetic to the mice and to flush the uterine horns for blastocyst recovery, respectively; also needed are scalpels and rubber sheets to make injection needles and 6 cm tissue culture dishes.

2.3. Dissecting Tools

1. Tools for mouse surgery: Scissors for microdissections (e.g., Roboz cat. nos. RS-5910 and RS-5940; straight and curved forceps (e.g., Roboz, cat. nos. RS-5130 and RS-5137); tweezers for microdissections (e.g., Roboz cat. no. RS-5055);

microclamps (e.g., Roboz, cat. no. RS-7423); 9 mm wound clip applier (e.g., Roboz, cat. no. RS-9260); 9 mm wound clips (e.g., Roboz, cat. no. RS-9262); clip-removing forceps (e.g., Roboz, cat. no. RS-9268).

2. Tools for uterus dissection: Operating scissors (e.g., Roboz, cat. no. RS-6814); Microdissecting scissors (e.g., Roboz, cat. no. RS-5960); Microdissecting spring scissors (e.g., Roboz, cat. no. RS-5650); straight forceps (e.g., Roboz, cat. no. RS-5130); tweezers for microdissections (e.g., Roboz, cat. no. RS-5055).
3. Warming tray: Needed to keep the mice warm after surgery for blastocysts transfer.

2.4. Microscope for Injection

Microinjection is most efficiently performed using an inverted microscope with an x/y mechanical stage and movable objectives **(Fig 1)**. Good-quality microscopes suitable for microinjection are available from different vendors (e.g., Zeiss, Olympus, Leica, Nikon). Magnifications of phase contrast objectives should be 5-, 10-, and 20-fold. An additional 10-fold magnification is provided by the eyepiece. The low-power magnification (50×) aides in manipulation of the embryos, whereas the injection can be performed at 100X or 200X. With phase-contrast objectives, it is possible to discriminate between dead and live ES cells. However, Nomarski optics allow for good-quality images of the cells. Some investigators recommend cooling the embryo during injection. We found that no significant difference is observed in the injection and viability of the embryos when performing the procedure at room temperature.

2.5. Micromanipulators

Two micromanipulators (e.g., Narishige, Leitz) are required to move the holding and injection pipets in all three dimensions. Mechanical joysticks are preferred over electric devices, as they offer a greater flexibility in manipulation of the injection pipet **(Fig. 1B, D)**. The micromanipulators should not be attached to the microscope stage. Several vendors provide attachments to the illumination pillar of the microscope **(Fig. 1B)**. The microinjection and the holding pipets are attached to a micrometer syringe through flexible tubing and an injection or holding pipet holder **(Fig. 1B, C)**. The tubing and the pipets should be filled with light paraffin oil to avoid air bubbles. The very end of both pipets should be filled with injection media.

2.6. Air Table

For stability, the microinjection setup should be mounted on an air table that buffers environmental vibrations. Air tables can be purchased from different vendors; however, they can also be supplied by the microscope vendor. The latter will ensure perfect fitting of the microscope and microinjection apparatus.

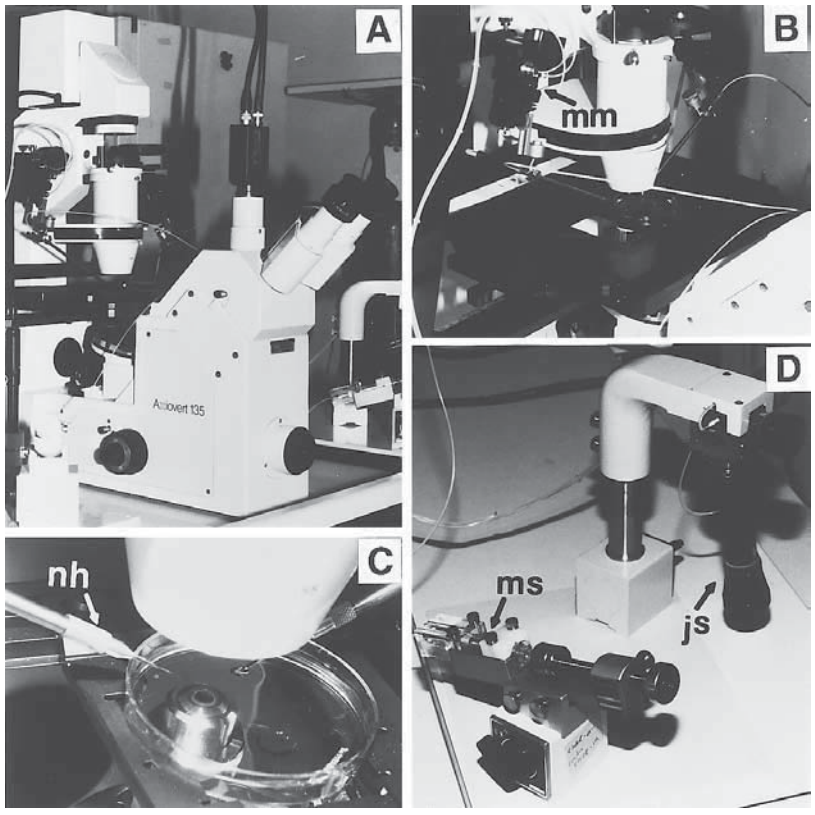

Fig. 1. Set up for blastocyst injection. **(A)** Inverted microscope. **(B)** Micromanipulators attached to the light pillar, and *xy* movable stage. **(C)** injection chamber and needle holders with needles. **(D)** Hanging joystick to control micromanipulators; micrometer syringe to control suction and injection pressure of holding and injection pipets. mm: Micromanipulator; nh: needle holder; ms: microsyringe; js: hanging joystick.

2.7. Surgical and Dissecting Microscope

A dissecting microscope that allows 25× and 50× magnifications (**Fig. 2A**) is used to handle blastocysts, for instance to collect them after flushing the uterine horns (*see* **Subheading 3.2.** and **Fig. 5**) or for grouping injected blastocysts to reduce the amount of media used during transfer to recipient females (*see* **Subheading 3.6.** and **Fig. 7**).

A surgical microscope (**Fig. 2B**) is used for transfer of manipulated blastocysts into recipient females. It should allow about 7× and 15× magnifications in order to be able to see the hole in the uterine horn created by the needle and to introduce the tip of the mouth-controlled pipet for blastocyst transfer (for

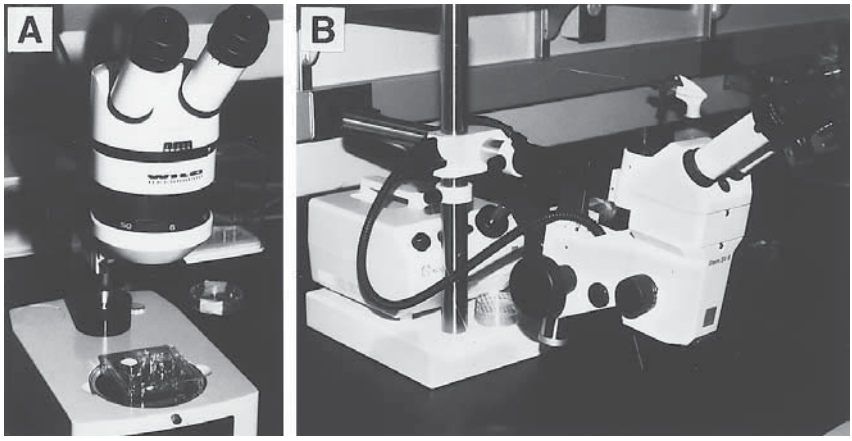

Fig. 2. Typical dissecting (A) and surgical (B) microscopes.

details, *see* **Subheading 3.6.** and **Fig. 7E, F**). Also, it should allow sufficient clearance to perform the operation.

2.8. Pipet Puller and Microforge

Different pullers are commercially available. We prefer a vertical pipet puller (e.g., Kopf instruments, model 720) because needles are pulled by gravity and once an optimal temperature setting is established, it is easy to reproduce needles with specific characteristics (**Fig. 3A, B**).

Most commonly available microforges are supplied with two heating elements of different size. We use a de Fonbrune-type microforge. Settings vary according to manufacturers protocols and individual preferences (**Fig. 3C**).

3. Methods

3.1. Preparation of Needles (see Note 4)

The microinjection procedure requires a holding and an injection pipet that can be prepared from 10–15 cm long 1 mm glass capillary. Both the holding and injection pipets are made from similarly pulled capillaries. Holding pipets are obtained from the thick part of the capillary (diameter of about 100 μm; the blastocysts have a diameter of about 180–200 μm), whereas the needle of the injection pipet is obtained from the tip of the capillary measuring about 20–25 μm in diameter (ES cells have a diameter ranging between 15–20 μm). The capillaries can be pulled consistently after appropriate setting of the pipet puller. This makes it fairly easy to identify the appropriate region to make a holding or an injection pipet.

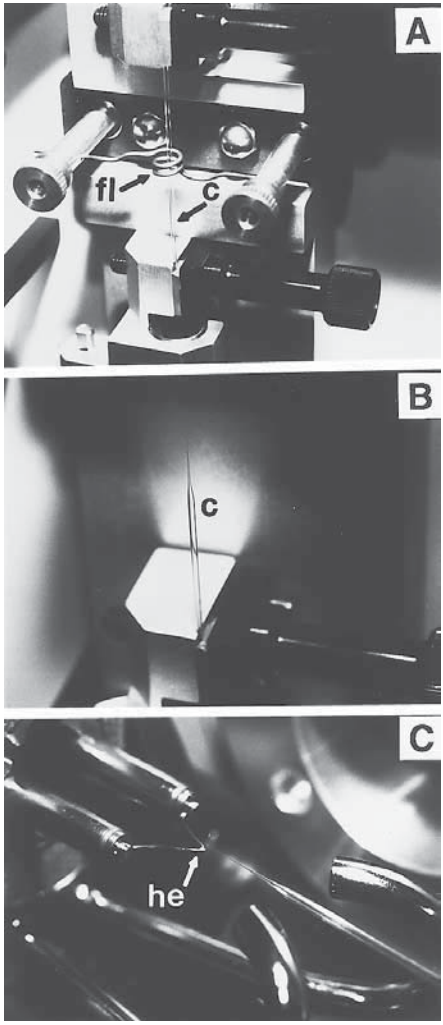

Fig. 3. Preparation of needles. **(A)** A capillary (c) is placed through the pipet puller filament (fl). **(B)** By regulating the temperature of the filament, pulled capillaries of different length will be generated. **(C)** View of a holding pipet during the bending by the microforge (also, *see* **Fig. 4E**). he: heating element.

3.1.1. Holding Pipets

Holding pipets are required to keep the blastocysts immobilized in place during ES cell injection. The blastocyst is held by gentle suction through the opening (20–40 μm) of the holding pipet. Holding pipets are made from pulled capillaries by using a microforge: (**Fig. 3C**).

1. The heating filament of the microforge carries a glass bead at the tip. The pipet should be positioned horizontally above the glass bead at the appropriate level of the capillary (about 80–100 μm external diameter).
2. Turning on the heat element will cause the glass bead to expand and move along side of the pipet (**Fig. 4A**). The heating has to be controlled carefully to ensure that the capillary will not bend or melt when contacting the heated glass bead. Exact parameters must be adjusted depending on the microforge used.
3. As soon as the pipet fuses slightly with the glass bead and extends in length, the heat should be switched off. Contraction of the glass causes the pipet to snap vertically (**Fig. 4B**).
4. The opening of the holding pipet is then placed in front of the filament with the glass bead at an appropriate distance to avoid contact during the polishing of the tip.
5. The pipet is moved carefully towards the glowing glass bead and the edges will start to melt inward creating a smoother, narrower tip (**Fig. 4C**).
6. The holding pipet is then bent (between 30 and 45° depending on the setting of the pipet holders) to allow an almost parallel position of the tip relative to the bottom of the injection chamber. The bend is introduced near the tip of the holding pipet by moving the capillary toward the filament.
7. Carefully controlling the temperature of the filament and its distance from the pipet, the tip is allowed to bend until the appropriate angle is achieved (**Fig. 4D, E**). Holding pipets can be used for several injections until breakage or blockage occurs.

3.1.2. Injection Pipets

The injection pipet has the shape of a very fine hypodermic needle (**Fig. 3A, B**). The inner diameter of the injection pipet must be slightly larger than the ES cells to be injected so that they can be collected without clogging the pipet. A reproducible method to make an injection pipet involves crafting it with a microforge. However, this requires experience and skill. A faster method requires only pulled capillaries, a scalpel, and a transparent rubber sheet:

1. Place the capillary on the rubber sheet under the dissecting scope
2. Cut the capillary with the scalpel at the level where the diameter is sufficient to allow the passage of ES cells (**Fig. 4F, G**). Although the choice of glass used for making holding pipets is not critical, it is a crucial factor in obtaining injection needles that form the right type of tip (shape of a hypodermic needle) when cut (Drummond microcaps).
3. Introduce a bend as previously done with the holding pipet but so that the opening faces the side of the injection chamber (**Fig. 4H, J**). This will allow the collection of ES cells without damaging the tip.
4. Store the injection and holding pipets such that the tips do not contact any surface.

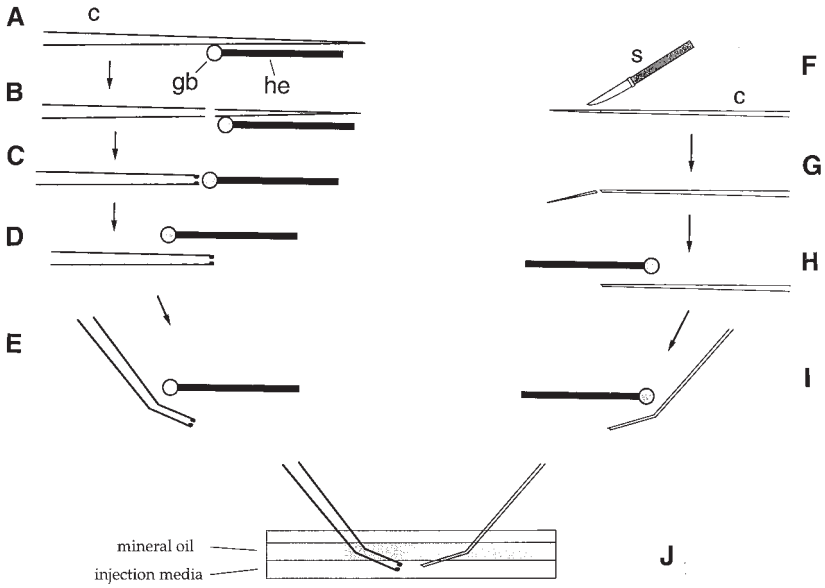

Fig. 4. Schematic illustrating the procedure to make holding **(A–E)** and injecting **(F–I)** pipets (see text for details). Representation of the injecting chamber **(J)**. he: heating element; gb: glass bead; c: pulled capillary; s: scalpel.

3.2. Isolation of Mouse Blastocysts

1. Set up female donor mice (C57Bl/6 strain) for natural matings.
2. The following morning they are checked for vaginal plugs. At this point, the plugged female is considered 0.5 d postcoitus (dpc).
3. Sacrifice donor females at 3.5 dpc by CO_2 asphyxiation.
4. Place the animal on its back on a gauze pad and expose the reproductive tract using a pair of scissors (**Fig. 5A**).
5. Isolate the uterus by holding the cervix with tweezers and cutting the connective vaginal side (**Fig. 5B**).
6. After isolation, place the uterus on a gauze pad (all uteri isolated should be laid on the pad with the same orientation) (**Fig. 5C**).
7. Drop some media on the uterus to keep it moist and carefully remove all the fat tissue (**Fig. 5D**). Trimming fat tissue is very helpful, as residual fat cells will obstruct vision while collecting the blastocysts.
8. Clip off the uterine horns very close to the cervix (leave the cervix next to the uteri for orientation) and also cut off a small piece at the level of the ovaries (**Fig. 5E**).
9. Insert a G25 needle (the tip of which has been rounded with sandpaper) of a syringe full of ES media at the ovary side of the uterine horn and flush the embryos into a 6-cm Petri dish with about 0.5 mL of media (**Fig. 5F, G**). The flushed embryos should then be checked under a binocular lens for a visible blastocoel cavity and intact zona pellucida and collected with a mouth pipet for injection.

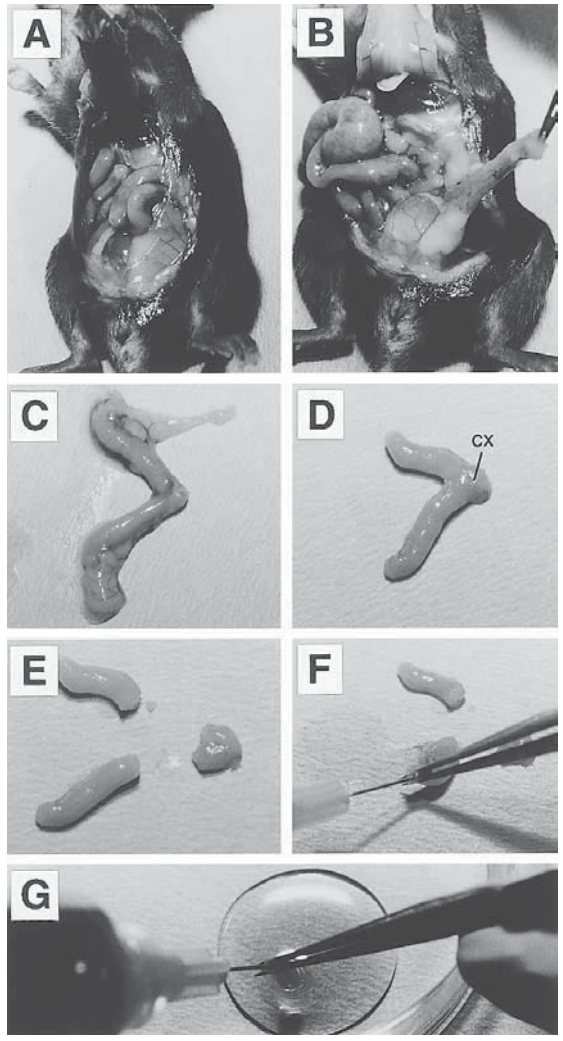

Fig. 5. Method employed to isolate and dissect the uterus from a pregnant female to recover mouse blastocysts (see text for detailed description of the procedure). cx: cervix.

Some investigators recommend superovulation protocols to increase the embryo recovery. However, embryos produced by superovulation have the disadvantage of being less synchronous with regard to their developmental stage.

3.3. Preparation of ES Cells for Injection

ES cells from a 60–70% confluent 6 cm plate are trypsinized as described in Chapter 4, this volume, and resuspended in 3–4 mL of ES media. The ES cell suspension should be fairly concentrated so that during the injection procedure

a few drops can provide a sufficient number of cells for a specific injection field. Cells can be kept at room temperature until they are ready to be injected.

3.4. Microinjection of Blastocysts

During the initial phase of the injection procedure, the quality of the needle will be apparent. Lack of sharpness and blockage of the needle by accumulation of cell debris can severely impact the success of injection. Cells should not escape from the opening produced by the tip of the injection pipet.

1. Microinjection of blastocysts is performed on a 6-cm Petri dish lid. Put about 3 mL of blastocyst injection media on the plate and cover the surface with embryo tested mineral oil (**Fig. 4J**).
2. Add a few drops of ES cells to the media followed by the addition of the blastocysts by a mouth pipet, being careful to keep them grouped on the plate.
3. Finally, set and position the holding and injection pipets on the plate and fill their terminal endings with media.
4. Load individual ES cells with good refraction (for viability) into the injection pipet. Any cells that look dark are dying and should not be used. To keep the volume of injected media as small as possible, cells in the injection pipet should be loaded at the very tip such to form trains of cells (**Fig. 6A**). Fifteen to 25 healthy-looking cells are picked up at a time.
5. Select a blastocyst for injection.
6. Using the holding pipet, maneuver the blastocyst into the correct position and then gently immobilize by suction.
7. Select the correct position to inject the blastocyst. The inner cell mass should be close to the opening of the holding pipet, but a slightly tilted position is also desirable (**Fig. 6**). The best spot for penetration into the blastocoel is between trophectoderm cells.
8. With one smooth push, bring the tip of the injection pipet into the embryo without collapsing or puncturing of the opposite wall (**Fig. 6B, C**). Once in the blastocyst, the injection needle should not touch the inner cell mass.
9. When the tip of the injection pipet is clearly visible inside the blastocoel, expel the ES cells (15–25 cells per blastocyst) by positive pressure (**Fig. 6D**).
10. Following injection, withdraw the needle from the embryo.
11. Slightly lift the holding pipet and move the injected blastocyst to a different field, so that injected and noninjected embryos are kept separated.
12. Repeat the process with a new blastocyst.
13. Collect injected blastocysts for their transfer to recipient females.

3.5. Preparation of Pseudopregnant Recipients

The injected embryos must be implanted into recipient females. Mating of recipients with male mice is required for the hormonal changes necessary to establish pregnancy and for proper development of manipulated embryos.

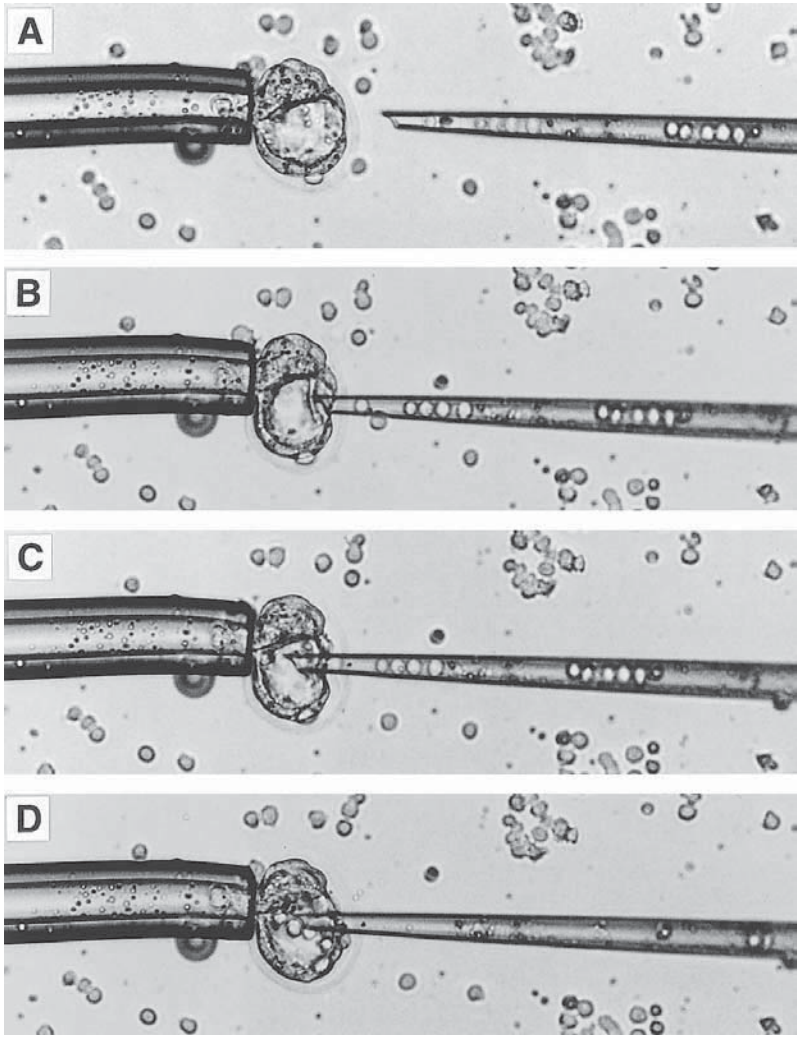

Fig. 6. Microinjection of blastocyst. **(A)** Cells are loaded into the injection pipet (right) and a blastocyst is immobilized near the inner cell mass by the holding pipet (left); note the trains of ES cells in the injection pipet. **(B)** The needle is moved close to the blastocyst and is now punturing its wall between trophectoderm cells. **(C)** The tip is now positioned at the center of the blastocoel. **(D)** Cells are injected.

Because recipients should not carry embryos of their own, females must be mated with vasectomized or genetically sterile males. To ensure availability of greater than 5 pseudopregnant females for a specific day of injection, we set up matings with about 30 females that are 6–8 wk old. Pseudopregnant females in

excess can be used again after a 2-wk resting period. Females at 2.5 dpc are used for transfer of the injected 3.5 dpc blastocysts. This 1-d difference is adopted to compensate for the delay in embryonic development caused by in vitro manipulations. Empirically, this results in a higher percentage of implantations.

3.6. Transfer of Blastocysts to Pseudopregnant Females

We usually carry out the transfer of manipulated blastocysts soon after the injection.

1. Anesthetize recipient females that are at 2.5 dpc by intraperitoneal injection of about 0.5 mL of Avertin.
2. Place the animal on its belly and shave its back.
3. Under a surgical microscope, using a pair of scissors, make an incision through the skin and the peritoneum on the right side of the back just along the bottom of the rib cage, avoiding cutting any blood vessels (**Fig. 7A, B**). The ovary should be visible right underneath the incision (**Fig. 7C**).
4. Grasp the ovary, oviduct, and uterus with tweezers by the fat pad that is attached to them, avoiding any pinching of the reproductive organs.
5. Carefully pull the uterus through the incision in the peritoneum and place it on a drape.
6. Attach a small clamp to the fat pad to hold the ovarian end of the uterus in place and prevent the uterus from sliding back into the peritoneum (**Fig. 7D**).
7. Pick up the blastocysts with a mouth-controlled transfer pipet. Up to 15 embryos can be transferred into one uterine horn.
8. Grasp the uterus very close to the uterus–oviduct junction with a pair of microdissection tweezers and make a small hole using a 30-gauge hypodermic needle (**Fig. 7E**).
9. Insert the tip of the transfer pipet and gently mouth pipet the embryos while monitoring the appearance of few air bubbles inside the uterus (**Fig. 7F**). Any resistance indicates that the pipet is touching the other wall of the uterine horn and the pipet should be pulled back gently.
10. When air bubbles are seen, all the embryos are assumed to be transferred into the lumen of the uterus.
11 Remove the clamp and gently push the uterus back into the peritoneum (**Fig. 7G**).
11. Staple the skin with two 9-mm surgical wound clips (**Fig. 7H**).
12. Finally, return the recipients to their cages onto a warm tray to ensure that they are kept warm until they regain consciousness. Pups from manipulated embryos should be born 16–18 d after the day of transfer.

4. Notes

1. Two criteria must be followed in choosing the donor strain of mouse blastocysts. The recipient embryo should be permissive to the ES cell lines used for injection; i.e., ES cells must be able to colonize and contribute to all cell lineages of the

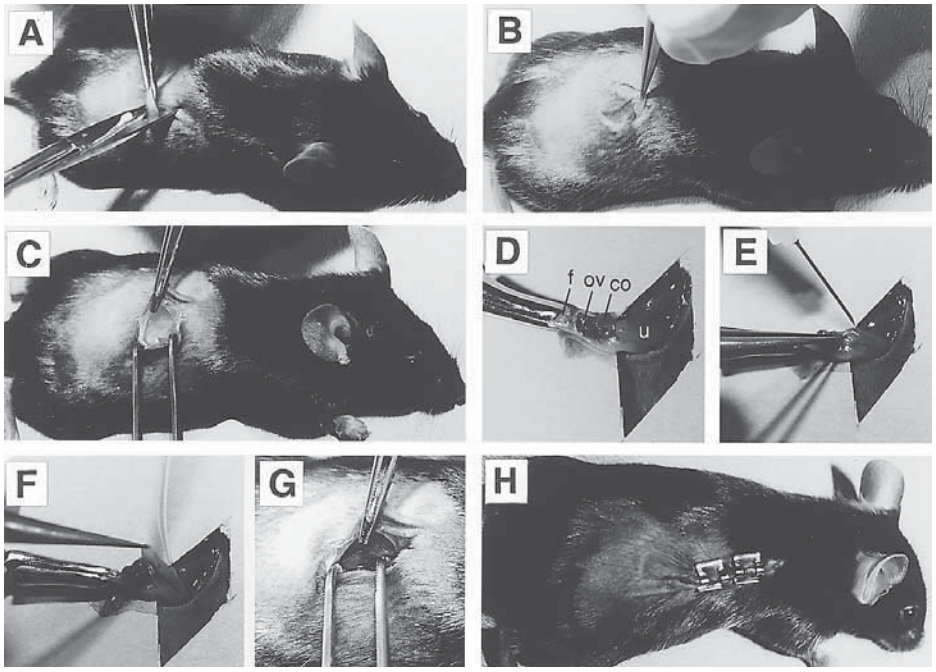

Fig. 7. Surgical procedure for transferring blastocysts into pseudopregnant females. See text for detailed description. f: fat pad; ov: ovary; co: coiled oviduct; u: uterus.

recipient embryo. Second, the recipient embryo should have markers that indicate the contribution of the ES cells in the resulting chimera. The majority of ES cell lines used in genetic manipulation experiments are derived from 129/Sv mouse strains. These strains have an agouti coat color. The most successful, and hence the most utilized, embryo donor mice are of the Balb/c and the C57Bl/6 strains. These two mouse strains are permissive to the 129/Sv-derived ES cells and have coat color-determining genes (white and black, respectively) that are recessive to the 129/Sv agouti gene. Furthermore, these strains breed well and produce a relatively large number of embryos.

2. Different strains of mice can be used as recipients of manipulated blastocysts as long as they tolerate foreign blastocysts. Usually, hybrid strains are favored, as they are physically stronger and mate at a fairly high frequency. We routinely introduce manipulated C57Bl/6 blastocysts into C57Bl/6xDBA/2 mice of the F1 generation.
3. Recipient females must be able to support the implanted embryos. The hormonal changes necessary for pregnancy and initial development of manipulated embryos are induced by mating with a vasectomized or genetically sterile mouse. Vasectomized mice from hybrid strains can now conveniently be purchased from commercial suppliers (Charles Rivers, The Jackson Laboratory). Alternatively, genetically sterile males can also be used. In particular, mice that are doubly

heterozygous for two recessive lethal haplotypes of the t complex ***(1)*** or males homozygous for a null c-*ros* targeted mutation (***2***; S.W.R. and L.T., unpublished result) can be used to generate pseudopregnant recipient females.

4. Some investigators recommend washing the capillaries in HNO_3 before use. However, we found that to be unnecessary.

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10

Aggregation Chimeras

Combining ES Cells, Diploid and Tetraploid Embryos

Mika Tanaka, Anna-Katerina Hadjantonakis, and Andras Nagy

1. Introduction

In recent years, chimeras have been providing a powerful way to study mouse development ***(1)*** in combination with invention and improvement of other techniques and materials, including embryonic stem (ES) cells ***(2)*** and tetraploid embryos ***(3,4)***. ES cells are pluripotent cell lines derived from late blastocyst-stage embryos, which are capable of differentiating into all derivatives of the primitive ectoderm (*see* **Fig. 1**) when aggregated with or injected into diploid embryos ***(5)***. In contrast, tetraploid embryos, which can be made by electrofusing two cell-stage diploid embryos ***(3,6,7)***, have been found to contribute preferentially to most of the extraembryonic cell lineages, i.e., the trophoblast (trophectoderm derivatives) and primitive endoderm derivatives (*see* **Fig. 1**) when aggregated with diploid embryos ***(3–8)***. Interestingly, ES cells show a deficiency in extraembryonic lineages, therefore these cells and tetraploid embryo derived cells have a complementary distribution in chimeras made between them. In such chimeras, the embryo proper, the amnion, the yolk sac mesoderm, the allantois and the chorionic mesoderm-derived part of the placenta are completely ES cell-derived, whereas the yolk sac endoderm and the trophoblast cell lineages are tetraploid embryo derived ***(3,7,9)***. It is certain that the ES cell⇔tetraploid embryo aggregates have an attractive feature in that they are a reliable and simple way of producing completely ES cell-derived embryos from developmentally competent cell lines ***(2–10)***. This feature is promoting their application in an increasing number of studies. In addition, chimeras between diploid cells (both embryo⇔embryo, and ES cell⇔diploid embryo chimeras) are going through a renaissance in addressing specific biological questions.

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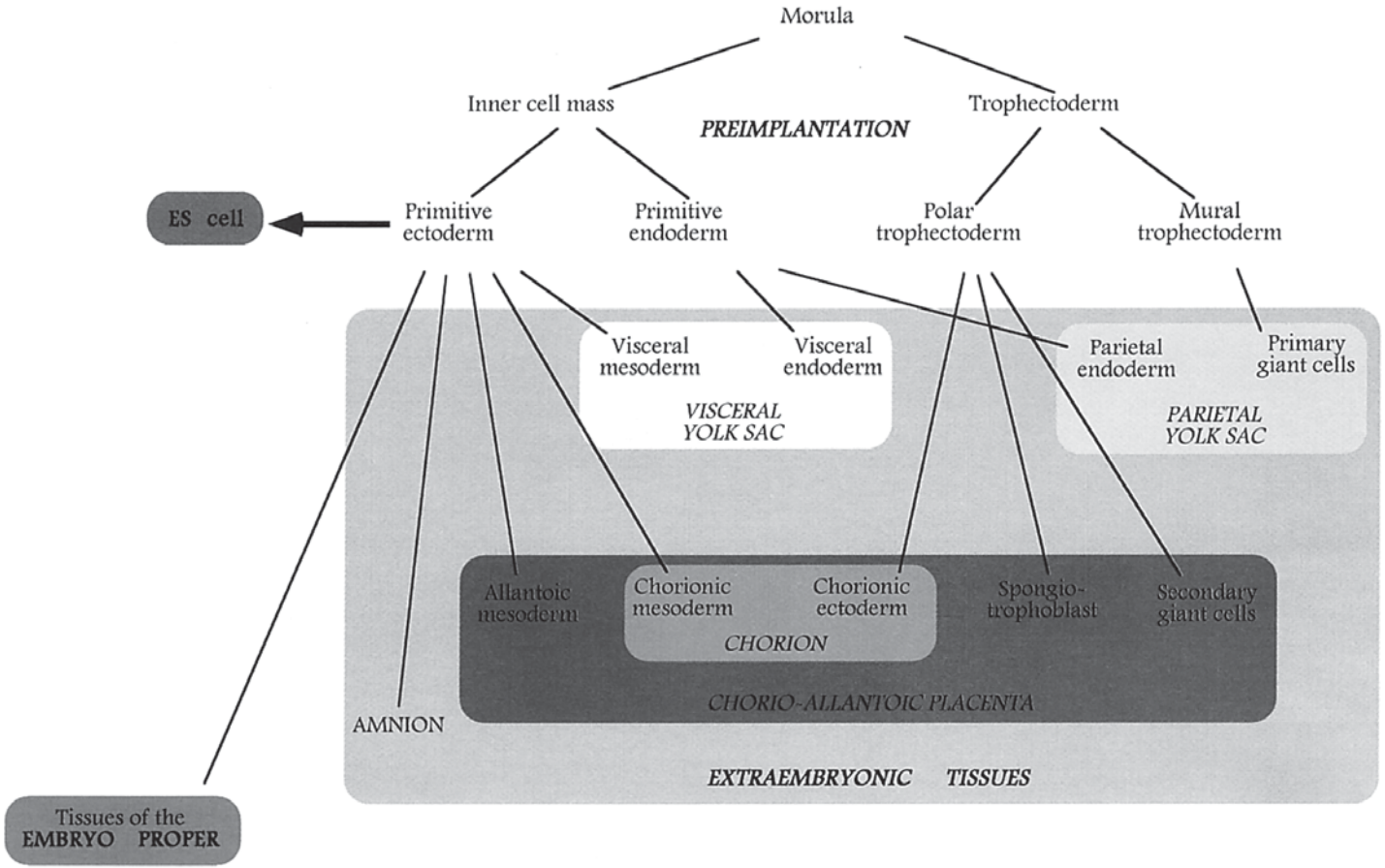

Fig. 1. Schematic representation of the various embryo proper and extraembryonic lineages and their relation to each other.

The embryo⇔embryo chimeras are made by the aggregation of two blastomere-stage embryos. ES cell chimeras have traditionally been produced by injecting the cells into blastocyst-stage embryos using micromanipulators. However, recently we have learned that a more simple way of producing such chimeras can be performed by aggregating ES cells with an eight-cell stage embryo ***(11)***. This alternative method is simple and also gives a high efficiency in chimera production under optimal culture conditions. In this chapter, we discuss general use of aggregation chimeras.

1.1. Uses of Aggregation Chimeras

There are four combinations by which postimplantation chimeric embryos can be produced in vivo (*see* **Fig. 2**), these being "diploid embryo and diploid embryo" (*see* **Fig. 2A**), "diploid embryo and ES cells" (*see* **Fig. 2B**) and "diploid embryo and tetraploid embryos " (*see* **Fig. 2C**) and "tetraploid embryos and ES cells" (*see* **Fig. 2D**). A suitable aggregation combination should be chosen depending on the aim of the experiment. The general use of such aggregations will be described as follows.

1.1.1. Germline Transmission of ES Cells by Aggregation

To obtain germline transmission of an ES cell genome, ES cells should be aggregated with diploid embryos in order to create viable and fertile chimeras ***(11)***. Due to frequent X chromosomal instability in female ES cell lines almost exclusively male ES cells are used for this purpose. In most cases, male chimeras that have a high contribution of ES cell derivatives, scored by using coat color markers, are ideal for obtaining germline transmission of an ES cell genome ***(12)***. It should be noted that there are cases in which male chimeras with strong ES cell contribution may not be ideal. With some ES cell lines, high ES cell contribution negatively correlates with viability and sterility. The success of germline transmission also depends on the quality of ES cells. In almost all situations it is essential to perform any genome alteration on ES cells that have a high developmental potential and germline compatibility. However, even in this case, a minor ratio of subclones will loose their original capabilities resulting in sterile or nontransmitter chimeras.

Certain types of mutations, such as dominant mutations or mutations in haploinsufficient or X-chromosome-linked genes, may directly affect the developmental potential of ES cells and are only able to contribute to viable chimeras at a low level. However, even in this situation it is not impossible to obtain germline transmission through chimeras exhibiting weak contribution of the ES cells ***(13)***.

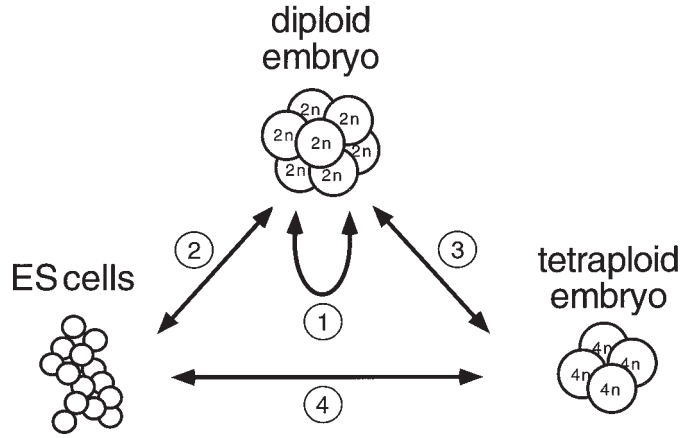

Fig. 2. The three components that can be combined to produce a chimera are diploid embryos, tetraploid embryos, are illustrated. 1. Diploid embryo⇔diploid embryo, 2. diploid embryo⇔ES cells, 3. diploid embryo⇔tetraploid embryos, and 4. tetraploid embryos⇔ES cells.

Other possible difficulties could arise if the mutation introduced into the ES cell line itself is the cause of the sterility resulted from for example a defect in spermiogenesis. A possible way to circumvent this problem can be provided by the occasional germline transmission through female chimeras ***(14)***. However, it is not clear what the status of the Y chromosome of these ES cells was in these cases. There is a possibility that the Y chromosome has to be lost or become nonfunctional in order to allow development through female gametogenesis.

Haploinsufficiency could also create an apparently surprising phenomenon, when in the case of germline transmission of the ES cell genome no heterozygous F1 animals are observed. In this case the heterozygotes might start developing but then die *in utero*. As a consequence, no mutant mouse line can be established. The consequences of the haploinsufficiency can be analyzed through the chimera-fathered embryos. The only possible way to access the homozygous null phenotype in a severe haploinsufficiency case is the production of homozygous null ES cells followed by ES cell⇔tetraploid embryos ***(13)***.

Genomic imprinting could create a similar situation where imprinting is the phenomenon in which the activity of a gene shows a difference depending on the parental origin. Maternally imprinted genes require transmission through the paternal germline for activation. Therefore, one may never find a viable progeny carrying the knockout allele of such a gene from a male chimera. In this case, again, female chimeras would be a choice to obtain germline transmission. The opposite, the knockout allele of a paternally imprinted gene, does not have germline transmission problem through male chimeras ***(15–17)***.

1.1.2. Determination of Cell Autonomy of Particular Mutations

Chimeric analysis has proven to be a powerful method for studying cell autonomous requirement of genes of interest ***(18–20)***. Mutant cells can be ES cells as well as diploid and/or tetraploid embryos depending on the question that one would like to address. The suitable aggregation combination for chimeric analysis to address cell autonomy will be discussed in further detail in **Subheading 1.2.**

1.1.3. Separation of Embryonic and Extraembryonic Phenotypes

Chimeras also provide an excellent way to separate the embryonic phenotype from any extraembryonic phenotype of a gene of interest ***(21)***. The complementary restricted developmental potential of ES cells and tetraploid embryos as mentioned in the introduction makes this feasible. For instance, if mutant diploid embryos die from placental failure or extraembryonic defect, one can rescue this defect by aggregating the mutant diploid embryo with wild-type tetraploid embryos. The latter provide functionally normal placentae to study the embryo proper phenotype of the mutation ***(14,15,22,23)***. It is also possible to rescue the embryonic phenotype to study the extraembryonic defect of the mutation by aggregating the mutant diploid embryo with wild-type ES cells that provide primitive ectoderm derivatives, but will never contribute to primitive endoderm or trophoblast cell lineages. The aggregation combination for this use will be discussed further in **Subheading 1.2.**

1.1.4. Accessing Phenotypes without Germline Transmission

ES cells carrying dominant genome alterations that may cause a phenotype in primitive ectoderm cell lineages can be aggregated with wild-type tetraploid embryos to study the phenotype directly, without going through the potentially problematic and time consuming germline transmission ***(13)***. In the case of recessive mutations, the production of ES cell lines homozygous for the mutation is required. The means to get homozygous ES cell lines for this use is reviewed in ref. ***(24)***.

1.2. Aggregation Combinations

Different aggregation combinations are required depending on the aim of experiments as discussed in the **Subheading 1.1.** In this section, all possible aggregation combinations using ES cells and diploid and tetraploid embryos (*see* **Fig. 3**) are listed and the expected contribution of mutant cells in resulting chimeras from each aggregation combination is discussed with examples of their practical use.

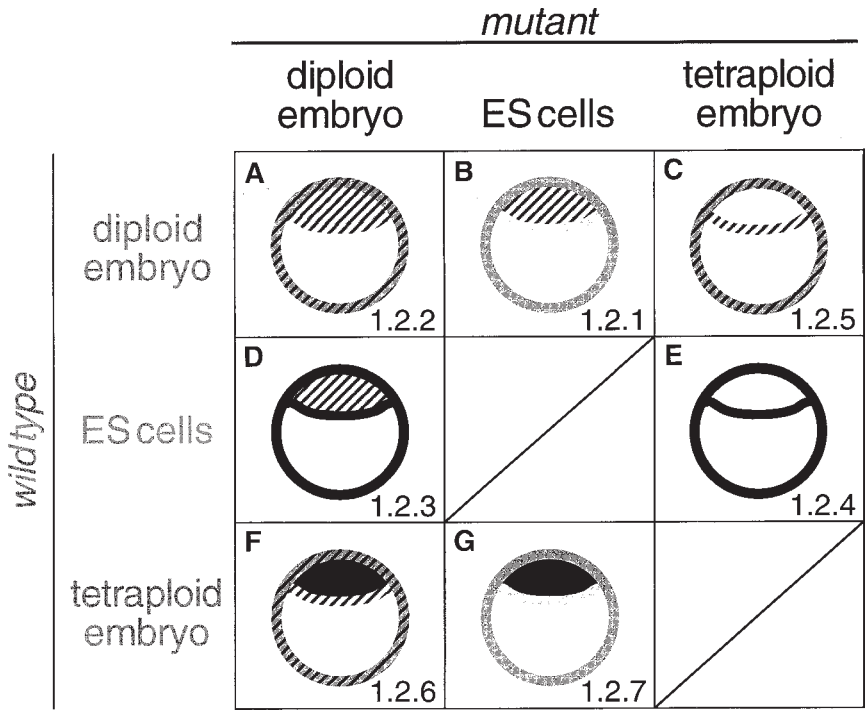

Fig. 3. Tissue contributions and lineage restrictions associated with the three components of chimeras.

1.2.1. Wild-Type Diploid Embryo⇔Mutant ES Cells

The contribution of mutant ES cells is restricted to primitive ectoderm derivatives in resulted chimeras (*see* **Fig. 3B**). This aggregation combination is suitable for all uses described in **Subheading 1.1.** To study cell autonomy during the development of primitive ectoderm derivatives, mutant ES cell lines carrying haploinsufficient or X-chromosome-linked or dominant mutations, or homozygous for recessive mutations are required. Chimeric embryos from this aggregation may also give phenotypes resulting from the mutation depending on the degree of ES cell contribution in the primitive ectoderm cell lineages.

1.2.2. Wild-Type Diploid Embryo⇔Mutant Diploid Embryo

The contribution of mutant diploid cells is expected to be in all cell lineages in chimeras made by this aggregation combination (*see* **Fig. 3A**) unless cells from the mutant embryo have developmental restrictions. This makes it possible to assess the question of cell autonomy of mutations of interest possibly in all cell lineages.

1.2.3. Wild-Type ES Cells⇔Mutant Diploid Embryo

The contribution of cells derived from mutant diploid embryo is expected to be in all cell lineages (*see* **Fig. 3D**) unless the mutation affects their developmental potential. The point of this aggregation combination is to make solely mutant embryo derived extraembryonic tissue, i.e. the trophoblast and primitive endoderm lineages, in combination with chimeric primitive ectoderm derivatives.

If mutant embryos of a gene of interest show phenotypes in both the embryonic and extraembryonic lineages, this aggregation is the choice to address whether the placental defects are cell autonomous or secondary to the embryonic defects, as the aggregation with wild-type ES cells can rescue the embryonic phenotype depending on the degree of chimerism without having any ES cell contribution in the extraembryonic tissues.

1.2.4. Wild-Type ES Cells⇔Mutant Tetraploid Embryos

The contribution of mutant cells is solely restricted to the trophoblast and primitive endoderm lineages in resulting chimeras (*see* **Fig. 3E**) so that the extraembryonic phenotypes can be separated from embryonic ones if the mutation affects both lineages. This is a clearer way to assess this question compared to other combinations, as there is no concern about the degree of chimerism because of the complementary distribution of ES cells and tetraploid embryo derivatives.

1.2.5. Wild-Type Diploid Embryo⇔Mutant Tetraploid Embryos

The contribution of mutant tetraploid cells is limited to the trophoblast and primitive endoderm derivatives (*see* **Fig. 3C**). The chimeras made by this aggregation are expected to have chimeric extraembryonic tissues with no contribution of mutant cells in the primitive ectoderm derivatives. This combination could be used in order to address cell autonomy of the mutation specifically in the extraembryonic lineages, when there are multiple cell autonomous defects in both extraembryonic and embryonic lineages.

1.2.6. Wild-Type Tetraploid Embryos⇔Mutant Diploid Embryo

There is no restriction for the contribution of mutant diploid cells, whereas the contribution of wild-type tetraploid cells is limited to the extraembryonic tissues (*see* **Fig. 3F**). This will result in chimeras that have chimeric extraembryonic tissues and exclusively mutant-embryo-derived primitive ectoderm derivatives. This aggregation will be the choice to study embryonic phenotype by rescuing the extraembryonic defects. The same question can be addressed by mutant ES cells⇔wild-type tetraploid embryos aggregation (*see* **Fig. 3G**), if such cells are available.

1.2.7. Mutant ES Cells–Wild-Type Tetraploid Embryos

If mutant ES cells are available, the aggregation with wild-type tetraploid embryos provides a powerful and the quick way to analyze embryonic phenotype without germline transmission. Normally, wild-type ES cells are capable of developing to form the primitive ectoderm derivatives (*see* **Fig. 3G**) with help of wild-type tetraploid embryos, which provide functional placenta and yolk sac. In the case of mutant ES cells, the phenotype is manifested in the completely ES cell-derived embryo proper. This aggregation also make it possible to assess pure embryonic phenotype with no influence from the extraembryonic lineages.

2. Materials

1. DMEM + (ES cell medium): Dulbecco's modified Eagle's (DMEM, Flow Labs, powder, cat. no. 430-1600) supplemented with the following:
   a. 0.1 m*M* Nonessential amino acids (100X stock, Gibco, cat. no. 320-1140AG),1 m*M* sodium pyruvate (100X stock, Gibco, cat. no. 320-1360).
   b. 100 m*M* β-Mercaptoethanol (100X stock stored as aliquots at –20°C, Sigma, Cat. No. 600564AG).
   c. 2 m*M* L-Glutamine (100X stock, stored as aliquots at –20°C, Gibco, cat. no. 320-5030AG).
   d. 15% Fetal calf serum (FCS). We regularly test several batches from different FCS manufacturers for ES cell culture.
   e. Penicillin and streptomycin (final concentration 50 μg/mL each, Gibco, cat. no. 600-564AG).
   f. Leukemia inhibitory factor (different sources, e.g., Gibco) 1000 U/mL.
2. 0.1% Gelatin: 1g (w/v) gelatin (Sigma or BDH) made up in 1 L water, autoclaved and stored at 4°C.
3. PBS (phosphate-buffered saline): Prepared without calcium and magnesium and used for all tissue culture work. one liter is made from 10 g NaCl, 0.25 g KCl, 1.5 g Na_2HPO_4, 0.25 g KH_2PO_4, pH 7.2. The solution is autoclaved and stored at 4°C.
4. Trypsin (0.1%): Dissolve 0.5 g trypsin powder (Gibco, cat. no. 0153-61-1) in 100 mL saline/ethylene diamine tetraacetic acid (EDTA) solution. Adjust the pH to 7.6, sterilize through a 0.22-μm filter and store at –20°C. This constitutes a 0.5% stock, which needs to be diluted to 0.1% on defrosting.
5. M2/M16 media for in vitro embryo culture: M2 and M16 media are prepared according to standard protocols ***(12)***.
6. KSOM: Our protocol for preparation of KSOM is modified from ***(25,26)***. It consists of the preparation of six stock solutions that are individually aliquoted and stored at –70°C. The six stocks are detailed in **Table 1**. Individual aliquots of stock solutions are thawed, then combined in the proportions detailed as follows. Bovine serum albumin (BSA) is then added, and the volume is made up using sterile water. The resulting solution is filter sterilized (0.2 μm filter), and stored at 4°C until use. The solution is equilibriated at 37°C/5% CO_2 overnight, just prior to use.

Table 1
Stock Solutions for the Preparation of KSOM

Stock A' (100 mL)	10X	NaCl	950 m*M*	5.55 g
(10 mL aliquots)		KCl	25 m*M*	0.186 g
		KH_2PO_4	3.5 m*M*	0.0476 g
		$MgSO_4 \cdot 7H_2O$	2.0 m*M*	0.0493 g
		Na·lactate	100 m*M*	1.87 g of 60% syrup
		D (+) glucose	2.0 m*M*	0.036 g
		Penicillin-G	600 μg/mL	0.06g
		Streptomycin·sulfate	500 μg/mL	0.05 g
Stock B (100 mL)	10X	$NaHCO_3$	250 m*M*	2.10 g
(10 mL aliquots)		Phenol Red		0.001 g or 1 mL of 1% solution
Stock C'	100X	Na·pyruvate	20 m*M*	0.022 g
(1 mL aliquots)				
Stock D (10 mL)	100X	$CaCl_2 \cdot {}^2H_2O$	17.1 m*M*	0.25 g
(1 mL aliquots)				
Stock F (10 mL)	10,000X	$Na_2EDTA \cdot 2H_2O$	100 m*M*	0.372 g
(10 μL aliquots)				
(1 mL aliquots)	Or 100X	$Na_2EDTA \cdot 2H_2O$		0.372g
Stock G	200X	Glutamine	200 m*M*	(comes in solution)
(0.5 mL aliquots)				

Stock Solution	Volume
10X Stock A'	10 mL
10X Stock B	10 mL
100X Stock C'	1 mL
100X Stock D	1 mL
100X Stock F	1 mL
200X Stock G	0.5 mL
Solid BSA	0.10 g
ddH_2O	to make volume up to 100 mL

7. O.3 *M* mannitol: Mannitol (Sigma, cat. no. M4125) prepared in water with 0.3% BSA (Sigma A4378). Filter sterilized and store in aliquots at –20°C.
8. Light mineral oil (embryo tested): Sigma, cat. no. M8410).
9. Acid Tyrode's solution: Sigma, cat. no. T1788.
10. Tissue-culture-treated plasticware (for cells and embryos): We routinely use Nunc, Corning, and Falcon plasticware.
11. Humidified incubators: Separate incubators for ES cell and embryo in vitro culture. Maintained at 37°C and 5% CO_2.

12. Stereo dissecting microscopes such as Leica M3b, MZ8, or MZ12: These are required for preimplantation embryo work, such as flushing embryos form oviducts/uteri, setting up the aggregations and for the transfer into recipient females.
13. Fine surgical instruments: Required for preimplantaion stage embryo recovery and embryo transfer into recipient females.
14. Needles: Suitable for making depressions in plates for aggregations.These should have the correct beveling such that a smooth depression is produced. Specially made needles can be purchased from BLS Ltd., H-1165 Budapest, Zsélyi Aladár u. 31, Hungary.
15. Pipet for handling embryos: For example a mouth pipet fitted with a drawn out Pasteur pipet.
16. Electrofusion apparatus for tetraploid embryo production: This specialized piece of equipment such as the CF-150 model available from BLS.

3. Methods

3.1. Preparing the Aggregation Plate

1. Place four rows of drops of M16/KSOM (approx. 3 mm in diameter) into a 35 mm tissue culture dish using a 1-mL syringe fitted with a 26G needle, with the first and fourth row comprising three drops and the second and third, having five (*see* **Fig. 4**).
2. Overlay the drops with mineral oil, so that they are totally submerged.
3. Sterilize the aggregation needle with ethanol, and immediately use it to make approximately six depressions per microdrop (*see* **Fig. 4**).
4. Put the plate into the incubator.

3.2. Obtaining the Embryos

1. Remove both oviducts from 1.5 d post coitum (dpc) females (for tetraploids) and 2.5 dpc (for diploids), and transfer to a drop of M2 medium in a petri dish.
2. Flush oviducts by inserting a flushing needle attached to a 1-mL syringe filled with M2 into the infundibulum.
3. Collect embryos and wash free of any debris using a mouth pipet in several drops of M2.
4. Wash embryos in several drops of M16 or KSOM (referred to hereafter as M16/KSOM) and then transfer to M16 or KSOM medium and place in an organ culture dish in an incubator for in vitro culturing, while the aggregation plate and cells are prepared.
5. Once the plate is prepared, proceed to remove the zona pellucida from the embryos.
6. Place a few drops of M2 and KSOM/M16 and two of Tyrode's in a Petri dish.
7. Transfer a group of embryos into the first drop of Tyrode's, rinse briefly, and then transfer to the second drop.
8. Continually observe embryos, and note when their zona has dissolved. Immediately transfer them to a drop of M2, and subsequently wash in several drops of M2.
9. Wash embryos in M16/KSOM. Embryos are now ready for transfer to the aggregation plate.

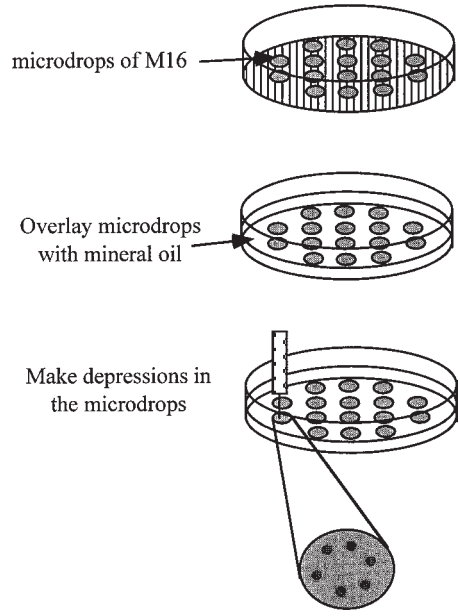

Fig. 4. Preparation of an aggregation plate involves making microdrops of media, overlaying them with mineral oil, followed by forging depressions for the placement of the aggregates. The place once set up is placed in a temperature controlled humidified incubator overnight.

3.3. Electrofusion to Generate Tetraploid Embryos

1. Turn on the pulse generator about 1 h beforehand in order to warm it up.
2. Place the electrode in a 10-cm Petri dish, connecting the cables from the electrode to the pulse generator and adjust all the parameters; then put this arrangement under a dissecting stereomicroscope. We routinely use two pulses (<repeat> set to 2) of 100 V, and 40 μs duration. These parameters, however, may vary between machines. Therefore, the optimum parameters should be experimentally determined for each.
3. Put a large drop of 0.3 *M* mannitol over the electrodes.
4. Place two drops of M2 medium and two drops of 0.3 *M* mannitol in a second 10 cm Petri dish placed under a second dissecting stereomicroscope.
5. Introduce 50–100 embryos to one drop of M2, from there take 20–25 embryos into a drop of the mannitol. After they have settled, place them between the electrodes of the chamber contained in the second Petri dish.
6. Carefully apply the orienting electric field. (If any embryos are not properly orientated, correct their orientation manually.)
7. When all embryos lie in the correct orientation, apply the pulse.
8. Then immediately transfer the embryos back to the other Petri dish into a drop of M2.
9. Repeat **steps 5–8** until all the embryos are subject to the pulsing.

10. When all embryos have been treated, rinse them briefly in M2 and then in M16/KSOM, and transfer them to an organ culture dish containing M16/KSOM or microdrops under mineral oil, and place in the incubator.
11. After approximately 1 h, separate the fused embryos and continue overnight culture.

3.4. Preparing the ES Cells

Extensive protocols for the maintenance and culture of ES cells are beyond the scope of this chapter and are described elsewhere *(27,28)*. The protocol for the preparation of ES cells for aggregation is briefly detailed as follows.

1. Day 1: Thaw cells 4 d before the aggregation onto a feeder cell containing plate.
2. Day 2: Change the medium on the plate.
3. Day 3: Passage cells onto gelatinized plates but instead of the usual 1:5 ration, split them 1:50 or to an even higher dilution.
4. Day 4: Change the medium.
5. Day 5: Trypsinize the cells briefly, just until the colonies begin to detach from the plate. Stop the action of the trypsin by adding DMEM+ to the plate. Select clumps of 10–15 loosely attached cells for the aggregation, and transfer into M16 microdrops contained on the aggregation plate.

3.5. Setting Up the Aggregation

3.5.1. Diploid Embryo⇔Diploid Embryo Aggregation

1. Transfer the zona free embryos into a microdrop not containing any depressions.
2. From there, place individual embryos of the first genotype into the individual depressions of the central two rows of microdrops.
3. Repeat **Steps 1** and **2** with the embryos of the second genotype.
4. After all the embryos have been assembled into aggregates (*see* **Fig. 5**), return the plate into the incubator and incubate overnight, thereby promoting the aggregation resulting in blastocyst formation by the following day.
5. The next day, most of the aggregates should have formed a single embryo that has progressed to the blastocyst stage and therefore be ready for transfer into recipient females.

3.5.2. Diploid Embryo⇔ES Cells Aggregation

1. Select several clumps of approx 10–15 ES cells (*see* **Fig. 5B**) and transfer them to the microdrops of the aggregation plate not containing depressions using a mouth pipet.
2. From there, place individual clumps into the microdrops harboring the depressions.
3. Set up the aggregation either by first placing the embryo in the well and then overlaying the ES cell clump or by putting in the cells first and then the embryo.
4. Follow **Steps 4** and **5**, as in **Subheading 3.5.1.**

3.5.3. Tetraploid Embryo⇔Diploid Embryo Aggregation

1. First, place embryos into the microdrops of the aggregation plate not containing depressions, then take a single tetraploid embryo within a depression.

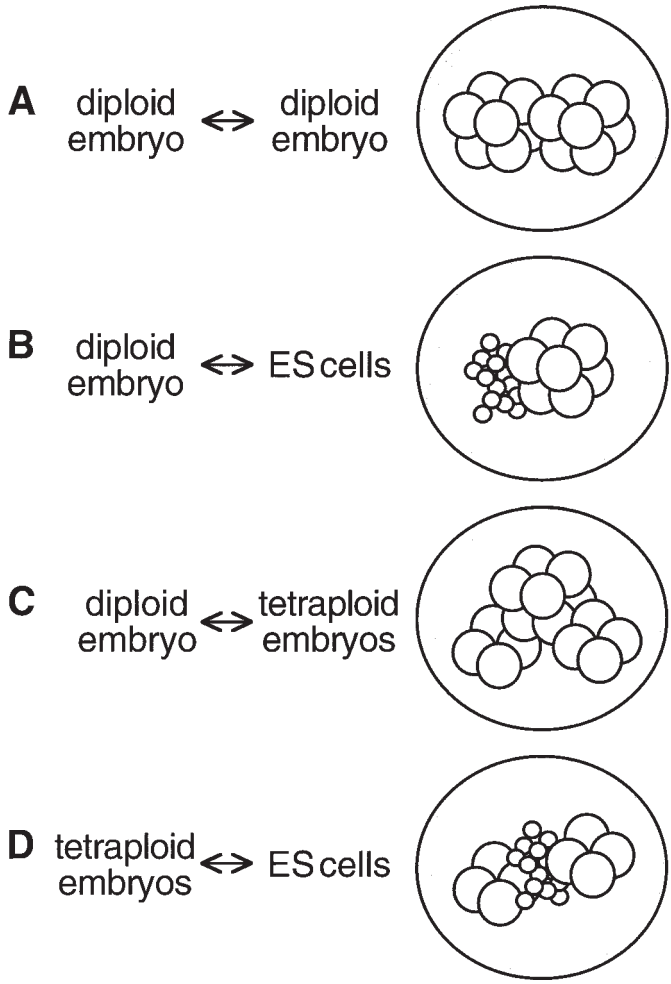

Fig. 5. Several different aggregate combinations can be set-up, compreising one or two of the three constituents of chimeras.

2. Following this, take the diploid embryos and transfer them to the microdrops of the aggregation plate not containing depressions (nor any tetraploid embryos!).
3. Carefully place a single diploid embryo next to the tetraploid embryo already positioned within the depression.
4. Follow **Steps 4** and **5** as in **Subheading 3.5.1.**

3.5.4. Tetraploid Embryo⇔ES Cell Aggregation

1. First, place embryos into the microdrops of the aggregation plate free from depressions.

2. Then take a single tetraploid embryo and place it within a depression.
3. Following this, select several clumps of approx 10–15 ES cells and transfer them to the microdrops of the aggregation plate not containing depressions (nor any embryos!).
4. Take a loosely attached clump of cells, and place it carefully next to the embryo already positioned within the depression.
5. Introduce the second embryo into the depression so that it lies on the side of the cells opposing the first embryo. This is best done by gently rolling this embryo over the rim of the depression, at the proper site.
6. Repeat **Steps 2–5** until all the sandwiches have been set up.
7. Follow **Steps 4** and **5**, as in **Subheading 3.5.1.**

3.6. Transfer of Blastocyst Stage Aggregates

On the day after aggregation the embryos should have reached the blastocyst stage (corresponding to 3.5 dpc of development) and are ready for transfer to pseudopregnant recipient females.

1. Optimally, 8–10 embryos are transferred into each uterine horn of a 2.5 dpc pregnant female. We routinely use CD1 or ICR outbred mice as recipients.
2. In the event of a shortage of recipient there are several solutions:
   a. Fourteen embryos can be transferred per uterine horn (a total of 28).
   b. Use recipients that are 3.5 dpc pregnant.
   c. To culture the leftover, usually less advanced, embryos, which may still be at the morula stage for another overnight.
   d. Transfer embryos into the oviduct of a 0.5–15 dpc pregnant female.
3. The embryo transfer procedure is detailed elsewhere ***(12)***.

4. Notes

4.1. Aggregation Details

4.1.1. Preparing the Aggregation Plate

1. A few microdrops are usually left without depressions (upper and lower rows) so that they can be used to introduce and briefly rinse ES cells and/or embryos just before assembly of the aggregation.
2. Plates can be prepared a few hours in advance and kept in the incubator until use. This allows enough time to obtain the embryos and prepare the cells.
3. The depression should have a clear smooth wall and be deep enough to hold the aggregates without a risk of disassembly of the aggregate or spilling over as the plate is placed in the incubator.

4.1.2. Obtaining the Embryos

4. Oviducts are removed by making incisions in upper part of the uterus and right below the ovary.
5. Flushing is performed from the infundibulum, resulting in embryos being expelled from the short length of uterus carried over.

6. Females are superovulated because of an outbred background such as CD1.
7. Flushing needles are made by cutting the tip off a 30 G1/2 needle, and then beveling the end with a sharpening stone.
8. The zona pellucida is a glycoprotein coat that encapsulates the embryo. Late blastocyst stage embryos will usually hatch out of their zona prior to and in order to implant. The zona is refractory to aggregation, as an intimate contact cannot be made between the cells and/or embryos in the aggregation sandwich.
9. Even though it does not matter whether embryos are in M2 or M16/KSOM prior to their Tyrode's treatment, M2 is used right after as it has a superior buffering capacity.

4.1.3. Electrofusion to Generate Tetraploid Embryos

10. The adjustable AC field is applied in order to allow for the correct orientation of embryos. Only the minimal necessary voltage should be used. If the field is too high it can cause lysis of the cells.
11. Electrofusion can be performed in an electrolyte or nonelectrolyte solution We favor, and have provided the protocol for, the nonelectrolyte method, as it allows for multiple embryos to be electrofused at the same time in addition to the ability of automatic orientation of the embryos with the high-frequency AC field.
12. It usually takes approximately half an hour for the blastomere fusion to occur. It is important to select only the fused (and therefore tetraploid embryos) approximately an hour after the pulsing. We recommend that embryos that have fused be transferred to a new organ culture dish or microdrop, and then cultured overnight.
13. After overnight culture in M16/KSOM medium, tetraploid embryos will have developed to the four-cell stage, which is equivalent to the eight-cell stage of diploid embryos. Tetraploids should be aggregated at the four-cell stage, as it is at this time that they will initiate compaction.

4.1.4. Preparing the ES Cells

14. A highly diluted plating of single cells is required in order to produce the optimal size clumps (10–15 cells) required for the aggregation. Clumps in which cells are loosely connected are favored for setting up the aggregate.
15. Care should be taken so as not too disaggregate the cell clumps by pipetting too vigorously or with extensive trypsin treatment.

4.1.5. Setting Up the Aggregation

16. Aggregates should be set up in such a way that there is maximal contact made between the cells and embryo, or between embryos. Therefore it is important that the clump of cells is not too compact, it is best if cells are loosely connected.
17. Aggregations involving tetraploid embryos are set up as a "sandwich," where two tetraploid embryos are used to flank either the ES cells or the diploid embryo.
18. When setting up the aggregates, especially if they are tetraploid "sandwich" types, take care not to jolt the plate and dissociate the intimately contacted embryos and/or cells.

4.2. Genotyping F2 Diploid Embryos in Weight Chimeras

19. Genotyping of chimeras is sometime a problem because of the mixed cell populations between mutant and wild-type embryos. One can avoid dealing with this problem by isolating the tissue that is expected to be solely mutant in origin , e.g., the yolk sac endoderm in the case of "mutant diploid embryo⇔ES cells" or "mutant tetraploid embryos⇔ES cells," and embryo proper in the case of "mutant diploid embryo⇔tetraploid embryos." In other cases, i.e., "mutant diploid embryo⇔wild-type diploid embryo" and "mutant tetraploid embryos⇔wild-type diploid embryo," the contribution of mutant cells can be mixed with wild-type cells in tissues and that makes genotyping very difficult. Here, practical approaches to solve this problem are discussed.

4.2.1. Genotyping of Potentially Chimeric Tissues

20. In the case of recessive mutation, homozygotes for the mutation have to be obtained from a cross between heterozygous female and male. Genotyping of chimeras can be performed by preparing genomic DNA followed by genomic Southern or PCR. The problem occurs if mutant cells are mixed with wild-type cells in the tissue, as the result from genotyping will be indistinguishable between chimeras containing heterozygous cells and mutant cells (*see* **Fig. 6A**).

    One solution for this problem is taking advantages of using two alleles for either wild-type alleles (*see* **Fig. 6B**) or mutant alleles (*see* **Fig. 6C**). In this way, one can distinguish chimeras made by mutant embryos from heterozygous littermates.

4.2.2. Isolation of Potentially Mutant Cells by Using Markers

21. It is possible to isolate potentially mutant cells (populations derived from het × het cross) from wild-type cells if one can design the cross to introduce a gene as a ubiquitous marker into all progenies. For this purpose, markers such as *lacZ* or green fluorescent protein (GFP) are suitable. In the case of *lacZ*, genotyping is performed with *lacZ*-stained tissues and/or by PCR. GFP is more straightforward because one can detect GFP without the use of a chromogenic substrate, and GFP positive cells can be collected manually using a drawn-out glass pipet (A. N., unpublished observation) or fluorescence-activated cell sorting (FACS) (***29*** and ***30***). In general, the use of the ubiquitously expressed markers makes genotyping chimeras more feasible and reliable.

5. Conclusion

Chimeric studies have been a feature of modern mouse embryology since its inception almost 40 yr ago. During the first half of this period embryo–embryo chimeras answered many questions about basic events during embryogenesis, such as cell movement and clonality. The 1980s bought a new component to chimera analyses, this being mouse ES cells. To date the majority of ES cell chimeras have been made by the technically demanding and labor-intensive method of

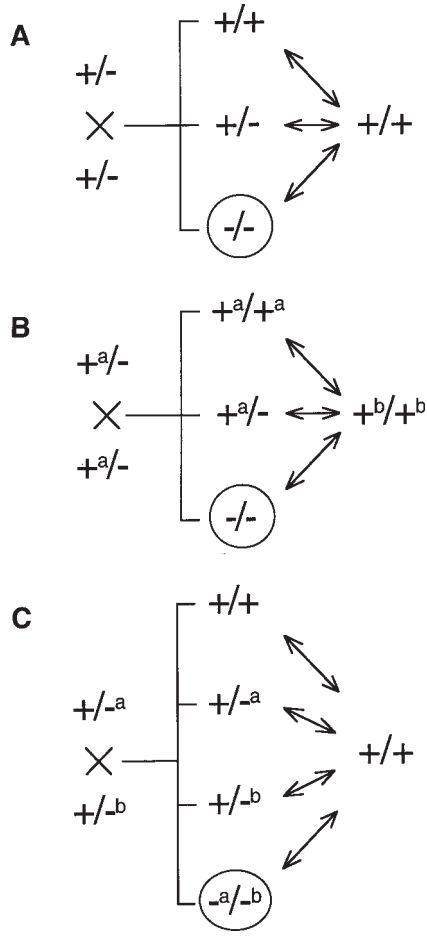

Fig. 6. If mutant cells are intermingled with wild-type cells in the tissues of a chimera **(A)** the result obtained from genotyping will be indistinguishable between chimeras containing heterozygous cells and mutant cells. To distinguish chimeras made by mutant embryos from heterozygous litter mates. One can employ two different tags for either wild-type alleles **(B)** or mutant alleles **(C)**.

blastocyst injection and have been used for the introduction into mice of genome alterations made in ES cells. Even so, the 1990s bought back the classic use of chimeras as tools employed to answer biological questions, as the means for generating homozygous mutant ES cells became available, thereby a recessive phenotype would already be represented in cell culture.

We now have an ease of producing ES cell chimeras after revisiting and refining the aggregation chimera technology. We have also learned that ES

cells are developmentally restricted, so that they are not able to differentiate into trophoblast and primitive endoderm leaneages, but have full potential in the primitive ectoderm lineage, e.g., in the embryo proper. A further component came into play after it was demonstrated that tetraploid embryos can provide a normal extraembryonic environment to an ES cell-derived embryo, such that tetraploid cells are selected against in the embryo proper if diploid cells (ES or embryo) are present.

Thus, today the three chimera components; diploid embryos, tetraploid embryos, and ES cells, whether mutant or wild type, open up a variety of possible combinations for creating specific chimeras, each tailor-made to address any relevant biological question posed. As a consequence over the past few years there has been an upsurge in the number of such studies reported in the literature. It is therefore reasonable to expect that we will enter the twenty-first century with a fully updated version of a classical tool that can be applied in many laboratories using genetic technologies in order to understand normal and disease life.

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11

How to Study Pathologic Phenotypes of Knockout Mice

Roderick T. Bronson

1. Introduction

It has become increasingly clear that when one knocks out a gene it is almost always impossible to predict the resultant phenotype. One must be prepared to look at every organ at every stage of development, from earliest embryonic life to old age. With any luck the knockout (KO) mice will have an obvious phenotype, such as an absent tail. Often the KO mice will have no apparent phenotype or will sicken and die prenatally, right after birth, during the first days or weeks of life, or later in life. The phenotype is critical. Thus, if a mutant is found to have no corpus callosum, that discovery can be published even if no one has figured out why the pups die shortly after birth ***(1)***. On the other hand, no one is interested in a mutant that simply dies or that has no phenotype. A common occurence is to make or acquire a second KO and to breed it with the first KO mice to make a double mutant. Sometimes breeding the mutated gene onto another genetic background by repeated backcrossings (i.e., making a new congenic strain) brings out a phenotype, as many genes are now known to be or are suspected to be modified by other genes.

A happenstance of scientific history is that most people who knock out genes know very little about histology, pathology, or physiology; they require help from someone who knows these medical sciences. It is also a happenstance of history that although many people know aspects of these sciences, few have broad knowledge of them and fewer still know about mice. Most people who know medical sciences have become specialists. They can help once a phenotype has been found in the particular tissue that they specialize in. For example, most research ophthalmologists can be quite helpful in elucidating eye abnormalities. Neuroscientists who know about mouse cerebellum, are happy

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to help solve mouse cerebellar problems. Some will be so eager to help that they will take the problem away from you altogether. At this time few people have had much experience in mutant mouse pathology. It is not clear what can be done about the paucity of trained and available mouse pathologists in the short term, though the National Institutes of Health as of Spring 1999 is developing new ways to fund mouse pathologists. For the moment it is possible at least to provide some pointers to people who have just made a mutant and are anxious or desperate to find a phenotype. This chapter describes the steps required to find a phenotype in a KO or spontaneous mouse mutant.

2. Clinical Observations

Clinical observations are usually critical to success in finding a phenotype. In fact most of the hundreds of spontaneously occurring mutants that have been found at The Jackson Laboratory (TJL, Bar Harbor, ME) were found by animal caretakers in the course of their daily rounds. How one studies the clinical phenotype of a mutant depends, of course, on when the phenotype is expressed.

2.1. Embryonic Phenotypes

You will suspect that you have an embryonic lethal phenotype when you fail to recover any null mice in born litters. Sometimes a few mutants survive to birth but die at that time. All mutants with embryonic defects have to be studied by time-mating dams and killing them at specific gestational times, best beginning in late gestation and working back to earlier developmental time points. The goal of such studies is to find and define lesions and to determine how they evolve. One looks at earlier and earlier time points until a developmental stage is found at which mutants and controls do not differ. Kaufman ***(2)***, Schambra and colleagues ***(3)***, and Theiler ***(4)*** are very useful guides to the anatomy and histology of embryonic mice. Altman and Bayer's atlas of embryonic rat brain is also helpful ***(5)***.

2.2. Perinatal and Infantile Phenotypes

1. Start with careful observations of mutant and control pups. Look for failure to thrive, loss of weight, and difficulty breathing. (In older mice you hold the mouse's nose up to your ear and listen for ìclickingî, evidence of pneumonia.)
2. Look for abnormal behavior such as weakness, ataxia and shivering, pallor or jaundice, diarrhea, failure of normal hair to grow in, or loss of hair later. (But many mice normally "barber" or chew off one another's hair.) As you study your mice, make sure you are ready to kill any that become ill, and be prepared to collect dead bodies, described as follows.
3. If mice are found dead after birth, it is essential to observe new litters of pups as soon after birth as possible. Are the mutants nice and pink after birth, or are they blue and breathing poorly? Are their bellies full of milk and is their mother look-

ing after them? Female mice that are null for FosB protein pay no attention to their pups, leaving them scattered around the cage ***(6)***. Often pups are found dead and their lungs are collapsed at postmortem. This suggests that they may never have breathed or that they had insufficient surfactant. A problem of interpreting the significance of collapsed lungs is that immature lungs of normal mouse pups tend to collapse when the chest is opened. Another common and nonspecific pathologic finding in mice that die within 24 h of birth is severe apoptosis in the thymus ***(7)***, presumably a response to stress. Mice that are abandoned by their mothers have this lesion.

4. A simple and direct way to determine if mice are able to begin to breathe on their own after birth is to time mate the dam and deliver the pups by caesarean section at E18.5 d. Each mouse is removed from the fetal membranes, the nose is cleaned off, and the snout is stroked to induce breathing. Normal mice squirm, struggle to breathe, throw their head back, and after a minute or so begin to breathe. A mutant we are studying now struggles to breathe, but never is successful and dies. We have yet to discover why this happens. In such cases the heart and all its vascular connections have to be studied in serial sections, as well as the entire respiratory tree.
5. Many other mutants fail to thrive, are runted, and die sometime during the first 3 wk of life or around the time of weaning because they cannot survive on their own. It has proven difficult to determine the precise cause of death in these wasting mutants. Sometimes it is possible to rescue such mutants by removing their normal litter mates so that the mutants get more of mother's attention. Mutants with movement disorders that tend to die at weaning age can be helped by putting food in the bottom of the cage. Mutants at any age may be perfectly normal and then are suddenly found dead because of fatal seizures. You have to be there to observe and document the seizures. Do not forget that failure to thrive may be because of something as simple as overgrowth or malocclusion of incisor teeth. Another issue has to be considered in studies of runted mice. What is the appropriate control? The runt may differ from normally developed wild-type litter mates in ways that reflect the runted state itself. The skin might be thinner; certainly there would be less body fat. Tooth eruption, eye and ear opening, and hair growth might all be delayed, but these abnormalities would not constitute a specific phenotype.

2.3. Adult Phenotypes

If mutants are perfectly normal at weaning, they next have to be mated to see if they are reproductively normal. If they pass that test, then the only hope for a phenotype is to let the mice age for many months, in hopes that a late-onset phenotype will appear ***(8)***. Mice null for E2F showed no phenotype until relatively late in life, when they showed quite a wide variety of lesions, including unusual tumors ***(9)***. For such studies of adult and aging mice, one must be prepared to encounter any of the many known age-related lesions of mice, many of which have been described in older literature ***(10–32)*** and is a nice introduc-

tion to general histopathology. Of course, to know what the abnormal looks like you have to know the normal. Popesko and co-workers ***(33)*** is a wonderfully illustrated text on mouse anatomy. The only book on mouse histology is by Gude and colleagues ***(34)***. Two mouse brain anatomy atlases are Franklin and Paxonos ***(35)*** and Sidman and colleagues ***(36)***, now out of print. (Richard Sidman, MD, is a name to know as the father of mouse mutant neuropathology. He and P. Rakic described most of the famous cerebellar during the 1960 and 1970.)

Despite the abundant literature on mouse pathology, studies of adult mutants are still plagued by the fact that few large-scale studies of the pathology of aging in the various 129 strains of mice ***(37)*** or of crosses between 129 and C57BL/6 (B6) have been performed. These are the genetic backgrounds on which most KO mice have been made. Exactly what kinds of lesions occur in these strains during aging is only gradually emerging as more long-term studies of KO mice are performed. It is already clear that 129 or 129-B6 crosses do sporadically develop some characteristic lesions. These include enlarged spleens that have excessive hematopoiesis, usually erythropoiesis but sometimes myelopoiesis. These mice quite often develop a severe inflammatory disease of blood vessels called polyarteritis nodosa. They also often develop membranous glomerulonephritis. Some males become enormously obese. Rarely, do mice of these genotypes develop teratomas. Another strain of mouse, FVB, has been used extensively in making transgenic mice because the pronucleus is easily injected. These mice are homozygous for retinal degeneration (rd-1) and are blind. They also develop a severe gliosis of white matter while still quite young. It is as severe as gliosis in quite old mice ***(38)***.

2.4. Neurological Phenotypes

A few neurological tests and observations have proven useful in studies of mouse mutants. Any mouse that has a head tilt or that spins around chasing its tail probably has a problem in the vestibular portion of its inner ears. If these mice are put in water they tend to swim directly down to the bottom of the container where they would drown. Many vestibular mutants have been discovered. Some of these are also deaf. Deafness can be diagnosed with a simple clap of the hands to induce a startle reflex, usually a very pronounced reaction in mice. Specialized auditory testing, usually using brain-stem auditory-evoked potentials, is necessary for detailed studies of hearing in mice. Blindness is difficult to evaluate in mice since even totally blind mice appear to behave normally. Many wild *mus musculus* are said to be homozygous for rd-1. Studies of blindness in mice require use of electroretinography. It is also quite feasible to observe lesions in eyes of mice by ophthalmoscopic examination. It takes practice but can be mastered. Any mouse that has a rapid tremor should be suspected to have a problem with myelination. Any mouse that

lurches, staggers, sways, or leans is likely to have a cerebellar abnormality, as reflected in the names of famous cerebellar mutants like staggerer and lurcher. These and other mutants are described in Lyon and co-workers ***(39)***. Information on all mouse mutants is kept up to date and on line at TJL's Web site, www.informatics.jax.org.

It is not easy to study reflexes in mice, but spasticity, suggestive of "upper motor neuron" spinal disease, can be diagnosed in some mice by manipulating the limbs. When you do this to normal mice they squirm and try to bite you. Mice with spinal disease hold their hind limbs stiffly. When you push on them with your finger to make them flex they resist in a somewhat springy fashion. We have seen this in mice with neuroaxonal dystrophy of various kinds ***(40)*** and with two forms of ceroid lipofuscinosis ***(41,42)***.

It must also be understood that some mice with severe neurological abnormalities have no obvious lesions. One neurological sign that is nonspecific and occurs in many neurological mutants for totally obscure reasons is clasping of the hind legs when the mouse is picked up by the tail ***(43)***. Another generalization is that if neurological abnormalities are seen in a mouse, the lesion responsible for the abnormality is likely to be in the brain stem, cerebellum, spinal cord, nerve, or muscle. Mice with only forebrain lesions are unlikely to exhibit any neurological signs other than seizures. It is very unlikely that anyone will find a mentally retarded mouse without special testing such as tests of performance in a water maze. In general, animals do not exhibit peculiar adventitious movements such as tardive dyskinesia, chorea, or athetosis, so one is not likely to find a spontaneously occurring model of such diseases as Parkinson's and Huntington's disease that involve extrapyramidal structures such as basal nuclei and substantia nigra.

Many behavioral tests have been used in studying KO mice. Most people who study KO mice have heard of these tests, the most famous of which is the water maze test. Other tests include rota-rod tests, tight wire cling, open field behavior, radial arm maze, and tail flick tests for pain response. One test is to put red ink on the left hind foot and blue ink on the right and have the mice walk up an inclined plane. The pattern of the mouse's foot prints can then be studied. A simple but effective way to evaluate a mouse's ability to smell is to bury peanut butter in the shavings of a mouse box and see if the mouse can find it.

A rule we have followed in studying mice with neurological phenotypes is that clinical signs may precede development of pathological changes. This rule is not unreasonable. Certainly it is assumed that metabolic and toxic diseases of the brain can be associated with reversible neurological signs, at least in the initial stages of disease. This is true of alcohol toxicity. More prolonged exposure to the insult leads to permanent damage to neurons that will show as definitive lesions. Thus, if you are studying a neurologic phenotype let the

disease ripen fully before harvesting your mice. Even then, some mice I have studied with the most horrific motor abnormalities have not yielded up any histopathologic lesions. One assumes that these mice have a defect in a neurotransmitter or membrane channel.

2.5. Clinical Chemistry and Hematology

Some simple clinical tests may prove useful in certain cases. Many labs are familiar with flow cytometry to evaluate blood samples. Conventional blood work such as red and white blood cell counts and blood smears for differential counts are easily performed. Impression smears of bone marrow can provide useful information about blood abnormalities, so-called "dyscrasias." Remember that the normal differential count varies from strain to strain. It is simple to collect urine from a mouse if one gets a container under the rear end of the mouse immediately after picking it up. Analysis of urine was useful in finding iron in urine in one mutant ***(44)*** and urate crystals in another. Urine specific gravity is very high in mice, precluding the use of that old clinical standby the refractometer, which is scaled too low for mouse urine. Clotting time is evaluated by anesthetizing a mouse, nicking the tip of the tail, and putting the tail in a beaker of water at body temperature. The blood will clot within a minute or so if clotting is normal. What about clinical chemistry? It is simple enough to collect serum and to send it to a reference diagnostic laboratory ***(45)***, but usually one must collect at least 0.5 mL of blood to get enough serum for a few assays. Mouse blood hemolyses easily, which can lead to false results for some clinical chemistry assays. In my experience, clinical chemistry has not proven very useful in most studies of mutant phenotypes.

3. Necropsy

Probably the first and most important rule in working up a mutant is to focus on just a few animals to start with. Do not bother looking at bits and pieces of many animals. The necropsy has four parts: euthanasia, gross observation of body surface and inner organs, dissection, and fixation.

3.1. Euthanasia

Do not chop off the mouse's head. It is crude, disrespectful of the process you are undertaking, possibly cruel, but most important it destroys the back of the brain, and the tissues of the throat and causes the lungs to become filled with blood. Use CO_2 to kill young and adult mice. Fill a container with CO_2 before you stick the mouse into the chamber. The mouse will hop around for about 15 s and will be dead by 30–45 s. Baby mice are more difficult to kill with CO_2. Here is a place for an anecdote. The morning after the second great fire at TJL, we went into mouse rooms that had been filled with smoke but had

not burned. Many dams had died but their litters were usually alive. It is best to kill baby mice with an overdose of an anesthetic such as Avertin. Do not even think of dropping a live baby mouse into fixative. Cool it first on ice till it stops moving and then put it in the fixative.

3.2. Gross Observations

1. Observe everything you can before you start dissecting. Look in the mouth, for example, to check the teeth. Check the eyes for cataracts. Careful gross inspection of pups and embryos must also be performed, but using a dissecting microscope, of course.
2. Open up the body cavities and inspect all organs.
3. Compare back and forth between the mutant and control, identifying tissues as you go along and checking to make sure they are the same color, size, and shape in mutants and controls.
4. Compare males with males and females with females. Adrenals are smaller in males than in females. The importance of careful observations of gross anatomy cannot be overstated. Any mutation that causes an alteration of an organ's size or shape can only be diagnosed by gross observation. Once the tissues have been dissected and histological slides made of them, it will be difficult or impossible to see the abnormality.
5. Brains from mutants and controls should be compared after fixation, as described in **Subheading 3.3.**, as unfixed brains are very soft.
6. If any tissue seems larger or smaller in mutants than in controls, organs should be weighed. On one occasion it was only after months of studying brains from a spontaneous mutant that we noticed that mutant brains were larger than normal controls ***(46)***. Otherwise weighing organs is not very useful.

3.3. Dissection and Fixation

After making gross observations, one next must dissect and fix tissues. This requires choosing a fixative and deciding whether to fix by perfusion or by immersion.

3.3.1. Choice of Fixative

This is a surprisingly controversial issue. Many investigators believe that only their favorite fixative should be used for all tissues at all times and for all procedures. This is not true. Because all fixatives have advantages and disadvantages, it is probably best to fix some mice one way for one procedure and another way for another. For example, it is generally agreed that 10% neutral buffered formalin, a 10% solution made from commercially available 37–40% formaldehyde, must be used to fix tissues for *in situ* hybridization. For immunochemistry, it really very much depends on the particular antigen as to which fixative can be used or if indeed only frozen sections must be used.

3.3.1.1. Aldehydes

Fixation in conventional aldehyde fixatives including formalin, paraformaldehyde and glutaraldehyde, makes mouse tissue very brittle and difficult to section. Paraffin blocks have to be soaked in water on cold plates before sectioning to prevent shattering when they are sectioned on the microtome. Note that buffering is essential. If tissues are fixed in unbuffered formalin an unsightly pigment called "formalin hematin" forms in tissues wherever blood is present.

3.3.1.2. Telly's Fixative

This fixative does not make mouse tissue too brittle. Used for years at TJL, it is made up of 160 mL of 37–40% formaldehyde, 80 mL glacial acetic acid and 1600 mL 70% EtOH. Telly's fixative has disadvantages, however. It causes red cells to hemolyse, formalin hemoglobin to deposit in tissues, and, worst of all, holes to develop in white matter of brain and spinal cord. This artifact seems to arise from some interaction between EtOH and myelin. Sometimes a birefringent gray material is observed in these holes. Obviously this artifact is not caused by short exposure to EtOH, as the few hours tissues spend in EtOH during the dehydration phase of tissue processing does not usually cause holes. I used to see these holes in brains from monkeys fixed in formalin immediately after killing. Formalin, itself, thus seems able to cause these holes. These holes are not seen in human and pet animal postmortem tissues, perhaps because such tissues are usually fixed hours after death. In any case, in at least one paper this artifact was published in *Nature* as a real fact ***(47)***; I have had to warn several people about this artifact before they published it.

3.3.1.3. Bouin's Solution

This fixative is commercially available or can be made by mixing 160 mL 40% formaldehyde, 80 mL glacial acidic acid, and 1700 mL saturated aqueous picric acid. Bouin's fixed mouse tissues do not become brittle as do aldehyde fixed tissues and so are easily cut on the microtome; the acid in the fixative demineralizes tissues, so after a week or 10 d in the fixative (shorter for young mice) skull, spine, and limbs can be trimmed with a razor blade and histological sections can be made that include bone and soft tissues left in their normal relationships to one another. M. H. Kaufman, in *Atlas of Mouse Development* ***(2)***, gives fixation times for Bouin's fixation of mouse embryos. His directions on pages 3–5 should be followed precisely. Of greatest importance, is the fact that Bouin's does not produce holes in white matter. Bouin's fixative, contrary to popular opinion, does not necessarily destroy antigens. In fact, antibodies to glial fibrillary acidic protein work very well with Bouin's fixed tissue.

Tissues fixed in Bouin's solution have traditionally been transferred to 70% ethanol before further processing. Ethanol removes Bouin's from tissues so that they can be processed into paraffin directly. However, as mentioned before, ethanol produces holes in the white matter. To remove Bouin's from tissues, wash them in slowly running water overnight before further processing into paraffin. Do not wash tissues for longer than 24 h or bacteria may grow in them while they wash. If you do not wash acid out of tissues they will not stain with hematoxylin.

3.3.1.4. Nonaldehyde Fixatives

Several companies are now producing fixatives in which aldehydes have been crosslinked, making them nontoxic. These fixatives can be discarded in the sink. It is likely that everyone will be using these fixatives for environmental reasons. Apparently they work well for immunohistochemistry and histochemistry.

3.3.2. Fixation by Perfusion

Fixation by intracardiac perfusion is essential if one wants good preservation of neural tissue, particularly the spinal cord.

1. Begin by anesthetizing the mouse with Avertin (stock solution is 1 g tribromoethanol in 0.5 mL amylene hydrate, also called tert amyl alcohol; working solution is 0.5 mL stock solution in 40 mL dH_20 or saline; injected intraperitoneally (i.p.) at 0.1 mL/5 g body weight).
2. To anesthetize mice more quickly I overdose with an i.p. injection, and begin the perfusion when the leg no longer reflexively withdraws when the foot is firmly pinched with forceps. You must make sure that the mouse is really in deep, deep anesthesia, or you will inflict pain and suffering.
3. Pin out the mouse on its back.
4. Open the chest; cut off the right atrium; insert the tip of a 21-gage butterfly needle (26 gage, in baby mice) straight down into the tip of the apex of the heart. You will be in the left ventricle at that point.
5. Flush in about 5 mL saline over the course of a minute or so. Then change to another syringe with Bouin's and flush in 30–50 mL fixative until the body is stiff and completely yellow. The whole perfusion should not take more than a few minutes. You want a fairly high rate of flow. Do not be alarmed by the twitching of muscle. That is how muscle reacts to fixation. However, if the mouse goes into a huge convulsion when the fixative first goes in, then it was not anesthetized deeply enough. Sometimes fluid flows out the nose if there has been a rupture of a blood vessel in the lungs, in which case you will not get a good perfusion. Proceed with the next stage, and open up the mouse for further fixation. Even well-perfused tissues require further fixation.

6. Open the abdomen completely on the midline. The abdominal and chest organs must be completely exposed. This can be done by turning the mouse a little bit inside out.
7. Incise the skin under the neck so that fixative can get directly to salivary glands, thyroid, and lymph nodes.
8. Turn the mouse over and slit the skin on the midline from head to tail.
9. Reflect the skin laterally to expose all of the muscles of the back and rump.
10. Carefully open the skull by following the midline and lateral sutures using a small extrasharp pair of scissors. Alternatively, cut around the sides of the skull with scissors. Do not smash the brain while you are cutting the skull.
11. Drop the whole adult mouse in Bouin's and leave it there for another week to 10 d; leave baby mice in fixative for several days. Fixation in Bouin's for longer than a month should probably be avoided. After the fixation period, transfer the body to tap water with a little fixative to prevent bacteria from growing.

You can get a pretty good fixation by following this procedure even with nonperfused mice. Just open everything up and drop the mouse in fixative. This is what you should do if you find a dead mouse. Open it up completely as described. If it is so far gone that it is cold, no longer stiff from rigor mortis and makes putrefied, discard it. Usually a mouse that was alive in the afternoon and is found dead in the morning the next day is worth saving, unless it has been cannibalized. Some information can be retrieved even from a fairly repugnant mouse. Such information may be critical in determining the cause of death, an important phenotype in itself.

3.3.3. Fixation by Immersion and the Traditional Necropsy

Traditionally, tissues collected during a necropsy or autopsy (the latter word is usually reserved for human postmortems) are fixed by immersion as a dissection proceeds. Immersion fixation has the advantage that organs can be weighed accurately. (Perfusion-fixed tissues can also be weighed and presumably normal and mutant tissues will have true relative weights but not true weights.) Immersion fixation also permits the dissector to see differences in color between normal and diseased tissue. Immersion fixation, however, has some disadvantages. Immersion-fixed tissues are never as well fixed as perfusion-fixed ones because autolysis, postmortem degradation because of enzymatic activity, proceeds to a certain extent before the fixative penetrates completely into the tissue. This is particularly important in the gut where bacterial digestion of tissue, in addition to enzymatic digestion, leads to sloughing of the tips of villi. In immersion-fixed mice the brain and spinal cord not only autolyze but also suffer from handling because they are soft and are easily deformed.

The most severe artifact of handling incompletely fixed or unfixed tissue is called "dark neuron artifact." Neurons shrink and stain darkly with any stain ***(48)***. This can happen even with perfusion fixed brains if they are handled

before fixation has been completed in the 24 h or more of immersion fixation after perfusion. If you do want to fix a brain by immersion, take off the skull cap without touching the brain and immersion fix the whole brain *in situ*. Do not dissect it out until after it has fixed. Dark neuron artifact, like holes in the white matter, has gotten the unwary into trouble in the past ***(47)***. It is particularly important not to confuse neurons that are shrunken because of dark neuron artifact with neurons that are shrunken because they are acutely necrotic as happens in ischemia. In hematoxylin- and eosin-stained sections the latter have bright red cytoplasm, and the former have red-purple cytoplasm. Perfusion is really the only way to fix the spinal cord. If you fix it by immersion, fixation will be quite poor, as the fixative has to penetrate the bone. If you try to dissect out the spinal cord there will be a lot of dark neuron artifact.

3.3.4. Mouse Necropsy

Feldman and Seely ***(49)*** is the only book available that describes small-animal necropsy technique.

1. Weigh the body.
2. Pin the mouse out on its back and open the skin from the jaw to the pubis. Do not hesitate to use a magnifying glass or dissecting scope at any time as you proceed.
3. Reflect the skin to the sides to expose the muscles beneath. Look for the axillary nodes in the subcutaneous tissue in under the arms and the inguinal nodes in front of the thighs: these are hard to find in normal mice. Check out the tissues under the neck. These include lymph nodes and salivary glands.
4. Dissect out the chain of glands and lymph nodes starting under one ear with the parotid gland, continuing down under the throat and up under the other ear.
5. Cut through the jaws on either side so that you can examine the upper and lower teeth and tongue.
6. Carefully expose the larynx and trachea by removing the muscles overlying them. Just behind the larynx and stuck to each side of the trachea you will see the pale brown thyroids on each side.
7. Cut across the trachea just above and just below the thyroids and save the entire piece. Later, the slice of trachea with attached thyroids will be cross-sectioned to include all three tissues.
8. Open the chest by cutting along the ribs on both sides to remove the sternum. Look for abnormal fluids in the chest or pericardial cavities. Observe the size of the heart. Damaged hearts are often larger in size than normal. The lungs should be a nice salmon pink color. However, lungs from mice that have died tend to collapse, and blood pools in the lungs, making them red in color. Lungs will have red patches if the mouse was killed by decapitation due to inhalation of blood. Dark red solid areas, however, might indicate pneumonia. Such areas will sink in formalin if pneumonia is present. Look for lung tumors in older mice. Check out the size of the thymus, and then dissect it out whole and drop it in fixative. Dissect

out heart and lungs whole and put them in fixative. Another way of doing this is to start at the head, cut into the soft tissues between the jaw, dissect out the tongue and follow back to the larynx, trachea, plus esophagus and on back to the heart and lungs. This entire tissue from tongue back to heart and lungs is called the "pluck" in slaughterhouse terms.

9. Now go to the abdominal cavity. Open the muscles to expose the entire cavity and look for abnormal fluids. There may be a lot of abdominal fat, which will tend to hide some tissues. Check out all organs, making sure each is the correct size, shape, and color and that all are present.
10. Start your dissection with the adrenals. If you dissect out other organs first you are likely to lose the adrenals. The right adrenal is tucked up under a lobe of liver and is hard to find. Dissect out both kidneys with adrenals attached, inspect them, and drop them in fixative. Look for pitting on the surface of the kidney and dilatation of pelvis, indicating hydronephrosis, common in mice.
11. Open the pelvis to expose the pelvic canal. Examine all reproductive organs. In males check out the testes, epididymus, seminal vesicles, prostate, urinary bladder, urethra, and penis. Old male mice often develop hugely dilated seminal vesicles. The preputial glands, located to either side of the penis under the skin, often become abscessed.
12. Dissect out the two testes and the rest of the genitourinary tract in one piece. In females check out the vagina, uterus, and ovaries. After examining these organs dissect them out in one piece and fix them.
13. Take out the spleen, which is often enlarged in mice.
14. Try to find the mesenteric lymph node located at the base of the mesentery.
15. Next identify the pancreas. It is a cream-colored tissue that is spread diffusely in the mesentery between the spleen and the duodenum. Dissect out most of it and drop it into fixative.
16. The gut is saved to the end so that the contents do not get all over everything. If you want good slides of gut, inject fixative directly into the lumen of the gut with a syringe and needle. This will stop autolysis. Do not cause the gut to balloon up. Inject small amounts of fixative into many places along the gut. Some people like to coil the gut into a flat roll, the way a sailor coils down a coil of rope. It is best to do this on a piece of paper towel so that the gut sticks down in a flat layer before dropping the gut and towel into fix. Fixation is not optimal if you do that, but you do get sections of the entire length of gut. The stomach and cecum have to be opened up and contents discarded before fixation. They are best fixed whole and later sliced into strips. Incidentally, you will see little round pale patches scattered along the gut. These are normal Peyer's patches. Fecal pellets should be removed from the colon by slicing over them and pushing them out. It is often best to place the fresh colon on a piece of paper to which it sticks. Then the colon on the paper is placed in fixative. This way the colon fixes flat and will be flat when embedded for sectioning. The liver and gall bladder should be examined. Make sure that the gall bladder is not thickened or enlarged. Often, mouse livers from

obese mice are very fatty and may even float in fixative. Slice the liver into 5 mm thick slices. While you are doing so, look for small rounded discolorations that might be tumors. Save a few slices, one containing sliced gall bladder, in fixative.

17. The head, spine, and legs are dealt with last. Open the skull with a extrasharp scissors following along the saggital and two lateral suture lines. Do not gouge the brain as you do so. Remove the skullcap and some additional bone from around the sides of the skull. You can carefully remove the brain, but it will have some dark neuron artifact. It is best to put the entire skull with brain into the fixative.
18. Remove the eyes.
19. Cut out the spine, removing as much muscle as you can. Do not try to dissect out the spinal cord; let it fix within the spine.
20. Skin out one foreleg and one hind leg and drop the entire legs into the fixative.

3.3.5. Special Handling of Tissues

3.3.5.1. Skin

If skin is fixed by immersion it has to be specially handled. The mouse is skinned out and the whole skin is spread out on a square of paper towel (pelt pad), hairy side up. The skin will stick to the paper. The skin and paper are then placed in fixative. If this procedure is not followed, the skin will twist and curl in the fixative and straight sections will be impossible to prepare. After fixation, 5 mm slices of skin are made and placed in tissue cassettes. It is useful to take both saggital and cross-sectional slices of skin so that hair follicles can be studied in both planes. Some histotechnicians dislike hair and want you to shave the skin. This is not necessary, but you can shorten it up a little with scissors.

3.3.5.2. Mammary Tissue

Mammary tissue is best studied along with the skin attached. The mammary tissue and subcutaneous fat can be carefully dissected away from underlying muscle as the skin is removed. The fat, mammary glands, and skin can then be placed on a pelt pad and fixed. The skin sections will have mammary tissue in the subcutis. Many people like to study mammary tissue as whole mounts, a very elegant method ***(39)***.

3.3.5.3. Lungs

Lungs have to be handled specially if detailed analysis of alveolar size or alveolar wall thickness must be assessed. Because lungs tend to collapse (atelectasis), it is necessary to fill alveoli with fixative by injecting fixative into the trachea with a needle and syringe. Do not overfill the lungs. Some people tie off the trachea after filling the lungs to prevent fixative from flowing out again. That is not usually necessary.

3.3.5.4. Eyes

Eyes may require special treatment. Eyes from perfused fixed mice usually do not develop artifactual retinal detachment. Immersion-fixed eyes always do. The retina may be so twisted that it is impossible to study. To get good histology from immersion-fixed eyes it is necessary to embed them in a plastic media like JB-4. The centers of mouse lenses never section well when embedded in paraffin; they always become shattered. Plastic embedding prevents this to some extent.

3.3.6. Clyde Dawe's Method of Fixation

The late Clyde Dawe, one of the foremost reseachers of mouse pathology, developed a very nice way of fixing mouse tissues ***(50)***. Slices and pieces of fresh tissue and whole tissues such as female reproductive tract are placed onto glass coverslips. The tissues are arranged just as they will appear on the histologic slide. The tissues stick to the coverslips, which are then immersed in Bouin's fixative for 3 d. The tissues are next transferred into 70% ethanol, which is fine for all tissues except central nervous system (CNS) tissue. The tissues fix with flat faces that are then placed into cassettes flat side down, as described in **Subheading 4.**

3.4. Storage and Shipment of Fixed Tissues

An important rule of thumb is that no tissues should ever be thrown away until the project is finished. Commonly one finds a lesion in a mouse after other mice have already been studied and wants to go back to see if the lesion was present in the earlier mice.

1. Bouin's fixed tissues should be stored in tap water with just enough fixative to make the water a little yellow to prevent overgrowth of bacteria. This is contrary to the tradition of storing Bouin's fixed tissue in 70% ethanol. Tissues stored in ethanol always dry out.
2. Formalin-fixed tissue should be stored in formalin, as should Telly's fixed tissue.
3. Do not store tissues in screwtop jars; they always dry out and take up too much room. Do not store tissues in zip-lock plastic bags; they invariably leak.
4. Store tissues in heat-sealed bags, just the way molecular biologists store gels in these bags. If the seal line has no wrinkles, the tissues in dilute fixative will be good for years.
5. If fixed tissues are to be shipped, they should be put in water in double heat-sealed plastic bags and then enclosed in two more layers of plastic bags. There is nothing more annoying to everyone than leaks of evil-smelling substances. The US mail and probably other mail services and such private carriers as FedEx will not deliver leaking packages.

4. Trimming and Cassetting

This important step is different for immersion- and perfusion-fixed tissues. If the mouse has been perfusion fixed, dissecting, trimming, and cassetting are accomplished at the same time. As one dissects, one takes appropriate slices of each tissue or of whole tissues and places them in plastic tissue cassettes. If the mouse is to be immersion fixed, tissues are dissected and placed in fixative. Later, the fixed tissue is trimmed and placed in cassettes. Whichever fixation procedure has been used, each trimmed tissue is placed into a cassette. Each cassette has the same dimensions as a glass histologic slide. What you put in each cassette is what you will get on the slide.

1. Label each cassette with the exact information that will be on the slide (it had better be correct!) using a sharpened #2 lead pencil.. And here we come to the most important point in this entire Chapter: *never, never use a conventional felt-tip pen to label cassettes*. The ink comes off in the xylene when the cassettes are processed. Special lab pens are available with ink that is indelible in xylene. It is best not to have felt-tip pens around. Someone inevitably will pick up the wrong pen and that is the end of that experiment.
2. Place tissues in the cassette with the flattest surface down. It is critical that each tissue in the cassette have a flat bottom because that bottom will be the face from which sections are cut after paraffin embedding. It is true that small tissues will turn over in the cassette during processing, but the histotechnician will embed the flattest surface down. Now what if you have no flat surface? Then the histotechnician will have to embed the tissue as best as possible and slice deeply into that tissue to get a large flat face. But if a small tissue such as an adrenal is in the same cassette, it will be sliced through completely and lost. Also, it is a huge waste of time slicing deeply into a tissue block.
3. Another essential rule is not to pack too much tissue in a cassette. Tissues must be put in a single layer. Some histotechnicians have firm opinions on which tissues should be put together and which should not. I think it makes no difference, except that bones should be put together in their own cassettes.
4. In some cases it makes a difference which face is placed down. For example, if a mouse brain is sliced five or six times in cross-section, and a section is to be made from each slice, one wants to make sure that the rostral face of each slice is sectioned. To know the difference between the rostral and caudal face of a tissue like brain, make a blue mark on the caudal face, after blotting, with a regular blue pencil before placing the slices in fixative. The histotechnician will embed the tissue with the blue markup.
5. Bouin's fixative is particularly useful because picric acid demineralizes bones. If formalin or Telly's fixative is used, the bones have to be demineralized. Immerse bones in 5% nitric acid for 24–48 h. Check from time to time until the bones are no longer gritty. Do not overdo demineralization in nitric acid or the bones will never stain with hematoxylin. Wash out the acid or Bouin's fixative by leaving

tissues overnight in running water. If you fail to do this, the tissues will not stain with hematoxylin.

6. Following demineralization, cut out 5 mm thick cross-sectional slices of spine in several places to study the spinal cord, ganglia, roots, nerves, spine, and lumbar muscle altogether. We usually take a longitudinal section of hind leg through the knee. We take several cross-sections of skull, often with the brain left in, if the head has been fixed with brain *in situ*.

5. Histotechnique

In hospitals, histology laboratories are staffed by professional histotechnicians whose major job is to embed tissues and cut paraffin sections. Staining and coverslipping are now automated in many laboratories. In most research centers, investigators who want to study the histopathology of their KO mice have unofficial access to hospital histology services. Usually such unofficial access involves an agreement with a pathologist who sneaks some animal tissues through the system or with a histotechnician who is paid to cut sections in her/his spare time. Some institutions have established core facilities where animal tissues can be sectioned on a fee-for-service basis. That sounds optimal, but curiously many investigators find such arrangements unsatisfactory. What often happens is that the histology lab eventually messes up a project. Some stain does not work, the sections are wrinkled or shattered, the serial sections are not oriented properly, critical sections or tissues are lost, or sections fall off the slide during staining. The investigator blames the histotechnician; the histotechnician blames the investigator for not fixing the tissues properly. The investigator buys his/her own equipment and hires a histotechnician, or more commonly puts a technician, or postdoctoral, or graduate student to work cutting sections. The investigator will run into all the same kind of histology problems that I have mentioned, but feels more in control of things. It seems wasteful to have equipment duplicated in many labs, and it semms inappropriate to use a graduate student's time to cut sections. Another curious problem is that if we make sections of someone's KO mice and charge for making them, we are told that it is not ethical for money to change hands between collaborators.

5.1. Embedding

Embedding is not quite as simple as it seems. The key to success is that each tissue has to be placed right at the bottom of the embedding mold in a single layer with the flattest side down. A special procedure is required for embedding whole-mouse brains for serial sectioning in the coronal or cross-sectional plane. The brain has to be stood up on a rostral or caudal tip for embedding, but it is longer than the normal embedding mold is tall. Thus special tall embed-

ding molds have to be contrived by soldering together two molds. For small brains, commercially available deep molds can be used. Why not just cut the brain in two and serial section each part? This is possible, of course, but invariably many sections are lost in the region where the cut was made. If you are a neuroscientist you probably want saggital and not coronal sections, so these problems will not be encountered.

5.2. Coronal Assymetry

Only in coronal sections is it possible to know if a brain is symmetrical. In neuropathology many diseases, such as cancer, cause asymmetries. In mutant pathology almost all disease is symmetrical because the defect is metabolic and affects the two sides of the brain equally. One exception to this is the asymmetry seen in some forms of exencephaly. Frontal sections can be useful in studies of newborn mice, as the relationships of organs such as liver, diaphragm, heart and lung can be visualized in one section.

5.3. How to Prevent Tissues from Coming Off Slides

Mouse tissue, and particularly CNS tissue and pancreas, has a peculiar tendency to come off the slide during staining. This happens sporadically for mysterious reasons. It rarely happens to slides of domestic animal tissue, except for poorly fixed brain tissue that does come off. To obviate this, special slides must be used. The least costly in materials but not labor is to rub slides with glycerin, egg white, or even white glue and let it dry. This is called ''subbing'' a slide. Slides coated with poly-L-lysine are commercially available, as are slides with an electrical charge, but these are quite expensive. Sometimes it is necessary to put an adhesive in the water bath. We use Chrome Alum Adhesive (American Histology Reagent Co.). The water bath temperature should be kept between 37° and 40°F. Brain tissues should be left in the bath only long enough to remove the wrinkles. Any longer and the tissue spreads too much. After picking up the tissues on slides, stand the slides up in a rack and drain for a half hour or more, after which they should be cooked in a 60°F oven for hours or overnight. Any of these steps can be left out, but from time to time tissues will still be lost. It still happens, for example, that in slides with spinal cord within spine, the bone will stay on the slide but the spinal cord will come off and be lost.

5.4. Staining

Hematoxylin and eosin (H & E) is still the best stain to screen all tissues for lesions. But do not forget the many kinds of classical histochemical stains for collagen, basement membranes, cross-striations in muscle, and so on. These stains are all described in Prophet and colleagues ***(51)***. Even for brain H&E is very useful. Three lesions show up best with H&E because they are "eosinophilic"; they stain

red. The lesions are dystrophic axons, small red balls ***(40)***, acutely dead neurons (red-dead neurons), and hypertrophic astrocytes, which look like splashes of red paint when a bucket of paint has been kicked over. These lesions do not show up in slides stained with cresylecht violet (for cell body detail) and luxol fast blue (for white matter tracts). We stain all nervous tissue with both H&E and CV-LFB. Serial-sectioned brains are stained with both stains—even numbered slides with ribbons of five or six sections are stained with H&E and odd slides with CV-LFB. Sometimes we stain sections with Bodian stain, a silver stain that stains axons. Bodian staining is particularly useful in studies of white matter or peripheral nerve disease to distinguish between lesions of myelin only or of both myelin and axon.

5.5. Immunohistochemistry

Certainly immunochemistry can be useful, but I advise against people buying every antibody kit in sight. There is a regrettable tendency to think that the fancier and more difficult a stain, the better it is. The worst use of immunochemistry is to use it as a diagnostic tool. An example of this is to the TUNEL staining technique to diagnose apoptosis. Apoptosis involves nuclear shrinkage (pyknosis) and nuclear fragmentation (karyorrhexis), which can be observed easily in H&E sections. Diagnose apoptosis first in H&E sections and then confirm the diagnosis with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) stain, which is notoriously unreliable. The overall rule in using such immunostains is that whatever stains must look like it does in conventional stains. Thus, if you think that an antibody to glial fibrillary acidic protein is staining hypertrophic astrocytes, the structures you are staining had better look like spiders and stars. The glial fibrillary acidic protein (GFAP) antibody we have been using stains astrocytes that look like spiders, so I have complete confidence in it. Another great antibody which works well on Bouin's fixed tissue is anti-bromodeoxyuridine which stains nuclei in s phase.

5.6. Labeling and Storing Tissues, Slides, and Blocks

You would think that this aspect of managing a pathologic study would be obvious; it is not. The first problem arises from the fact that any histology lab, even an in-house lab, will invariably process tissues from many different mice, many different projects, and at least several different investigators. It does not take long before a great many tissues, slides, and blocks begin to pile up. To keep track of things, obviously all tissues, slides, and blocks from each individual mouse have to be labeled with the same label. The conventional way in a diagnostic lab is to maintain an accession log book into which information on each specimen is entered and a sequential pathology number

assigned, usually 99-1 for the first specimen of the year and so on. Tissues, bottles, blocks, and slides are archived according to their accession number. Another way to do it is to label the blocks and slides with whatever designation the investigator wants to use, preceded by the investigator's initials. Even then, the investigator should have his or her own specimen log book with some kind of unique designation for each animal. It is amazing how many investigators submit mice in bottles labeled –/– #1, –/– #2, +/– #1, +/– #2, and then weeks later submit mice with the exact same designations.

6. Histopathologic Examination

Finally we come to studying slides and hopefully finding lesions. The only way to learn pathology is by doing it. The more slides you look at from more animals, more species, and more types of diseases the better you will be. Of course after studying a set of slides yourself you will have to show your findings to someone more experienced than you to check yourself. Even very experienced pathologists benefit from showing slides to others. There seems to some kind of innate factor involved in learning pathology. Some people never seem able to look at a slide and figure out what is going on. Others get it almost right away. Apart from slide reading there is, of course, a book-reading component to learning pathology.The References section includes citations on mouse pathology.

In the setting of KO mouse studies, analysis of a mutant mouse's phenotype always requires a collaboration between a pathologist and a molecular biologist. How such a collaboration works out will be determined by the individuals involved. Searching for a new phenotype is extremely exciting—but often very difficult—even for an experienced pathologist. If both parties mutually feel the excitement and challenge and work together to find the answer the collaboration is fun and often successful. If, however, the pathologist treats the problem as just another diagnostic exercise, and the investigator treats the pathologist's work simply as a professional service, the collaboration has a good chance of failing. In the best collaborations pathologist and investigator sit down and study slides with a double-headed microscope. The pathologist teaches pathology to the investigator; the investigator teaches the pathologist molecular biology, immunology, tumor biology, and the like. After a while the best investigators become pretty good histopathologists themselves, at least in the areas in which they have had experience. It is possible that some of my pathology colleagues feel threatened by nonpathologists learning their trade. These are the colleagues that will look at tissues and, in the fullness of time, will send a report with histopathological findings written up in traditional Armed Forces Institute of Pathology format:

Heart: inflammation, subacute, mild, locally extensive
Liver: necrosis, coagulative, peripherilobular, chronic, severe
Brain: inflammation, nonsuppurative, multifocal, moderately severe

You must make sure that your mutant is not missing anything. Finding something that is not there can be tricky. For example, I was once studying histologic sections of KO mice at embryonic day (E) 18.5. They looked perfectly normal. I was asked if the kidneys had anything wrong with them. I said I had not seen any kidney lesions. Indeed I had not, because as it turned out the mice had no kidneys at all.

7. Special Procedures

7.1. Skeletal Preparations

Many people have published mouse skeletons by clearing soft tissues and staining hard tissue with elizarin red ***(52,63)***.

1. Skin and eviscerate the mouse.
2. Put body into 1% KOH for 5 d.
3. Put it into another solution of 1% KOH to which a few drops of 0.5% alizarin red S has been added. Leave for another 5 d.
4. Store the body in glycerin. To stain embryos to show both cartilage and bone, a number of quite different procedures have been published ***(52,53)***.

7.2. Clearing Petrous Bones for Examination of Inner Ears

Earlier I mentioned that examination of whole structures is essential before sections are studied. In sections the three-dimensional anatomy of a structure is very difficult to envision even if it is serially sectioned. A method used for years by L. Erway, at the University of Cincinnati ***(54)***, involves clearing ear bones so that both vestibular and cochlear portions of the petrous temporal bones can be examined.

1. Start by dissecting out the ears. The petrous bone is much denser than surrounding bones so that it pops out quite easily. Open the bulla ossea to release air so that the bone does not float in clearing solutions.
2. Fix at least overnight in 70% ethanol. You can also fix in formalin for a few days if you later want to process the tissue for plastic embedding and sectioning.
3. Dehydrate using two changes of 95% ethanol and two changes of 100% ethanol, soaking for at least 30 min at each step. Hours or even overnight at each step is not a problem.
4. Immerse dehydrated ears in methyl salicylate (oil of wintergreen) overnight. They should be completely clear at that point. If not, you have not dehydrated them enough. Go back to alcohol and start again.
5. After clearing, put the ear in a little dish with methyl salicylate so it is covered, and put it on a conventional microscope stage or on a dissecting microscope.
6. Arrange it so that there is a polarizing lens below and above the specimen in the light beam.
7. Now turn one lens so that the field is darkened. The entire ear glows and you can see the entire three-dimensional structure—semicircular canals, utricle, saccule,

and cochlea. Best yet, you will see the maculae of the utricle and saccules because otoconia (otoliths) have white granular birefringence. As it happens, Erway has found that many mouse mutants have vestibular problems because they have defective or absent otoconia.

8. I have heard of a technique used by K. Steele of the MRC Laboratory in Harwell, England. She fills the inner ear with fast-drying latex paint and then digesting bone away, leaving a cast. This sounds like a wonderful way of examining the three-dimensional anatomy of the ear.

7.3. Electron Microscopy

Twenty-five years ago many people were trained in electron microscopy (EM), which has become something of a lost art. There are electron microscopes costing thousands of dollars for service contracts. But it is very difficult to find anyone who can cut and stain sections. It has become a vicious circle—people do not get good results when they submit specimens to the EM lab, so they use other ways of studying their tissues, often immunochemistry or confocal microscopy. But if no one is using the lab, an institution has little incentive to hire and reward a good EM technician. Some rules to follow in using EM are:

1. First, do not hope that you will find a lesion by EM if you cannot find one by light microscopy. You might, but it is a wild goose chase. EM is particularly useful once you have found something and want to define it at higher magnification and resolution.
2. Next, start with large semithick sections stained with toluidine blue. It is perfectly possible to embed and thick section slices of tissue 5 × 5 × 2 mm. Thus large pieces of mouse brain can be thick sectioned; this is essential for orientation. You must not just hack out a chunk of brain.
3. If the lesion is present in the semithick sections, the epon block can be trimmed down to the lesion and thin sections cut from the new small block face.

As for scanning EM, it is generally not very useful, but J. Sundberg has been using it successfully to examine the hair of hair mutants ***(55)***. K. Steele uses scanning EM to examine the hair cells in the organ of Corti.

8. Microscopy

Many people do not know how to use a microscope; poor-quality pictures are still being published. Common mistakes are to have the condenser diaphragm closed or to have the condenser racked down too low. These mistakes result in a very refractile image. Often the image is out of focus, particularly low-power images, the light source is not centered, and color pictures often are murky and have a sickly green hue. It is easy to get around these problems.

8.1. Setting Up the Microscope

1. Begin by aligning the light beam. Put a slide on the stage and choose a low magnification objective. Do all the adjustments by looking down the appropriate eyepiece for taking pictures. Many microscopes have an eyepiece up near the camera. Other microscopes let you use the regular binocular oculars. You usually have to focus on double cross hairs in the field of view for any irregularities for your eyes. Once you have a good focus on the double cross-hairs, you can leave that adjustment alone.
2. Close the bottom (substage) diaphragm until you can see its edges in the light beam as you look into the microscope. As you open and close the diaphragm you will see the edges retract and advance. Adjust the height of the condenser up and down. You will see that at some point the image of the diaphragm is very sharp. Then, as you rack the condenser up and down through that focal point, you will see the edges around the diaphragm turn from red on one side of the focal point to blue on the other. Make it a little blue and leave it.
3. Go to the top diaphragm in the condenser; you will see graduations on it. Look at the objective lens you are using. It will read something like 0.30 for a 10× objective. That is the setting for the condenser diaphragm. You can check this: if you peer down the eyepiece and open and close the condenser diaphragm you will see that the image darkens slightly when it is more closed than 0.30. The point here is that you are closing down the diaphragm as much as necessary to prevent scattered light. That is what you did for the substage diaphragm, too. Each objective lens has its own diaphragm setting so this has to be changed when you change lenses.
4. Focus on the image on the slide. Usually focusing is accurate, as long as you have focused the eyepiece on the cross-hairs. Wear your glasses at all times, even if it is a little uncomfortable. Otherwise you will make focusing adjustments that will throw the image off, particularly if you are astigmatic. If you are shooting low-magnification pictures, pretend as you look down the microscope that you are looking across a valley miles away. That will cause you to relax your ciliary muscles. In fact, for general use of the microscope it is always best to look across the valley; this prevents squinting and eye fatigue.

8.2. Photomicroscopy

Most people now realize that the way to get publishable-quality color pictures is to have 35 mm slides scanned into a computer and then to use Photoshop or similar program to compose a color plate. The colors will be close to the real thing. In the last year or so digital imaging has come of age, so that high-quality images can be scanned directly into the computer from the microscope. High-resolution cameras and image capture boards are still rather expensive, however. If you use film follow these rules:

1. For color pictures use a slow daylight film, say ASA 100. What about print color film? Never use it. Print film simply cannot distinguish the reds and blues of H & E. The pictures always come out with a sickly green hue. For black- and-white pictures use a slow film, even ASA 50, to get maximum resolution.

2. Next, set the light intensity. For black- and-white pictures set the light intensity up about midway. Some microscopes have a mark on the light-power indicator showing where the light should be set. For black and white, use a green filter. For color 35 mm slides use a 80B blue filter for halogen lamps as well as for older tungsten lamps. The light intensity should be very high. If a particular image at low power is so bright that the camera refuses to take a picture, do not turn the light intensity down. Use a neutral density filter to reduce the intensity. The reason for this is that the higher the intensity of the bulb the closer the emission spectrum is to true daylight.
3. Frame images attractively. For example, if you are taking a picture of retina, make it a cross-section or longitudinal section, do not have the retinal slant from lower left to upper right. Arrange pictures so that is up is up and down is down. Conventionally, pictures of gut have mucosa up and serosa down; for skin epidermis is up, dermis is down. If you are taking a low-power image of kidney, arrange the image so that the capsule is at the top curving around with a rim of background nonkidney above it.
   Low-power pictures of complete cross-sections of brain, heart, kidney, or femur, for example, are very nice. Many cameras do not have a low-enough magnification for this. High-quality dissecting microscopes can be used with substage illumination for some low-power shots.
4. A final note on taking pictures of whole organs might be helpful. Because mice are so small you have to use a dissecting microscope with a camera attachment for this. Most people take successful pictures of embryos by immersing the embryo in water or glycerin. A ring-type illumination usually works to light the image without causing highlights on the water surface. Do not use the substage light when photographing embryos. Whole brains or other organs should be placed dry on a black surface. Ring lights should not be used because you cannot control the highlights. It is better to use two fiber-optic lights and adjust them until you have the right shadows and highlights to show significant hills and valleys in the tissue. Fiber-optic lights can also be preferable to ring lights in photographing embryos.

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12

Analysis of Hematopoietic Phenotypes in Knockout Mouse Models

Ivan Bertoncello and Brenda Williams

1. Introduction

The hematopoietic and vascular system of the developing embryo share a common mesodermal precursor, the hemangioblast ***(1–3)***. The primitive hematopoietic system arises in the extraembryonic blood islands of the yolk sac and the aorta–gonad–mesonephros (AGM) region of the embryo. Multipotent hematopoietic stem cells from these primary sites then migrate through the developing vascular system to colonize the fetal liver. Late in gestation, fetal liver-derived stem and progenitor cells seed the developing bone marrow, spleen, and thymus. In the adult, the hematopoietic system comprises an hierarchically ordered, concatenated series of stem and progenitor cell compartments of progressively restricted potentiality and proliferative capacity ***(4)*** (*see* **Fig. 1**). These processes are precisely regulated by the complex interplay of cytokines, accessory cells, stromal cells, and matrix. This involves the coordinated expression of many genes that directly or indirectly govern hematopoietic stem and progenitor cell maintenance, maturation, lineage commitment, and differentiation; cell migration, mobilization, and sequestration; and mature blood cell function ***(5–7)***.

Although targeted gene disruption can result in very clear and precise phenotypes exemplified by the asplenic Hox 11 knockout mouse model ***(8)***, this is an exception. The pleiotropic activity of many cytokines and gene products, and the intersection of signaling pathways ***(5,7,9)***, will often result in prenatal or perinatal mortality, multiorgan system involvement, and complex, sometimes counterintuitive hematopoietic phenotypes when genes are deleted. The following selective examples of naturally occurring and knockout mouse models illustrate the diversity of responses to the targeted disruption of a single gene.

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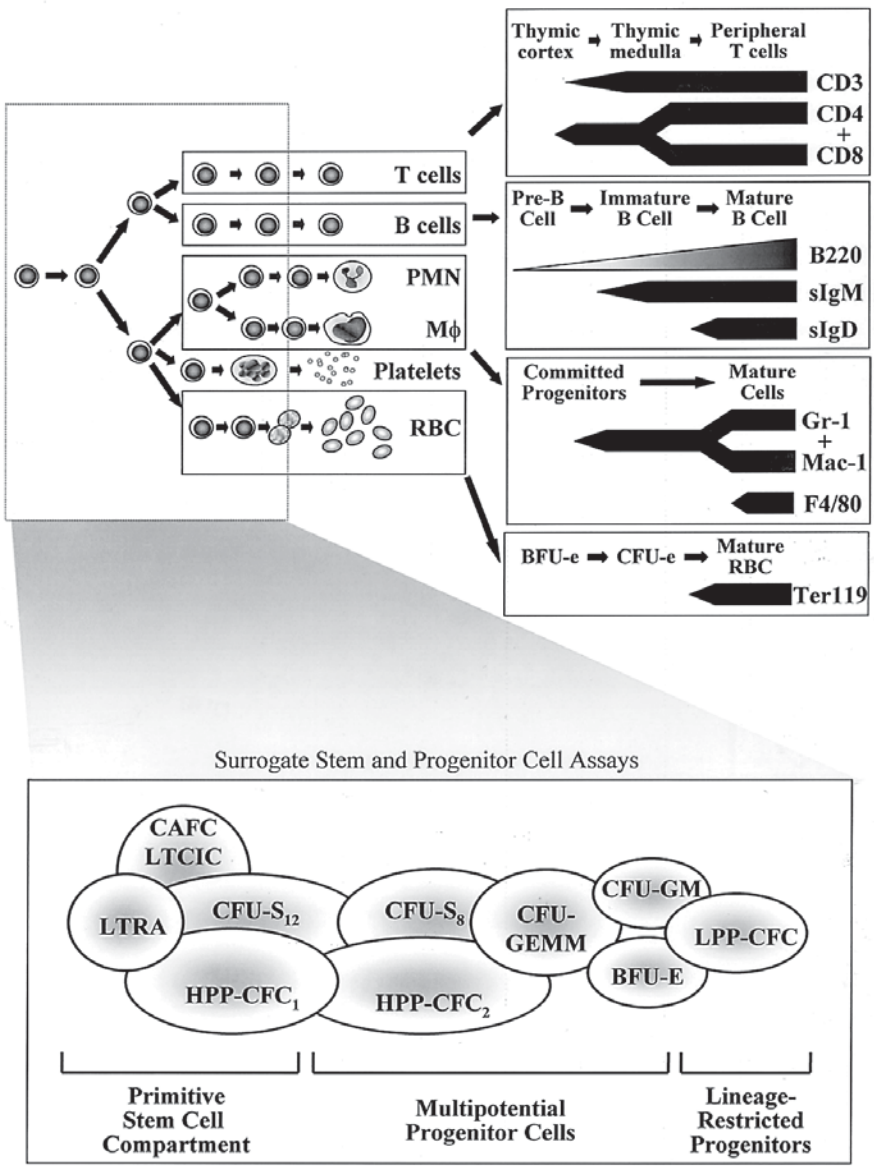

Fig. 1. Schematic representation of the hierarchical organization of the hematopoietic system, surrogate stem, and progenitor cell assays ***(34–36)***, and the level of expression of hematopoietic lineage antigens during lymphoid **(33)**, myeloid, and erythroid cell maturation and differentiation. Abbreviations: LTRA-stem cells with long-term reconstituting ability in vivo; CAFC-cobblestone area-forming cell; LTCIC- long-term culture-initiating cell; CFU-S_{12}-colony-forming unit-spleen measured 12 d posttransplantation; HPP-CFC_1 and $_{-2}$-high-proliferative potential colony-forming cells stimulated by IL1α + IL3 + CSF-

Hematopoiesis in Mdr-1 –/– mice is overtly normal but hematopoietic stem cells are more sensitive to cytotoxic drugs than their wild-type littermates ***(10)***. In telomerase–/– mice hematopoiesis remains unaffected until the sixth generation, where a statistically significant decrease in the total progenitor cell, and high proliferative potential colony-forming cell (HPP-CFC) content of the bone marrow was the only abnormal hematopoietic parameter ***(11)***. Hematopoietic deficiencies in W/W^v and Sl/Sl^d mice are the consequence of the deletion of the pleiotropic c-kit ligand and the c-kit receptor, respectively ***(12,13)***. Both mutants are are characterized by a severe macrocytic anemia ***(12)***. However, these mice exhibit differing and abnormal responses to the induction of thrombocytopenia ***(12,14)***; and whereas W/W^v mice can be cured by transplantation of marrow from either wild-type or Sl/Sl^d mice, hematopoietic stem cells do not engraft and cure Sl/Sl^d mice ***(12)***. P- and E-selectin –/– mice are characterized by a marked leukocytosis, splenomegaly, elevated cytokine levels, elevated numbers of myeloid precursors, and increased susceptibility to infection ***(15)***. These mice survive neutropenia induced by sublethal irradiation as well as wild-type mice, but the homing of transplanted hematopoietic progenitors to the marrow of lethally irradiated knockout mice is significantly impaired (16). CD34–/– mice have normal bone marrow cellularity and peripheral blood cell parameters, and respond normally to sublethal irradiation ***(17)***. However, the numbers of HPP-CFC and myeloid and erythroid progenitors in the marrow are significantly reduced, and the capacity of these cells to expand ex vivo is also significantly impaired. The c-cbl–/– mice exhibit splenomegaly with large numbers of megakaryocytes and normoblasts in the spleen ***(18)***. However, with the exception of elevated circulating platelet levels, peripheral blood parameters are normal, as are the bone marrow cellularity and CFC content. Analysis of the thymus, reveals normal T-cell numbers but altered T-cell receptor expression and signaling ***(18)***. GM-CSF–/– mice have normal peripheral blood and bone marrow parameters, but are characterized by an alveolar accumulation of surfactant lipids and protein causing infiltration and the accumulation of B cells, macrophages, and neutrophils in the lung ***(19,20)***. Later studies have shown that these mice also have an increased tolerance to endotoxin ***(21)***.

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1 and IL3 + CSF-1 respectively; CFU-GEMM-colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte; CFU-GM-colony-forming unit-granulocyte/macrophage; BFU-E-burst-forming unit-erythroid; LPP-CFC-low proliferative potential colony-forming cell.

The naturally occurring CSF-1 deficient osteopetrotic (op/op) mouse *(22–24)*, and the fos–/– *(25)* and cathepsin K–/– *(26)* mice are examples of knockout mouse models where hematopoietic defects are primarily the indirect consequence of the occlusion of bone marrow cavities as a result of impaired osteoclast function. The op/op *(22–24)* and (op/op × GM-CSF–/–) mouse models *(28)* also demonstrate the plasticity of hematopoietic regulation *(9)*, showing that macrophages and monocytes are produced despite the absence of these two cytokines considered essential for macrophage lineage commitment and differentiation. Significantly, the hematopoietic deficiencies of op/op mice progressively correct with age even though macrophage subpopulations in the peritoneum and tissues remained impaired *(27)*. As Metcalf *(9)* pointed out, this model shows that it may be necessary to analyze knockout mice at quite restricted times following gene deletion, and conversely, that it is also possible that genuine defects resulting from gene deletion may be missed if analysis is delayed too long.

Analysis of targeted gene disruption in developmental hematopoiesis poses unique problems *(2,30–32)*. The targeted disruption of genes encoding transcription factors or signal transduction pathways often result in lethality during embryonic life. If this occurs in mid- to late gestation as observed in the myb –/– *(29)*, scl –/– *(30)*, and sos1–/– *(31)* mouse models, a hematopoietic phenotype can be determined by analyzing the hematopoietic organization of yolk sac, fetal liver, peripheral blood, and the developing bone marrow and spleen. On the other hand, where gene deletion results in lethality prior to maturation of the embryonic hematopoietic and vascular system, determination of a hematopoietic phenotype may require the analysis of hematopoiesis in adult mice established from aggregation chimeras *(32)*.

These examples illustrate that prediction, exposition, and interpretation of hematopoietic phenotypes in knockout mouse models is rarely straightforward and requires systematic and unbiased analysis of hematopoietic organization and function. It is precisely such considerations that has prompted recent "Requests for Applications" by various National Institutes of Health agencies (e.g., RFAs: HL-99-010 and HD-99-007) to develop rapid, and large-scale systematic screening and phenotyping protocols for high throughput analysis of mouse phenotypes in mutant and genetically modified mice. However, such comprehensive "gold-standard" screening systems are impractical, and beyond the means of the majority of investigators.

The systematic approach we have developed for the hematopoietic analysis of viable knockout mouse models encompasses a series of investigations calculated to reveal all but the most subtle hematopoietic phenotypes resulting from targeted gene disruption (*see* **Tables 1** and **2**). This approach utilizes standard techniques for the gross pathological and histological examination of hematopoietic tissues, robust surrogate assays of hematopoietic stem and

Table 1
Description of the Hematopoietic Tissues and Parameters Analyzed in Knockout Mouse Models

	Peripheral Blood	Bone marrow[a]	Thymus	Spleen	Peritoneal fluid	Lymph nodes
Organ weight			•	•		•
Paraffin block		•	•	•		•
Cytospin	•	•	•	•	•	•
Organ cellularity	•	•	•	•	•	•
Cell differential	•	•		•		
Immunophenotyping	•	•	•	•	•	•
Reticulocyte count	•	•		•		
Clonal agar assay		•		•		

[a] Femoral cellularity.

Table 2
Description of the Immunophenotypic Analysis of Knockout Mouse Models

		Peripheral blood	Bone marrow	Thymus	Spleen	Peritoneal fluid	Lymph nodes
T-lymphocyte	CD3	•		•	•		•
	CD4/CD8	•	•	•	•	•	•
B-lymphocyte	B220/IgM	•	•		•	•	•
	B220/IgD	•	•		•	•	•
Neutrophil	Gr-1	•	•		•	•	
Macrophage	Mac-1	•	•		•	•	
	F4/80					•	
Erythroid	Ter-119	•	•		•		

progenitor cell activity, and immunophenotyping using a panel of readily available antibody reagents to monitor the status and maturity of all hematopoietic lineages (*see* **Table 3**, and **Fig. 1**). This protocol entails:

1. Gross examination of the mouse for evidence of (a) enlargement or attrition of hematopoietic organs including the thymus, spleen, and lymph nodes, (b) inflammatory exudates, and (c) increased susceptibility to infection.
2. Histological examination of hematological organs.
3. Determination of peripheral blood parameters including total and differential cell counts, and examination of blood smears.
4. Determination of bone marrow, hematopoietic organ, and peritoneal cellularity.
5. Flow cytometric immunophenotyping of thymus, spleen, peripheral blood, and bone marrow cells.

Table 3
Immunophenotyping Antibody Panel

Antibody[a]	Specificity and Alternate Nomenclature	Clone	Supplier cat. no.	Isotype	Supplier cat. no.
CD4 - FITC	L_3T_4 Expressed on thymocytes and mature T helper cells	GK1.5	Pharmingen 09424D	Rat IgG2b,κ	Pharmingen 11184C
CD8 - PE	Ly2 Expressed on thymocytes and mature T suppressor cells	53–6.7	Pharmingen 01045A	Rat IgG2a,κ	Pharmingen 11025A
CD3 - FITC	Expressed on thymocytes and mature T lymphocytes	145–2C11	Pharmingen 01084A	Hamster IgG, group1, κ	Pharmingen 11154C
B220 – PE	CD45R Expressed at all stages of B cell maturation from the pro–B-cell stage through to mature and activated B cells, but not plasma cells	RA3.6B2	Pharmingen 01125B	Rat IgG2a,κ	Pharmingen 11025A
IgM - FITC	Expression of IgM is restricted to B lymphocytes, first appearing on the cell surface at the immature B-cell stage, and precedes the expression of cell surface IgD	R6–60.2	Pharmingen 02084D	Rat IgG2a	Pharmingen 11024C

Table 3 (cont.)
Immunophenotyping Antibody Panel

Antibody[a]	Specificity and Alternate Nomenclature	Clone	Supplier cat. no.	Isotype	Supplier cat. no.
IgD - FITC	Immunoglobulin D is expressed on the cell surface of mature B cells. Relative expression of IgM and IgD can be used to identify B cell subsets	11–26c.2a	Pharmingen 02214D	Rat IgG2a,κ	Pharmingen 11024A
Gr-1- FITC	Ly6G Expressed by peripheral neutrophils, and transiently by cells of the monocyte lineage in bone marrow	RB6–8C5	Pharmingen 01214A	Rat IgG2b,κ	Pharmingen 11184C
Mac-1 – FITC (CD11b)	CD11b Expressed on macrophages; and at varying levels on granulocytes, dendritic cells, NK cells, and peritoneal exudate cells	M1/70	Pharmingen 01714D	Rat IgG2b,κ	Pharmingen 11184C
F4/80 - FITC	Expressed on cells of the mononuclear phagocyte lineage, but not in macrophages of thymus and white pulp of spleen	CI:A3–1	Serotec MCA497F	Rat IgG2b	Pharmingen 11184C

Table 3 (cont.)
Immunophenotyping Antibody Panel

Antibody[a]	Specificity and Alternate Nomenclature	Clone	Supplier cat. no.	Isotype	Supplier cat. no.
Ter-119-PE	Ly76 Expressed by erythroblasts through to mature erythrocytes, but not on BFU-e and CFU-e progenitors	Ter-119	Pharmingen 089085B	Rat IgG2b	Pharmingen 11185A

[a] All antibodies are rat anti-mouse, with the exception of anti-CD3, which is Armenian- hamster anti-mouse.

6. Measurement of the incidence and content of stem and committed progenitor cell populations in bone marrow and spleen using surrogate in vitro clonogenic stem cell assays ***(34,35)***, and committed progenitor cell assays.

For each knockout mouse model, this strategy would be supplemented by additional assays and investigations chosen on the basis of the known biology of the targeted gene (*see* **Note 1**). Similarly, the hematopoietic profile of the knockout mouse model determined by this analysis would then be used to develop a rational approach to further screening and experimentation addressing the consequences, and the cellular and molecular mechanisms of gene disruption.

2. Materials

1. Ammonium Chloride lysing buffer (10X): 80.2 g NH_4Cl (1.5 *M*), 8.4 g $NaHCO_3$ (100 m*M*), 3.7 g disodium EDTA (10 m*M*). Dissolve in 900 mL distilled H_2O, adjust the pH to 7.4 using HCl (1 *N*) or NaOH (1 *N*) as required, and adjust to 1 L. This 10X stock solution can be stored at 4°C for up to 6 mo. Make up single-strength NH_4Cl lysis buffer fresh prior to use by dilution of stock with distilled H_2O, and equilibrate to room temperature prior to use. Discard unused buffer at the end of the day (*see* **Note 2**).
2. Phosphate-Buffered Saline (PBS): The following ingredients are required for 5 L of PBS: 40 g NaCl, 1.0 g KCl, 5.75 g Na_2HPO_4, 1.0 g KH_2PO_4, 1.0 g glucose. Dissolve NaCl in approximately 4 L of distilled H_2O. Add the remaining salts and glucose separately, ensuring that each has fully dissolved before adding the next ingredient. Adjust the buffer to pH 7.4 using HCl (1 *N*) or NaOH (1 *N*), then adjust the volume to 5 L. Check the osmolarity using an osmometer and adjust to 310 mosmol as necessary with distilled H_2O. Filter sterilize the buffer (0.2 μm) and store refrigerated.
3. Peripheral blood differential staining**:** Diff Quik Stain Set (Lab Aids Pty Ltd.). This is a modified Wright–Giemsa stain for the rapid preparation of peripheral blood differentials; Coplin jar or equivalent staining device; microscope slides.
4. Retic-COUNT™ (Becton Dickinson, cat. no. 349204): Used for flow cytometric enumeration of reticulocytes.
5. 1% New methylene blue: Dissolve 1 g in 100 mL citrate-saline solution (1 part 3% w/v sodium citrate to 4 parts 0.9% w/v sodium chloride) for use in manual enumeration of reticulocytes by phase contrast microscopy (*see* **Note 3**). Filter (0.2 μm) prior to use to remove small particulate matter.
6. Fluorescence-activated cell sorter (FACS)-fix buffer: 45 mL PBS, 1.0 g glucose, 1.25 mL formalin for fixing of fluorescent antibody-labeled cell populations for subsequent flow cytometric analysis.
7. Heat-inactivated serum (HiSe)**:** Fetal calf serum (FCS), or newborn bovine serum (NBBS) used for addition to PBS for tissue dissaggregation and immunophenotyping is heat inactivated at 56°C for a minimum of 1 h. Cool the HiSe, filter through coarse filter paper to remove denatured protein, then filter through filters of progressively smaller pore size, and then finally sterile filter (0.2 μm), aliquot and store frozen for use as required.

8. 1% and 0.66% Agar gels**:** Stocks of agar are prepared in batches sufficient for use over one month (*see* **Note 4**). Weigh agar (Difco Bacto-agar) into a sterile conical screw-topped flask containing a sterile magnetic stirrer bar. Add the requisite amount of sterile distilled water, stir and heat until the agar is melted and just commences to boil. Cool the agar to room temperature and store at room temperature until required. It is important to make sure the caps are loose during melting and boiling to prevent shattering of the flasks.
9. Concentrated alpha-modified Eagles MEM Stock (*see* **Note 5**): Dissolve powdered alpha medium without nucleosides sufficient for 10 L of medium in approximately 1500 mL sterile distilled water by stirring for 4–6 h. Add 100 mL Eagle's MEM vitamins (100X) and 10 mL phenol red (1% aqueous). Determine the osmolality of the concentrated medium and dilute to 1300 mosmol (*see* **Note 6**), and gas with CO_2. Sterile filter (0.22 μm filters) and store frozen at –20°C.
10. Double–strength medium for agar cloning: Concentrated alpha-modified Eagles MEM stock 32 mL, 2 mL sterile 200 m*M* glutamine, 40 mL sterile serum (*see* **Note 7**), 8 mL sterile 5.6% sodium bicarbonate, 8000 U gentamycin, 18 mL sterile distilled water to make 100 mL of double-strength medium ready for use (*see* **Note 8**).
11. Antibody reagents**:** The panel of hematopoietic lineage antibody reagents and isotype control antibodies are listed in **Table 3**. Dilute antibodies in PBS-2% HiSe containing 0.1% sodium azide (w/v), and pretiter to establish optimal working concentrations for immunophenotypic analysis. Typically, we have found the optimal dilutions for the commercial sources of antibodies listed to range between 1:80 and 1:640 (v/v) (*see* **Note 9**).
12. Cell strainers: Cell strainers (40 μm nylon mesh) to fit 50 ml disposable tubes (Falcon, cat. no. 2340) and cell strainers (35 μm nylon mesh) to fit 6 mL (12 × 75 mm) disposable tubes with strainer cap (Falcon, cat. no. 2235).
13. Stainless steal mesh sieves: 7.5 cm diameter with a 200 μm pore size (*see* **Note 10**).
14. Tubes for peripheral blood collection: Microtainer brand diagnostic tubes for blood collection coated with K_2-EDTA (Becton Dickinson, cat. no. 365974). Alternatively, 6 mL (12 × 75 mm) polystyrene Falcon tubes can be rinsed and coated with 10% disodium EDTA (w/v) prior to blood collection.
15. Microhematocrit tubes: Clay Adams, cat. no. 1020 (75 mm; o.d: 1.5 mm; i.d.: 1.1 mm); and "seal-ease" microhematocrit tube sealer and holder. (Clay Adams, cat. no. 1015) or equivalent.
16. FACS tubes: 4 mL Falcon #1008 (Becton Dickinson).

3. Methods

3.1. General Considerations

The genetic background of knockout mice, the exquisite sensitivity of the hematopoietic system to perturbation, and environmental and experimental conditions are significant sources of variability in the measurement of hematopoietic parameters both within and between batches of wild-type and knockout mice. It is important that these sources of variability, and their impact on

hematopoiesis, be understood in order to obtain meaningful data, and accurately evaluate the function of targeted genes.

Inbred strains of mice were initially developed to establish the existence and influence of genetic factors on the incidence of cancer ***(37)***. Coincidentally, these genetic factors also influence hematopoiesis, immune surveillance, and inflammatory responses. Recent data have demonstrated that stem cell pool size and stem cell cycling characteristics are under genetic control ***(38,39)***. Different strains of mice vary markedly in total and differential leukocyte counts ***(40)***, in their susceptibility to irradiation ***(41)***, and in their propensity to mobilize stem and progenitor cells in response to cytokine administration ***(42)***. Genetic background also conditions the response to genetic manipulation. The different rates of lymphomagenesis observed in transgenic Eμ–myc mice of differing genetic background is an example of this phenomenon ***(43)***.

Husbandry and other environmental factors are also important variables, and often ignored ***(44,45)*** (*see* **Note 11**). Hematopoietic parameters and responses of both wild-type and mutant animals will be conditioned by the spectrum of environmental pathogens present in individual mouse colonies. The consequences of the targeted disruption of genes involved in immune surveillance may not be manifested in mice housed under pathogen-free conditions.

In order to mimimize the impact of these variables, it is essential that:

1. Hematopoietic parameters in knockout mice be analyzed and compared with those of wildtype control mice of the same age and genetic background, subjected to the same conditions of husbandry, housing and handling, and sampled at the same time. Historical control and published data should only be used as a guide.
2. Sufficient individual knockout and wild-type mice be analyzed to minimize the impact of individual variation and to establish statistical significance. For subtle phenotypic differences, it may be necessary to acquire data from large numbers of animals over a period of time. In situations where analysis of additional mice is required in order to confirm a subtle phenotype, or in order to stabilize data where there is significant individual variation within groups, hematopoietic analysis of additional mice may be restricted solely to analysis of the parameter in question.
3. Where practicable, knockout and wild-type mice should be randomized and evaluated "blind" in order to minimize observer bias.

3.2. Tissue Sampling

3.2.1. Peripheral Blood Cell Collection

1. Lightly anesthetize the mouse, which is restrained by the neck.
2. Collect peripheral blood from the orbital plexus behind the eye. Insert a microhematocrit tube into the orbital plexus with a twisting motion, and collect peripheral blood into a Microtainer tube and immediately mix taking care to prevent clotting (*see* **Note 12**).
3. Hold blood samples at room temperature for subsequent analysis.

3.2.2. Peritoneal Lavage

1. Pin the euthanased mouse to a dissecting board.
2. Make a skin incision taking care not to pierce the peritoneum.
3. Collect peritoneal cells by injecting 10 mL of PBS containing 2% HiSe into the peritoneal cavity using a syringe fitted with a 23-gage needle.
4. Massage the peritoneum gently with the needle in place, and aspirate peritoneal cells.
5. Collect the remaining peritoneal lavage fluid by making a small incision, and aspirate the residual cells while clasping the edge of the peritoneum with forceps and holding the intestine aside (*see* **Note 13**).

3.2.3. Harvesting of Bone Marrow Cells

1. Strip both femoral shafts of each mouse of all tissue and flush from one end and then the other with chilled PBS-2%HiSe using a 1-mL syringe fitted with a 23-gage needle (*see* **Notes 14** and **15**).
2. Collect femoral bone marrow cell suspensions at a concentration of one femur equivalent per milliliter, and keep on ice until required.

3.2.4. Harvesting of Hematopoietic Organs

1. Excise spleen, thymus, and lymph nodes of individual mice using sterile instruments.
2. Remove extraneous tissue and fat and rinse tissues in PBS to remove contaminating blood.
3. Place the individual organs in sterile PBS 2% HiSe in tared containers and record organ weight.

3.3. Peripheral Blood Analysis

An automated cell counter is the method of choice for the determination of peripheral blood cell counts and hematopoietic parameters. In this laboratory, we use a Sysmex K-1000 fully automated quantitative hematology analyzer which, in addition to the total cell count, will accurately quantitate platelet count, erythrocyte count, hematocrit, hemoglobin, mean cell volume (MCV) and mean hemoglobin content (MHC). Alternatively, cells can be counted manually using a hemacytometer chamber.

3.3.1. Hematocrit

1. Collect peripheral blood from the orbital plexus behind the eye using a microhematocrit tube as described in **Subheading 3.1.1.**
2. Place filled microhematocrit tubes in a seal-easy tray prior to loading into a microhematocrit centrifuge.
3. Centrifuge for 5 min and read the hematocrit by determining the volume of packed cells (PCV) as a percentage of total blood volume.
4. Calculate the mean cell volume using the formula: MCV(μm^3) = (PCV(%)/erythrocyte count ($10^6/mm^3$)).

3.3.2. Blood Film — Differential Count

1. Place a drop of whole blood on a microscope slide, and a make a blood film by gently, but firmly, pushing blood along the slide using the edge of a second slide held at a 45° angle.
2. Air dry the film, fix and stain using the Diff Quik stain set following the manufacturer's instructions.
3. Scan the slide using a 100X oil immersion objective and determine the leukocyte differential by noting the percentages of neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

3.3.3. Reticulocyte Enumeration by Flow Cytometry

Reticulocyte counts are determined as a percentage of total erythrocytes in peripheral blood by flow cytometry as follows using thiazole orange. (Retic-COUNT reagent, Becton Dickinson).

1. Set up two 6-mL polystyrene tubes (12 × 75 mm) for each determination: One tube for the stained sample, the other for the unstained (control) sample.
2. Aliquot 0.5 mL of Retic-COUNT reagent into one set of tubes (stained samples); and 0.5 mL PBS to the other set (unstained control samples).
3. Add 5 μL of whole blood to each tube and mix by gentle vortexing.
4. Incubate both tubes at room temperature for 30 min in the dark. Analyze the samples within 3.5 h of incubation.

3.3.4. Manual Enumeration of Reticulocytes

1. Add 2–3 drops of New methylene blue stain to 2–4 vol of whole blood, mix well, and incubate at 37°C for 15 min.
2. Prepare a blood film is as described in **Subheading 3.2.2.**
3. Examine the film when dry without fixation or counterstaining using an oil immersion objective. The reticular material in reticulocytes is stained deep blue, and nonreticular cells shades of pale greenish-blue (*see* **Note 16**).
4. Score reticulocytes and mature erythrocytes in successive fields until 100 reticulocytes have been counted. Reticulocyte counts are expressed as a percentage of the erythrocyte count, and the absolute number of reticulocytes per milliliter is calculated using the formula:

$$\text{Reticulocytes/mL} = \text{erythrocytes/mL} / 100 \times \%\text{reticulocytes}$$

3.3.5. Ammonium Chloride Lysis

Flow cytometric immunophenotyping of peripheral blood cells is performed following lysis of erythrocytes using ammonium chloride (*see* **Note 17**):

1. Aliquot whole blood into a centrifuge tube and add 3 mL of fresh NH_4Cl lysing buffer per 200 μL of whole blood.
2. Incubate the cell suspension at room temperature for 10 min, then centrifuge at 300*g* for 5 min at room temperature to pellet nucleated cells.

3. Wash the nucleated cell pellet twice by discarding the supernatant, resuspending the cells in 2 mL PBS-2%HiSe, and recentrifuging at 400*g* for 5 min at room temperature.
4. Resuspend the cell pellet in PBS-2%HiSe and aliquot into 6 mL polystyrene tubes (12 × 75 mm) for antibody labeling.

3.4. Hematopoietic Organ Analysis

3.4.1. Histological Examination

1. Dissect hematopoietic tissues from each mouse, weigh then sever a portion of each using a sterile scalpel blade, and place in fixative for histological analysis.
2. Reweigh the remaining tissue prior to dissaggregation for subsequent determination of total organ cellularity.
3. Process tissues, embed in paraffin wax, and stain with hematoxilin-eosin for light microscopic examination of tissue architecture and cell composition using standard histological techniques ***(46)***.

3.4.2. Tissue Dissaggregation

Hematopoietic tissues are mechanically disaggregated by mincing using fine scissors or scalpel blades.

1. Place tissues on a sterile metal-mesh sieve (*see* **Note 10**) resting in a sterile 90 mm Petri dish and gently mince using fine scissors or scalpel blades.
2. Gently extrude minced tissues through the metal sieve immersed in 10 mL PBS-2% FCS using the plunger from a disposable 10 mL syringe.
3. Rinse the sieve twice with 5 mL of PBS-2% HiSe.
4. Further disaggregate the pooled cell suspension by gentle pipetting.
5. Pellet the cells by centrifugation at 300*g* for 5 min at room temperature.
6. Discard the supernatant and resuspend the cells in 5 mL PBS-2%HiSe and centrifuge at 300*g* for 5 min at room temperature.
7. Wash the cell suspension a second time as described in **Step 6**.
8. Filter the cell suspension through 40 μm nylon mesh cell strainers to remove aggregates and debris prior to cell counting and antibody labeling.
9. Rinse the cell strainers with an additional 5 mL of buffer and keep the cell suspensions on ice prior to further analysis.

3.5. Flow Cytometric Immunophenotyping

The panel of hematopoietic immunophenotyping antibodies, and relevant isotype controls are listed in **Table 3** (*see* **Notes 18–23**). Antibody labeling procedures are carried out on ice. All reagents and buffers must be kept chilled, and the centrifuge must be operated cold.

1. Set up and label nonsterile 4 mL FACS tubes
2. Aliquot appropriate number of cells into FACS tube (ideally 1×10^6 cells per tube resuspended in 0.5 – 1.0 mL PBS-2%HiSe + 0.1% sodium azide)

3. Centrifuge tubes at 400*g* for 5 min at 4°C .
4. Decant the supernatant using a Pasteur pipet to give a dry pellet. Resuspend the cells in the residual solution by gently flicking the base of the tube.
5. Add 50 μL of conjugated monoclonal antibody to the appropriate FACS tube and mix well.
6. Incubate cells on ice for 30 min.
7. Add 2 mL PBS-2%HiSe, 0.1% sodium azide to each tube .
8. Centrifuge tubes at 400*g* for 5 min at 4°C.
9. Decant the supernatant, resuspend cell pellet in 200–300 μL PBS-0.25%HiSe, 0.1% sodium azide for flow cytometric analysis (*see* **Note 24**).

3.6. Surrogate Stem and Progenitor Cell Assay

Bone marrow cells can be cloned in nutrient agar using either a double-, or a single-layer system. We routinely use a double-layer culture system with growth factors included in a 1-mL underlay, and cells plated in a 0.5-mL overlay, as we find that colony formation is better in this system than in the single-layer system.

3.6.1. HPP–CFC Assay

The HPP–CFC assay is used as a surrogate assay to determine the status of the hematopoietic stem cell compartment (*see* **Note 25**). These cells are operationally defined by their resistance to 5-fluorouracil; their obligatory requirement for multiple cytokines acting in synergy; and their ability to form macroscopic colonies in agar larger than 0.5 mm in diameter and containing at least 50,000 cells. This method has been described at length in another volume of this series ***(36)***.

1. Add sterile growth factors directly to 35-mm Petri dishes to give the desired final concentrations. Typically we find the following concentrations of recombinant growth factors to be optimal for HPP–CFC growth in the 1.5-mL double-layer culture system: rhCSF-1, 1×10^3 U/dish; rhIL-lα, 4000 U/dish; rmIL-3, 25 U/dish; rrSCF, 100 ng/dish. However, all growth factor preparations should be tested for the concentrations necessary to achieve optimal colony formation.
2. Melt the 1% agar in a conical flask to just boiling and put in a 37°C bath to cool.
3. When the agar is cooled to approximately 37°C (test it by agitating it in the flask and feeling the temperature) add an equal volume of agar to the double-strength medium and mix thoroughly (either by pipette or with a sterile magnetic stirrer bar previously placed in the flask)
4. Dispense 1 mL aliquots to each of the dishes, shaking the dishes from side to side to ensure complete coverage of the dish and mixing with growth factors. This basal layer should gel within a few minutes at room temperature (20°C).
5. For the overlay, melt and just boil 0.66% agar and place in the 37°C water bath to cool.
6. Add the required number of target cells from bone marrow or spleen to prewarmed double-strength medium and immediately add an equal volume of

agar. We routinely plate 2.5×10^3 cells per dish, and 1×10^4 cells per dish, for bone marrow and spleen cell suspensions respectively.
7. Mix thoroughly and dispense 0.5 mL aliquots to the dishes — no shaking is required.
8. Allow a few minutes for the upper layer to gel before incubating the dishes.
9. Incubate the cultures at 37°C in a humidified incubator at low oxygen tension (*see* **Note 26**).
10. Score colonies at 14 d of incubation using a dissecting microscope at ×20 magnification with a calibrated grid in one eyepiece to measure colony diameters. Normally the colonies originating from HPP-CFC are clearly visible without magnification but are checked to ensure that they are tightly packed with cells.

3.6.2. Committed Progenitor Cell Assay

The committed progenitor cell compartment is assayed by determining the incidence of lineage-restricted colony-forming cells (CFC) in clonal agar cultures stimulated solely by single lineage-restricted growth factors. We routinely use the growth of macrophage–lineage restricted CFC of low proliferative potential as a simple index of the status of the progenitor cell compartment. Cultures are set up exactly as described for HPP–CFC assay, but using CSF-1 as the sole stimulus. Colonies of at least 50 cells are scored at 14 d incubation exactly as described above.

If analysis of other hematopoietic parameters in knockout mice, or flow cytometric immunophenotyping, indicates involvement of other lineages the appropriate progenitor cell assay can be undertaken (*see* **Note 8**).

4. Notes

1. Early in the generation and establishment of a knockout mouse colony, it may not be practical or desirable to euthanase precious founder mice in order to undertake comprehensive hematopoietic analysis. In this instance analysis may be limited to periodic sampling of peripheral blood from the retroorbital sinus, and monitoring peripheral blood parameters, and performing flow cytometric immunophenotyping.
2. In the formulation of NH_4Cl (10X stock) solution, $NaHCO_3$ (8.4 g) may be replaced by $KHCO_3$ (10 g), and disodium EDTA (3.7 g) may be replaced by tetrasodium EDTA (3.66 mg; 0.82 m*M*) ***(47)***. If desired, the NH_4Cl lysing buffer may be adjusted to a specific pH using HCl (1 *N*) or NaOH (1 *N*). Working solutions of NH_4Cl should be prepared fresh, as prolonged storage at less than 10X concentration will result in formation of ammonium carbonate, rendering the lysing buffer ineffective.
3. Water-soluble brilliant cresyl blue can be used as an alternate stain for manual reticulocyte enumeration, but new methylene blue stains more deeply, uniformly, and less variably than brilliant cresyl blue. New methylene blue should not be confused with methylene blue, which is chemically different, and a poor reticulocyte stain.
4. Various agarose preparations may be used instead of agar, provided that they gel adequately at room temperature. However, we have found that cloning efficien-

cies and colony growth in agarose tends to be lower than in agar. Sterilization of agar by autoclaving should be avoided, as it reduces colony formation ***(48)***.

5. We use the alpha-modification of Eagle's MEM, but any basic medium that can be made to double strength may be used. Alpha medium has consistently given better results than most other media. Because we use large quantities of medium, we routinely prepare a more concentrated stock with vitamin supplementation
6. It is important to measure the osmolality of media and sera to be used in order to ensure reproducibility and uniformity of culture conditions. A final medium osmolality of 280–300 mosmol is optimal for mouse bone marrow colony formation.
7. We routinely use medium supplemented with 20% serum. Batches of sera must be tested to choose a pool suitable for the next 6 mo to 1 yr work. We often find batches of newborn calf serum to be better than fetal calf serum for clonal agar culture. Hyclone iron-loaded newborn calf serum has consistently given good results in this assay.
8. Methodologies for the clonal assay of other myeloid, erythroid, lymphoid, and megakaryocytic progenitor cell populations have been described ***(49)***. Pretested and preprepared media containing relevant cytokines can be purchased from suppliers such as Stem Cell Technologies (Vancouver, BC, Canada). This is an economical and practical option, avoiding the necessity to source cytokines and pretest media if these progenitor cell assays are not used routinely or often.
9. We have routinely used fluorescently labeled antibody reagents sourced from Pharmingen for immunophenotyping of knockout mouse models. These reagents can also be obtained from other commercial suppliers. Hybridoma cell lines expressing most of these antibodies are in the public domain, and can be obtained from organizations including the American Type Culture Collection. We have also used our own purified and labeled antibody reagents from these hybridoma cell line conditioned media prepared using standard methodologies ***(50,51)***.
10. A fresh sieve should be used for each tissue sample. Mechanical dispersal of tissues is preferred to enzyme degradation to maintain cell surface phenotype. The sieves for tissue dissaggregation were manufactured in our workshop (*see* **Fig. 2**). The 36-gage mesh (A.W.G; Brown and Sharp America) has 30 perforations per linear centimeter (900/cm^2) giving a pore size of 200 μm, and is made of stainless steel wire of 0.005-in. diameter (0.127 mm). If suitable sieves cannot be procured, tissues can be finely minced with scalpel and scissors and then gently, and repeatedly pipetted to break up aggregates prior to washing. Tissue fragments and particulate debris can be removed by decanting, having allowed the suspension to stand for a few minutes in the tube.
11. The excellent references (44,45) describing the impact of phenotypic, environmental and experimental variability in animal research can be obtained from The Australian and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART). Details and addresses are available on the Web sites: http://www.adelaide.edu.au/ANZCCART/ or http://anzccart.rsnz.govt.nz
12. Intracardiac puncture or throat bleeding can also be used to exsanguinate mice if they are to be exsanguinated prior to euthanasing for collection of hematopoietic

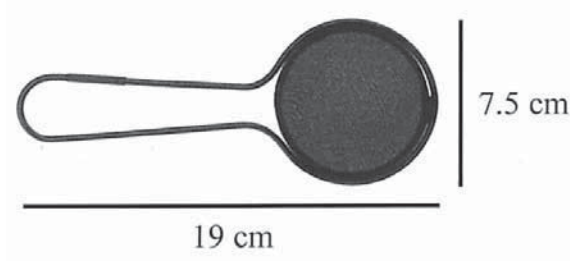

Fig. 2. Diagram of stainless-steel mesh sieve used for tissue dissaggregation.

organs. It is critical that blood samples are taken with speed and precision in order to avoid clotting and platelet aggregation leading to inaccurate counts. The blood sample should be maintained at room temperature, and should not be held for extended periods prior to processing. When EDTA is used as an anticoagulant, it is recommended that myeloid immunophenotyping be done immediately, and that lymphocyte immunophenotyping and reticulocyte counts should be performed within 72 h of sampling ***(52)***. It should also be noted that methods of handling have been identified as a source of variation in total and differential counts in mice.

13. The volume of peritoneal cell exudate, and the number of peritoneal cells collected are operator dependent. It is advisable that the one person be responsible for the harvesting of peritoneal exudates from individual knockout and wild-type mice in each experiment to mininize this source of variability.
14. The typical femoral bone marrow count for an adult mouse ranges from $12 – 20 \times 10^6$ nucleated cells per femur. Care should be taken to ensure that the femurs are removed intact, and that they are not fractured. Severing of the ends of the femur results in significant variability in femoral bone marrow counts. Care should also be taken to avoid frothing bone marrow cell suspensions when flushing femurs. It is also advisable to keep bone marrow cell suspensions chilled to avoid cell aggregation and clumping.
15. In mouse models where the femoral shaft may be occluded due to excessive bone formation or impaired bone resorption, it may not be possible to flush femurs effectively. In this case, bone marrow cells can be harvested by grinding femurs in a small volume of PBS-2%HiSe using a sterile mortar and pestle. The cell suspension is decanted, vortexed to free cells adhering to bone fragments, and allowed to settle for 5 min. The supernatant cell suspension is then decanted, and filtered into 6-mL sterile tubes with 35-μm strainer caps to remove particulate debris prior to further processing of the cell suspension.
16. Manual counting of reticulocytes and platelets can be determined by microscopy as follows. For platelet counts: 20 μL of whole blood is diluted in 1 mL of a 1% ammonium oxalate solution (1:50 dilution). Platelets are counted under phase contrast on a hemacytometer chamber using a ×40 objective. Platelets are distinguished by the presence of blue-black refractile granules in the cytosol. For

reticulocyte counts: 20 μL whole blood is diluted in 50 μL of reticulocyte stain, and incubated at 37°C for 15 min. A blood film is prepared, and scanned using a ×100 oil-immersion objective. Reticulocytes will be stained pale blue with violet inclusions, whereas erythrocytes are stained pale blue. Eight to 10 fields are scored in an area containing 100 – 200 erythrocytes per field, and the number of reticulocytes is estimated as a percentage of total erythrocytes. The erythrocyte count for the sample is determined by hemacytometer using phase contrast and a ×40 objective following serial dilution of whole blood in PBS (1:500 final dilution). The reticulocyte count is then calculated from the total cell count (retics × 10^3/mm^3).

17. NH_4Cl lysis of peripheral blood cells should be carefully controlled to avoid variability in cell recovery and antigen expression. Exposure to NH_4Cl may alter the incidence of defined hematopoietic cell subpopulations; the level of surface antigen expression ***(53)***; and the osmotic fragility of hematopoietic cell subpopulations are known to differ ***(54)***.
18. The concentration of antibody required for optimal staining is generally independent of the number of cells stained, but dependent on the stain volume. When adding a combination of conjugated monoclonal antibodies to the same tube, the titer of each individual antibody must be adjusted to achieve the optimal antibody concentration.
19. Aliquots of thymus cells individually labeled with CD4-FITC and CD8-PE are used to carry out fluorescence compensation for two-color flow cytometric immunophenotyping.
20. Isotype controls should normally be used to determine the level of nonspecific background labeling. Cells must be labeled with the relevant isotype control antibody reagent at the same antibody concentration, and under the same conditions as cells labeled with primary antibodies.
21. Nonspecific labeling can be minimized by incubating cells for 30 min on ice in 5% skim milk (w/v) made up in PBS, and then washed in PBS-2%HiSe prior to antibody labeling.
22. Care needs to be taken when quantifying the level of F4/80 expression on hematopoietic cells. The F4/80 antibody is a rat IgG2b but has an isoelectric point lower than pH = 7.0 and is somewhat sticky, so a perfect isotype control reagent is not available. This problem led to the erroneous claim that F4/80 is present on eosinophils ***(55)***.
23. In collating data on defined cell subsets in each tissue, it is essential to determine and report the content as well as the incidence of target cell populations. This also applies to the measurement of stem and progenitor cell populations in the bone marrow and spleen.
24. It is often inconvenient to undertake flow cytometric analysis of freshly labeled cells. Fluorescently labeled, and control cell populations can be fixed following centrifugation by resuspending the cell pellet in 200 μL of FACS-fix solution. Fixed cell suspensions are then stored refrigerated until required for analysis. Prior to analysis, fixed cells are pelleted by centrifugation and the pellet resuspended in an appropriate volume of PBS.

25. The HPP–CFC compartment of murine bone marrow comprises a number of subpopulations that can be distinguished, and hierarchically ordered on the basis of their differing obligatory requirements for multiple cytokines acting in synergy. HPP-CFC with an obligatory requirement for at least IL-1 plus IL-3 plus CSF-1 (HPP-CFC-1) share many of the properties of primitive hematopoietic stem cells and are a reliable surrogate assay for determination of the status of the hematopoietic stem cell compartment. Determination of the relative incidence of HPP-CFC with more restricted cytokine preferences (e.g., IL-3 plus CSF-1; HPP-CFC-2) can be used to more rigorously assess the status of the hematopoietic stem cell compartment ***(34–36,56)***.
26. Incubation of hematopoietic cells at low oxygen tension (5% O_2, 10% CO_2, 85% N_2) is preferable to using a standard gas phase of CO_2 in air for the determination of colony-forming ability. This results in higher cloning efficiencies and improved colony growth ***(57)***. If an incubator that regulates O_2, CO_2, and N_2 is not available, dishes can be incubated in sealed, humidified boxes gassed with gas mixture ordered from regular commercial gas suppliers ***(36)***.

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13

Isolation of Embryonic Fibroblasts and Their Use in the In Vitro Characterization of Gene Function

Paul J. Hertzog

1. Introduction

The use of primary-embryo-derived fibroblasts and established cell lines derived from them has played an important role in the characterization of gene function in knockout and overexpression transgenic mice. This is for several reasons. First, these have been widely used for a variety of applications including the elucidation of molecular mechanisms and analyses of growth, senescence, transformation, apoptosis, and differentiation. These cells are also susceptible to infection and transfection with expression constructs. Furthermore, primary embryo fibroblasts are one of the first cell lines available during a gene knockout experiment, as heterozygous mice can be mated and pregnant mice killed as early as d 12 of gestation without waiting for pups to be born. Also, if the gene modification results in lethality late (i.e., >d 12) in the fetal period, then an analysis of the "phenotype" or of gene function could still be undertaken at the cell biological or molecular level using the embryonic fibroblast cells.

Some cells in the body, when placed in culture, will undergo a defined number of generations before entering a stage of senescence, where no further growth occurs. The initial period of growth prior to senescence refers to primary cultures, the duration of which varies for cells of different origin. Primary fibroblast cultures can be grown for up to about 30 generations before senescence, depending on the source, subculturing protocol, and the strain of mouse. Unlike some other species, murine embryo fibroblasts have the tendency to spontaneously develop into an established "immortalized" line after a period of senescence. The first to describe this phenomenon was Todaro and Green ***(1)*** who established the "3T3" protocol, which entailed subculture

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of the line every 3 d at a density of 3×10^5 cells per 5 cm diameter dish. The 3T3 cell line differs from primary fibroblast cultures in many important features including morphology, growth rate, ploidy, (plus ill-defined molecular changes), ability to undergo spontaneous and induced transformation, and ability to be stably transfected with plasmid constructs. Both primary embryo fibroblasts cultures and established cell lines have been generated and studied from many genetically modified mice.

This chapter outlines a protocol for the establishment of embryo fibroblast cell lines and reviews applications of these cells in biochemical and biological studies of gene function, particularly as applied to cells derived from knockout mice. These cells are, of course, crucial to the generation of gene-targeted cells because of their use as a feeder layer for the growth of embryonic stem cells. This application has been covered elsewhere in this book and will not be addressed in this chapter.

2. Materials

1. Mice: For the analysis of cells from gene-targeted mice, we routinely mate heterozygous mice, so that there will be embryos and thus cell cultures obtained from all three, +/+, +/–, –/–, genotypes, usually within the same litter. Because of the potential for variation between individual cell lines from normal mice, it is important that any molecular or cell biological differences between primary embryo fibroblasts (PEF) from wild-type (+/+) and gene-targeted (–/–) mice are established for multiple cell lines if the results are to be significant
2. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 10% fetal calf serum (batch tested for suitability for use with embryo fibroblasts and heat inactivated at 56°C for 30 min) and antibiotics (100 units per mL penicillin and 100 μg/mL streptomycin).
3. Phosphate-buffered saline (PBS): Dissolve 0.2 g KCl, 0.2 g K_2HPO_4, 2.86 g $Na_2PO_4 \cdot 12H_2O$, 8.0 g NaCl in 800 mL H_2O MilliQ water, adjust the pH to 7.4, make to 1000 mL then autoclave to sterilize.
4. 70% Ethanol.
5. Trypsin/EDTA : Dissolve 2.5 g trypsin and 0.4 EDTA in 1 L PBS, Filter through a 0.22-μm filter to sterilize, aliquot, and store at 0–4°C for up to 4 wk or at –20°C for longer-term storage.
6. Dimethyl sulphoxide (DMSO).
7. Trypan blue.
8. Dissecting instruments: Sterile fine scissors, fine forceps, and large scissors.
9. Laminar flow hood.
10. Sterile plastic centrifuge tubes, 50 mL and 15 mL.
11. Sterile Petri dishes, tissue-culture grade, 6 cm and 10 cm diameter.
12. Cryovials.
13. Sterile pipets: 1mL transfer pipets, 5 and 10 mL graduated pipets.

14. Hemocytometer.
15. Liquid N_2 container with boxes.
16. Freezer: –80°C.
17. Water bath : 37°C.

3. Methods

3.1. Establishment of Primary Embryo Fibroblast Cultures

1. Kill pregnant female mice from timed matings on day 13 or 14 of pregnancy by cervical dislocation or by CO_2 asphyxiation (*see* **Note 1**). The mouse is thoroughly swabbed with 70% alcohol and in a laminar flow hood, the abdomen dissected to expose the uterine horns. The uterine horns are removed into a 50 mL sterile tube containing sterile PBS.
2. Place the uterus into a 10 cm dish in fresh PBS, cut the walls with fine scissors and remove the embryos together with the attached placentae and fetal membranes into a dish of fresh PBS.
3. From this point it is important to treat each embryo individually in a separate 6 cm dish and with clean instruments to avoid cross contamination of cell lines some of which will be of different genotype.
4. Using fine forceps and scissors, dissect the membranes and placenta away from the embryo and wash the latter with PBS. Then dissect out the liver, heart, kidney, intestines and head and discard these.
5. Wash the remainder of the fetus in 2× in 5 mL of PBS to remove as much blood as possible, then transfer to a fresh 6 cm dish and mince with fine scissors into 1–2 mm^3 pieces. Before mincing, take a small piece of tail, eg 3 mm and place in a sterile tube for genotyping.
6. Add 1 mL of Trypsin/EDTA to the minced tissues and transfer the pieces to a sterile 10 mL centrifuge tube. Incubate at 37°C for up to 30 min with regular mixing and twice pipetting the suspension up and down vigorously with a transfer pipet.
7. When the pieces are digested, add 9 mL DMEM containing 10% FCS. Mix and allow to stand for one min to let any large clumps settle. Aspirate the supernatant into a fresh 10 mL centrifuge tube and centrifuge at 1000*g* for 10 min (*see* **Note 2**).
8. After centrifugation, aspirate the supernatant and resuspend the cells in DMEM + 10% FCS for counting. Using a haemocytometer, add Trypan Blue and count viable cells.
9. Plate cells into 10 cm dishes at a density of 0.5 to 1×10^5 per cm^2 and incubate at standard conditions, at 37°C and 5% CO_2. In a typical experiment 1 to 2 × 10 cm dishes per embryo would be used at this stage, depending on the size of the starting embryo. This concentration of cells is much higher than is used at later passages because many cells will not adhere to dishes.
10. After 24 h, change the medium to remove cellular debris and non-adherent cells.
11. The cultures should be confluent in 2 to 3 d. This is called Passage 0. Cultures can be passaged or frozen as described in **Subheading 3.2.** below.

3.2. Routine Passage of Primary Embryo Fibroblast Cultures (see *Note 3)*

1. Aspirate the medium from a confluent culture, rinse twice in PBS, and add approx. 1 mL of trypsin/EDTA per 10 cm dish. Incubate at 37°C for 1 to 5 min or until all the cells can be detached from the surface of the dish when the dish is lightly tapped.
2. Pipet cells up and down in the dish using a transfer pipet. Add 5mL DMEM + 10% FCS and pipet up and down vigorously 4 × using a 10 mL pipet to break up cell clumps disperse the cells.
3. Distribute the cell suspension into 3–5 plates (i.e., ca. 1.5×10^6 cells per 10 cm dish) and incubate as above.
4. Change the medium 24 h after seeding.
5. Perform passages at or before the cells are confluent. Cultures should not be left at confluence for any length of time.

3.3. Establishment and Culture of "3T3-Like" Fibroblast Cell Lines (see *Note 4)*

1. Harvest embryos and cells as described in **Subheading 3.1.**
2. Trypsinize passage 0 cells as described in **Subheading 3.1.** and plate at a density of 1.5×10^4 cells per cm^2.
3. Change the medium after 24 h of growth.
4. Three days after plating, trypsinize the cells, count and plate again at a density of 1.5×10^4 cells per cm^2. Change the medium after 24 h (*see* **Note 5**).
5. Perform subsequent passages as outlined in **Steps 2–4**.
6. Once an established cell line is obtained it can be passaged indefinitely according to the above protocol and frozen according to standard procedures outlined below in **Subheading 3.4.** Cultures should not be left at confluence as they can undergo spontaneous transformation into tumorigenic cell lines.

3.4. Freezing and Thawing of Embryo Fibroblast Cultures

Both primary embryo fibroblast cultures and "3T3-like" fibroblast cell lines can be frozen according to standard protocols.

3.4.1. Freezing Cells

1. Prepare cells at 60–80% confluency.
2. Trypsinize as described in **Subheading 3.1.**, and resuspend in 10 mL DMEM + 10% FCS for each 10 cm dish.
3. Take an aliquot and count the number of viable cells using trypan blue.
4. Centrifuge the cell suspension at 1000*g* for 10 min.
5. Aspirate the supernatant and resuspend the cells in freezing medium (10% DMSO in FCS) at a concentration of $2 - 5 \times 10^6$ cells per mL.
6. Aliquot 1 mL cells in freezing medium per vial and label with passage number and date.

7. To freeze the cells, place them in a styrofoam box with walls approximately 1 cm thick and store at –80°C for 24 h.
8. After 24 h at –80°C, place transfer vials into liquid nitrogen for long-term storage.

3.4.2. Thawing Cells

1. Equilibrate medium (DMEM + 10% FCS) at room temperature and aliquot 12 mL into 15 mL centrifuge tubes.
2. Remove a vial from liquid nitrogen and transfer immediately to a 37°C waterbath. Hold the vial for a minute or two and remove from the water when it is almost completely thawed.
3. Swab the vial thoroughly with 70% alcohol, open the vial, mix the contents gently with a transfer pipet, then transfer the contents to the tube of medium from **Step 1**. Wash out the contents of the vial, mix the tube contents gently, but thoroughly, then centrifuge at 250 g for 10 min.
4. Aspirate the supernatant, resuspend the pellet in growth medium, and centrifuge again in **Step 3**.
5. Aspirate the supernatant, resuspend the pellet in growth medium, count viable cells and plate at 1.5×10^4 cells per cm^2, or 1.5×10^6 viable cells per 10 cm dish (1–2 dishes per freezing vial depending on the success of the freeze/thaw procedure).
6. Remove the medium 24 h after thawing.
7. Passage cells as described in **Subheading 3.2.**

3.5. The Use of Embryo Fibroblast Cultures to Study the Function of Genes Involved in Interferon Action (see Note 6)

3.5.1. Cytopathic Effect Reduction Bioassay for Antiviral Activity of Interferons

The cytopathic effect reduction bioassay for antiviral activity of interferons (IFNs) was performed essentially as described previously for other cell lines *(5)*. In the application detailed, the induction of antiviral state by type I IFNs in PEF from IFNAR 1–/– mice is examined. This was required to determine whether this receptor component was essential for transmitting the antiviral activity of all IFNs including α subtypes as well as β, or whether a partial activity remained in its absence.

1. Trypsinize almost confluent (80–90%) cultures of PEF derived from IFNAR 1 +/+, +/–, and –/– mice and count viable cells as described in **Subheading 3.2.**
2. Resuspend cells at a concentration of 7.5×10^5 cells per mL in DMEM + 10% FCS, then distribute into wells of a microtiter plate using a multichanel pipet to dispense 190 μL per well. Incubate for at least 4 h at 37°C in 5%CO_2 in air to ensure adherence to the wells. The low-serum concentrations that are usually used in the bioassay are not used with PEF assays.
3. The remainder of the assay procedure, which involves serial titration of IFN samples, then addition and incubation with a cytopathic virus (e.g., Semliki forest

virus or encephalomyocarditis virus), are performed the same as in assays using other cells except that 10% FCS is used throughout the assay. The cytopathic effects of the virus can be scored by light microscopy or the plates can be stained with a vital dye such as monotetrazolium as described elsewhere ***(6)***.

The results obtained from these studies demonstrated that virus grew well in these cells and that the PEF from IFNAR 1 +/+ or +/– mice were equally sensitive to the antiviral effects of IFNs, with 50% protection achieved by 1–10 IU/ml IFNα or β. By contrast, several PEF cultures from IFNAR 1 –/– mice were not responsive to up to 10^4 IU/mL IFNα or β.

*3.5.2. Assay of IFN-Inducible 2'-5' Oligoadenylate Synthetase (OAS) (*see ***Note 7****)*

1. Trypsinize almost confluent (80 – 90%) cultures of PEF derived from IFNAR 1 +/+, +/–, and –/– mice and count viable cells.
2. Resuspend cells at a concentration of 7.5×10^5 cells per mL in DMEM + 10% FCS then distribute into wells of a 24-well plate, 1 mL per well.
3. Incubate cells for at least 4 h at 37°C in 5% CO_2 in air to ensure adherence to the wells.
4. Incubate PEF cultures in the 24-well plates for 48 h in the presence or absence of 1000 IU per mL of IFNα.
5. Remove the culture supernatants, wash the wells three times with 1 mL cold PBS, then add 20 μL of lysis buffer per 10^5 cells.
6. Incubate on ice for 15 min, harvest lysates, and centrifuge at 10,000*g* for 15 min at 4°C.
7. Collect the supernatants and store in 10 μL aliquots for determination of 2'-5'OAS activity and protein concentration as described previously ***(8)***.
8. Results from these assays are shown in **Table 1**. Other studies have demonstrated that PEF are suitable for the study of IFN signal transduction using other techniques such as Northern blots, gel shifts for ISGF3, and GAF activity in response to IFNs α and γ ***(7,9,10)***.

3.6. Studies of Gene Functions in Growth, Differentiation, and Tumorigenicity

Primary, immortalized, and transformed PEF have been important tools in studies of the genes involved in the regulation of cell growth, senescence, apoptosis, and transformation. Indeed, the "immortalization'' process that occurs in the recovery from "crisis" in the 3T3 protocol has been shown to frequently involve alterations in the tumor-suppressor gene, *p53*. These alterations include deletion of the first six exons in both alleles, mutation to introduce a premature stop codon, and reduced or undetectable mRNA levels ***(11)***.

Mice with a null mutation in the tumor-suppressor gene, *p53*, are susceptible to tumor development, which confirms p53 as a potent tumor-suppressor gene

Table 1
2'-5' Oligoadenylate Synthetase Activity in PEF from IFNAR-1 +/+ and –/– Mice

Interferon treatment	0	IFNα	0	IFNβ
IFNAR-1 +/+	189 +/– 54	1,312 +/– 305	86 +/– 44	365 +/– 120
IFNAR-1–/–	23 +/– 9	17 +/– 5	6 +/– 4	3 +/– 1

Enzyme activity is expressed as mean μmoles Pi incorporated per μg protein +/– S.E.M for at lest three replicates.

In PEF from normal mice the basal levels of 2'-5' OAS were increased 5- to 10-fold after IFN treatment. By contrast, in PEF from IFNAR-1 –/– mice the basal enzyme activity was virtually zero, and there was no induction by either IFNα or β. These results demonstrate that IFNAR-1 is necessary for mediating the induction of synthesis of this enzyme by type I IFNs and furthermore, that the basal levels of this enzyme are dependent on IFNAR-1, presumably due to the action of autocrine type I IFN production.

(12). Studies of PEF from these mice have been very important in elucidating the mechanism of action of this effect. Studies of early passage *p53* –/– PEF showed that, compared with wild-type cultures, they divided at a faster rate and had fewer cells in G0/G1, consistent with the proposal that *p53* mediated a G1 block. On continuous passage, the *p53* cell line grew for over 50 passages and did not undergo crisis as observed in +/+ PEF. Furthermore, the *p53* –/– cells were genetically unstable showing a high degree of aneuploidy ***(13)***. Interestingly, *p53* –/– PEF show intermediate-growth characteristics and a moderate degree of chromosomal abnormalities. These studies confirm the important role of *p53* as a negative growth regulator and that its absence promotes genomic instability, which in turn might result in genetic alterations that directly produce immortality.

Another example is the studies of PEF from mice with a null mutation in the *Msh2* gene that is one of three mismatch repair genes that are involved in hereditary nonpolyposis colon cancer (HNPCC) ***(14)***. Spontaneously immortalized PEF demonstrated a higher rate of mutation than +/+ cells. Furthermore, in PEF lines transformed with E7 oncoprotein, *Ras* or mutant *p53*, the mutator phenotype measured by mutation rates, microsatelite instability, and sensitivity to DNA damaging agents (as in HNPCC), was higher in *Msh2*–/– cf *Msh* +/+ cells. These data are consistent with the model that inactivation of the *Msh* gene is a critical step for tumorigenesis.

It had been proposed that the IRF1 gene was an "antioncogene," based on its ability to reverse the transformation of 3T3 cells achieved by expression of IRF2 ***(15)*** and in leukemias and the deletion of the region of human chromosome 5 where the IRF1 gene is localized ***(16)***. However, definitive evidence

was lacking, and mice with a null mutation in the IRF1 gene did not develop spontaneous tumors ***(17)***. Tanaka and colleagues then performed elegant experiments, in PEF from the IRF1 –/– mice, that support the role of IRF1 in the inhibition of tumorigenesis ***(18)***. They demonstrated that IRF1 –/– PEF could be transformed by expression of an activated c-Ha-ras, whereas normal PEF or those from IRF2 –/– mice could not. The transformed PEF demonstrated anchorage-independent growth and growth as tumors in nude mice. Furthermore, the IRF1 –/– PEF were resistant to the c-Ha-ras–induced apoptosis observed in +/+ PEF. Thus, IRF1 appears to be a critical determinant of oncogene-induced apoptosis as well as transformation. These results are very similar to the *p53*-dependent apoptosis of murine PEF expressing EIA and ras oncogenes ***(19)***.

Collectively, the studies in this section demonstrate that embryo fibroblasts can be used to obtain supportive evidence for a role for particular genes in regulating cell growth and death (apoptosis) and tumorigenicity.

4. Notes

1. In the literature mice from days 12–17 are commonly used. We have had success with the earlier times but have not rigorously tested the effect of the day of pregnancy on the resultant cultures. The original 3T3 protocol used older mice that were 17–19 d pregnant.
2. If the suspension becomes viscous at this stage, some protocols recommend the addition of DNase I to degrade DNA. We have not found this necessary if the described protocol is strictly followed.
3. The literature contains many different protocols for the passage of primary embryo cultures depending on the purpose of the cultures. For example, when used as feeder layers to support the growth of embryonic stem cells, primary cultures are often split into many dishes (up to 10), then frozen to obtain the most number of vials at early passage numbers and with minimal handling. For establishment of a 3T3-like cell line, it is important to plate the cells at a precise density according to the original protocol of Todaro and Green ***(1)*** as described in **Subheading 3.** For routine use of early passage cultures, we have used a protocol outlined below. Using this procedure, PEFs from normal mice usually survive as "healthy" cultures for at least 10 passages (sometimes as many as 20). The exact number of passages varies with the strain of mouse, the age of the starting embryos and the passage conditions.
4. Primary fibroblast cultures can be developed into an established, but nontumorigenic cell line by repeated passage according to a strict protocol developed by Todaro and Green to establish the 3T3 cell line ***(1)***. The name 3T3 is derived from the protocol of plating cells at $\underline{3} \times 10^5$ cells per 5 cm plate and passaging every $\underline{3}$ d. In this procedure, cells proliferate at a steady rate for a number of generations (up to 20 depending on the strain of mouse and other parameters) and

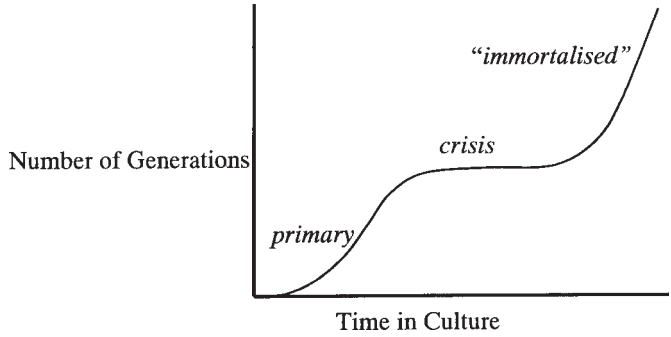

Fig. 1. After proliferating at a steady rate for a few generations, the culture enters the crisis period. Once the culture passes the crisis period it becomes immortalized.

then enter a period of senescence or "crisis." After crisis, the culture changes to become "immortalized" and capable of indefinite growth in culture (*see* **Fig. 1**). These immortalized cell lines show morphological, karyotypical, and molecular differences from primary cultures. Of the various protocols in the original paper (3T3, 6T3, and 12T3) the 3T3 protocol gave a reasonable frequency of cell lines entering a well-defined crisis and finally becoming an established cell line which was nontumorigenic. The nontumorigenicity is important if the cell lines are to be used for studies in oncogenic transformation. Further details of the growth requirements of 3T3 cultures are available elsewhere ***(2)***.

5. The number of generations the culture has undergone in one passage can be determined from the final cell density after 3 d and the initial cell density. Thus, it is important to keep accurate records of all cell counts.
6. Our group has generated mice with a null mutation in the IFNAR 1 component of the type I IFN receptor ***(3)***. PEFs were used to elucidate the role of this receptor component in mediating signals from the various ligands that constitute the type I IFNs, i.e., IFNα subtypes and IFNβ. Signaling studies were conducted at the biological and molecular level. It is known that type I IFN signaling interaction involves phosphorylation and activation of Janus kinases, JAK1 and tyk2, which phosphorylate the receptor and latent signal transducers and activators of transcription (STATs 1 and 2). Phosphorylate STATs translocate to the nucleus where they activate the transcription of IFN-stimulated genes (ISG)***(4)***. The protein products of the IFN stimulated genes are responsible for the biological changes that IFN causes in the cells such as inhibition of proliferation, induction of an antiviral state, and, in some cells, modulation of differentiation and apoptosis ***(4)***.
7. In order to examine the effects of IFNAR-1 deficiency on signaling by IFN ligands, we and others have compared the effects of IFNs α and β on the induction of IFN-responsive genes in PEF derived from IFNAR-1 +/+ and –/– mice ***(3,7)***. Although these studies usually use Northern blotting procedures, we have also examined the IFN-inducible 2'-5' OAS by measuring the activity of the

enzyme. This enzyme catalyzes the formation of oligo-A (n = about 10–15) with the unusual 2'–5' linkage from ATP. This oligonucleotide induces the latent enzyme RNase L. Both the 2'–5' OAS and RNase L are dependent on double-stranded RNA for activity ***(4)***. The benefits of measuring IFN-induced enzyme activity are that (a) a quantitative measure of basal activity can be obtained, (b) the assay is not species specific like the probes for Northern blots, so that the same assay can be used for murine and human cells, and (c) only relatively small numbers of cells are required.

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14

Influence of Genetic Background on Knockout Mouse Phenotypes

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1. Introduction

Once a knockout allele generated by gene targeting has been introduced into the germline of a mouse, the primary concern is to efficiently screen the animal for mutant phenotypes. This is not necessarily a trivial exercise given the high frequency of unexpected or lack of phenotypes. With the number of published knockout mouse strains approaching 1000, considerable experience has accumulated with respect to these concerns. Some of the most obvious approaches involve developmental surveys at the morphological and histological levels, physiological studies, perturbation of homeostatic balances, introduction of stress or injury, and analysis of mutant organs, tissues and cells in vitro. However, the history of mouse genetics, which in itself can be termed the study of strain-dependent phenotype variability, tells us that as a backdrop to these approaches the genetic background onto which the targeted allele is placed can cause considerable variation in phenotype. This variation can present itself as completely different phenotypes, as variations in penetrance of phenotype, or as variable expressivity of phenotype.

In this chapter we provide examples from the gene-targeting literature showing each of these types of phenotype variation. In addition, we discuss ways in which modifier genes can affect the phenotype of a mouse with a mutant gene, and we give examples in which such background-dependent phenotype differences have been used to localize and identify modifier genes. Finally, we discuss the advantages and disadvantages of performing the first analysis of a knockout mouse on a mixed genetic background. We conclude that a mixed background provides the quickest preview of possible strain-dependent phenotypes.

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2. Influence of Genetic Backgrounds on Knockout Mouse Phenotypes

The genetic background of the mouse has been shown to dramatically influence the phenotype of single-locus changes brought about by gene targeting. For example, the postnatal phenotypes of TGFβ1 null mice display considerable strain dependence (*see* **Table 1**). On the mixed backgrounds studied (129/Sv X CF-1, 129/Sv X C57Bl/6, or C57Bl/6J/Ola X NIH/Ola) approximately half of the mutant animals survive to birth and develop a multifocal autoimmune-like inflammatory disorder that affects most organs ***(1,2)***. The distribution of the affected organs varies slightly between these backgrounds and is discussed later as an example of background-dependent variation in penetrance of the inflammatory phenotype. The remaining half of the TGFβ1 null animals die before birth. The phenotypes of the embryonic lethality in these mice are quite strain dependent. On the C57BL/6J/Ola X NIH/Ola background, approximately 50% of the embryos die around embryo day (E) 10.5 due to defective hematopoiesis and yolk sac vasculature ***(3,4)***. However, on the 129/Sv X CF-1 background about 50% of the homozygous mutants die before implantation. Finally, only about 1% of TGFβ1 null animals on the C57BL/6 inbred background live to birth and develop the inflammatory disorder. It is unclear that yolk sac lethalities on this strain are above background levels (*see* **Table 1**).

Other examples of background-dependent phenotype variation include the keratin 8, A-raf, and EGF-receptor knockout mice. On the C57BL/6 X 129/Sv background a null mutation in the keratin 8 gene causes embryonic lethality due to abnormal liver development ***(5)***. However, when placed on an FVB/N background, the mutation leads to colorectal epithelial hyperplasia in adults ***(6)***. Similarly, the knockout of the A-Raf protein kinase gene, which is ubiquitously expressed during development, causes neurological and intestinal anomalies leading to weanling-stage lethality on a C57BL/6 background ***(7)***. Alternatively, when the A-Raf null allele is maintained on the 129/Ola background, the animals survive until adulthood, are fertile, and show no obvious intestinal disorder, though they do retain a subset of the neurological defects. Finally, targeted mutation of the EGF-receptor gene leads to diverse strain-dependent lethalities that occur at the time of implantation on the CF-1 background, that involve placental defects in the 129/Sv strain, or that occur postnatally from multiorgan failure in CD-1 mice ***(8,9)***.

3. Variation in Penetrance

Not only can different genetic backgrounds result in distinct phenotypes, they can also vary the penetrance of, or proportion of animals affected by, a single phenotype. The multifocal inflammatory disorder of TGFβ1 knockout

Table 1
Strain Dependency of Lethal Phenotypes in TGFβ1 Knockout Mice

Strain	Pre-yolk sac lethality	–/– Yolk sac lethality	Autoimmune lethality	+/+
129/Sv X CF-1[a]	69			142
			113	280
C57BL/6[b]		1		20
NIH/Ola X C57BL/6J/Ola[c]		28	22	57
C57BL/6J/Ola[c]		1	0	39

Data represent numbers of animals.
[a]Unless otherwise denoted.
[a]Analysis of E3.5-d blastocysts.
[b] It is important to note that 4 TGFβ genotypes had yolk sac defects (1,+/+; 1, +/–; –/–).
[c] From **ref. *(4)***.

mice on the 129/Sv X CF-1 background affects the heart, lung, liver, stomach, pancreas, skeletal muscle, spleen, lymph nodes, and salivary and lacrimal glands, but not the colon, brain, and kidney ***(1,10)***. However, not all of these organs are always affected in each mutant animal. Hence, the penetrance of this phenotype in the diaphragm is not as complete (50%) as it is in the stomach (80%) (*see* **Table 2**). When the penetrance levels of inflammatory phenotypes on the 129/Sv X C57BL/6J ***(2,11)*** vs 129/Sv X CF-1 ***(1,10)*** backgrounds are compared, differences can be seen. TGFβ1 gene ablation on the 129/SV X C57BL/6 background results in a higher penetrance level of salivary gland inflammation than occurs on the 129/Sv X CF-1 background (80% vs 30%), but a lower penetrance level in liver (70% vs 90%) (*see* **Table 2**).

The Hoxb-4 knockout mouse provides another example. Hoxb-4 gene ablation causes two obvious skeletal changes: a partial homeotic transformation of the axis to an atlas and defective morphogenesis of the sternum ***(12)***. Both phenotypes show incomplete penetrance when assayed in 129/SvEv X C57BL/6J hybrids, yet the sternum defect is completely penetrant when the targeted allele is placed on the inbred 129/SvEv background.

The BMP-4 knockout mouse is noteworthy because, although it presents background-dependent penetrance variability, it shows less than expected background-dependent variation in the types of phenotypes presented ***(13)***. The phenotypes involve embryonic lethalities that occur at the egg cylinder, headfold, and embryo-turning stages of development. Putting the targeted allele on several backgrounds does not appreciably change the types of phenotypes observed, with the exception that an increase in contribution of C57BL/6 in the genetic mix leads to an increased penetrance of the early, egg cylinder lethality and a loss of the late, embryo-turning stage lethality.

Table 2
Variations in Phenotype and Penetrance of Phenotype in TGFβ1 Knockout Mice[a]

Organ	129/Sv X CF-1[b]	129/Sv X C57BL/6[c]
Stomach	80	80
Colon	0	70
Liver	90	70
Pancreas	70	70
Heart	90	90
Lung	90	100
Salivary gland	30	80
Kidney	0	30
Brain	0	20
Mesentery	80	80

[a]Data represent percent of TGFβ1–/– animals that had inflammatory lesions in each organ.
[b] From **ref.** ***(10)***.
[c] From **ref.** ***(16)***.

All TGFβ2 knockout mice on the 129/Sv X Black Swiss background die within a few hours before or after birth. They have multiple developmental defects that can affect the cranial, axial, and appendicular skeleton, and the eyes, ears, heart, and urogenital tract ***(14)***. Of the 25 phenotypes analyzed, 9 were less than 50% penetrant, whereas the remainder were 90–100% penetrant. Although a thorough analysis of TGFβ2 null phenotypes on other backgrounds has not been done, our expectation, based on phenotype and penetrance variation in other knockout mouse strains, is that the penetrance of some of these phenotypes will vary, whereas other phenotypes will be lost or gained.

4. Variation in Expressivity

Not only can phenotype penetrance vary with genetic background, but its expressivity can also be background dependent. The TGFβ3 knockout mouse dies shortly after birth of a cleft plate ***(15,16)***, and on all backgrounds tested the penetrance of the cleft palate phenotype is 100%. However, on the 129 X CF-1 background, only 2% of the homozygous knockout animals have a complete posterior to anterior cleft palate, whereas on the 129 X C57BL/6 background nearly half are complete ***(15)***.

Similarly, dramatic background dependency is also seen in phenotype expressivity in APRT and plakoglobin-knockout mice ***(17,18)***. APRT-deficient mice on a C57BL/6J background develop signs of overt illness earlier (3–4 wk vs 3–4 mo) and die sooner (75 d vs 180 d) than APRT null mice on an out-bred

Black Swiss background ***(17)***. Plakoglobin is a member of the armadillo gene family and is a constitutive component of the desmosomal plaque. It is also a part of the cadherin–catenin cell adhesion complex in adherens junctions. Plakoglobin-null animals on the 129/Sv background die from E10.5 onward with severe heart defects. In contrast, some mutant embryos on the C57BL/6 background die perinatally due to cardiac dysfunction ***(18)***.

5. Identification of Modifier Genes in the Mouse

Allelism in genes that modify the phenotypic outcome of a specific genetic alteration are likely to be the basis for nearly all of the genetic background dependence of knockout mouse phenotypes. Such genes are termed modifier genes and can be identified either by a subjective approach in which a mutant allele is combined with the knockout allele to determine their combined effect on a knockout phenotype, or they can be identified through an objective approach in which they become the object of a search for a gene that alters the knockout phenotype.

We can return to TGFβ1 null mice to provide examples of the subjective approach. Placement of a TGFβ1 null mutation on a severe combined immunodeficiency (SCID) ***(19)*** or RAG2 knockout background (unpublished observations), or combination of a TGFβ1 null allele with the major histocompatibility class II allele ***(20)*** modifies the phenotype by eliminating the autoimmune-like inflammatory disorder that would otherwise kill the TGFβ1 knockout mice around weaning age. From these combined mutations it becomes clear that both lymphocytes and antigen presenting cells are required for development of the inflammatory disorder. However, several of the phenotypes observed in the TGFβ1 null mice, such as increased cardiac expression of ICAM-1 (intracellular adhesion molecule 1) and hyperplastic growth of many epithelial and cardiac cell types, are eliminated when the inflammatory response is inhibited or removed ***(19)***. A TGFβ1 deficiency alone does not cause excessive proliferation of epithelial and heart cells. For these phenotypes to develop, a lack of TGFβ1 must be combined with severe inflammation. Consequently, although *Scid*, *Rag2*, and *Mhc2* can be considered modifiers of *Tgfb1*, the relationship is indirect at best.

The objective approach involves a search for the identity of the modifier gene(s) causing background-dependent variation in the knockout phenotype. Consequently, the identity of the modifier becomes the object of the investigation. Recently, papers describing the genetic basis of such background effects have begun to narrow in on the identification of modifiers. With respect to the TGFβ1 null mouse, Bonyadi and colleagues ***(4)*** have mapped a major genetic modifier of the yolk sac-related embryonic lethality in these mice to the distal region of mouse chromosome 5. They estimate that this locus accounts for

about 75% of the variability and that one or more minor loci account for the rest. The gene has not been identified to date, but it is in a region syntenic with a human chromosomal region associated with the human hemangioblastic telangectasia gene, a disease with some phenotypic similarity to the yolk sac phenotype of the TGFβ1 null mouse.

In humans, mutations in the adenomatous polyposis coli (APC) gene are associated with a number of familial colon cancer syndromes ***(21)***. *Min*/+ mice (multiple intestinal neoplasia) mice are heterozygous for a chemically induced nonsense mutation in exon 15 of the *Apc* gene ***(22)***. Following loss of heterozygosity, these mice develop intestinal and colonic adenomas resembling human APC and Gardener's syndromes ***(23)***. However, the number of intestinal tumors in *Min*/+ mice is affected by the genetic background of the mice. Although C57BL/6 *Min*/+ mice average 29 intestinal tumors, *Min*/+ AKR X C57BL/6 F_1 mice average only 6 tumors ***(23)***. These data suggest that the AKR background possesses a modifier gene(s) which inhibits the tumorigenic effect of the *min* mutation ***(24)***. Backcrosses of the F_1 *Min*/+ with C57BL/6 suggested that only 2 modifier loci were influencing this tumor multiplicity ***(23,24)***. A modifier locus called *Mom-1* (modifier of min) was mapped to the distal arm of chromosome 4 ***(24)***.

It must be cautioned that the identification of a modifier gene by either the subjective or objective approach does not necessarily indicate that the modifier gene is directly acting on the tissue affected. As mentioned before, the TGFβ1 knockout mouse experiment in which hyperplasia of cardiac and epithelial cells was eliminated in the absence of inflammation suggests an indirect relationship between TGFβ1 and SCID, RAG2, or MHC2 gene products. Whether genes with such indirect effects should be considered true biological modifiers will not be discussed here. Operationally, however, no distinction can be made until the modification pathway is delineated. What is important is that regardless of the strength of the relationship between the targeted and modifier genes, the identification of operationally defined modifier loci will reveal novel molecular, cellular, and physiological interactions between genes and their products in the context of complex organisms.

6. Value of Initially Analyzing Null Phenotypes on a Mixed Genetic Background

Knockout mice are nearly always generated by crossing a germline chimera, in which the knockout allele is on a 129 background, with an animal of any desired background, including 129. The resulting offspring are then intercrossed to generate homozygous mutant animals that will either be inbred 129 strain or F_2 generation mice with a 50/50 mixture of 129 and other desired background. With the exception of doing the gene targeting in an ES cell of

another background, putting the targeted allele on a background other than 129 requires a standard backcrossing scheme. For convenience, the first homozygous mutant animals can be produced both on a 129 inbred background and on a mixed background. The question arises as to which background is better for phenotype analysis. Obviously, the more backgrounds the better; however, with limited resources, one must make choices.

We argue here that the background most likely to provide the widest range of phenotypes is a mixed background. This is due to the considerable background dependence of knockout phenotypes discussed previously. On a mixed background this phenotype variation could often play itself out as incomplete penetrance and variable expressivity. These, in turn, would likely decrease as the targeted allele were moved to a more inbred state. Consequently, the mixed background knockouts potentially display a wide range of phenotypes, and those phenotypes with incomplete penetrance and variable expressivity would be candidate phenotypes upon which a modifier gene search could be based. If resources allow phenotype analysis of a second knockout strain, the 129 strain knockout would be most appropriate because it would reveal which of those mixed strain phenotypes may have 129 strain modifiers, and, by elimination, which phenotypes may have modifiers on the other strain of the original mixture. It is for these reasons that we always make our first phenotype screen on a mixed strain. The TGFβ1 knockout mouse provides an important case in point. Had the decision been made to put the knockout allele on the C57BL/6 background, the preorganogenesis lethality would have precluded discovery of the yolk sac and autoimmune disorders.

7. Conclusion

The study of mouse genetics has taken an exciting step forward with the advent of gene targeting via homologous recombination in ES cells. The knockout mice are wonderfully informative because of the unexpected phenotypes that often result. The continued utilization of mixed genetic backgrounds for the analysis of knockout phenotypes will accelerate the identification of modifier loci and lead to a better understanding of the complexities of gene function and interaction at the whole animal level.

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15

Lineage Allocation During Early Embryogenesis

Mapping of the Neural Primordia and Application to the Analysis of Mouse Mutants

Gabriel A. Quinlan, Bruce P. Davidson, and Patrick P. L. Tam

1. Introduction

Fate-mapping and lineage-tracing studies that were performed on mouse embryos maintained in culture have provided a unique insight into the morphogenetic processes that take place during gastrulation and early organogenesis. These experiments allow the analysis of the developmental fate and the differentiation potency of cells that are allocated to different tissue lineages during postimplantation development. Results of lineage analyses using *in situ* labeling and cell transplantation have led to the construction of maps that describe the regionalization of cell fate in the embryonic germ layers and their derivatives at various stages of mouse development. From this spatial description of where cells of a particular lineage are localized at successive developmental stages, it is possible to track the most likely pattern of morphogenetic movement of cell populations in the normal and mutant mouse embryos during gastrulation and organogenesis.

Fate-mapping studies of the mouse embryo at the onset of gastrulation has revealed that the progenitor populations of major germ-layer derivatives such as neuroectoderm, paraxial, and lateral mesoderm and gut endoderm are already present in defined regions in the epiblast ***(1,2)***. Such regionalization of cell fate can be demonstrated by fatemapping even though no morphological differences among cells in the epiblast could be discerned. There is, however, distinct localization of gene transcript in the epiblast, but as yet there has not been any simple and direct correlation between the cell fate and the expression of specific

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genes ***(3)***. There is experimental evidence suggesting that cells in the epiblast are pluripotent and display no lineage restriction ***(1,4–6)***. In view of this observation, the regional differences in cell fate may reflect, instead of lineage determination, the order of allocation of cells to different tissues during embryonic development. Such an order could be either spatial (depending on the relative position of the cells to the site of active morphogenetic movement) or temporal (the timing of their recruitment to the component parts of the final body plan), or both. It is possible to reconstruct from the fate maps the pattern of cellular recruitment and tissue movement during gastrulation and organogenesis. The task now is to demonstrate by direct *in situ* tracking of cell movement in live embryos that such morphogenetic processes indeed occurs. With the technical advance of using the green fluorescent protein (GFP) as a vital cell marker ***(7)***, the possibility of such studies is certainly not remote.

The experimental strategies outlined here detail the methods used to map the location and the morphogenetic movements of the neural primordia during gastrulation and neurulation. In this chapter, we focus on the some aspects of the application of some recently developed cell labeling reagents to lineage analysis and provide a protocol to introgress a *lacZ* transgenic marker onto a mutant background for the analysis of neural differentiation of the mouse embryo. More in-depth discussions of the application of *lacZ* transgenic mice in fate-mapping experiments and the techniques for cell marking and transplantation can be found in chapters written by Beddington and Lawson ***(8)***, Trainor and co-workers ***(9)***, and Quinlan and colleagues ***(10)***.

2. Experimental Strategy

2.1. Dissection of Embryos

Decidua are isolated from pregnant mice at the appropriate gestational ages. Embryos are at the gastrulation stages at 6.5–7.5 d post coitum (p.c.) Neurulation begins with the formation of neural tube at 8.25 d p.c. and the neural tube is completely closed in the cranial region by 9.5 d p.c. Neural crest cells begin to migrate before neural tube closes and colonise the craniofacial tissues by 10.5 d p.c. ***(11)***. The techniques of microdissection and *i*n vitro culture of mouse embryos have been described by Tam ***(12)*** and Sturm and Tam ***(13)***.

2.1.1. Gastrulation-Stage Embryos

The decidua are pear shaped and embryos are located in the antimesometrial half (narrower end) of the decidua. The decidua is cut longitudinally from the broader to the narrower end using a pair of Wecker's iridectomy spring scissors (FST #15011-11) to expose the embryo. The embryo is then lifted out of the decidua and Reichert's membrane is then removed. This is done by first pinning down the membrane one side of the embryo near the junction between the

embryonic and extraembryonic part. Using a second pair of dissecting forceps, the membrane is grasped immediately adjacent to where it is pinned down and then torn towards the distal end of the embryo. Once the membrane is torn beyond the tip of the cylindrical embryo, it will retract to the extraembryonic part of the embryo. Any excessive membrane is then trimmed off along the rim of the ectoplacental cone.

2.1.2. Early-Somite-Stage Embryos

The early-somite-stage embryos are three to four times the size of the egg cylinder stage embryos; therefore it is difficult to cut the decidua using irrecdectomy scissors without puncturing the yolk sac. If the yolk sac is damaged during dissection, fluid leakage results and the embryos will not culture normally. To expose the embryo, the top of the decidua is removed by making a circular cut using sharp dissection forceps and a longitudinal slit is made along one side of the decidua. The embryo can then be dissected by removing the surrounding decidual tissues and Reichert's membrane.

2.2. In Vitro Culture of Embryos

In vitro culture systems have been developed so that experimental manipulation can be performed directly on the postimplantation embryos. **Table 1** summarizes the conditions for culturing mouse embryos of different postimplantation stages.

Postimplantation embryos isolated from pregnant mice can be cultured for a maximum of 48 h without compromising development. Embryos of 6.5-d p.c. can be cultured in the same medium for the duration of culture. Embryos that have commenced gastrulation prior to dissection culture better than pre-streak-stage embryos. Culturing embryos of 7.5 d p.c. onward requires the medium to be changed after 24 h. Embryos cultured in vitro develop at a slower rate than their in vivo counterparts. However, the majority of cultured embryos displays normal morphology and expresses the appropriate molecular markers, such as *Brachyury*, *Mox1*, *En2*, *Otx2*, *Hnf3*β, and *Lim1* that are indicative of proper tissue differentiation.

2.3. Method of Lineage Tracing

There are essentially two methods of lineage tracing; *in situ* labeling of a population of cells or transplantation of cell populations. *In situ* labeling involves the intracellular injection of horseradish peroxidase (HRP) or fluorescent dyes directly to label single cells. Alternatively, groups of cells can be labeled by introducing cell membrane markers such as lipophilic dyes to the immediate vicinity of the cells. *In situ* labeling allows the precise labeling of a defined population of cells with minimum disruption to cellular contacts. The

Table 1
The Composition of Culture Media and In Vitro Conditions for Whole-Embryo Culture from 6.5-day Early Gastrula to 9.5-day Forelimb Bud Stage

Media type	DMEM	Sera	Supplement
DRH (1:2:1)	0.5 mL	1.0 mL (rat serum) 0.5 mL (human cord serum)	Glutamine (2 m*M*) Penicillin (50 IU/mL) Streptomycin (50 μg/mL)
DR (1:1)	0.5 mL	0.5 mL (rat serum)	As above
DR (1:3)	0.25 mL	0.75 mL (rat serum)	As above

Vessel	Volume	Gas ratio*	No. of embryos	Starting age (d p.c.)	Maximum culture time
4-Well chamber	0.8 mL per well	5% CO_2 95% air	8–10 per well	6.5	48 h
Roller bottle	1.2 mL per bottle	20% O_2 5% CO_2, 75% N_2	5–8 per bottle	7.5	72 h
Roller bottle	1.2 mL per bottle	5% O_2 5% CO_2, 90% N_2	3–4 per bottle	8.5	48 h

*Gas ratio varies during culture as indicated.

major shortcoming of *in situ* labeling for lineage analysis is that the label may not last for the whole course of the experiment. As cells divide, the label is distributed between the daughter cells and may result in the label being diluted to below the sensitivity of detection.

Cell transplantation has been employed to test the developmental fate of embryonic cells in an environment akin to their original location in the embryo. To track the descendants of the transplanted population, cells have to be marked so that they can be distinguished from the host cells. Marking cells for transplantation is accomplished by labeling the cells with exogenous markers such as wheat germ agglutinin-conjugated collodial gold and lipophilic dyes. The transplanted cells may also be obtained from transgenic embryos that express a reporter construct such as *lacZ*, alkaline phosphate, and GFP.

For both cases the tissue of interest is isolated from the donor embryo. If marked by exogenous labels, the tissue explant is first incubated in the colloidal gold preparation or lipophilic dyes. These labeled cells are then transplanted to the host embryo. With the transplantation of cells from transgenic donor embryos, no incubation step is required. After isolation the cells can be transplanted directly to the host embryo. Cell transplantation results in more disrup-

tion of cellular contacts than does *in situ* labeling, due to the insertion of a foreign population of cells. However, the advantage of cell transplantation is that cells could be tested for their developmental potency by transplanting them to heterotopic sites. In addition, other experimental manipulation of the cells such as incubation with growth factors and transient coculture with other cells may be performed prior to cell transplantation, depending on the experimental design. A significant advantage of the transplantation approach is the ability to exploit the potential of genetic markers to follow cell fate. When donor cells of transgenic embryos are used, there is no dilution of the label during the course of culture. Furthermore, different transgenic lines ranging from ubiquitous expression, to tissue-specific expression are available for different experiments. Further discussion of the use of transgenically marked cells for lineage analysis can be found in Trainor and colleagues ***(9)***.

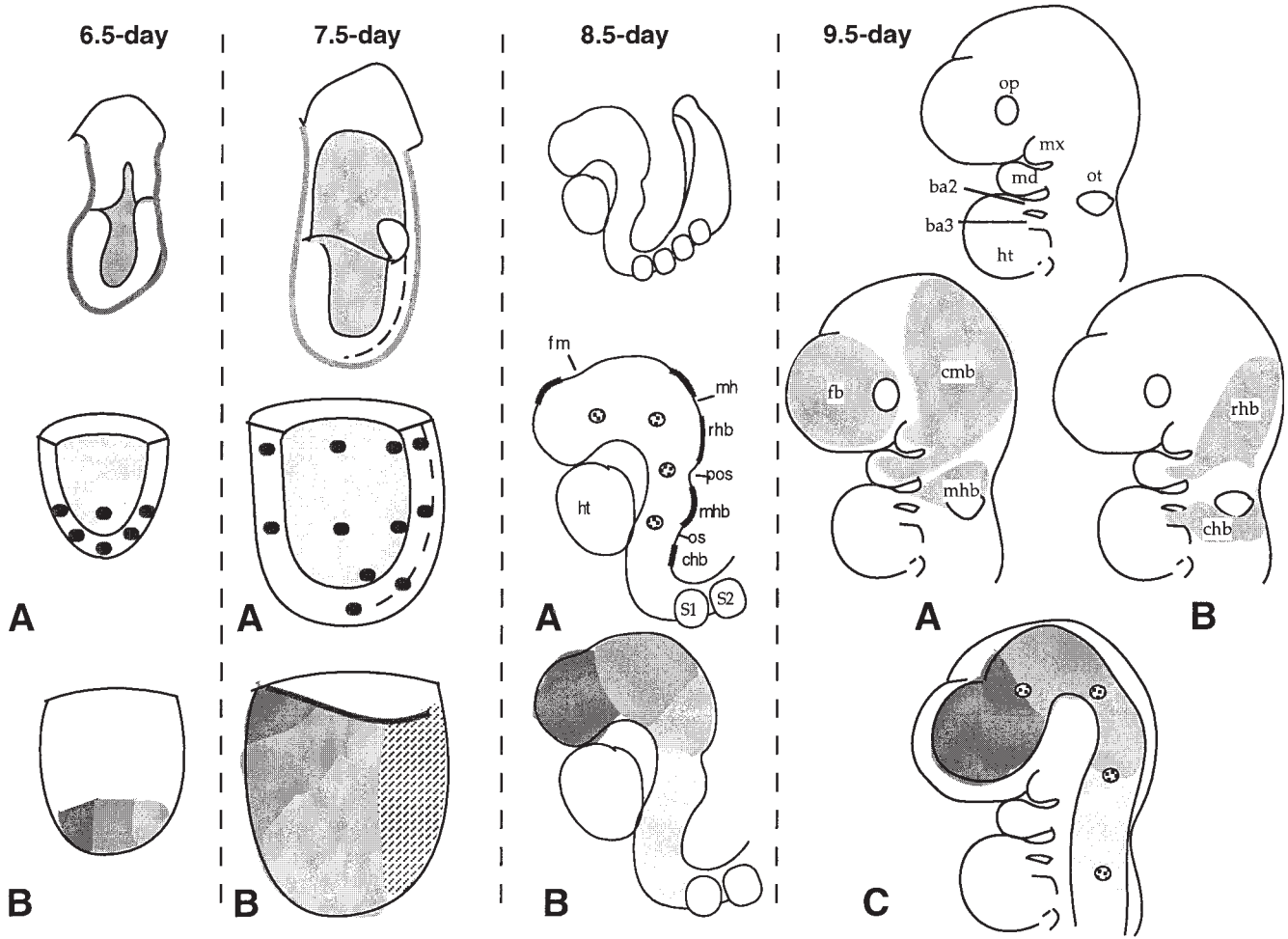
2.4. Requirement for Lineage Tracing Experiments

2.4.1. Identification of the Population To Be Tested

Before *in situ* labeling or cell transplantation can be carried out a reliable method for reproducibly identifying the population of interest must be established. There are essentially two methods available: the use of morphological landmarks or coordinate reference points. The method used depends on the age of the embryo that is to be labeled. Often a combination of both methods provides the most efficient system for fate mapping.

2.4.1.1. Early-Streak-Stage Embryos

At the onset of gastrulation, the best morphological reference point is the primitive streak. The primitive streak identifies the anterior-posterior axis. Any further precision in labeling early- to mid-streak-stage embryos requires the use of coordinate reference points. The first extensive fate mapping study of the epiblast carried out by Lawson and colleagues ***(1)*** by marking cells that are localized to one of the 11 sectors in the epiblast. Since endodermal cells in the extraembryonic region of the embryo tend to remain in constant relative position in the yolk sac during development, they are labeled at the same time when the epiblast cell is labeled to provide a spatial reference of the original location of the epiblast cells (*see* **ref. *1*** for a detailed discussion of the labeling strategy). More extensive mapping studies of the distal region of the epiblast (which contains the neural primordium) require finer subdivision of this region (*see* **Fig. 1**). To carry out labeling of cells in this region the distal most point of the epiblast was used as a reference point (the distal site). Two other sites are delineated in the distal epiblast that are at a 30° angular coordinate anterior and posterior to the distal site, respectively ***(14)***.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2.4.1.2. Late-Streak-Stage Embryos

By 7.5 d p.c. the streak is fully extended and the node has formed, thus providing other morphological landmarks for cell fate studies. As the node can be identified as a morphological structure, it is possible to label either the dorsal or ventral surface for lineage analysis studies ***(15,16)***. Labeling cells within the streak requires the use coordinate reference points ***(17)***. Embryos of the same developmental stage may vary in size, and therefore the length of the streak also varies. Sites within the streak can be labeled according to a ratio of the entire streak length, for example, $^1/_3$ to $^1/_2$ of the proximal-distal length of the streak (Quinlan and Tam, unpublished).

Fig. 1. The fate-mapping studies of the neural primordia in embryos during gastrulation and neurulation. The figure at the top of each panel is a schematic representation of respectively the 6.5-, 7.5-, 8.5-, and 9.5-day embryos. Cell populations that are destined for specific parts of the neural tube are given the same shading patterns in the germ layers and the neural tube at the different stages of development. 6.5-day early gastrula: The fate mapping studies of the neural primordia at the onset of gastrulation. **(A)** The black circles indicate the sites, in the distal epiblast, that have been mapped in order to identify the location of the neural primordia. These fate-mapping studies indicate that the neural primordia are restricted to the distal region of the gastrulating embryo ***(1)***. Further refinement of these studies have indicated that there is a bias in the craniocaudal colonisation of the segments of the neural tube even at this early stage of development ***(14)***. In **(B)**, the shaded regions indicate the prospective domains of the neural tube that different regions of the distal epiblast may colonize. 7.5-day late gastrula: The fate-mapping studies of the prospective neural domains in the ectoderm of the late-streak-stage embryo. In **(A)**, the black circles indicate the sites that have been mapped in the embryonic ectoderm. There is a distinct pattern of distribution of clonal descendants of cells at different sites of the ectoderm to specific segments of the neural tube along the craniocaudal axis, which is depicted in the fate map shown in **(B)**. 8.5-day early-somite embryo: In **(A)** The spots in the neural plate indicate the sites that were labeled for mapping the boundary between major brain segments. In **(B)** and 9.5-day **(C)**, results of this mapping experiment show that four sites that are identified by the position of transverse sulci in the early neural tube mark the junction between forebrain and midbrain (fm), midbrain and rostral hindbrain (rhb = rhombomere 1–2, mh = junction) and rostral and middle hindbrain (mhb; pos = preotic sulcus) and middle and caudal hindbrain (chb; os = otic sulcus; from **ref. *18***, s = somite). When the neural crest cells associated with each of the four major brain parts are tested for their developmental fate, they are found to colonize the craniofacial structures of the 9.5-day embryo in a segmental manner [9.5-day **(A)** for neural crest cells of forebrain (fb), caudal midbrain (cmb), and middle hindbrain (mhb); 9.5-day **(B)** for neural crest cells of rostral hindbrain (rhb) and caudal hindbrain (chb)].

A Cartesian coordinate system has been used to locate the sites of fate mapping for the late-streak-stage embryos. Essentially, a grid system is mapped onto the embryo and the site of interest is determined by its relative position along the proximal-distal and anterior posterior axes of the embryo (*see* **Fig. 1**). However, fate mapping of the late-streak-stage embryo is complicated because gastrulation is complete and the embryo now consists of three germ layers. It is difficult to label cells within, or transplant cells to, one of the germ layers without inadvertent contamination of cells in the adjacent layer. This makes analysis of such fate mapping studies more difficult.

2.4.1.3. Early-Somite-Stage Embryos

Once the embryo has reached the early-somite-stage of development, there are many morphological landmarks that can be used for cell fate lineage analysis experiments. For mapping the cranial paraxial mesoderm or the prospective brain segments, there are conspicuous constrictions in the neural plate that partition the cephalic neural tube into distinct segments. The neuromeric junctions are used to delimit the territory of each neuromeres (*see* **Fig. 1**) and the corresponding somitomeric segments in the paraxial mesoderm ***(18,19)***.

2.4.2. Cell Labels for In Situ *Lineage Analysis Studies*

There are a number of labels available for *in situ* labeling studies. There are a range of fluorescent carbocyanine dyes (*see* **Table 2**) and carbocyfluorescein diacetate succinimidyl ester compounds (CFSE) (Molecular Probes). CFSE is a cytosolic marker and can be visualised either by fluorescence using fluorescein isothiocyanate, conjugated (FITC) filter set or by immunostaining of the fluorescein moiety of this compound ***(20)***. **Table 2** summarizes the method of preparation and the characteristics of these fluorescent markers.

2.4.3. Analysis of Results of Fluorescent Labeling

Embryos labeled with fluorescent dyes can be analyzed by fluorescence microscopy. The filter sets required for detection of the carbocyanine dyes is listed in **Table 2**. For a more accurate analysis of the patterning of staining, confocal microscopy can be used. Such analysis allows the determination of the depth of labeling and therefore provides a preliminary identification of the tissue layers containing labeled cells in whole-mount specimens. However, to obtain precise information on the location of labeled cells in the tissue layers of mouse embryos requires histology. To preserve the fluorescent labels, this can be performed on cryocut sections of embryos ***(21)***.

One of the problems associated with using fluorescent dyes is that the signal fades over time and with exposure to UV light, which is necessary for detec-

Table 2
Carbocyanine Fluorescent Dye Used in Lineage-Tracing Experiments

Dyes	Color of solution	Fluorescent color	Excitation wavelength (nm)		Filter for visualization	Solubility in ethanol
Carbocyanine dyes			Abs	Ems		
CM-DiI	Pink	Red	553	570	Rhodamine	Moderate
$DiOC_{18}$	Orange	Green	484	501	FITC	Low
SP-$DiOC_{18}$	Dark orange	Green	497	513	FITC	Poor
5,5'-Ph_2-$DiOC_{18}$	Orange	Green	496	513	FITC	Moderate
DiA/4-Di-16-ASP	Red	Green	491	613	FITC	Good

The range of lipophilic dyes available is constantly expanding (http://www.probes.com). **Table 2** consists of a list of those dyes that have been tested in our laboratory. All stock solutions are prepared at a concentration of 5 mg/mL. The first choice of carbocyanine dyes for *in situ* labeling in embryos is DiI. It has moderate solubility and can be diluted in 0.3 *M* sucrose without precipiation problems. The difficulty has been identifying a second carbocyanine dye, with different fluorescent properties, that can be used in double-labeling experiments. $DiOC_{18}$ was the original candidate; however, it has low solubiltiy in both ethanol and on dilution in sucrose. These properties have made it difficult to work with for *in situ* labeling. DiA is highly soluable; however, its adherence propertities means that it does not attach to the glass coverslip in the hanging-drop setup. Furthermore, results using this dye suggest that the dye diffuses from the site of injection making it unsuitable for *in situ* labeling experiments. From the new range of DiO solutions available, 5,5'-Ph2-DiOC18 has working properties most like that of DiI. Abbreviations: Abs, absorption; Ems, emission.

tion of the signal. In the past, to obtain a permanent signal the fluorescent label was photooxidated, this involves the precipitation of a diaminobenzidine (DAB) reaction product by exposure to UV light. There are several limitation to this system including lengthy exposure to UV light for precipitation to occur. This means that only one embryo can be processed at a time making it a very inefficient detection method. Furthermore, the detection is also plagued with the problem of sensitivity, high background, and inconsistent results of staining ***(15,20)***. A more efficient system for obtaining a permanent record of dye-labeled embryos has been developed. This system involves the specific binding of an antibody to the fluorescein portion of CFSE. Once the primary antibody is bound, an insoluble reaction product is generated using an HRP-conjugated antibody and DAB. This method is more efficient, as it is possible to perform bulk processing of many embryos. There is less of the background problem associated with photooxidation and the label survives histology and paraffin sectioning ***(20)***.

2.4.4. Multiple Fluorescent Labeling

The major advantages of using fluorescent dyes for *in situ* labeling is that a variety of color reagents are available, making it possible to perform multiple labeling experiments. Several cell populations can be labeled within the one embryo. After culturing, different combination of excitation and transmission filters are used to analyze the morphogenetic movement of these two populations, relative to one another, during the course of development ***(14)***.

2.4.5. A Protocol for Confocal Analysis of Double-Labeled Primitive-Streak-Stage Embryos

The micromanipulation setup consists of two manipulators (Leitz, cat. nos. 335-520-137 and 335-520-138) that are clamped to a base plate (Leitz, #335-520-139). Each manipulator holds up two instrument holders (Leitz, cat. nos. 335-520-142 and 335-520-143). One instrument holder is attached to micrometer syringe (Alga, Wellcome or Gilmont), which is used for holding the embryo, the other instrument holder is attached to a de Fonbrune syringe (Alcatel), which is used to inject dye or transgenic cells into the embryo. The 6.5- and 7.5-d embryos can be injected in hanging drops of culture medium in a manipulation chamber or in a paraffin oil-covered drop in a Nunc (two- or four-) chambered slide that has the plastic cover removed. For older-stage embryos, manipulation may be performed in drops of medium kept under paraffin oil on the lid of a Petri dish.

After dissection, dye labeling and culture, embryos were fixed overnight at 4°C in 4% paraformaldehyde, in preparation for confocal analysis. For confocal microscopy, embryos were analyzed in whole mount. As the embryos are relatively clear, it is possible to analyze thick tissue fragments effectively. For 8.5-d embryos, the extraembryonic tissue are dissected away and the embryo is mounted flat and dorsal side up. Both 6.5- and 7.5-d embryos are analyzed intact and mounted in a sagittal plane, with the anterior-posterior axis marked on the slide for reference of the location of the dye-labeled cells. As the embryos are relatively thick, they cannot be mounted flat under a coverslip. Therefore, to prevent excessive distortion of the embryo's small blue-tac feet are placed in the four corners of the coverslip. This provides sufficient space between the slide and the coverslip for the embryo. The slide was filled with phosphate-buffered saline (PBS) and sealed with nail polish.

The confocal microscope consists of a scanning device linked to a standard Lietz Diaplan microscope. The microscope is linked to a computer system for analysis. The computer consists of a modular computer, based on a Motorola 68020 processor with VersaModule Eurocard (VME) bus that runs the control units. The Leica confocal laser scanning microscope (CLSM) software is

responsible for image recording and evaluation and allows the construction of the images. The microscope can be used in three ways: as a conventional fluorescent microscope; as a nonconfocal laser scanning microscope (this is used for transmission light microscopy and produces higher contrast images than conventional microscope); and as a confocal laser scanning microscope.

The laser scanning unit is linked to the standard microscope, the region of the embryo containing the fluorescent label can be preselected using conventional fluorescent techniques. The DiI-labeled cells are detected with a rhodamine filter, which has an absorption peak wavelength of 574 nm. The DiO-labeled cells are detected with a FITC filter, which has an absorption peak wavelength of 495 nm. Once the region of interest is identified, the microscope is simply switched from conventional to scanning microscopy. Scanning movements in the XZ plane are achieved by a special Z stage. This stages moves the embryo with high precision through the Z plane. Optical sections are taken at the required sites through the embryo. Using nonconfocal scanning procedures, transmission images of the embryonic region that contains the labeled cells are taken. The optical sections of the fluorescent dyes are compiled and overlaid on the transmission image. The resulting image depicts the location of the labelled cells within the embryo (**Fig. 2C** and **D**).

2.4.6. Analysis of Cell Fate by Cell Transplantation

For cell transplantation studies to be heritable, a cell autonomous marker is required. The most commonly used is the reporter gene β-galactosidase, which is encoded by *lacZ*. b-galactosidase is easily detected by a sensitive X-gal histochemical assay ***(9)*** (**Fig. 2A** and **B**). In addition to X-gal, there are now many histochemical enzyme substrates available that may be used in transplantation experiments involving transgenic lines. These substrates include magenta-gal, red-gal, and salmon-gal (Biosynth AG). Following the histoenzymatic reaction, these substrates produce differently colored products in the transgenic cells.

In addition to the commercially available ROSA26 mice (The Jackson Laboratory, cat no. JR2073) produced by gene trap integration of a β-geo reporter into the mouse genome, another lineage-ubiquitous transgenic line is available in which the *lacZ* gene is regulated by the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase promoter and the transgene is expressed in all lineages at high levels during development ***(22)***. Perhaps one of the most exciting discoveries for cell transplantation analysis is the mutation of the green fluorescent protein (GFP), which suppresses its thermosensitivity enabling it to be detected at 37°C ***(23)***. A stable embryonic stem (ES) cell line that expresses GFP has been established. These stem cells have been introduced into living embryos and, although this has yet to be demonstrated, the differentiation of GFP-marked cell clones in postimplantation chimeras may be examined over

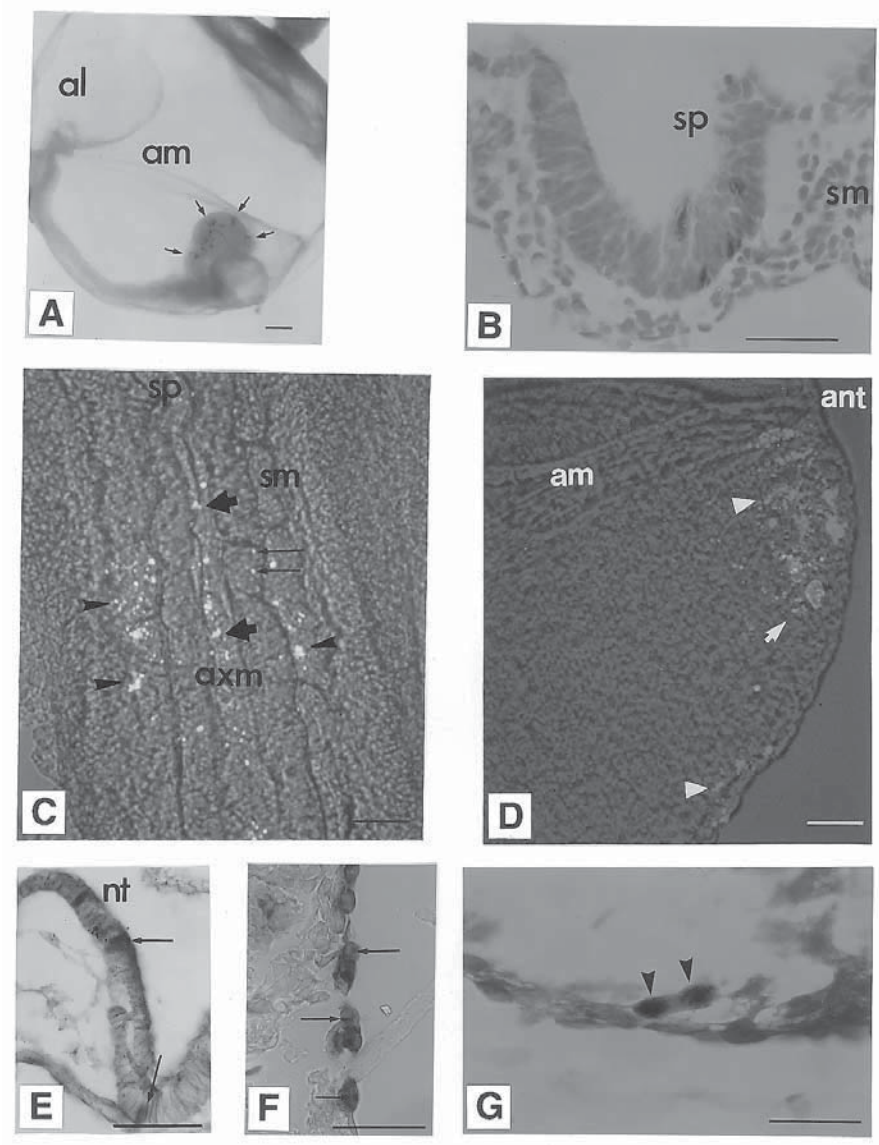

Fig. 2. **(A)** The localization of X-gal-positive cells (arrows) in the forebrain and midbrain of an embryo after grafting the transgenic cells to the anterior region of the distal cap of a 6.5-d embryo and culturing for 48 h to the early-somite stage. Bar = 50 μm. **(B)** Histological section showing the localisation of X-gal-positive cells in the spinal cord of an embryo after grafing the transgenic cells to the posterior region of the distal cap of a 6.5-d embryo and culturing for 48 h to the early-somite-stage. Bar = 50 μm. **(C)** Confocal image of the spinal cord of and early-somite-stage embryo with DiI-labeled cells in the somite (arrowhead), the axial mesoderm (large arrow), and the

time. A dynamic fate-mapping study such as this gives a more complete understanding of the morphogenetic movements that take place during development *(7)*. The generation of mouse strains that stably expresses the GFP transgene will be an extremely useful source of marked cells for lineage analysis. The precise expression pattern of the GFP marker has not yet been fully investigated. To obtain a nonlineage bias GFP marker, the transgene may have to be driven by the promoter of a housekeeping gene encoding HMG or β-actin and coupled with an ubiquitous enhancer such as the CMV or SV40 enhancer. It is also important to establish that the expression of the GFP marker is sufficiently robust for repetitive fluorescence detection and that the expression of this marker does not alter the developmental potency and the morphogenetic behavior of the transgenic cells.

Figure 3 outlines the protocol used for cell transplantation studies. Cells are isolated from the donor transgenic embryos and then grafted to the host wild-type embryo. After grafting the embryos are cultured and then stained, using the X-gal histochemical assay, to identify the location of the graft-derived cells. **Figure 3** depicts an orthotopic transplantation that is used for fate-mapping studies. In these transplantations, cells are isolated from one region in the donor embryo and then grafted to the same site in the host embryo. In the case of heterotopic transplantation, the transgenic cells are isolated from one site in the donor embryo and then transplanted to a different location in the host embryo. These heterotopic transplantations can be used to test the potency of cells by determining if there is a restriction in the types of tissues to which the donor cells can contribute.

Cell transplantation studies can be used not only to map the fate of cells in particular regions of the embryo, but can also be used in conjunction with studies cell potency and specification. Cells transplanted to a particular location in

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neural tube (small arrow). Bar = 100 μm. **(D)** Confocal image of a doubly labeled late-streak-stage embryo showing the localization of DiI and DiO in the anterior region of the embryo. Bar = 100 μm. **(E)** Histological section showing the localisation of X-gal-positive cells in the cranial neural folds of the early-somite-stage embryo (arrows). The embryo was stained for both β-galactosidase and alkaline phosphatase (APase) (dark blue background staining. Bar = 50 μm. **(F)** Immunhistochemical staining of *LacZ*-positive cells in the endoderm, also showing a cytoplasmic spot of APase staining in the primordial germ cells (arrow). Bar = 50 μm. **(G)** Histological section showing X-gal-positive cells in the myocardium of an early-somite-stage embryo. Cytoplasmic staining indicates the expression of sarcomeric tropomyosin in both the host and graft derived cells located in the myocardium. Bar = 25 μm. Abbreviations: al, allantois; am, amnion; ant, anterior; axm, axial mesoderm; nt, neural tube; sm, somite; sp, spinal cord.

the embryo may reflect the fate of cells in that region. However, it is also possible that they do not differentiate according to that fate. Cell transplantation studies in combination with *in situ* gene expression studies can be used to determine the specification state of the graft derived cells (**Fig. 2E–G**).

3. The Mapping of Neural Primordium

3.1. Identification of the Location of the Neural Primordia at the Onset of Gastrulation

The fate-mapping techniques outlined in this chapter have been used to successfully map the location of the neuroectodermal precursors at the onset of gastrulation, and their subsequent expansion during gastrulation and neurulation (*see* **Fig. 1**). *In situ* labeling and grafting studies have demonstrated that the precursors of the neuroectoderm are mostly confined to the distal cap region of the epiblast at the onset of gastrulation ***(1)***. Furthermore, cells in this distal cap region display a bias in their craniocaudal colonization of the neural tube. Cell located in the anterior region of the distal cap tend to colonize cranial regions of the neural tube, whereas cells located in the posterior region tend to colonize more caudal regions of the neural tube in the early-somite-stage embryo ***(14)***.

3.2. Regionalization of the Brain

Cell transplantation studies using wheat germ agglutinin (WGA)-gold-labeled cells has identified the regionalisation of the prospective brain segments in the late-primitive-streak-stage embryo. The anterior-posterior polarity of the neural tube identified in the early-streak-stage embryo persists in the late-streak-stage embryo ***(24)***. Cells labeled in the distal cap region, at the onset of gastrulation, and cultured to the late-streak-stage, expand to occupy the prospective neural domains of the ectoderm in a regionalised fashion that was demonstrated by previous ***(24)*** cell transplantation studies ***(14)***. Different brain segments are, however, not represented in the final tissue proportions. This early pattern therefore requires subsequent differential expansion of its component parts.

3.3. Segmentation of Neural Progenitor Tissues

The cranial–caudal bias of the neural primordia present at the onset of gastrulation is also present in the cranial paraxial mesoderm at a later stage of development, in the 8.5-d early-somite-stage embryo. Fate-mapping studies of the cranial paraxial mesoderm have shown that there is a strict cranial–caudal distribution of the paraxial mesoderm to the craniofacial structures during morphogenesis. The branchial motor neurones and the distribution of the rhombencephalic neural crest cells display a two-segment periodicity, this is also observed for the paraxial mesoderm during the formation of the branchial

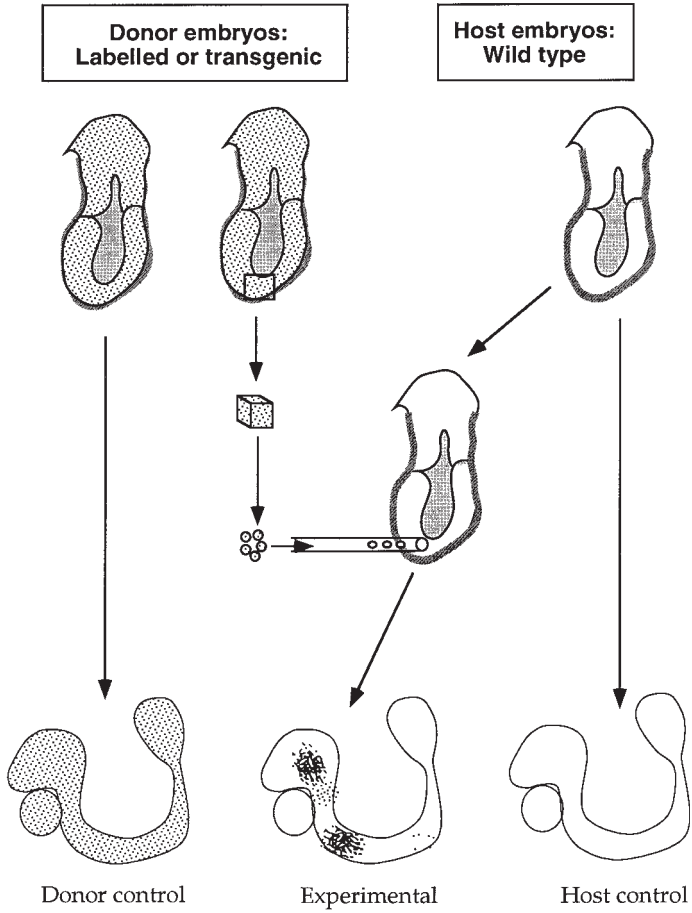

Fig. 3. Schematic representation of the protocol used for lineage analysis by cell transplantation experiments. The tissue of interest is isolated from the transgenic donor embryo. In this example, the donor cells are isolated from the distal cap region of a 6.5-day embryo. Once the tissue fragment is isolated, it is cut up into clumps of approximately 5–10 cells. These donor cells are grafted orthotopically to the host wild-type embryo, which is then cultured to the early-somite-stage. The transgenic cells from the host embryos can be detected by incubation in X-gal staining solution. The donor control, and experimental and host control embryos are all incubated in X-gal staining solution. All the cells of the donor control produce a blue reaction product when stained with X-gal. The host control embryos that do not carry the transgene remain clear after incubation in X-gal. In the experimental embryo, only those cells originally derived from the transgenic donor embryo stain blue after incubation in X-gal staining solution. Using this method, the fate of cells in the distal cap region can be determined. Transplantation of transgenic donor cells to other regions in host wild-type embryos can be used to map the fate of cells in the epiblast of a 6.5-d embryo.

arches ***(18)*** (*see* **Fig. 1**). Furthermore, paraxial mesoderm and neural crest cells arising at the same segmental position share common destinations, suggesting that a basic meristatic pattern is established globally in the neural plate ectoderm and the paraxial mesoderm during early mouse development.

In the mesenchyme adjacent to the brain and the primordia of the sense organs (e.g., optic evagination and otic vesicles), neural crest cells intermingle with cells derived from the paraxial mesoderm (the somitomeres). However, in the branchial arches, neural crest cells are localized to the subectodermal region and are peripheral to the somitomere-derived mesenchyme in the core of the arch ***(19)***. The mixing and segregation of the neural crest cells and the somitomeric mesenchyme, respectively, in the cranial mesenchyme and the branchial arch may be critical to the patterning of the skeletogenic and myogenic tissues during craniofacial morphogenesis.

Taken together, the results of these extensive fate-mapping studies have identified the location of the neural primordia and the expansion of this population during gastrulation. They have also demonstrated the spatial relationship between the specific brain segments and the paraxial mesoderm during morphogenesis of the neural plate.

4. Analysis of Neural Development Using Targeted Null Mutants

4.1. General Consideration

Targeted mutation of genes by homologous recombination has provided a rich and diverse source of mouse mutants. In these mutants, insertional or replacement mutagenesis often results in the loss of function by the creation of a null mutation in the genes of interest. In **Table 3**, we have listed a few mutations that, when presented in the homozygous state, lead to significant morphological defects of the craniofacial structures. These defects include the complete absence of major parts of the brain such as the disruption of tissue patterning and lack of overt segmentation of the neural tube (*Hnf3*β), the loss of major parts of the brain (*Lim1, Otx2*) or specific regions of the brain (*En1, Krox20*) and the failure of neural tube formation (*Pax2, twist).* These mutants are chosen because the developmental defects are manifested at the neurulation and early organogenesis stage, and therefore are amenable to the types of lineage analyses that can be performed using the in vitro experimental strategy outlined in this chapter.

Apart from the description of the abnormal morphology of the mutant embryos, very little is known of the cellular and molecular basis of these mutant phenotype. **Table 3** lists the target tissues that might be affected by the mutations. These include the neuroectoderm, the cranial mesoderm, and the neural crest cells. The mutation may impact on the development of the craniofacial structures in several ways. First, the mutation may affect the specification of

the progenitor population of a specific cell lineage and thereby results in the deficiency of, e.g., the neural plate cells. Second, the mutation may disrupt the morphogenetic movement of specific cell population, resulting in the misappropriation of tissue types that must be assembled for neurulation or the abnormal migration of the neural crest cells. Third, the mutation may compromise the competence of the tissues to undertake inductive interactions that are critical for neural specification, neural tube morphogenesis, and the patterning of neural and mesoderm tissues. Fourth, the mutation may affect the developmental potency of the cells such that they cannot complete differentiation even after normal migration and induction.

The normal allele of some mutant genes affecting craniofacial development encodes gene products that regulate cellular growth and differentiation. Depending on the nature of the gene product and the immediate downstream response to the activity of the mutant gene, the gene may act either autonomously within the cell population that normally expresses this gene or it may affect other cell populations that are not directly involved and do not require the activity of the normal allele of this gene, i.e., a non-cell-autonomous effect. The mutations illustrated in **Table 3** primarily involve genes that encode for transcription factors and therefore would be acting a priori in a cell-autonomous manner. As such, the impact of the mutation on cell potency and lineage specification in the heterotopic tissue environment is conceptually easier to interpret in transplantation experiments.

To carry out these transplantation experiments, two important issues need to be considered. One is the availability of the tissue to be transplanted and the second, is the ease of delivery of this tissue to specific tissue sites. Availability is important when considering the number of donor embryos that may be required to obtain the appropriate null mutant genotype for transgenic transplantation studies. To ensure a high rate of success of delivery, it is important that the site of gene expression can be easily targeted for transplantation. These sites may include the germ layers, the primitive streak, the node or specific segmental structures in the neural tube, and the paraxial mesoderm.

4.2. A Protocol for the Introgression of a Null Mutant Allele onto a Transgenic lacZ Background

To perform transplantation studies, it is necessary to generate donor embryos that contain both the transgenic marker gene and the null mutation of interest. The strategy used for the generation of donor embryo is determined by the homozygous phenotype of the marker strains used. Often, mice carrying the null mutation of interest are inflicted with an early embryonic lethality and therefore have to be maintained as heterozygous individuals. The strategy required for the generation of null mutant donor embryos carrying transgenic reporter genes is

Table 3
Selected List of Mutations That Affect the Early Development of the Cephalic Neural Tube

Gene	Gene Product	Expression domain	Neural phenotype	Likely target tissue affected
Otx2	*Orthodenticle*-related homeodomain transcription factor	Entire epiblast (prestreak) Anterior region of all three germ layers (midstreak) Fore and mid brain (10.5 d p.c.)	Forebrain and midbrain structures absent	Epiblast, neural plate and axial mesendoderm
Lim	LIM box containing homeodomain transcription factor	Organizer (prestreak) Mesodermal wings, primitive streak, and anterior endoderm (early to midstreak) Primitive streak and prechordal mesoderm (late streak)	Forebrain and midbrain structures absent. Node absent No head process or prechordal mesoderm	Node and axial mesendoderm
En1	Homeodomain transcription factor	Dorsolaterally in the anterior epiblast (one somite stage). Later in presumptive midbrain/hindbrain junction	Deletion of midbrain and portion of hindbrain region	Embryonic ectoderm
*Hnf3*β	Forkhead related winged helix transcription factor	Organizer (prestreak) Node, notochord, and midline mesoendoderm	Dorsal-ventral patterning of neural tube disrupted. Node and head process absent	Node and node derivatives (head process and notochord)
Krox20	Zinc-finger transcription factor	Two transverse strips prior to segmentation of the neural tube. Rhombomeres r3 and r5 after formation of the hindbrain	Reduction or absence of rhombomeres r3 and r5 Fused segmentation of the hindbrain (r2/r4/r6).	Neurectoderm
Pax2	Paired box-containing transcription factor	Neural plate encompassing forebrain and anterior hindbrain (late streak 7.5-day) Restricted to midbrain/hindbrain boundary (9.5-day)	Failure of neural tube closure in the mid brain region	Neurectoderm

References: *Hnf3*β ***(29)***, *Lim1* ***(30)***, *Krox20* ***(31)***, *Otx2* ***(28,32–34)***, *Pax2* ***(35,36)***, *En1* ***(37)***.

therefore influenced by the phenotype of the null mutant. Several transgenic strains have been established expressing the marker gene under the control of various promoters.

4.2.1. Generation of a Breeding Nucleus

The site of integration of the *lacZ* transgene is generally not well characterized in transgenic marker strains; therefore, it is difficult to distinguish homozygous and hemizygous individuals by genotype analysis or reporter gene expression. In transgenic strains where it is not possible to determine tissue expression without culling the animal, polymerase chain reaction (PCR) or Southern analysis can be used to determine if the *lacZ* transgene is present. A breeding nucleus of mice homozygous for the reporter gene is the most efficient means of introgressing a mutant locus onto a transgenic marker line.

In situations where the marker gene is autosomally integrated, backcrossing individuals potentially homozygous at the transgenic marker locus to a wild-type strain would result in hemizygous progeny. The genotype of these progeny can be identified by β-gal staining, PCR, or Southern analysis. If progeny not carrying the reporter transgene are produced, then hemizygosity of the transgenic parent can be assumed. Progeny testing for an X-linked marker locus is more easily achieved. Males expressing the marker gene are of a known hemizygous genotype; however, females require testing by mating with known hemizygous males. If any male offspring not carrying the reporter gene are produced, this indicates that the female parent is a hemizygous. Once the genotype of marker carrying animals has been determined a close-bred line of the specific marker genotype can be maintained.

Most null mutations are genetically characterized (specifically those generated by targeted gene disruption) and PCR genotyping assays are available to identify homozygous and heterozygous individuals. For the majority of null mutants, the phenotype is fully expressed only when homozygous and it is often these individuals that are required as donor embryos for cell transplantation studies. As the genotype of most null mutations can be determined by PCR analysis, the maintenance of heterozygous breeding pairs and the screening of their offspring is relatively straightforward.

4.2.2. Mating Strategy

Once the genotype of both the mutant and marker lines has been established, an appropriate breeding structure is required to introgress the mutant allele onto the homozygous or hemizygous marker background. By using a homozygous marker line, introgression of the mutant allele is most easily achieved by an initial crossing of a heterozygous null mutant with an individual homozygous for the reporter transgene (*see* **Fig. 4**). Approximately 50% of the off-

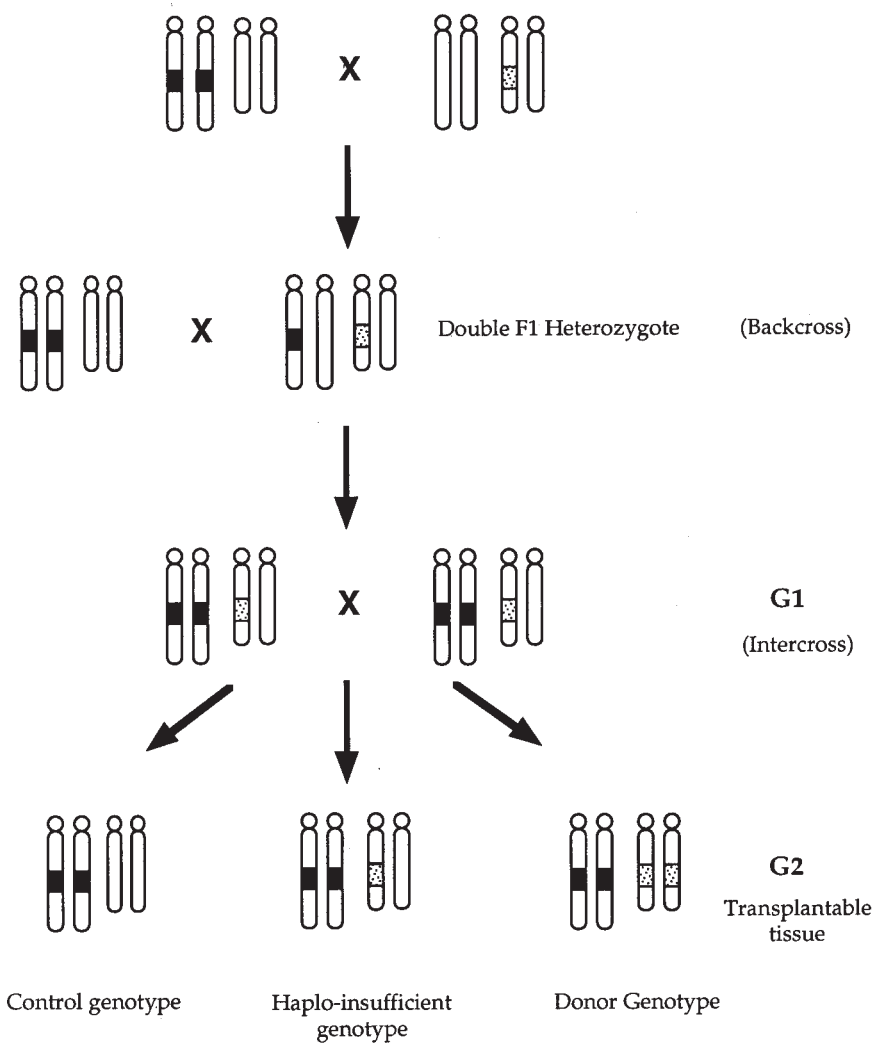

Fig. 4. A mouse-breeding strategy to obtain transgenically marked cells from donor embryos that are homozygous for the null mutation of interest. The filled box indicates the transgenic locus that encodes the ubiquitous cell marker that could be either *lacZ* or GFP (see text). The mutant allele is indicated by the stippled box. It is assumed that (a) homozygosity of the transgenic locus does not have any significant impact on lineage differentiation and embryonic development, (b) heterozygous mutant mice are viable and fertile, and (c) the transgene and the mutant gene are localised on different chromosomes. Mating of the homozygous transgenic (marker) mice and the heterozygous mutant mice produces a number of genotypes. Only the double-heterozygous F1 progeny are selected for the backcross to the homozygous marker mice. Progeny from this mating (G1) are then selected for homozygosity at the transgenic locus and heterozygosity at the mutant locus. These mice

spring that are produced will be doubly heterozygous at both the mutant and marker loci. These can be identified by β-gal staining for the presence of the *lacZ* locus and assayed for heterozygosity at the mutant locus using PCR. Once these individuals have been identified, a backcross can then be established by mating these double heterozygotes to the original homozygous marker line. Approximately 50% of the progeny produced from this cross will be homozygous at the marker locus, whereas the remaining 50% will be hemizygotes. About 50% of these homozygous marker progeny should also be heterozygous for the null mutation of interest.

Identifying the heterozygous null mutation on a homozygous marker background is required next to ensure that homozygosity of the marker locus is maintained. This may be achieved by further progeny testing to identify individuals homozygous for the marker locus that also carries the mutant allele. Once identified, these animals can be intercrossed to generate donor embryos of the appropriate doubly homozygous genotype at a frequency of approx 25%. Embryos homozygous for the marker transgene but wild type at the mutant locus will also be present at a similar frequency and may be used as control transplant tissue.

4.2.3. Determination of the Transplanted Tissue Genotype

The collection of embryonic tissue for genotyping is coincident with the transplantation of donor tissue. This allows DNA extraction from unused embryonic tissue by a number of methods. The homozygous marker genotype can be assayed *in situ*, after culturing, by β-gal staining. The mutant genotype of the donor embryo can be determined by PCR analysis.

4.2.4. Potential Problems

The change of genetic background associated with the introgression of the mutant allele onto that of the marker strain may result in a modification of the mutant phenotype. A strain-dependent expressivity for several null mutations has been reported ***(25–28)***. A phenotypic analysis of the homozygous mutant after introgression onto the marker background may be

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are then intercrossed to produce the G2 progeny. If the progeny are examined before the onset of embryonic lethality, about 25% of them would be homozygous for both the transgene and the mutant allele. These embryos will be suitable for *in situ* analysis of cell fate and tissue pattern, and cells can be isolated from these embryos for transplantation analysis of lineage potency and organising activity. Embryos that are homozygous for the transgene but heterozygous for the mutation may be used to tested the haplo-insufficiency effects if a heterozygous phenotype has been detected in the original mutant strain. The remaining embryos that only carry the transgenic marker may be used as controls.

required to confirm that the null mutant phenotype is maintained. Other potential problems in generating double mutants may occur due to the chromosomal location of the marker and mutant loci which, if closely linked, will decrease the frequency of the doubly heterozygous genotype.

5. Summary

The methods outlined in this chapter discuss a range of techniques that have been employed for lineage analysis studies of the neural primordia from the onset of gastrulation and during neurulation. As the mouse has been extensively mapped, lineage analysis during normal morphogenesis is well understood. Attention is now focused on the tissue interactions that are essential for gastrulation and neurulation to proceed normally. The key to understanding these tissue interactions lies in the study of mutant embryos where abnormal development of specific tissue types affects the processes of gastrulation and neurulation. Lineage analysis and tissue potency experiments on particular mutant embryos will provide insight into these essential tissue interactions. As the first step toward undertaking such analysis of the neural derivatives, we have outlined the mutant strains available and detailed a protocol for the introgression of the *lacZ* transgene onto the mutant background.

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16

Generation of Double-Knockout Embryonic Stem Cells

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1. Introduction

The inactivation of selected genes in the prokaryotic and eukaryotic genome is a powerful tool in studying their function both at the level of individual cells and in the context of a complete organism. The method of gene inactivation relies on the ability of virtually every cell type to exchange DNA sequences with a high degree of sequence similarity by homologous recombination. In general, the procedure involves the generation of a targeting construct that consists of a selectable marker gene flanked by DNA sequences largely identical to the chromosomal locus to be modified. On entry of the targeting construct into the cell, exhange of the flanking sequences with their chromosomal counterparts will result in the introduction of the marker gene into the chromosome, thereby disrupting the gene of interest. This procedure can be efficiently applied on mouse embryonic stem (ES) cells, which, on fusion to recipient blastocysts, give rise to chimeric mice that can transmit the disrupted allele to their offspring ***(1)***. Inbreeding of heterozygous animals will yield offspring carrying the disruption in both alleles. The phenotypic consequences of a complete gene knockout in the mouse germ line can provide pivotal information on the function of the gene in development and maintenance of the organism. Moreover, valuable mouse models have been generated for a number of recessive genetic disorders in humans.

For a number of reasons, however, it can be desirable or even necessary to achieve complete gene inactivation in ES cells. For many genes, only limited information is gained from their complete inactivation in the mouse germ line. This is the case when a complete loss-of-function mutation leads to early

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embryonic lethality, which precludes studying its phenotyping consequences at later stages of development and during adult life or, on the other hand, does not cause any overt phenotype at all. To circumvent the first problem, both copies of a gene can be inactivated in ES cells which can be used to generate chimeric mice in which embryonic lethality might be prevented by the presence of wild type cells. This approach has been succesfully used in studying the developmental and tumorigenic consequences of an embryonic-lethal loss-of-function mutation in the retinoblastoma gene *Rb* ***(2)***. The second problem may indicate that loss of function of a particular gene is complemented by other genes, thus requiring a combination of loss-of-function mutations in different genes. This can be achieved by crossing the relevant knockout mice ***(3)***, a complicated effort, however, when the number of genes involved increases. The generation of chimeric mice using an ES cell line carrying multiple gene knockouts can then be a valuable alternative. Besides the generation of chimeras, mutant ES cell lines can also be studied as to their differentiation capacity in vitro or in vivo after subcutaneous implantation in syngeneic mice ***(4)***. Finally, the generation of double-knockout ES cell lines can be highly instrumental in studying genes expected to function in a cell-autonomous way, i.e. independent of the context of a complete (developing) organism. This is the case for genes functioning in various DNA repair pathways ***(5)***.

Clearly, the generation of double-knockout ES cells has become a valuable addendum to the generation of knockout mice. Unfortunately, gene targeting only rarely produces double knockouts in a one single experiment. Thus, in a gene-targeting experiment aimed at disrupting the *Rb* gene with a hygromycin-resistance marker, 80% of the selected colonies resulted from correct integration of the marker into *Rb*. Surprisingly, however, in none of the colonies that were analyzed was a double knockout observed ***(6)***. Although targeted ES cell clones can often be obtained far more efficiently than cells with random integrations of the targeting construct, homologous recombination in an individual cell is apparently resticted to a single event.

Therefore, alternative protocols have been developed to introduce a disruption in the remaining wild-type allele of a single-knockout ES cell line. These are schematically illustrated in **Figure 1**.

1.1. Method 1: Selection at High Drug Concentrations

ES cells carrying a disruption in one allele of a particular gene are subjected to a second round of selection at drug concentrations largely exceeding that used to obtain the single knockout. Highly resistant colonies often appear to result from duplication of the targeted allele and concomitant loss of the wild-type allele. This method has originally been described for ES cells carrying a

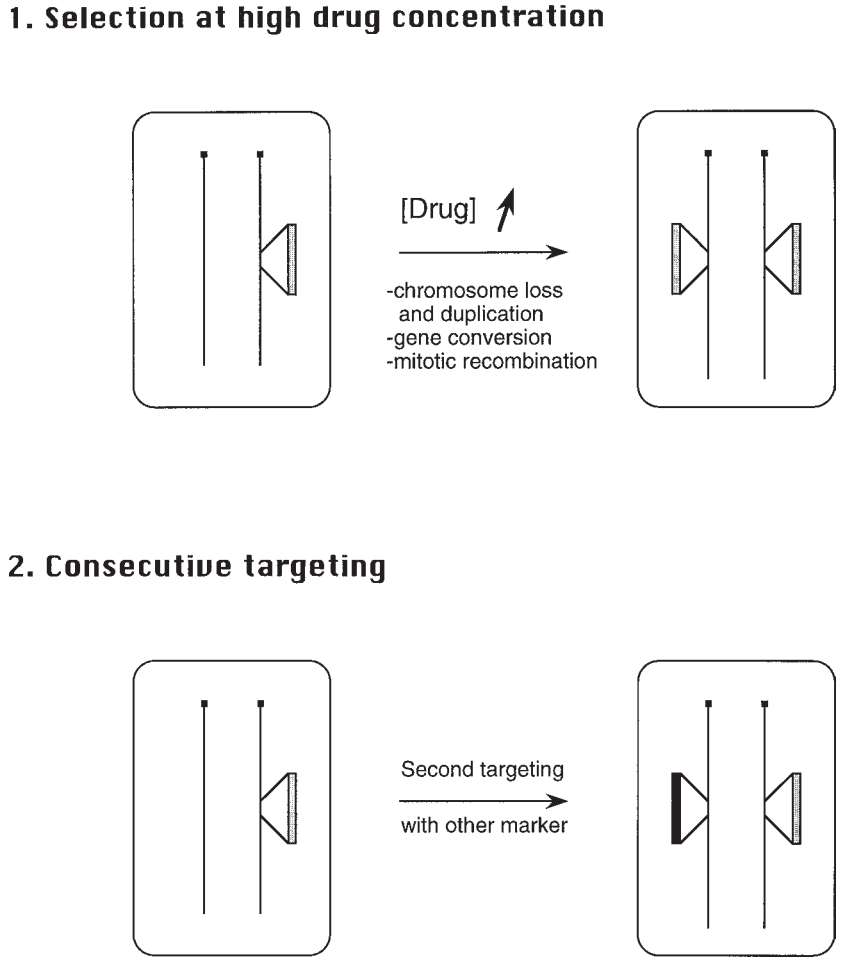

Fig. 1. Two methods to generate double knockout ES cells.

neo gene conferring resistance to G418 *(7)*, but also works with hygromycin B and puromycin resistance genes. The mechanism by which double-knockout ES cells spontaneously arise in a culture of single-knockout cells is not known but may involve nondisjunction and duplication of the targeted chromosome, gene conversion or mitotic recombination. The procedure is relatively simple but often fails when the resistance level of the single knockout is already high or when other mechanisms of gene amplification dominate. Another potential danger of the method is that it would also lead to homozygosity of an inadvertant mutation linked to the targeted gene. Therefore, double knockouts should ideally be generated from several independently obtained single knockouts.

1.2. Method 2: Consecutive Targeting

The wild-type allele in a single-knockout ES cell line is targeted with a new targeting construct carrying another selectable marker as previously used. This procedure requires the construction of a new targeting vector, but is highly reliable ***(8)***. Importantly, unlike the first method, a second round of gene targeting with a new marker gene will easily reveal lethality of a complete gene inactivation at the cellular level. In that case, only single knockouts will be obtained in which the first marker gene is exchanged for the second, leaving the wild-type allele unaltered. Various marker genes are available (*see* **Table 1**). The most widely used are *neo*, *hyg*, *pur*, *his*, and *Hprt*, driven by promoters which are highly expressed in ES cells. These include the *3-phosphoglycerate* (*Pgk*) gene promoter ***(9)*** and the HSV-*Tk*/*Py*F441 promoter/enhancer combination from the pMC1*neo* vector ***(10)***. Note that the *Hprt* marker gene can only be used in *Hprt*-deficient ES cells. A number of other markers may be potentially useful in ES cells but have not been extensively tested. These include *bleo*, conferring resistance to Phleomycin ***(14)***, and *bls*, conferring resistance to Blasticidin S ***(15)***.

In principle, it should be feasible to combine methods 1 and 2, i.e. to introduce into single knockout ES cells the *same* targeting construct as was previously used, followed by selection at high drug concentration. Whether this method really increases the number of double knockouts remains to be tested.

In case of cellular lethality of a complete gene inactivation, conditional/inducible double knockouts could be made by using the Cre/lox or Flp/Frt site-specific recombination systems.

Figure 2 presents a flow scheme for the generation of double-knockout ES cells. Single-knockout ES cell clones should always be subcloned, in particular when method 1 is applied. This is because ES cell cultures obtained after outgrowth of selected colonies are often bi- or even polyclonal: targeted ES cell clones are contaminated with cells carrying random integrations of the targeting construct. These may preferentially grow out at high drug concentration. Then, the resistance level of single-knockout cells is determined. If this is more than tenfold the concentration that is normally used (*see* **Table 1**), direct selection for double knockouts at high drug concentration is likely to fail and method 2 should be used. In selected clones, the status of the target gene is determined by Southern blot analysis. ES cell clones that have lost the wild-type allele are karyotyped to verify the number of chromosomes. It is higly recommendable to derive subclones of the double-knockout line, which should be checked again for genotype and chromosome number.

2. Materials

ES cells are routinely cultured on irradiated primary mouse embryo fibroblasts (MEFs). However, during drug selections, cells are cultured on gelatin-

Table 1
Marker Genes for Gene Targeting in Embryonic Stem (ES) Cells

Marker gene	Selective drug concentration[a]		Reference
neo	G418	200 μg/mL	***(10)***
hyg	Hygromycin B	150 μg/mL	***(6,8)***
pur	Puromycin	1.8 μg/mL	***(11)***
his	L-Histidinol	2 m*M*	***(12)***
Hprt[b]	HAT medium:		***(13)***
	Hypoxanthine	100 μ*M*	
	Aminopterin	800 n*M*	
	Thymidine	20 μ*M*	

[a]For ES cell line E14 ***(17)***.
[b]To be used in *Hprt* -deficient cells.

coated plates in buffalo rat liver (BRL)-conditioned medium. This is because mouse embryonic fibroblasts or other feeder layers resistant to the drugs to be used are not always readily available or are not always tolerant to the high concentrations that are required.

Protocols for the preparation of MEF feeder layers, BRL-conditioned medium, and culturing of ES cells are given elsewhere. This chapter provides protocols for subcloning of ES cell lines and the generation of double knockout mutants.

2.1. Media and Solutions

1. CM (complete medium): 1X GMEM (Gibco-BRL, cat. no. 21710-025) 500 mL, 100 m*M* sodium pyruvate (Gibco-BRL, cat. no. 11360-0395) 5 mL, 100X non-essential amino acids (Gibco-BRL, cat. no. 11140-0355) 5 mL, fetal calf serum 50 mL (FCS is tested for optimal growth of ES cells).
2. CM + β + leukemia inhibitory factor (LIF): CM 100 mL, β-Mercaptoethanol (1000X) 0.1 mL, LIF (1000X) 0.1 mL.
3. BRL medium: CM conditioned on a monolayer of BRL cells.
4. BRL + β + LIF (60% medium): BRL medium150 mL, CM 100 mL, L-glutamine 200 m*M* (Gibco-BRL, cat. no. 25030-024) 1.5 mL, β-Mercaptoethanol (1000X) 0.25 mL. Filter using a 0.22 μm Millex-GV then add LIF (1000X) 0.25 mL.
5. β-Mercaptoethanol (1000X, 0.1 *M*) : 0.1 mL 2-mercaptoethanol (14.2 *M*, Merck, cat. no. 15433) and 14.1 mL water. Sterilize by filtration through 0.22 μm Millex-GV filter. Working concentration (conc.) 0.1 m*M*.
6. LIF (1000X): 10^6 units/mL complete medium of ESGRO-LIF (Gibco, cat. no. 3275SB). Working conc.: 10^3 U/mL.
7. Water: Triple-distilled water tested for optimal growth of ES cells, 500 mL bottles. (Flow, cat. no. 16-960-54, Gibco-BRL, cat. no. 15230-089)
8. Gelatin (10X): 1% gelatin (Sigma, cat. no. G1890) in water. Sterilize by filtration through 0.22 μm Millex-GV filter.

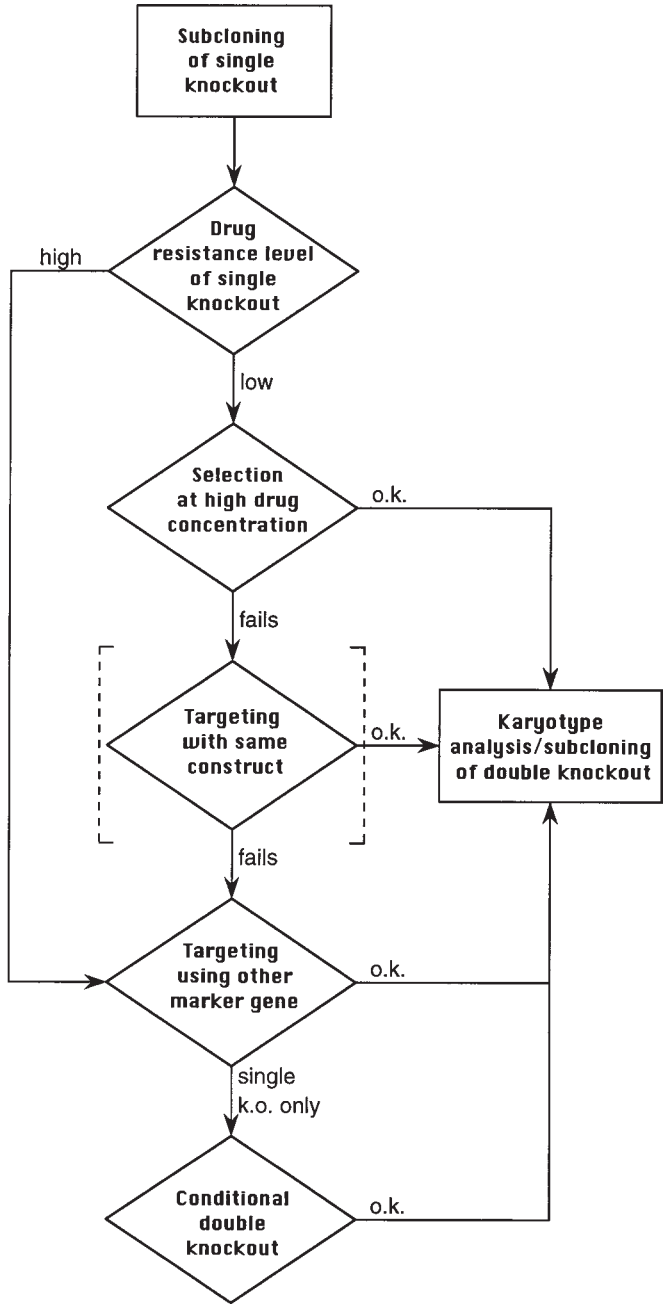

Fig. 2. Flow scheme for the generation of double knockout ES cells.

9. Phosphate-buffered saline (PBS): 1 tablet (Flow, cat. no. 28-103-05) in 100 mL water or one tablet (Gibco-BRL, cat. no. 18912-014) in 500 mL water. Sterilize by filtration through 0.22 μm Millex-GV filter.
10. TVP: PBS 191 mL, 40 m*M* ethylene diamine tetraacetic acid (EDTA), 5 mL, chicken serum (Flow, cat. no. 29-501-49, Gibco-BRL, cat. no. 16110-033), 2 mL trypsin 2.5%, (Flow, cat. no. 16-893-49, Gibco-BRL, cat. no. 25090-028) 2 mL. Sterilize by filtration through 0.22 μm Millex-GV filter.
11. 2X trypsin-EDTA-PBS (TEP): 10 mL TVP plus 0.1 mL trypsin 2.5%.
12. 10X TEP: 10 mL TVP plus 1 mL trypsin 2.5%.
13. Lysis Buffer: 100 m*M* Tris pH 8.0, 5 m*M* EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 m*M* NaCl, 100 μg/mL (freshly added) Proteinase K (Merck, cat. no. 24568.0100).

2.2. Selective Drugs

Solutions are sterilized by filtration through 0.22 μm Millex-GV. Use only plastic disposables.

1. G418 (100X): 20 mg G418 (Gibco-BRL, cat. no. 11811-031) per mL 60% medium, store at 4°C. Working conc.: 200 μg/mL.
2. Hygromycin B (100X): 15 mg Hygromycin B (Calbiochem, cat. no. 400050 or 400051) per mL 60% medium, store at 4°C. Working conc.: 150 μg/mL.
3. Puromycin (1000X): 18 mg Puromycin (Sigma P, cat. no. 7255) per 10 mL PBS, store at –20°C. Working conc.: 1.8 μg/mL.
4. Histidinol (100X) 250 m*M* : 53,525 mg L-Histidinol.2 HCl (Sigma H, cat. no. 6647) per mL 60% medium, store at 4°C. Working conc.: 1.5–2.5 m*M*.
5. HAT (100X): Hypoxanthine 10 m*M* in water, neutralized with 10 *M* NaOH, store at –20°C.
6. Aminopterin: 80 μ*M* in water, store at –20°C.
7. Thymidine: 2 m*M* in water, store at –20°C.
8. HAT (50X): HAT supplement (50X) Gibco-BRL, cat. no. 043-01060H 100 mL.

2.3. Tissue Culture Plastics

ES cells are grown on standard tissue culture supports. We commonly use:

1. T25: 25 cm^2 tissue culture flask (Costar, cat. no. 3055).
2. T75: 75 cm^2 tissue culture flask (Costar, cat. no. 3275).
3. T150: 162 cm^2 tissue culture flask (Costar, cat. no. 3150).
4. 96-Well multidish: 0.3 cm^2 Flat bottom (Falcon, cat. no. 3072).
5. 4-Well multidish: 1.8 cm^2 (Nunc, cat. no. 176740).
6. 12-Well multidish: 4.0 cm^2 (Costar, cat. no. 3512).
7. 6-Well multidish: 10 cm^2 (Costar, cat. no. 3506).
8. TC Petri dish: 100 × 20 mm (Falcon, cat. no. 3003).

3. 1. Subcloning of Single Knockout ES Cell Clones

3.1.1. Subcloning

1. Prepare a 96-well plate (flat bottom) with a MEF feeder layer. (Use a multichannel pipet when handling 96-well plates).
2. Culture an ES cell line at a small scale (e.g. on a 4-well plate).
3. Trypsinize the ES cell culture and count the number of cells.
4. Add 50 cells to 20 mL of CM + β + LIF.
5. Fill the 96-well plate with 200 μL per well.
6. Grow individual colonies (15–20 will appear after 7 d).
7. Wash the wells with 100 μL PBS and trypsinize with 25 μL 10X TEP for 5 min at 37°C.
8. Add 150 μL CM + β + LIF, resuspend and transfer the cells to a new 96-well plate with MEFs.
9. Culture the cells overnight and refresh the medium (150 μL).
10. Culture the cells to (semi)confluency (2–3 d).
11. Wash the cells with 100 μL PBS and trypsinize with 50 μL 10x TEP for 5 min at 37°C.
12. Add 150 μL CM + β + LIF and resuspend.
13. Transfer two 100 μL portions of cell suspension to two new 96-well plates with MEFs and 100 μL CM + β + LIF medium.
14. Culture one plate semiconfluency (2–3 d) and processed for freezing (*see* **Subheading 3.2.**). The other plate is cultured as dense as possible for DNA isolation (*see* **Subheading 3.3.**).

3.1.2. Freezing Procedure

1. Prepare CM (without LIF) with 20% dimethylsulfoxide (DMSO) at 4°C.
2. Aliquot 100 μL CM plus 20% DMSO in individual tubes of a 96-tube freezing box (Greiner, cat. no. 975561).
3. Wash the cells with PBS and trypsinize with 25 μL 10X TEP.
4. Add 75 μL complete medium and resuspend carefully.
5. Add 100 μL of trypsinized cells to 100 μL 20% DMSO in the freezing tubes and resuspend.
6. Store in liquid nitrogen (or at –80°C for a maximum of 4 wk).

3.1.3. DNA Isolation

1. Wash the cells twice with PBS.
2. Optional: Cells can be stored dry in a 96-well plate at –20°C.
3. Add 100 μL lysis buffer with 100 μg/mL Proteinase K.
4. Carefully seal 96-well plate and incubate overnight at 55°C.
5. Add to the lysates 100 μL 2-propanol and mix in a shaker (Amersham) at RT for 60 min.
6. Centrifuge plates at 1800*g* for 30 min at RT.
7. Remove the supernatant with a Gilson pipet, each well individually.
8. Add 150 μL 70% ethanol, shake for 10 min, centrifuge at 1800*g* for 15 min at RT.

9. Remove the supernatant with a Gilson pipet, allow the DNA precipitates to dry.
10. Add 100 μL TE and incubate overnight at 55°C.
11. Use 40 μL DNA for Southern analysis.

3.1.4. Thawing Procedure

1. Cut the tube with the desired clone out of the freezing box and thaw immediately at 37°C.
2. Transfer the cells to a sterile eppendorf tube or a 15 mL Falcon tube containing 1 mL CM.
3. Centrifuge the cell suspension (1200 rpm).
4. Resuspend the cells in CM + β + LIF and transfer the cells to a 96-well or 4-well multidish (with MEFs).
5. Culture the subclone to the appropiate surface for DNA analysis and long-term storage in liquid nitrogen.

3.2. Generation of Double Knockout ES Cells

3.2.1. Determination of Drug-Resistance Level

1. Seed single-knockout ES cells onto a gelatin-coated 6-well plate at a density of 10^5 cells per well in 3 mL of BRL + β + LIF (60% medium).
2. Refeed the cells the next day with fresh BRL + β + LIF (60% medium), adding to each well increasing concentrations of the drug to be tested:
   a. G418: 0, 0.4, 0.8, 1.2, 1.6, 2.0 mg/mL
   b. Hygromycin B: 0, 0.3, 0.6, 0.9, 1.2, 1.5 mg/mL
   c. Puromycin: 0, 4, 8, 12, 16, 20 μg/mL
3. Culture the cells for at least 7 d. Determine the drug concentration at which complete cell death is observed. If cells are surviving the highest concentration, method 2 (*see* **Subheading 4.3.**) should be used.

3.2.2. Selection for Spontaneous Double Knockouts at High Drug Concentration

1. Culture a subcloned single-knockout ES cell line in CM + β + LIF on one 6-well plate with MEFs. A confluent well contains approximately 3×10^6 cells.
2. Trypsinize the cells with 10X TEP and feed onto four 10-cm petri dishes, coated with gelatin, in BRL + β + LIF (60% medium).
3. Refeed the cells the next day with fresh BRL + β + LIF (60% medium), and start selection: add to two dishes the concentration of drug at which all cells died within 7 d; add to one dish a somewhat lower, to the other a somewhat higher, concentration.
4. Refeed the cells with selective medium every 2–4 d. Surviving colonies arise after 8–12 d.

3.2.3. Generation of Double Knockouts by Consecutive Targeting

3.2.3.1. DNA

1. Linearize the targeting vector or purify the DNA targeting fragment (50–100 μg per experiment) from an agarose gel (Pharmacia NA) by electroelution (*see*

ref. *16*). Extract DNA with phenol, phenol/chloroform, and chloroform, and precipitate with alcohol.
2. Dry the DNA pellet in a flow cabinet and dissolve in 300 μL of PBS (sterile).
3. Check the DNA concentration by agarose gel electrophoresis of 1–2 μL.

3.2.3.2. ES Cells

1. Trypsinize ES cells cultured on one T75 flask.
2. Wash the cells with a small volume of PBS.
3. Add 3 mL 10× TEP per T75, incubate 5 min at 37°C.
4. Detach the cells.
5. Add 3 mL CM and resuspend to single cells with a 10-mL pipet.
6. Count the cells, approximately 2×10^7 cells are present.
7. Spin down the cells for 5 min at 275*g* at RT.
8. Resuspend the cell pellet in 300 μL PBS.

3.2.3.3. Electroporation (*see* **Note 1**)

1. Mix the cells ($\approx 2 \times 10^7$) with the DNA (≈ 50–100 μg) Adjust the volume with PBS to ± 600 μL and leave 5 min at RT.
2. Transfer the mixture to a 0.4-cm electroporation cuvet (Biorad, cat. no. 165-2088).
3. Electroporate the cells with a Biorad gene pulser model, cat. no. 165-2078: voltage 0.8 kV, capacitance: 3 μF. The time constant of a pulse should be 0.1 ms.
4. Leave 5 min at RT.
5. Resuspend the cells in 48 mL of BRL + β + LIF (60% medium) and spread 6 × 8 mL in 10-cm gelatin-coated TC dishes.
6. Incubate ON at 37°C/ 5% CO_2.

3.2.3.4. Drug Selection

1. Refeed the cells with 8 mL BRL + β + LIF (60% medium) plus the appropriate drug (*see* **Notes 2**,**3**):
   a. G418: 200 μg/mL.
   b. Hygromycin B: 150 μg/mL.
   c. L-Histidinol:1.5–2.5 m*M*.
   d. HAT: Hypoxanthine 0.1 m*M*, aminopterin 0.8 μ*M*, thymidine 20 μ*M*.
   e. Puromycin: 1.8 μg/mL.
2. Refeed the cells with selective medium every 2–4 d.
3. After 6–8 d, individual colonies can be seen.

3.4. Culturing of Selected Colonies on 96-Well Plates

1. Prepare a 96-well plate (U-bottom, Falcon, cat. no. 3918) with 15 μL PBS per well using the multichannel pipet.
2. Scrape off individual colonies with a Gilson P10 pipetman and transfer them to the PBS in the 96-well plate. Pick a series of 24–36 colonies.
3. Add 15 μL 2X TEP to each well and incubate 5 min at 37°C.
4. Add 50 μL CM + β + LIF medium and resuspend.

5. Transfer trypsinized colonies (80 μL) to a 96-well plate with MEFs (flat bottom) and 100 μL CM + β + LIF.
6. Culture the cells overnight and refresh the medium (150 μL).
7. Culture the cells to semiconfluency (2–3 d).
8. Wash the cells with PBS and trypsinize with 50 μL 10X TEP for 5 min at 37°C.
9. Add 150 μL CM + β + LIF and resuspend.
10. Transfer two 100 μL portions of cell suspension to two new 96-well plates with MEFs and 100 μL CM + β + LIF.
11. One plate is cultured to semiconfluency (2–3 d) and processed for freezing (*see* **Subheading 3.2.**).
12. The other plate is cultured as far as possible for DNA isolation *see* **Subheading 3.3.** and **Note 4.**

4. Notes

1. Electroporation can be done at a smaller scale, e.g., mix cells from a T25 with 25–30 μg DNA in a total volume of 200 μL PBS. Eelectroporate with a capacitance of 1 μF giving a time constant of 0.1. and spread the cells onto two 10-cm dishes.
2. In some cases, cross-resistance can occur. E.g. in the presence of a *hyg* marker, selection for *neo* requires a somewhat higher G418 concentration (250 μg/mL).
3. Double selections are not recommended, as this would obscure lethality of complete gene inactivation.
4. Double-knockout ES cell clones, as identified by Southern analysis, are karyotyped to verify the chromosome number. For obvious reasons, it is strongly recommended that you subclone double-knockout ES cell lines before using them for in vitro experiments or the generation of chimeric mice.

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17

In Vitro Differentiation of Embryonic Stem Cells and Analysis of Cellular Phenotypes

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1. Introduction

Embryonic stem (ES) cells, the totipotent cells of early embryos established as permanent lines ***(1,2)*** retain their developmental capacity in vivo ***(3)*** as well as in vitro *(see **4–6**)*. The totipotent properties of ES cells are the basis of the gene-targeting technology to create mutant mice strains with inactivated genes by homologous recombination ***(7)***.

ES cells cultivated as embryo-like aggregates, so-called embryoid bodies (EBs), differentiate in vitro into cellular derivatives of all three primary germ layers of endodermal, ectodermal, and mesodermal origin. The pluripotent/totipotent ES cell lines develop from an undifferentiated stage resembling cells of the early embryo into terminally differentiated stages of the cardiogenic ***(6,8–11)***, myogenic ***(11–13)***, neurogenic ***(14–17)***, hematopoietic ***(5,18,19)***, or adipogenic ***(20)*** lineage, as well as into epithelial ***(21)***, endothelial ***(22)*** and vascular smooth muscle cells ***(22–24)***. We found that the terminally differentiated cells showed also pharmacological and physiological properties of specialized cells: in vitro differentiated cardiomyocytes resemble characteristics of atrial-, ventricle-, purkinje- and pacemaker-like cells ***(9,10,25,26)***, neuronal cells are characterized by inhibitory and excitatory synapses ***(14,17)*** and the neuronal, cardiac and vascular smooth muscle cells express functional receptors ***(8,14,24,25)***.

During the last years, several differentiation protocols for optimal development of ES cells into cardiomyocytes, skeletal muscle cells, neuronal cells, or vascular smooth muscle cells have been established. It was found that the

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following parameters influence the developmental potency of ES cells in culture: (1) number of cells differentiating in the embryoid bodies, (2) medium quality of fetal calf serum, growth factors, and medium additives, (3) ES cell lines used, and (4) the time of embryoid body plating ***(6)***.

In principle, the different ES cell lines show pluripotent developmental capacities in vitro, i.e., they differentiate in the EB outgrowths into many differentiated cell types. But to obtain maximal differentiation of a defined cell type, specific cell lines and cultivation conditions were employed ***(6)***.

2. Materials

2.1. Cells

Examples of cell lines used for in vitro differentiation into the cardiogenic, neurogenic, and myogenic lineages and into vascular smooth muscle and epithelial cells:

1. Cardiogenic differentiation: D3 ***(4,8–11,25,27–29)***, R1 ***(29)***, Bl17 ***(8)***, AB1, AB2.1. ***(23)***, CCE, and E14.1 ***(30)***.
2. Myogenic differentiation: D3 ***(11,31)***, BLC6 ***(12,13)***, AB1, AB2.1 ***(23)***.
3. Neuronal differentiation: BLC6 ***(14)***, D3 ***(15,31)***, CGR8 ***(16)***, J1 ***(17)***.
4. Endothelial and vascular smooth muscle cell differentiation: D3 ***(22,24)***, AB1, AB 2.1 ***(23)***.
5. Epithelial differentiation: D3 ***(21)***.

2.2. Media, Reagents, and Stock Solutions

2.2.1. Solutions for Cell Culture (see **Notes 1** and **2**)

1. Dextran T500 (Pharmacia)
2. 0.01 *M* Tris-HCl, pH 8.0.
3. Activated charcoal (Serva)
4. Phosphate-buffered saline (PBS): 10 g/L NaCl, 0.25 g/L KCl, 1.44 g/L Na_2HPO_4, $KH_2PO_4 \cdot 2H_2O$ 0.25 g/L.
5. Trypsin solution: 0.2 % trypsin 1:250 (Gibco-BRL) in PBS.
6. Ethylene diamine tetraacetic acid (EDTA) solution: 0.02 % EDTA (Serva) in PBS.
7. Trypsin:EDTA: Mix trypsin solution and EDTA solution at 1:1.
8. Gelatin solution: 1% gelatine (Fluka) in *Aqua tridest*., autoclave and 1:10 dilute with PBS. Coat Petri dishes with 0.1% gelatin solution for 1–24 h at 4°C before use.
9. MC buffer: Dissolve 2 mg mitomycin C (Serva) in 10 mL PBS, filter sterilize. From this stock solution, dilute 300 µL into 6 mL of PBS. **Caution!** Mitomycin C is carcinogenic.
10. β-Mercaptoethanol (β-ME): Prepare a stock solution from 7 µL of β-ME (Serva) in 10 mL of PBS, add 1 mL to 100 mL medium. Make fresh at weekly intervals and store at 4°C.

11. Nonessential amino acids (NEAA): 100X (Gibco-BRL)
12. L-Glutamine solution: 100X (Gibco-BRL)
13. Cultivation medium I: Dulbecco's modification of Eagles MEM (DMEM, 4.5 g/L glucose, Gibco-BRL) supplemented with 15% fetal calf serum (FCS) for feeder layer cells (*see* **Note 1**).
14. Additives I: To 100 mL media add 1 mL L-glutamine stock, 1 mL β-ME stock, 1 mL NEAA stock.
15. Additives II: To 400 mL medium add: 40 µL of a 3×10^{-4} *M* stock solution of Na-selenite (Sigma), 10 mL of 7.5% stock solution of bovine serum albumin (BSA, Gibco-BRL) and 1 mL of stock solution (4 mg/mL) of transferrin (Gibco-BRL).
16. Cultivation medium II: DMEM supplemented with 15% FCS (selected batches) and Additives I for ES cell cultivation.
17. Differentiation medium I: DMEM or Iscove's modification of DMEM (IMDM) supplemented with 20% FCS and additives I (*see* **Note 2**) for EB differentiation into cardiomyocytes.
18. Differentiation medium II: DMEM supplemented with 15% DCC-FCS and additives I and additives II for EB differentiation into myogenic and neuronal cells.
19. Retinoic acid (RA): Prepare in the dark a 10^{-3} *M* stock of all-*trans* retinoic acid (Sigma) in 96% ethanol. Store aliquots in the dark at –20°C and thaw a fresh aliquot for each experiment.
20. Dibutyryl-cyclic adenosine monophosphate (db-cAMP, Boehringer Mannheim): dilute in *A. tridest.* to a 0.1-*M* stock solution. Store aliquots at –20°C.

2.2.2. Solutions for Reverse Transcription/Polymerase Chain Reaction (see ***Notes 3*** *and* ***4****)*

1. Diethyl pyrocarbonate-treated water (DEPC-H_2O): Add 1 mL DEPC (Fluka) to 1 L double-distilled or Milli-Q water and stir overnight. DEPC is inactivated by heating to 100°C for 15 min, or autoclaving for 15 min.
2. Lysis buffer: Add 23.6 g of guanidiniumthiocyanate to 5 mL of 250 m*M* Na-citrate, pH 7.0, 2.5 mL 10% Sarcosyl, and add DEPC-H_2O to a total volume of 49.5 mL and mix carefully. Add 0.5 mL of β-ME before use.
3. 2 *M* Na-acetate, pH 4.0: Dissolve 27.2 g of Na-acetate · 3 H_2O in 0.1% DEPC-H_2O, adjust the pH to 4.0 with glacial acetic acid, and adjust to 100 mL with DEPC-H_2O. Treat the buffer with 0.1% DEPC-H_2O at 37°C for at least 1 h and heat to 100°C for 15 min, or autoclave for 15 min.
4. Acidic phenol: Phenol is saturated with DEPC-H_2O instead of Tris. The saturated acidic phenol contains 0.1% hydroxyquinoline (antioxidant, partial inhibitor of RNase, and a weak chelator of metal ions; its yellow color provides a convenient way to identify the organic phase). Stored at 4°C for up to 2 mo.
5. Chloroform: Isoamylalcohol (24:1).
6. 75% Ethanol: Prepare in DEPC-H_2O.
7. 25 m*M* $MgCl_2$.
8. 10X PCR buffer II: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl.

9. RNase inhibitor: 20 U/μL.
10. Oligo $d(T)_{16}$: 50 μ*M* in 10 m*M* Tris-HCl, pH 8.3.
11. Random hexamers: 50 μ*M* in 10 m*M* Tris-HCl, pH 8.3.
12. MuLV reverse transcriptase: 50 U/μL.
13. A*mpli*Taq DNA polymerase: 5 U/μL.
14. 5 m*M* dNTP mix: 250 m*M* of each dNTP (dGTP, dATP, dCTP, dTTP; Pharmacia) dilute to 20 mM with DEPC-H_2O and freeze at –20°C. 5 m*M* dNTP mix is freshly made by mixing the equal volumes of 20 m*M* of each dNTP before use.
15. Select PCR primer pairs: Synthetic oligonucleotides diluted to 10 m*M* with DEPC-H_2O freeze at –20°C (a critical step in the PCR reaction, *see* **ref. *32***).
16. Paraffin wax (Fluka Chemie AG).
17. Glycogen: 20 mg/mL (Boehringer Mannheim).
18. 5 *M* NaCl: Dissolve 29.2 g of NaCl in distilled water, adjust to 100 mL with water and autoclave.
19. TE buffer: 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA (pH 8.0).
20. 6X Loading buffer: 0.25% bromophenol blue (Sigma), 0.25% xylene cyanol FF (Sigma), 30% glycerol in water.
21. 5X TBE: Dissolve 54 g Tris-base and 27.5 g boric acid in distilled water, add 20 mL 0.5 *M* EDTA, pH 8.0, and adjust to 1 L with distilled water.
22. Ethidium bromide aqueous solution (Serva): 1% w/v = 10 mg/mL.
23. Agarose gels: Melt electrophoresis grade agarose (Gibco-BRL) in 1X TBE by gentle boiling in a microwave oven. Cool until hand warm and pour into prepared gel former. Run small gels at around 80–100 V by using bromophenol blue and xylene cyanol FF in the stop mix as an indicator of migration.

2.2.3. Solutions for Immunohistochemical Analysis

1. Paraformaldehyde (PFA) 3.7%: dissolve 3.7 g PFA in PBS and adjust to 100 mL with PBS, heat the mixture to 95°C and stir until the solution becomes clear, cool to room temperature (**PFA is toxic!** Work under a fumehood and use gloves!).
2. Methanol: acetone (7:3) fixative.
3. Ten percent goat serum (Sigma) in PBS for blocking unspecific binding of antibodies.
4. Mounting medium: Vectashield (Vector, Burlingame, CA).
5. 0.02% Triton-X 100 in PBS.
6. 0.5% BSA in PBS for dilution of second antibodies.

2.2.4. Solutions for Physiological Analysis

1. Supplemented low-Ca^{2+} medium: 120 m*M* NaCl, 5.4 m*M* KCl, 5 m*M* $MgSO_4$, 1 m*M* EGTA, 5 m*M* Na pyruvate, 20 m*M* glucose, 20 m*M* taurine, 10 m*M* HEPES-NaOH, pH 6.9 at 24°C, supplemented with 1 mg/mL collagenase B (Boehringer Mannheim, selected batches) and 30 μ*M* $CaCl_2$.
2. KB medium: 85 m*M* KCl, 30 m*M* K_2HPO_4, 5 m*M* $MgSO_4$, 1 m*M* EGTA , 5 m*M* Na pyruvate, 5 m*M* creatine, 20 m*M* taurine, 20 m*M* glucose, freshly added 2 m*M* Na_2ATP; pH 7.2 at 24°C.

2.3. Equipment

1. Tissue culture plates: 35, 60, and 100 mm (Nunc).
2. 24-Well microwell plates (Falcon).
3. Pasteur pipets, 2, 5, 10, and 25 mL.
4. Bacteriological Petri dishes (Greiner): 60 mm for EB mass culture, 100 mm for EB hanging-drop culture.
5. Tissue culture plates (60 mm) with sterilized cover slips ($n = 4$) for immunofluorescence.
6. 2 mL glass pipets for preparing single-cell suspensions.
7. For feeder layer culture: sterile dissecting instruments, screen or sieve (about 0.5 diameter), Erlenmeyer flasks with stir bars, centrifuge tubes.
8. Magnetic stirrer.
9. Laboratory centrifuge.
10. Tissue culture incubator with 37°C and a 5% CO_2 atmosphere.
11. An inverted microscope Diaphot-TMD (Nikon) is used for routine morphological analysis of ES cells or EB outgrowths; for pharmacological measurements the inverted microscope is equipped with a 37°C heating plate and a CO_2 chamber; for pharmacological measurements with the computer-assisted imaging system (*see* **Subheading 3.3.3.2.**) the inverted microscope Diaphot-TMD (Nikon) is coupled via a one-chip CCD camera (Sony, Tokyo, Japan) to a computer imaging station (Pentium CPU, 100 MHz) running the LUZIA Laboratory Imaging System including the ''HEART'' application (Nikon).
12. DNA thermal cycler (Perkin-Elmer/Cetus).
13. 0.5 and 1.5 mL microtubes (Eppendorf) and 20, 100, and 1000 μL filter tips (Biozym).
14. Lightly powdered latex exam gloves.
15. Electrophoresis equipment (Bio-Rad).
16. Polaroid film.
17. E.A.S.Y. system (Herolab GmbH, Wiesloch, Germany).
18. TINA2.08e software (Raytest Isotopenmebgeräte GmbH, Straubenhardt, Germany).
19. Axioscope fluorescence microscope (Carl Zeiss, Jena) coupled to a microscope-photometer system MPM20D (Carl Zeiss, Jena).
20. Inverted Confocal Laser Scanning Microscope (CLSM) LSM-410 (Carl Zeiss, Jena) equipped with an argon–ion laser.

3. Methods

3.1. Cultivation of Undifferentiated ES Cells on Feeder Layer

3.1.1. Feeder Layer Culture

1. Remove embryos from pregnant mice (i.e., NMRI strain) between d 15–17 of pregnancy; rinse in PBS; remove placentae and fetal membranes of the head, liver, and heart; rinse the carcass in trypsin solution.
2. Mince the embryonic tissue in 5 mL of fresh trypsin solution and transfer to an Erlenmeyer flask containing a stir bar.

3. Stir on magnetic stirrer for 25–45 min (use longer incubation time if the embryos are older), filter the suspension through a sieve or a screen, add 10 mL of medium and spin down.
4. Resuspend the pellet in about 3 mL of cultivation medium I and plate on 100 mm tissue culture plates (about 2×10^6 cells/100 mm dish) containing 10 mL medium I, incubate at 37°C and 5% CO_2 for 24 h.
5. Change the medium to remove debris, erythrocytes, and unattached cellular aggregates, cultivate for additional 1–2 d.
6. Passage the primary culture of mouse embryonic fibroblasts: split 1: 2–1: 3 on 100 mm tissue culture plates, cultivate in medium I for 1–3 d. The cells are a secondary culture suitable as feeder layer for undifferentiated ES cells.
7. Incubate feeder layer cells with MC buffer for 2–3 h (MC stock solution should be freshly prepared at weekly intervals), aspirate the MC buffer, wash three times with PBS, trypsinize feeder cells, and replate to new gelatin (0.1%)-treated microwell plates or to Petri dishes. Feeder layer cells prepared 1–2 d before ES cell subculture are optimal!

*3.1.2. Culture of Undifferentiated ES Cells (*see ***Note 5****)*

It is important to passage ES, EC and EG cells every 24 or 48 h. Do not cultivate longer than 48 h without passaging, or the cells may differentiate and be unsuitable for in vitro differentiation studies. Selected batches of FCS have to be used for ES cell culture.

1. Change the medium 1–2 h before passaging. Suck off the medium, add 2 mL of trypsin: EDTA, incubate at room temperature for 30–60 s.
2. Remove carefully the trypsin-EDTA mixture, add 2 mL of fresh cultivation medium II.
3. Resuspend the cell population with a 2-mL glass pipet into a single-cell suspension and split 1:3 to 1:10 to freshly prepared (60 mm) feeder layer plates (*see* **Subheading 3.1.1.**).

3.2. In Vitro Differentiation

3.2.1. Preparation of FCS for Differentiation of ES Cells into Myogenic or Neuronal Cells

For some experiments, FCS must be treated with dextran-coated charcoal to remove high-molecular-weight proteins, growth, and differentiation factors from FCS. DCC-FCS is prepared as follows:

1. Dissolve 0.45 g Dextran T500 in 1800 mL 0.01 M Tris-HCl, pH 8.0, add 4.50 g activated charcoal and stir the mixture at 4°C in a tightly closed Erlenmeyer bottle overnight.
2. Inactivate FCS by incubation at 56°C for 30 min.
3. Fill 50 mL DCC solution in plastic centrifuge tubes and centrifuge at 2000*g* for 20 min, remove and discard the supernatant, repeat the procedure in the same tube without removing the pellet (= double pellet).

4. Add 50 mL of FCS to the tube with a double pellet, transfer the mixture to a clean glass bottle, and incubate for 45 min at 45°C in a water bath under shaking.
5. Centrifuge the mixture at 2000*g* for 20 min, and transfer the supernatant to another centrifuge tube. Repeat **Steps 3–5**, if necessary.
6. Collect the FCS supernatant in a clean centrifuge tube and sterilize through a 0.22 μm filter (low protein binding) to sterile flasks, add at required concentration to culture medium.

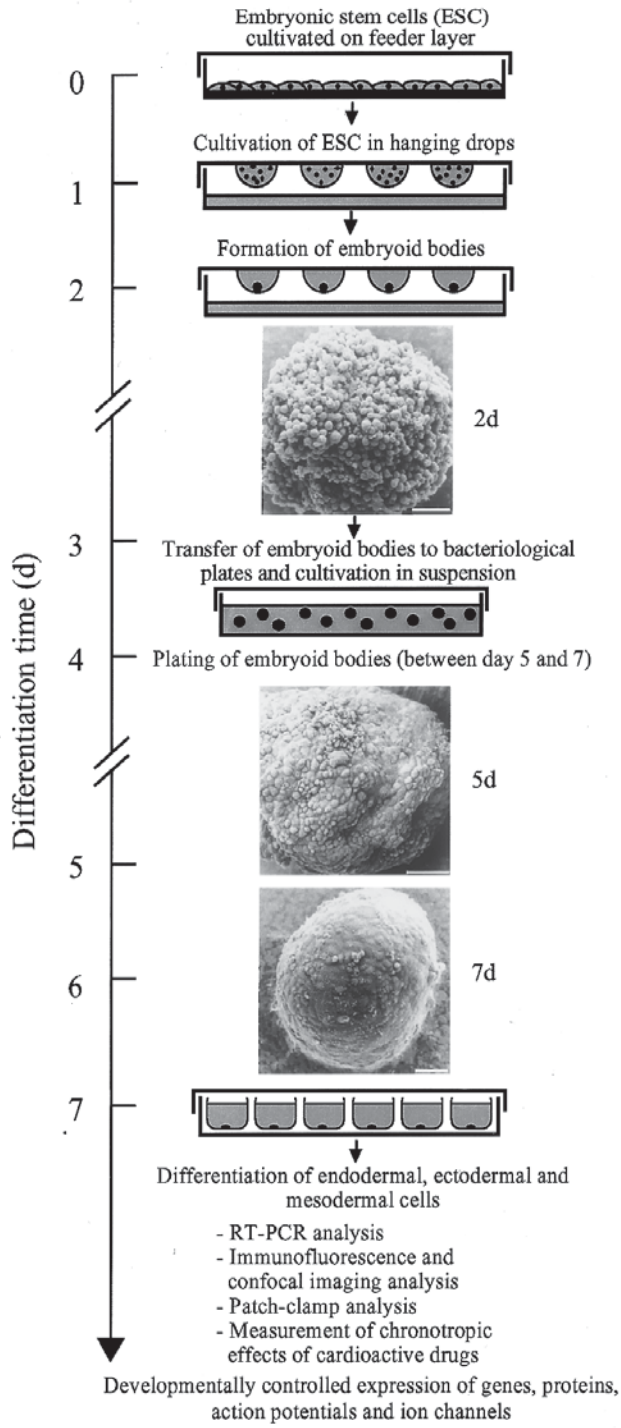
3.2.2. Differentiation Protocols

For the development of ES, EC, or EG cells into differentiated phenotypes of cardiogenic, myogenic, and neurogenic lineages or into vascular smooth muscle cells, pluripotent cells have to be cultivated in three-dimensional aggregates or EBs, by the hanging drop method *(see **8,33*** and **Fig. 1***)*, by mass culture *(see **4*** and **Note 6**), or by differentiation in methylcellulose (*see **18,19,*** and **Note 6**). The differentiation of various cell types requires different conditions and these may vary for the particular ES cell line used (*see* **Note 2**). In the following protocol (*see* **Note 6**) the basic protocol for the hanging-drop method is described. The detailed protocols for obtaining the differentiated phenotypes are described in **Subheadings 3.2.2.1.–3.2.2.4.**

1. Prepare an ES cell suspension containing a defined ES cell number of 400, 600, or 800 cells (depending on the differentiation protocols) in 20 μL differentiation medium.
2. Place 20 μL drops (n = 50–60) of the ES cell suspension on the lids of 100 mm bacteriological Petri dishes, and put the lid on the dish containing 10 mL PBS.
3. Cultivate the ES cells in hanging drops for 2 d. The cells will aggregate, forming one EB per drop.
4. Rinse the aggregates carefully from the lids with 2 mL of medium, transfer into a 60-mm bacteriological Petri dish with 5 mL of differentiation medium, and continue cultivation in suspension for 2, 3, or 5 d until the time of plating.
5. Transfer one EB separately into one well of gelatin (0.1 %)-coated microwell plates for morphological analysis, or transfer 20–30 EBs onto 60 mm tissue culture dishes containing four coverslips for immunofluorescence, or transfer 15–20 EBs onto 60 mm tissue culture dishes for RT-PCR with EB outgrowths.
6. The EB outgrowths are characterized by morphological analysis; calculate the percentage of EBs with the specific differentiated cell type.

3.2.2.1. Cardiogenesis

1. Use of 400–600 cells of ES cell lines D3, R1, or CCE for preparation of EBs is optimal for cardiac differentiation.
2. Culture with differentiation medium I (*see* **Note 2**).
3. At d 5–7, plate EBs onto gelatin-coated tissue-culture plates. (The first beating clusters in EBs can already be seen in 7-d-old EBs, but maximal cardiac differentiation is achieved only after plating. The first clusters of spontaneously beating cardiomyocytes appear 1–2 d after EB plating ***(8–10)***.


Embryonic stem cells (ESC) cultivated on feeder layer
0
Cultivation of ESC in hanging drops
1
Formation of embryoid bodies
2
2d
3
Transfer of embryoid bodies to bacteriological plates and cultivation in suspension
4
Plating of embryoid bodies (between day 5 and 7)
5d
5
7d
6
7
Differentiation time (d)
Differentiation of endodermal, ectodermal and mesodermal cells
- RT-PCR analysis
- Immunofluorescence and confocal imaging analysis
- Patch-clamp analysis
- Measurement of chronotropic effects of cardioactive drugs
Developmentally controlled expression of genes, proteins, action potentials and ion channels

4. For physiological investigation (*see* **Subheading 3.3.4.**) of early cardiac stages, plate EBs at d 5. EC cells of line P19 are induced by treatment with 1% DMSO or 10^{-8} *M* RA between the first 2 d of EB differentiation, and plating at d 5–7 ***(34)***.

3.2.2.2. Myogenesis

1. Use 600 cells of ES cell line BLC6 ***(12)***, 800 cells of line D3 or EG-1 ***(35)*** for optimal myogenic differentiation.
2. Culture with differentiation medium I or differentiation medium II.
3. Plate EBs at d 5. The first myoblasts appear 4 (line BLC6) or 5–6 d (D3) after EB plating. Skeletal muscle cells begin to fuse into myotubes in the EB outgrowths 1–2 d later.
4. A specific differentiation induction of skeletal muscle cells from ES ***(31)*** or EC ***(36)*** cells is achieved by 10^{-8} *M* RA or 1% DMSO.

3.2.2.3. Neurogenesis

The frequency of spontaneous differentiation of BLC6 and D3 cells into neuronal cells amounts to about 20–30%, whereas differentiation induction by RA ***(14–16)*** or other differentiation and growth factors ***(17)*** increases the differentiation rate of neuronal cells to nearly 100% of the EBs. Neuronal cells have been differentiated from ES ***(14–17)***, EG ***(35)***, and EC ***(37)*** cell lines.

1. Use 400 ES cells of lines BLC6 ***(14–17)*** and D3 ***(31)***, or 600 cells of line EG-1 ***(35)*** and cultivate in hanging drops or differentiate ES or EC ***(37)*** cells as mass culture for 5 d to prepare EBs.
2. Culture EBs of BLC6 cells in differentiation medium II with induction by 10^{-7} *M* RA between the first two days of EB culture, 2 d of suspension culture (without RA) and plating at d 4 ***(14)***. Differentiate D3 cells as EBs in differentiation medium I or II with induction by 10^{-8} *M* RA between days 2–5 and plate at d 7 ***(31)***.
3. Another protocol: Cultivate D3 cells as EBs (mass culture, *see* **Note 6**) for 4 d followed by a 4-d suspension culture in the presence of 5×10^{-7} *M* RA and plating at d 8 ***(15)***. Protocols for other ES cell lines are available ***(16,17***; *see* **Note 7**).

Fig. 1. Protocol for ES cell differentiation in vitro. Undifferentiated ES cells are cultivated as embryoid bodies (EBs) in hanging drops for 2 d and in suspension for additional days followed by plating onto gelatine-coated tissue culture plates. The morphology of 2-, 5-, and 7- d-old EBs is shown by scanning electron microscopy (Bar = 50 μm). The differentiation of cellular phenotypes derived from endodermal, ectodermal and mesodermal lineages is characterized by specific properties using the following methods: (a) gene expression patterns by semiquantitative RT-PCR, (b) protein formation by immunofluorescence and/or confocal laser scanning microscopy, (c) expression of action potentials and ion channels of excitable cells by patch-clamp analysis, and (d) chronotropic responses of cardiomyocytes.

3.2.2.4. Vascular Smooth Muscle Cell Differentiation

1. ES cells of line D3 ($n = 800$) are differentiated as EBs in hanging drops in differentiation medium I.
2. Plate EBs at d 7 and induce differentiation of vascular smooth muscle (VSM) cells by a combined treatment with 10^{-8} *M* RA and 0.5×10^{-3} *M* db-cAMP between d 7 and 11.
3. The first spontaneously contracting VSM cells that express the vascular-specific splice variant of smooth muscle myosin heavy-chain gene appear in the EBs 1 wk after plating ***(24)***.
4. Change the medium during the following differentiation period every day or every second day ***(24)***.

In another protocol using ES cells of lines AB1 and AB2.1, EBs were cultivated in supplemented DMEM medium for 4.5 d and after plating, the medium is partially exchanged every third day ***(23)***.

3.3. Characterization of Differentiated Phenotypes

3.3.1. Semiquantitative RT-PCR Analysis (see Note 8)

3.3.1.1. Preparation of Cell Samples (*see* **Note 9**)

The transcripts of genes which are specifically expressed during ES cell differentiation are analyzed by RT-PCR with primers of tissue-specific genes.

The following steps are used to harvest ES cells or EB outgrowths:

1. Discard the medium and wash twice with PBS.
2. Add 400 µL lysis buffer per 60-mm culture dish. Allow the lysis buffer to spread across the surface of the dish and transfer the lysate into a 1.5 mL microcentrifuge microtube.

The following steps are used to harvest EBs from suspension:

1. Collect EBs by centrifugation at 2000*g* for 3 min.
2. Wash the EBs twice by resuspension in PBS.
3. Add 100 µL lysis buffer per 10 EBs and lyse the cells completely.
4. Store samples at –20°C or –80°C.

3.3.1.2. Isolation of Total RNA (*see* **Note 10**)

The method described here is based on the use of a chaotropic agent (guanidine salt) for inactivation of ribonucleases ***(38)***.

1. Thaw lysate (400 µL) and vortex for 15 s.
2. Add 40 µL (1/10 vol) of 2 *M* Na-acetate, pH 4.0. Mix carefully.
3. Add 400 µL of acidic phenol (phenol saturated with DEPC-H_2O instead of Tris) and vortex vigorously.

4. Add 80 µL chloroform-isoamylalcohol (24:1) and vortex again.
5. Store for 15 min on ice.
6. Separate the organic and aqueous phases by centrifugation at 16,000*g* for 10 min at room temperature.
7. Transfer the upper aqueous phase carefully to a fresh tube, add an equal volume of isopropanol, and mix well. Store for 1 h at –20°C (*see* **Note 10**).
8. Centrifuge at 16,000*g* for 10 min at room temperature. Carefully discard the supernatant.
9. Dissolve the pellet in 300 µL of lysis buffer. If the pellet is difficult to dissolve, heat to 65°C for several min. Add an equal volume (300 µL) of isopropanol and mix well. Store at –20°C for 1 h.
10. Centrifuge at 16,000*g* for 10 min at room temperature. Carefully discard the supernatant.
11. Wash the pellet with 500 µL of 75% ice-cold ethanol (made with DEPC-H_2O), vortex briefly, recentrifuge at 16,000*g* for 10 min, discard supernatant, and allow the pellet of nucleic acid to dry in the air.
12. Dissolve RNA pellet in 30 µL DEPC-H_2O and freeze at –80°C.
13. The concentration of RNA is determined by measuring the optical density at 260 nm (OD_{260}). Dilute 1 µL RNA with 100 µL of DEPC-H_2O, measure OD_{260} nm and the concentration of RNA using GeneQuant RNA/DNA calculator (Pharmacia LKB Biochrom) or a suitable spectrophotometer, adjust all samples to the same RNA concentration (i.e., 0.2 µg/µL) with DEPC-H_2O, measure again to ensure the same RNA concentration of all samples. The yield of RNA from EBs ($n = 20$) is in the range of 20 to 100 µg.

3.3.1.3. Reverse Transcription Reactions (*see* **Note 11**)

All solutions for RT and PCR are offered by commercial suppliers ready to use. RT reactions are performed in 20 µL reaction volumes using 0.5 mL microcentrifuge tubes.

1. Label a number of PCR reaction tubes equivalent to the number of samples. Add the same amount of RNA (0.5–1.0 µg in 3 µL) to each tube.
2. Prepare the following RT-Mastermix for 25 reaction or a smaller quantity as required containing: 100.0 µL DEPC-H_2O, 50.0 µL 10X PCR buffer II, 100.0 µL 25 m*M* $MgCl_2$, 100 µL 5 m*M* dNTPs mix, 25.0 µL RNase inhibitor, 25.0 µL specific antisense primers or random hexamers or oligo $d(T)_{16}$ 25.0 µL MuLV reverse transcriptase to a total volume of 425.0 µL.
4. Add 17 µL of RT-Mastermix to each tube, and mix carefully.
5. Add two drops of paraffin wax (Fluka) to each tube.
6. Centrifuge briefly to separate the phases (20 s).
7. Transfer tubes to PCR machine and perform RT reactions for 1 h at 42°C then heat to 99°C for 5 min.
8. Cool the samples to 4°C or store at –20°C until use.

3.3.1.4. PCR Reactions (*see* **Note 12**)

1. Prepare a PCR-Mastermix for 25 reactions or a smaller quantity as required containing: 725 µL ddH_2O, 120 µL 10X PCR buffer II, 90 µL 25 m*M* $MgCl_2$, 40 µL dNTPs mix, 50 µL 10 µ*M* 5' sense primer 1, 150 µL 10 µL 3' antisense primer 1, 12.5 µL *Ampli*Taq DNA polymerase, 12.5 µL DMSO to a total volume of 1100 µL.
2. Label new PCR reaction tubes and add 2.0 µL of RT reaction product as template DNA.
3. Add 44 µL of PCR-Mastermix to each tube.
4. Mix by vortexing.
5. Add two drops of paraffin wax to each tube.
6. Centrifuge briefly to separate phases (20 s).
7. Transfer tubes to PCR machine and amplify cDNA by PCR for 35–45 cycles. The conditions will depend on the primers and thermal cycler used. Standard conditions are denaturation at 95°C for 40 s, annealing at 55–68°C for 40 s and extension at 72°C for 40 s.
8. Equal aliquots (4 µL) of second primer sets (antisense primer 2 and sense primer 2) were added at the appropriate cycle number by the „primer-dropping" method ***(39)***.
9. After the PCR machine cools down to 4°C, take tubes out or store at –20°C.

3.3.1.5. Post-PCR TReatment of Samples

1. Transfer the PCR products to 1.5 mL microcentrifuge.
2. Add 2.5 µL of a 1:4 mixture of glycogen:5 *M* NaCl and 150 µL ice-cold ethanol to each tube.
3. Incubate at –20°C for at least 1 h.
4. Centrifuge at 16,000*g* for 15 min.
5. Dissolve the pellet in 25 µL TE buffer, add 5 µL 6X loading buffer and store at 4°C.

3.3.1.6. Electrophoresis and Quantitative Analysis of Gene Expression

1. Separate one-third of each PCR reaction (10 µL) by electrophoresis on a 2% agarose gel in 1X TBE containing 0.35 µg/mL of ethidium bromide at 5–10 V/cm for 70 to 100 min.
2. Illuminate the gel with UV light and photograph using Polaroid film.
3. Quantitate the ethidium bromide fluorescence signals of gels. We use the E.A.S.Y. system (Herolab GmbH) and analyze the data using TINA2.08e software (raytest Isotopenmebgeräte GmbH.).

*3.3.2. Immunofluorescence Analysis (*see ***Note 13****)*

The formation of tissue-specific proteins in the EB outgrowths is analyzed by immunofluorescence (IF) with a normally equipped fluorescence microscope. For quantitative evaluation of immunolabeling a photometer system, and for the investigation of structural organization of proteins in EBs, a CLSM is necessary (*see* **Note 13**).

3.3.2.1. Plating of Embryoid Bodies

1. Place four sterile coverslips (18 × 18 mm) in one 60 mm Petri dish.
2. Incubate coverslips with 2.0 mL 0.1% gelatine for approximately 16 h (or at least 1 h) at 4°C.
3. Aspirate gelatin solution and add 3 mL of the appropriate differentiation medium.
4. Transfer EBs from suspension culture, and plate 20–40 EBs per dish using a transfer pipet.
5. Cultivate plated EBs in the incubator at 5% CO_2 and 37°C for several days according to the experimental differentiation protocol.

3.3.2.2. Immunofluorescence for the Detection of Tissue-Specific Proteins

For the characterization of the ES cell-differentiated phenotypes, monoclonal antibodies (mAbs) against tissue-specific proteins (i.e., intermediate filament proteins, sarcomeric proteins, synaptic vesicle proteins) are suitable (*see* **Table 1**).

1. Rinse coverslips containing EB outgrowths with PBS two times.
2. Fix cells onto coverslips with methanol: acetone (7:3) at –20°C for 10 min (for intracellular proteins), or alternatively, with 3.7% paraformaldehyde in PBS at room temperature for 10 min (depending on the antibody used).
3. Rinse cover slips with PBS at room temperature three times for 5 min.
4. Incubate fixed material with 10% goat serum in PBS in a humidified chamber at room temperature for 30–60 min to prevent unspecific immunostaining.
5. Incubate with the primary antibody at 37°C for 30–60 min, or at 4°C overnight (final concentration according to manufacturers instructions).
6. Rinse coverslips with PBS for three times at room temperature for 5 min.
7. Incubate with the secondary antibody (i.e., dilute DTAF-labeled goat antimouse IgG 1:100 in PBS with 0.5% BSA; or prepare DTAF-labeled goat antirat IgG, at 12 μg protein/mL final concentration, depending on the primary antibody) in a humidified chamber at 37°C for 45 to 60 min.
8. Rinse coverslips with PBS for three times at room temperature 5 min.
9. Rinse coverslips quickly with distilled water at room temperature.
10. Embed coverslips in mounting medium and analyze immunolabeled cells with a conventional fluorescence microscope at 100× magnification.

3.3.2.3. Immunofluorescence for the Analysis of Tissue-Specific Proteins on EBs (Quantitative IF)

1. Cultivate EBs in suspension for 3 d up to 20 d or even longer, according to the experimental protocol.
2. Transfer about 20 EBs per sample from suspension culture into an 1.5-mL microcentrifuge tube, allow the EBs settle to the bottom of the tube and aspirate the medium.
3. Wash EBs with 200 μL PBS at room temperature for two times and remove the PBS.
4. Fix EBs with methanol: acetone (7:3) at –20°C for 10 min. Alternatively, use 3.7% paraformaldehyde in PBS at room temperature for 10 min (depending on the antibody used).

Table 1
Antibodies for the Analysis of Tissue-Specific Proteins on ES Cell-Derived EB Outgrowths

Cell type	Cell-specific antibody	Antibody	Source	Ref.
ESC, ECC, EGC	Stage-specific embryonic antigen	SSEA-1	Developmental Studies Hybridoma Bank, University of Iowa	*(49)* *(50)*
Parietal endoderm	Cytokeratin K8[a]	TROMA-1	Hybridoma supernatant	*(51)*
Visceral endoderm	α-Fetoprotein		DBS, Fremont, CA	*(52)*
Cardiac and skeletal muscle cells	Desmin[a]	DE-U-10	Sigma	*(53)*
	Sarcomeric proteins			
	Titin	T11, T12	Sigma	*(54)*
	Sarcomeric myosin heavy chain	MF20	Hybridoma supernatant	*(55)*
	α-Sarcomeric actin	5C5	Sigma	*(56)*
Mesenchymal cells	Vimentin [a,b]	VIM-13.2	Sigma	*(57)*
Epithelial cells	Cytokeratin K8[a]	Ks 8.7	Boehringer Ingelheim	*(21)*
	Cytokeratin K19[b]	Ks19.1 (A53-B/A2)	Boehringer Ingelheim	
	Involucrin	BAbCo	Cambridge Bioscience	
Neuronal cells	Neurofilament protein (68 kDa)[a]	NR4	Boehringer Mannheim	*(14)*
	Neurofilament protein (160 kDa)[a]	NN18	Boehringer Mannheim	*(14)*
	Neurofilament protein (200 kDa)[a]	NE14	Boehringer Mannheim	*(14)*
	Synaptic vesicle protein			
	Synaptophysin	SY38	Boehringer Mannheim	*(58)*
	Neuronal cell adhesion molecule			
	N-CAM	NCAM-OB11	Sigma	*(14)*
Glia cells	Glial fibrillary acidic protein (GFAP)[a]	G-A-5	Boehringer Mannheim	*(14)*
Smooth muscle cells	Smooth muscle α-actin	1A4	Sigma	*(59)*
	Smooth muscle myosin heavy chain (fast)	MY-32	Sigma	*(60)*

[a]Intermediate filament protein.
[b]Vimentin, a marker protein of mesenchymal cells, is expressed in many cultivated cells.

5. Wash EBs with 200 µL PBS at room temperature for two times, and with 200 µL PBS containing 0.1% Triton X-100 at room temperature once.
6. Incubate fixed EBs with 100 µL of 10% goat serum in PBS at room temperature for 30–60 min to prevent nonspecific binding.
7. Incubate EBs with the primary antibody, then wash EBs twice with 200 µL PBS at room temperature.
8. Incubate with the secondary antibody.
9. Wash EBs three times with 200 µL PBS with 0.1% Triton X-100 at room temperature.
10. Embed EBs in mounting medium
11. For quantitative immunofluorescence we use an Axioscope microscope (Carl Zeiss, Jena) coupled to a microscope photometer system MPM20D (Zeiss).
12. Adjust the photometer system before each measurement.
13. Select an appropriate aperture to measure total fluorescence of one EB at 10-fold magnification (varies according to the size of EBs), measure at least 20 EBs per sample.
14. Fluorescence values are given by the photometer system as relative percentage values.
15. Calculate the mean values of relative fluorescence (in %) in Sigma Plot 3.0 (Jandel Scientific) graphs in correlation to the time of differentiation of EBs.

3.3.3. Pharmacological Studies for Characterization of ES Cell-Derived Cardiomyocytes

Cardiomyocytes that are differentiated from ES and EC cells of the mouse develop cardiac-specific physiological properties, as well as cardiac-specific receptors and signal transduction mechanisms ***(8–10,34)***. The ES cell-derived cardiomyocytes are suitable to investigate chronotropic effects of cardioactive substances.

3.3.3.1. Measurement of Beating Activity of Cardiomyocytes

1. Plate EBs separately onto 24-well microwell plates at d 5 or 7, cultivate the EB outgrowths for further 5–7 d until 85–100% of the EBs contain clusters of rhythmically contracting cardiomyocytes. Beating cardiomyocytes should be in 5–30% of the EB outgrowths. Change medium 1 d before measurements and add exactly 1 mL per well.
2. Place the 24-well microwell plate on the inverted microscope equipped with a 37°C heating plate and a CO_2 incubation chamber, localize independently beating areas (n = 20), measure the spontaneous beating frequency by counting the basal beating rate and calculate beats per min.
3. Add different concentrations of the test substance and incubate for 3 min before measurement. Determine dose-dependent effects of the beating frequency after cumulative application of increasing concentrations of the test substance, i.e., by adding positive chronotropic drugs (e.g., the Ca^{2+} channel opener BayK 8644 may be used as a positive control) or negative chronotropic drugs e.g., the Ca^{2+}

channel inhibitor Diltiazem may be used as a positive control), at final concentrations in the range of about 10^{-9} to 10^{-5} *M* depending on the test substances.
4. Count the beating rates, and estimate beats per min, calculate the mean values (± standard error of the mean) of beats per min for each data point from the pulsation rates of ES cell-derived cardiomyocytes without and with treatment of cardiotropic drugs, test for significance with the Mann–Whitney *U*-test and calculate dose-response curves.

3.3.3.2. Use of a Semiautomatic Screening System for the Measurement of Chronotropic Responses (Fig. 2)

1. Prepare EB outgrowths with beating clusters of cardiomyocytes.
2. Select different areas ($n = 20$) of pulsating cardiomyocytes by visual control under the inverted microscope (Diaphot-TMD, Nikon) coupled via a one-chip black- and white- CCD camera to a computer imaging station (Pentium CPU, 100 MHz) running the LUCIA Laboratory Imaging System including the HEART application (Nikon). The coordinates (x,y,z) of selected areas are collected by the LUCIA HEART System and the sponteanous beating activity is automatically determined for each area.
3. Add the test substance according to **Subheading 3.3.3.1.** Measure the beating frequency by the automatic imaging system of the LUCIA HEART system, increase cumulatively the concentration of test substances.
4. Final processing of data is done by the LUCIA HEART application, resulting in tables and graphs showing an increase or decrease in number of beats per minute including standard deviation (SD) or standard error of the mean (SEM), evaluate dose–response curves of chronotropic activity.

3.3.4. Preparation of ES Cell-Derived Cells for Physiological Analysis

Ion channels of excitable cell types are specific markers of differentiated cells. The whole-cell patch-clamp analysis permits an investigation of the developmentally controlled expression of ion channels during differentiation of ES cells into cardiomyocytes ***(9)***, skeletal muscle ***(12)***, neuronal ***(14,16)***, and VSM cells ***(24)***.

Whereas myocytes and neuronal cells can be directly analyzed by patch clamping on EB outgrowths, cardiomyocytes that differentiate in clusters have to be isolated as single cells by the following procedure (a modification of the method described in ***40***):

1. Isolate the beating areas of EBs ($n = 10$) mechanically using a microscalpel under an inverted microscope and collect the tissue fragments in PBS Dulbecco in a centrifuge tube at room temperature. Centrifuge at 1000*g* for 1 min and aspirate the supernatant.
2. Incubate the pellet in collagenase B-supplemented low-Ca^{2+} medium at 37°C for 25–45 min dependent on the collagenase activity.

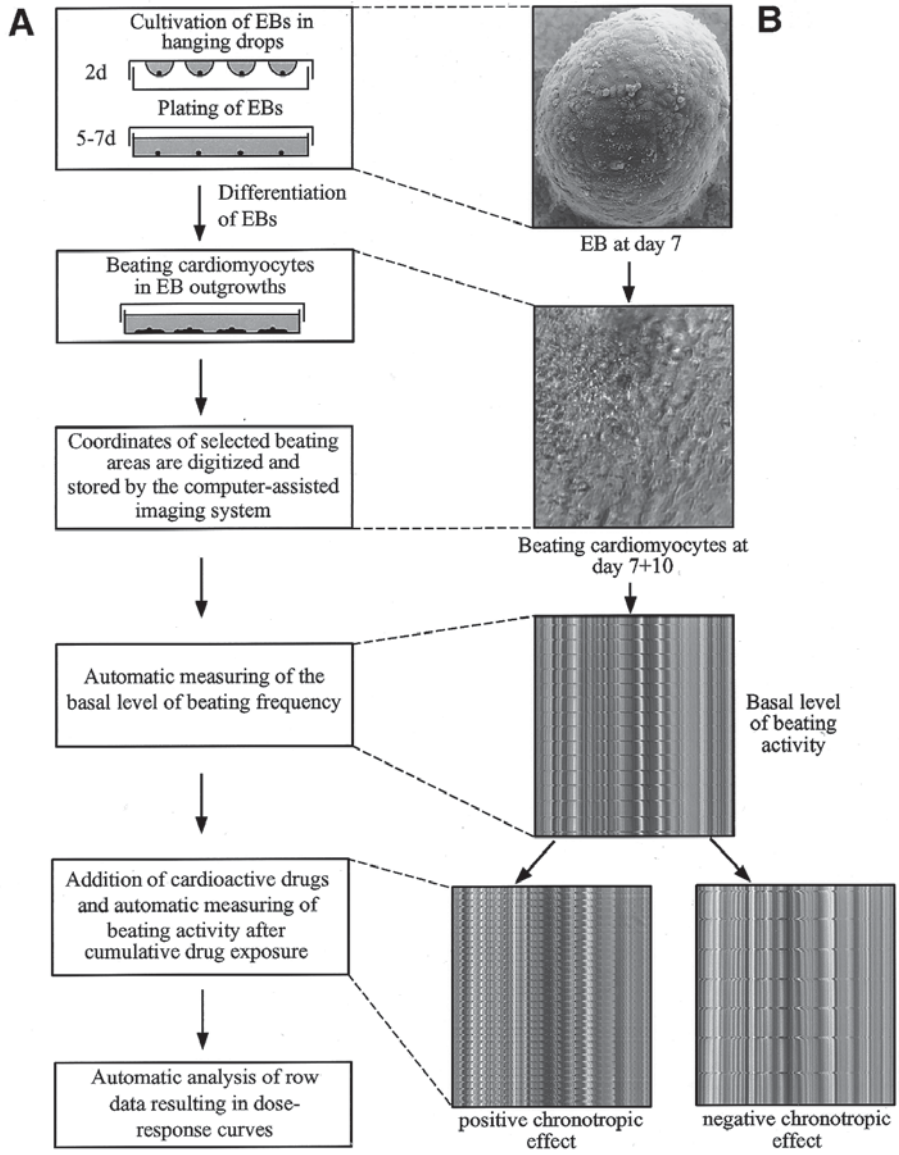


Fig. 2. A computer-assisted imaging system for analysis of pharmacological responses of ES cell-differentiated cardiomyocytes. ES cells are cultivated via EBs into the cardiogenic lineage (**A**, **B**, top). EBs are then plated onto tissue culture plates, areas of beating cardiomyocytes are selected and the coordinates digitized and stored by the computer-assisted imaging system (LUZIA HEART). The levels of the beating frequencies of cardiomyocytes are automatically measured before (= basal level) and after addition of positively or negatively acting cardiotropic agents (**B**, bottom).

3. Aspirate the enzyme solution, resuspend the cell pellet in about 200 μL KB medium and incubate at 37°C for 60–90 min.
4. Transfer the cell suspension into tissue culture plates with gelatine-coated small glass slides (5 × 8 mm, suitable for patch-clamp analysis) containing differentiation medium I and incubate at 37°C over night. The KB medium is diluted at least 1:10 with differentiation medium.
5. Change the medium with differentiation medium I, cardiomyocytes begin rhythmical contractions and are ready for patch-clamp analysis after a 3 h recovery time.
6. The whole-cell configuration of the patch-clamp-technique ***(41)*** is used for the characterization of ion channels of differentiated cells (for cardiomyocytes, *see* ***10***; for neuronal cells, *see* ***14***) according to electrophysiological standard techniques ***(42)***.

3.4. Loss-of-Function Analysis In Vitro and Modulation of Differentiation

The developmental pattern of EBs differentiated in vitro may be modulated by exogenous factors, i.e., RA ***(25,31)***, growth factors ***(30)*** or by genetic means: the targeted inactivation of genes (=loss of function, ***23,29***), or the overexpression of genes in ES cells (=gain of function, ***43***).

The in vitro differentiation of mutant totipotent ES cells has been used as an alternative and supplement to in vivo studies to analyze the phenotypes of mutant cells during early embryonic development. The strategy is especially useful in those cases, where the mutation results in early embryonic death in vivo. This has been successfully employed to the analysis of cellular differentiation of desmin- and β_1 integrin-deficient ES cells ***(23,29)***.

For the in vitro loss-of-function assay, the generation of homozygous mutant ES cells is necessary. This has been made possible by selection at high G418 concentrations or by using a second targeting vector in combination with, e.g., hygromycin selection.

4. Notes

1. ES cell lines should be cultivated without antibiotics. In some cases, i.e., for selection procedures, the addition of a penicillin/streptomycin mixture or gentamycin (Gibco-BRL, 1 mL of stock solution to 100 mL medium) may be helpful.
2. Both DMEM and Iscove's modification of DMEM (IMDM) can be used for efficient cardiac and myogenic differentiation of cell lines D3 and R1. IMDM is supplemented with 20% FCS and additives I, with the exception that α-monothioglycerol 3-mercapto-1,2-propandiol (MTG, Sigma, final concentration 450 μ*M*) instead of β-ME is used for differentiation induction experiments.
3. DEPC is a suspected carcinogen and should be handled with care.
4. If possible, the solutions should be treated with 0.1% DEPC at 37°C for 1 h and then heated to 100°C for 15 min or autoclaved for 15 min. DEPC reacts rapidly with amines and cannot be used to treat solutions containing buffers, such as Tris.

5. Whereas EC cell lines are cultivated without feeder cells, ES and EG cell lines need feeder cells for growth in the undifferentiated state. Some ES or EG cell lines (e.g., EG-1 cells, ***44***) need both feeder cells and LIF (= leukemia inhibiting factor) to keep growth in the undifferentiated state. LIF is commercially available (i.e., Gibco-BRL), or may be prepared from LIF expression vectors (*see* ***45–47***).
6. For preparation of EBs, three different protocols may be used: the hanging-drop method ***(8,33)***, the mass culture ***(4)*** or the methylcellulose technique ***(18,19)***. The hanging-drop method generates EBs of a defined cell number (and size). Therefore, this technique is used for developmental studies, because the differentiation pattern is dependent on the number of ES cells that differentiate within the EBs. For mass culture plate 5×10^5 to 2×10^6 cells (depending on ES cell lines used) into 6 cm bacteriological Petri dishes containing 5 mL differentiation medium. After 2 d culture, let the aggregates settle down in a centrifuge tube, remove the medium and transfer the aggregates carefully with 5 mL fresh differentiation medium into a new bacteriological dish. Change the medium every second day. Mass culture of EBs may be used for differentiation of a large number of differentiated cells. For hematopoietic differentiation, the methylcellulose method is used (*see* ***18,19***). Methylcellulose (e.g., MethoCult H4100; Stem Cell Technologie Inc., Vancouver, BC, Canada) is added to the differentiation medium at a final concentration of 0.9%.
7. Other protocols for neuronal differentiation that can be used are (1) 5×10^5 ES cells of line CGR8 are grown for 2 d in suspension culture in the presence of 10^{-6} *M* RA in 100 mm bacteriological plates, and replating of EBs in RA- and LIF-free medium at d 2 ***(16)***. (2) Another method describes differentiation factors (insulin, transferrin, selenium), growth factors (bFGF), and extracellular matrix proteins (fibronectin, laminin, polyornithine) as efficient neuronal differentiation inducers ***(17)***.
8. Use Gloves and Filter tips throughout the whole procedure.
9. Do not leave lysis buffer in Petri dishes longer than 5 min, as polystyrene is not resistant to lysis buffer.
10. Never mix and disturb the organic and the aqueous phases.
11. For RT-PCR, rTth DNA polymerase can also be used as both reverse transcriptase and DNA polymerase ***(48)***. In this case, the components of both RT- and PCR-Mastermix are different from using MuLV reverse transcriptase and *Taq*DNA polymerase.
12. Semiquantitative RT-PCR is used to detect the reletive levels of mRNA expression and includes at least two sets of primer pairs in coamplification reactions. A housekeeping gene, i.e., β-tubulin or HPRT, is used as an internal standard to control variations in product abundances due to differences in individual RT and PCR reaction efficiencies. The relative signal strenghts of PCR products of the target genes and the internal standard gene can be controlled by using varying numbers of cycles in the multiplex reactions ***(39)***.
13. Confocal laser scanning microscope (CLSM) analysis can also be used to study EBs. Confocal microscopy expands the visualization of small substructures beyond the limits of conventional light microscopy. For immunofluorescence

image analysis of EBs cultivated in suspension, it is necessary to use a CLSM because EBs are three-dimensional aggregates that require an extended depth of focus. Some EBs achieve up to 400 μm in diameter. Therefore, EBs are scanned in thin sections (0.5–10 μm) starting from the bottom and going to the top. The resulting single pictures are used to evaluate high-resolution images or stereoscopy images, or perform an image analysis. Appropriate filter combinations are selected depending on the fluorescent dye used. For example, FITC/DTAF-labeled samples can be excited with a 488-nm argon laser beam, light passes a beam splitter at 510 nm, and emitted light larger than 510 nm goes through a band-pass filter of 515–565 nm. Emitted light is collected by a photomultiplier and converted into signals for imaging analysis.

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18

Embryonic Stem Cells in the Study of Hematopoiesis

Francesca Lazner, Ismail Kola, Elizabeth Stadler, and Andrew G. Elefanty

1. Introduction

The hematopoietic system is a complex mix of stem cells, committed progenitors, and mature effector cells of at least nine different lineages. Although these cells perform very different functions, they arise from a common ancestor, the hematopoietic stem cell (HSC) ***(1–8)***. HSCs may be defined functionally by their ability to repopulate a myeloablated host. In order to achieve this, these cells must be capable of both self-renewal and commitment to different cell lineages. It has been suggested that lineage commitment involves the activation of a small subset of ''master genes'' that then initiate a cascade of events influencing the pattern of gene expression (and thus the phenotype) in the daughter cells ***(9–11)***. As consequence of these changes in gene expression, the functional diversity of cell types formed is increased, but the spectrum of lineage potentials for any given progenitor cell is diminished. The processes of lineage commitment and differentiation are regulated by an array of soluble cytokines ***(12)*** and growth factors ***(13–15)*** and by intercellular interactions ***(16)***.

1.1. Early Hematopoiesis

In the mouse embryo, hematopoiesis commences shortly after gastrulation when ventral mesoderm migrates into the developing yolk sac at embryonic day (E) 7.0 and forms aggregates of hemangioblasts ***(17–19)*** likely to represent a common precursor for endothelial and hematopoietic cells ***(20–22)***. These progenitor cells develop into blood islands, which consist of an outer layer of endothelium enclosing primitive hematopoietic cells.

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It was originally postulated that HSCs formed in the yolk sac seeded the fetal liver *(23)*, which remained the dominant hematopoietic organ until shortly before birth when hematopoiesis shifted to the bone marrow. However, the realization that definitive hematopoietic cells in avians and amphibians were derived from an intraembryonic source *(24)* lead to a search for analogous regions in the mouse embryo. These studies culminated in the discovery of a hematopoietic stem cell pool in the para-arotic splancnopleura/aorta–gonad–mesonephros (AGM) region of the E8–11 mouse embryo (*25–29,* reviewed in *30*). It now appears likely that the definitive HSCs in the mouse may also arise from an intraembryonic source, either exclusively or in conjunction with cells of yolk sac origin *(31)*.

1.2. Embryonic Stem Cell Differentiation Recapitulates Early Embryonic Development

Embryonic stem (ES) cells, which are derived from the inner cell mass of the preimplantation embryo *(32,33)*, provide a useful tool to dissect the processes of hematopoietic commitment and differentiation. Because ES cells can give rise to most hematopoietic cell types under the appropriate in vitro conditions *(34)*, they have proved very helpful in characterizing the role of many genes implicated in hematopoiesis.

When ES cells are cultured in the presence of leukemia inhibitory factor (LIF), they grow as aggregates of undifferentiated, pluripotent adherent cells. However, if disaggregated ES cells are cultured in suspension in the absence of LIF, they form spherical, endoderm-coated structures termed embryoid bodies (EBs), composed of differentiating cells derived from all three germ layers *(35)*. The rich diversity of tissue types that may form from EBs was exemplified by the studies of Chen and Kosco who transplanted EBs subcutaneously or under the kidney capsules of mice and showed that they formed a range of tissues including bone, blood vessels, cardiac muscle, and skin *(36)*. Hematopoietic cells arise in the EB in a temporal pattern that closely resembles that observed in the developing embryo. Primitive hemangioblasts, which can give rise to hematopoietic and endothelial cells can be grown from EBs after only 3–4 d of differentiation *(21,22)*. Committed hematopoietic progenitors for primitive and definitive erythroid colonies and myeloid colonies appear shortly afterwards from d 4–8 of EB differentiation *(37)*. Eventually, some embryoid bodies develop visible hemoglobinization or even endothelial-lined cysts harboring clumps of hematopoietic cells, analogous to the blood islands of the yolk sac (*see* **Fig. 1**). This similarity in the kinetics of hematopoietic development observed in the EB and early embryo *(34,37,38)* has validated the use of ES cells as a tool to study genetic events that are associated with hematopoietic differentiation.

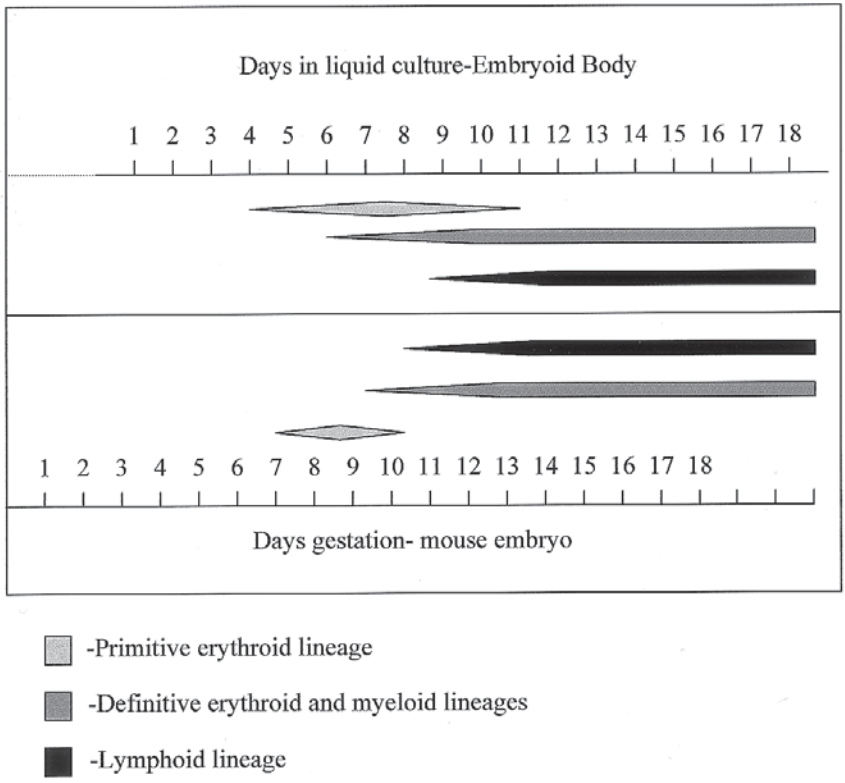

Fig. 1. The sequential appearance of primitive and definitive erythroid, myeloid, and lymphoid lineages in embryoid bodies grown in liquid culture closely follows the normal pattern of their emergence in the developing embryo. Top panel data from Chen and Kosco ***(36)***.

1.3. Generation of Embryoid Bodies in Culture

The formation of EBs can be induced by culturing ES cells in the absence of LIF in methylcellulose cultures or in liquid cultures as a cell suspension *ab initio* or following initial aggregation in hanging droplets ***(34)***. The culture of ES cells in hanging drops ***(39,40)*** promotes the formation of EBs because of the close association of the ES cells within the droplets. This method is most suitable in situations where the ES cell lines do not efficiently form EBs by other methods ***(34)***.

The in vitro development of mesoderm and hematopoietic progenitors in the EBs proceeds in the absence of exogenous growth factors in serum-containing cultures, but seems to require the addition of mesoderm inducers such as bone morphogenetic protein 4 or activin in serum-free differentiation cultures ***(41)***. Supplementation of serum-containing differentiation cultures

with a range of hematopoietic cytokines does not dramatically influence the onset of hematopoiesis within embryoid bodies, but Kit ligand and IL-11 do seem to improve the survival or promote the expansion of hematopoietic precursors once they form ***(37)***.

In suspension cultures, EBs are grown in bacterial-grade (hydrophobic) petri dishes or with an agar underlay to minimize adherence of the cells to the culture dish. The EBs shown in **Fig. 2** were generated in suspension culture in the presence of Iscove's modified Dulbecco Medium (IMDM) supplemented with 15% fetal calf serum (FCS), 5% protein-free hybridoma mix II (Gibco), 2 m*M* glutamine, 50 μg/mL ascorbic acid (Sigma), 200 μg/mL iron-saturated transferrin (Boehringer Mannheim) and 4.5×10^{-4} M α-monothioglycerol (α-MTG) (***20*** and G. Keller, personal communication).

1.4. Generation of Hematopoietic Cells from Embryoid Bodies

Although the molecular events that occur at early stages of hematopoietic differentiation are accessible through the analysis of primary EBs, the isolation and culture of erythroid, myeloid, and endothelial precursors from this heterogeneous mixture of cell types is necessary for the study of subsequent phases of hematopoietic differentiation. Committed progenitors for erythroid, myeloid, and mixed hematopoietic lineages can be cultured from dissociated embryoid bodies. Typically, day 4–5 embryoid bodies yield several hundred primitive erythroid colonies per 10^5 cells plated in the presence of erythropoieitin. It seems that the growth of primitive erythroid colonies is enhanced by the use of plasma-derived serum ***(37)***. Culturing later-stage embryoid bodies (optimally from d 6–12) allows the identification of definitive erythroid, macrophage, and granulocyte macrophage, mixed lineage, and mast cell progenitors.

Dissociation and in vitro culture of cells derived from d 3–4 primary EBs demonstrated that primitive and definitive erythropoiesis could arise in vitro from a common precursor ***(20)***. In these experiments, blast cell colonies were generated by culturing dissociated EBs in methylcellulose in the presence of vascular endothelial growth factor (VEGF), c-kit ligand (KL), and the supernatant from a transformed endothelial cell line, D4T ***(20)***. These blast cell colonies could be induced to differentiate into many hematopoietic lineages when cultured in methylcellulose in the presence of a cocktail of cytokines.

In follow-up studies, the same workers cultured blast colonies on matrigel-coated wells in media supplemented with hematopoietic and endothelial growth factors to show that a subset of these blast colonies also contained endothelial precursors ***(22)***. This provided experimental confirmation for the existence of a common endothelial and hematopoietic presursor — the hemangioblast, an elusive cell whose existence had long been postulated ***(42)***.

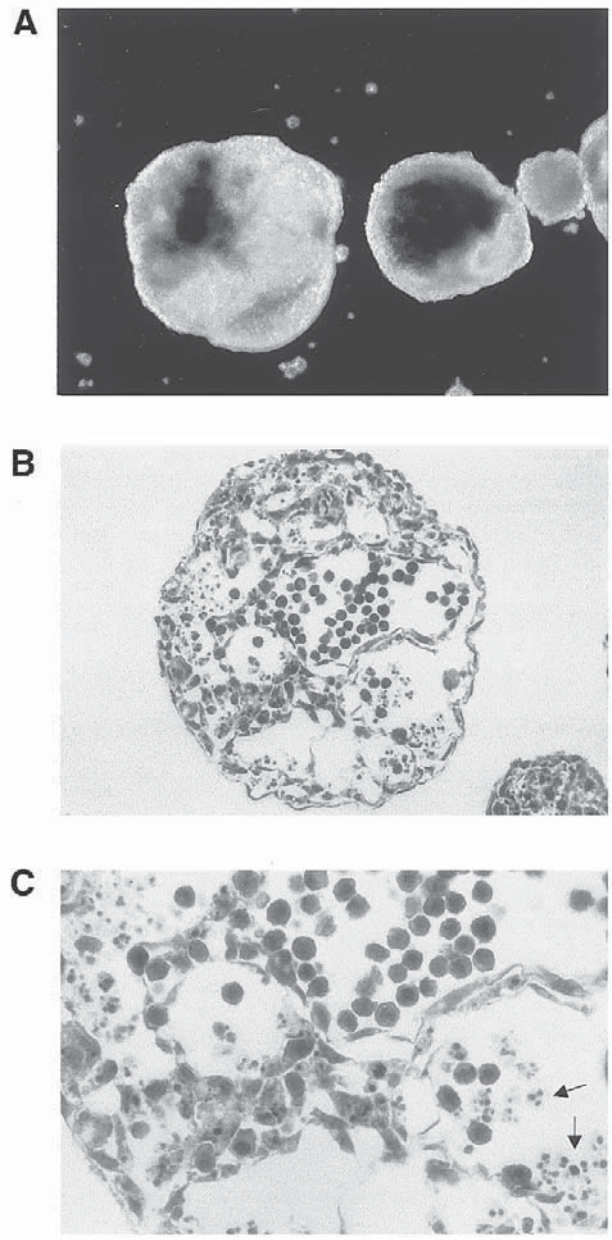

Fig. 2. **(A)** Cystic embryoid bodies differentiated for 13 d displaying foci of hemoglobinization. **(B,C)** Hematoxylin and eosin-stained sections of an 8-d embryoid body showing the development of cystic structures containing hematopoietic cells. Note the apoptotic cells (arrows) visible in the higher power view. Original magnification for **(B)** ×100, **(C)** ×200.

It is also tantalizing to speculate that the blast colonies just described may harbor hematopoietic stem cells. Along these lines, it is pertinent that Hole and colleagues found primitive hematopoietic precursors in day 4 embryoid bodies that were able to provide radioprotection and multilineage hematopoietic reconstitution in recipient mice ***(40)***.

1.5. Differentiation of ES Cells in the Absence of EB Formation

A number of workers have demonstrated that a three-dimensional structure, such as an EB, is not mandatory for the differentiation of ES cells. In these instances, ES cells deprived of LIF have been cocultured with stromal cell lines or on tissue culture flasks coated with collagen ***(21,43–47)***. These data imply that sufficient differentiative signals may be generated through soluble growth factors in the culture medium and through the cell–cell interactions of the ES cells with the stromal monolayer, the collagen matrix, and/or each other.

For example, Nakano and colleagues have reported the generation of primitive and definitive erythroid, myeloid, and B lymphoid cells from ES cells cocultures with OP9 cells as a stromal cell layer ***(43,47)***. The OP9 stromal cell line was derived from the calvaria of newborn osteopetrotic (op/op) mice, a spontaneous mouse mutant that lacks functional M-CSF due to a mutation in the M-CSF gene ***(48)***. Immature hematopoietic colonies derived from ES cells cultured on OP9 cells for 10 d were further differentiated by culturing in methylcellulose in IL-3 and Epo or IL-7 to generate cells of erythroid, myeloid, or B lymphoid lineages.

The bone marrow-derived ST2 preadipocyte cell line is useful for the differentiation of cells of the monocyte–macrophage-related lineages, at least partly because it produces significant amounts of M-CSF ***(49)***. Yamane and co-workers ***(44)*** showed that ST2 cells supplemented with dihydroxyvitamin D3 and dexamethasone could support the differentiation of osteoclasts (a macrophage-derived lineage that requires M-CSF for its generation ***[50]***) from ES cells, whereas OP9 cells were inefficient for osteoclast generation even when the culture medium was supplemented with M-CSF.

ES cells have also been induced to differentiate into pro-T and pro-B lymphocyte precursors, by coculture with bone marrow-derived RP.0.10 stromal cells ***(51)*** in the presence of recombinant IL-3, IL-6, and IL-7 ***(45)***. These precursor cells were reported to reconstitute of B- and T-lymphocyte populations in the thymus, spleen, and bone marrow after injection into sublethally irradiated mice. Palacios and colleagues ***(46)*** described culture conditions that supported differentiation of hemtopoietic stem cells from ES cells. Coculture of ES cells with RP.0.10 stromal cells, in the presence of IL-3, IL-6, and cellfree supernatant from FLS4.1 fetal liver stromal cells resulted in the formation of PgP-1 + c-Kit + Lin- stem cells after 21–25 d.

In a series of experiments that paralleled the blast cell colony assay described by Keller and colleagues *(20,22)*, Nishikawa and co-workers *(21)* initially differentiated ES cells on a matrix of type IV collagen for 4 d and observed that approximately 30% of the cells now expressed FLK-1, a tyrosine kinase receptor previously identified on vascular endothelium *(52)* and on immature mesodermal cells with hematopoeitic potential *(53,54)*, but not on undifferentiated ES cells. This first step was analogous to primary EB formation and displayed similar kinetics of FLK-1 induction *(52)*. To permit further differentiation of these FLK-1$^+$ cells, Nishikawa and colleagues *(21)* found that it was necessary to sort them away from the rest of the culture, a step that was similar to the dissociation of primary embryoid bodies that was required to generate blast colonies, as described earlier. These FLK-1$^+$ cells were cultured further on a collagen matrix to generate erythroid and myeloid hematopoietic cells or on OP9 stromal cells, on which FLK-1$^+$ VE-cadherin$^+$ endothelial cells proliferated in addition to hematopoietic cells. Single-cell deposition experiments demonstrated the existence of some FLK-1$^+$ cells, which could give rise to both endothelial and hematopoietic progeny — the hemangioblast also detected by Keller *(20,22)*.

1.6. Selection for Specific Cell Types by FACS Sorting

As alluded to above, a variety of different hematopoietic developmental stages can be recognized by their cells' surface phenotype using flow cytometry. The use of fluorescence-activated cell sorter (FACS) analysis to sort cells of a particular lineage adds another dimension to the potential studies which may be performed with differentiated ES cells. FACS sorting to obtain specific populations can be used after dissociation of primary EBs or during differentiation on stromal cultures.

1.7. Conclusions

The ability of ES cells to differentiate in vitro has been invaluable in characterizing their developmental potential and in allowing the generation of hematopoietic progenitors and mature cells. At this stage, the combination of growth factors required, signal transduction pathways used, and transcription factors involved in the differentiation of ES cells to hematopoietic cells are still largely unknown. The understanding of these events at a molecular level is clearly of great relevance scientifically and potentially therapeutically, as it may provide a means whereby cell fate may be manipulated in vitro. Even without this knowledge, the study of ES cells has been highly informative, especially in dissecting the function of genes whose absence leads to fetal lethality in the homozygous state and in overexpression studies evaluating the function of immortalizing or transforming genes *(55,56)*.

2. Materials

1. Trypsin/ethylene diamine tetraacetic acid (EDTA)/chicken serum: 0.25% Trypsin 1:250 (Sigma, cat. no. T-4799), 1 m*M* EDTA. This is supplemented with 1% heat-inactivated chicken serum (Life Technologies, cat. no. 200-6110PG), which significantly improves viability of hematopoietic progenitors cultured from replated embryoid bodies. Stored in 10 mL aliquots at –20°C. Although freshly thawed solutions are used to disaggregate embryoid bodies, trypsin solution may be stored at 4°C for 1–2 wk for use in routine passaging of ES cells.
2. Glutamine: 200 m*M* stock (Life Technologies, cat. no. 25030-081), stored in single use aliquots at –20°C.
3. Ascorbic acid: prepare stock solution of ascorbic acid (Sigma, cat. no. A-4403) at 5 mg/mL in water. Filter and store as single use aliquots at –20°C.
4. α-Monothioglycerol: Prepare a fresh dilution of 13 μL α-monothioglycerol (Sigma,cat. no. M-1753) per 1 mL of IMDM. This diluted solution is used at 0.3% in ES cell differentiation medium to give a final concentration of 450 μ*M*.
5. Protein-free hybridoma medium II (PFHM-II, Life Technologies, cat. no. 23600-026): The reconstituted solution is filtered and stored in aliquots at –20°C, stable for at least several weeks at 4°C.
6. ES cell differentiation medium: IMDM supplemented with 15% fetal calf serum, 5% PFHM-II, 2 m*M* glutamine, 50 μg/mL ascorbic acid, 200 μg/mL transferrin (supplied as 30 mg/mL solution in sterile IMDM from Boehringer Mannheim, cat. no.652 202), 450 μ*M* α-monothioglycerol (Sigma, cat. no. M-1753). The FCS is batch tested for its ability to support hematopoietic differentiation in embryoid bodies and for its ability to support the growth of hematopoietic progenitor cells from murine fetal liver and bone marrow. Heat inactivation (56°C for 30 min) is optional. The transferrin and ascorbic acid are optional. The inclusion of PFHM-II enhances hematopoietic differentiation in embryoid bodies.
7. Collagenase: To make 5% stock solution, dissolve 1 g of collagenase type I (Sigma, cat. no. C-2674) in 20 mL phosphate-buffered saline (PBS). Store at –20°C in single use aliquots of approx 0.5 mL. Make up 0.25% collagenase working solution by diluting stock solution 1:20 with PBS/20% FCS. Filter sterilize before use. As an alternative, make up 0.25% collagenase/PBS/20% FCS solution, filter and store in 5–10 mL single-use aliquots at –20°C.

3. Methods

3.1. 2% Methylcellulose (2X Stock)

1. Record the weight of a sterile 2 L Erlenmeyer flask and a magnetic stirrer.
2. Add 230 mL of sterile pure water to the flask and boil (in a microwave oven, before adding the magnetic stirrer).
3. Add 10 g of methylcellulose (Methocel MC, Fluka Biochemika, cat. no. 64630) while swirling the mixture. Sterilize by reboiling for several minutes.
4. Add the sterile magnetic stirrer, cover the methylcellulose mixture, and allow the mixture to cool to below 40°C while stirring. It forms a slightly opaque slurry.

5. Warm 250 mL of sterile 2X IMDM to 37°C and add this to the methylcellulose mixture in a tissue culture hood.
6. Adjust weight of IMDM-methylcellulose to 500 g with water.
7. Leave the mixture covered and stirring overnight in a cold room. The mixture should become completely clear. It may stop stirring because of the high viscosity.
8. Aliquot into sterile tubes (e.g., 15 mL/50 mL Falcon tube) and freeze at –20°C before use. The initial freezing is necessary for the methylcellulose to acquire its correct gel-like quality. Thaw aliquots at 4°C or at room temperature. These can be stored at 4°C for up to 4 wk.
9. The 1% methylcellulose working stock is generated by diluting the 2% stock with IMDM plus batch-tested FCS to a final concentration of 10%, 2 m*M* glutamine and 450 μ*M* α-monothioglycerol. The inclusion of 5% PFHM-II may enhance growth of some hematopoietic colonies. Specific growth factors are required for the growth of different colony types as outlined in **Note 4**.

3.1.1. In Vitro Differentiation of ES Cells into Embryoid Bodies in Liquid Culture

1. Passage ES cells 1–2 d prior to initiation of differentiation, aiming for a 25–50% confluent culture. The yield from a 25-cm^2 flask should be between 2 and 10×10^6 ES cells (*see* **Note 1**).
2. Change medium approx 2 h prior to trypsinization.
3. Trypsinize ES cells; they should completely detach within 3 min and be readily dispersed into a single-cell suspension. It may be necessary to disaggregate residual clumps by pipetting the cells several times through the blue tip of a Gilson or syringing through a-21 gage needle.
4. Neutralize the trypsin/EDTA solution with IMDM/10% FCS, spin the cells at 150*g* for 3 min in a refrigerated centrifuge, and resuspend the pellet in the same medium.
5. For a 25-cm^2 flask of ES cells cultured on feeder layers, the washed cells are transferred onto a 10 cm diameter tissue culture dish and incubated at 37°C for 45–60 min in 10 mL of IMDM/10% FCS to allow feeder cells to reattach.
6. Harvest nonadherent cells, spin down, and resuspend in 5 mL of fresh IMDM/10% FCS and count cells in a hemacytometer. Viability should be greater than 95%.
7. Dilute cells in ES cell differentiation medium and plate in bacterial-grade Petri dishes (e.g., 55 mm Petri dishes [Fisher Scientific] cat. no. 8-757-13-A). Sufficient cells are plated to allow the formation of 50–150 primary embryoid bodies per mL of culture. Depending on the ES cell line, this usually requires between 500 and 5000 ES cells per mL of culture. For many experiments, it is convenient to set up 5 mL cultures in 55 mm dishes.
8. Cultures are incubated in a humidified atmosphere of 8% CO_2 in air at 37°C. (The optimal CO_2 concentration depends on the bicarbonate concentration of the medium used.) Spherical embryoid bodies can be identified from 2 d of differentiation onward. Overt hemoglobinization is visible after 6–10 d.
9. At different time points, embryoid bodies can be harvested for disaggregation and replating for hematopoietic progenitor assays or for a range of analyses including

gene expression studies. Embryoid bodies are easily harvested by allowing them to settle by gravity in a 10-mL conical-bottomed test tube. This usually takes only approx 5 min. Very small embryoid bodies or those grown in methylcellulose (*see* **Subheading 3.1.2.**) will need to be harvested by centrifugation (150*g* for 3 min).

3.1.2. In Vitro Differentiation of ES Cells into Embryoid Bodies in Methylcellulose

1. Perform **steps 1–6** as before and mix the required number of cells with methylcellulose differentiation medium by briefly vortexing.
2. Allow the medium to settle for approx 5 min before dispensing as approx 1 mL aliquots to 35 mm Petri dishes using an 18-gage needle and syringe. For example, approx 4.5 mL of methylcellulose dispensed from a 5-mL syringe is needed to make three replicate 1 mL cultures in 35 mm Petri dishes.
3. Incubate the methylcellulose cultures are described in **Subheading 3.1.1., step 8**.
4. Harvest embryoid bodies by diluting the 1% methylcellulose approx 1:6 with IMDM/5% FCS, followed by centrifugation.

3.2. Detection of Hematopoietic Progenitor Cells in Disaggregated Embryoid Bodies

1. Harvest embryoid bodies from liquid or methylcellulose primary differentiation (*see* **Note 2**).
2. Wash embryoid bodies twice in PBS by centrifugation at 150*g* for 3 min.
3. Embryoid bodies below 8 d of differentiation are dissociated by brief trypsinization, whereas older embryoid bodies may require collagenase for dissociation.

3.2.1. Trypsin

1. Resuspend embryoid bodies from up to 15 mL of differentiation medium in 0.5 mL PBS and transfer to a small tissue culture dish (one well of a six-well plate is a convenient size).
2. Add 3 mL of freshly thawed trypsin/EDTA chicken serum solution and leave undisturbed for approx 2.5–3 min (*see* **Note 3**).
3. Neutralize trypsin by adding 3 mL of IMDM/10% FCS and disaggregate embryoid bodies by trituration for approx 1–2 min. It may be necessary to break up residual clumps by pipetting the cells several times through the blue tip of a Gilson or syringing them through a 21-gage needle.

3.2.2. Collagenase

1. Resuspend embryoid bodies from up to 15 mL of differentiation medium in 5 mL of 0.25% collagenase/PBS/20% FCS solution in a tissue culture dish.
2. Incubate at 37°C with intermittent swirling. It may take 30–60 min for the embryoid bodies to break up. Break up clumps by pipetting the cells several times through the blue tip of a Gilson or syringing them through a 21-gage needle.
3. Pellet cells by centrifugation, resuspend in IMDM/10% FCS, and count cells. The viability of cell suspensions derived from smaller embryoid bodies (less than 5 d) should be 90% or better (*see* **Note 4**).

4. Plate cells in 1% methylcellulose supplemented with appropriate hematopoietic growth factors (*see* **Note 5**) and incubate in a humidified atmosphere of 8% CO_2 in air at 37°C. Blast colonies and primitive erythroid colonies are visible in 3–5 d and definitive erythroid and myeloid colonies are usually scored after 7 d culture.

4. Notes

1. It is best to use ES cells within 2–3 wk of thawing for differentiation studies. Both feeder-layer dependent and independent cell lines can be used. We find that the former often give better hematopoietic differentiation, although a brief feeder cell depletion step is required.
2. Depending on the age of the embryoid bodies, different hematopoietic progenitor activity is observed. The earliest progenitors form blast cell colonies (2.5–4.5 d), followed by primitive erythroid colonies (4–6 d), definitive erythroid and myeloid colonies (6–12 d).
3. Embryoid bodies are very sticky and can readily clump together and the cells lyse unless left undisturbed during the trypsinization. The duration of trypsinization is as brief as possible. If prolonged, hematopoietic progenitor survival is compromised (even if cell viability seems very good at the time of plating).
4. Viability of cells disaggregated from older embryoid bodies will be poorer because of the apoptosis that accompanies embryoid body formation. The viability of cell suspensions derived from embryoid bodies dissociated with collagenase may be as low as 50–60%.
5. Blast colonies are stimulated by 5–10 ng/mL vascular endothelial cell growth factor (VEGF) and 100 ng/mL stem cell factor (SCF). At low cell concentrations, formation of these colonies is said to be enhanced by the inclusion of the conditioned medium of a transformed endothelial cell line, D4T, at 25% ***(20)***. Primitive erythroid colonies grow in the presence of 2–5 U/mL erythropoietin (epo). Growth of primitive erythroid colonies is enhanced by the substitution of fetal bovine plasma derived serum (Antech) for FCS ***(36)***. Definitive erythroid colonies require 100 ng/mL SCF in addition to epo and different myeloid colonies grow in the presence of 1000 U/mL of IL3, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) or macrophage colony stimulating factor (M-CSF). A cocktail of growth factors including epo, SCF, IL 3, GM-CSF and M-CSF will stimulate a broad range of hematopoietic progenitors.

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Interferon-Inducible ES Cell Expression Systems

Linda M. Whyatt and Peter D. Rathjen

1. Introduction

1.1. Embryonic Stem Cells as an In Vitro Assay System

The ability to culture homogeneous populations of pluripotent embryonic stem (ES) cells in the presence of leukemia inhibitory factor (LIF) or IL-6 (interleukin) family cytokines provides an experimentally tractable system for the investigation of gene function in pluripotent cell biology. The availability of a range of in vitro differentiation protocols ***(1–7)***, in combination with systems for the generation of gain- or loss-of-function mutations in ES cells, provides an unparalleled opportunity for investigation of the genetic control of cell differentiation and early mammalian development (*see* **Fig. 1**). Gain-of-function mutations are generally generated by cDNA overexpression and in this chapter we review the status of heterologous gene expression systems that can be used for this purpose in ES cells.

1.2. ES Cell Gene Expression Systems

1.2.1. Constitutive Gene Expression Systems

Viral and eukaryotic promoters have been used to direct constitutive heterologous gene expression in ES cells. Unmodified retroviral promoters are often unable to function in undifferentiated EC or ES cell lines, possibly due to lack of enhancer activity, *de novo* methylation, intragenic sequences, or negative *trans*-acting factors ***(8)***. Grez and co-workers ***(8)*** produced a recombinant mouse retroviral-based system termed murine embryonic stem cell virus (MESV), which could direct constitutive gene expression in ES cells. However, the utility of retroviral promoters is compromised by the fact that they can act unpredictably in differentiated cell types ***(9)***.

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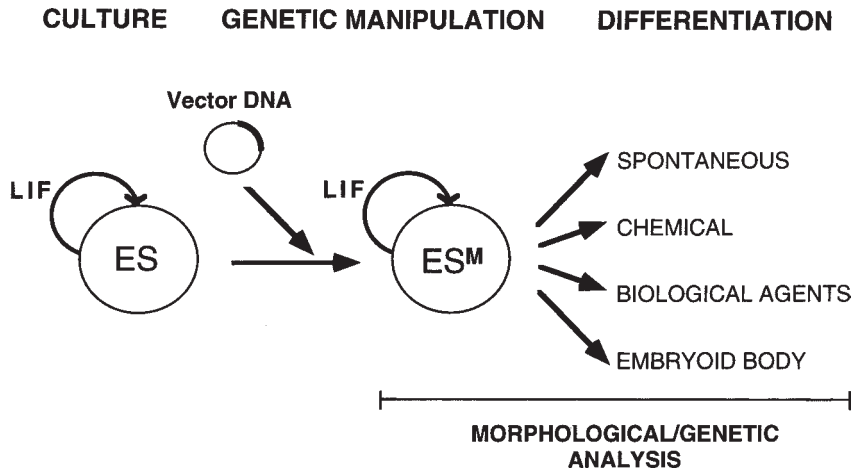

Fig. 1. Diagram illustrating use of the ES cell system for analysis of the genetic control of pluripotent stem cell renewal or differentiation. Loss- or gain-of-function mutations are generated in stable ES cell lines (ES^M) by genetic manipulation. The cellular consequences of altered gene expression are assessed in ES cells, or in their differentiated progeny produced by a variety of techniques in vitro.

Eukaryotic promoters such as that of the *Pgk-1* gene ***(10)*** can be used to direct constitutive, high-level gene expression in ES cells and behave more predictably in differentiated cell types. The *Pgk-1* promoter and the engineered viral promoter MC1 ***(11)*** have found extensive use in stable ES cell lines generated by gene targeting. High-level constitutive expression in transiently transfected ES cells can be achieved with promoters such as cytomegalovirus (***12***; P. Rathjen and A. Smith, unpublished observations), but these have not been characterized extensively in stably transfected ES cells. Although the choice of constitutive promoter allows some control over the levels of heterologous gene expression in ES cells, precise temporal and quantitative regulation of expression is not possible. This limits the application of these systems to the investigation of ES cell biology outlined as follows.

1.2.2. The Requirement for Inducible ES Cell Expression Systems

Overexpression of developmentally important genes in ES cells can result in altered cell proliferation, differentiation, and/or viability. In many cases these are irreversible processes, making it difficult to demonstrate that an altered cell phenotype is a direct result of the gain- or loss-of-function mutation and not the result of transfection or clonal selection. Inducible gene expression systems can potentially be used to circumvent this problem. Stable ES cell lines can be

generated in the absence of heterologous gene expression and divided into parallel clonal cultures. The effects of induced expression of the exogenous gene can then be compared with an uninduced control. Alterations in cell phenotype or behavior in the induced cultures can then be attributed directly to expression of the exogenous gene.

Full exploitation of the ES cell system for in vitro analysis of gene function requires the development of inducible ES cell gene expression systems with the following features:

1. Minimal/absent basal (uninduced) expression: An expression system with a tightly controlled on/off switch is required for analysis of gene products that can exert irreversible biological effects at low thresholds such as cytotoxic molecules or growth factors.
2. High levels of induced expression.
3. Quantitative control over the level of induced expression: Quantitative control over the level of induced expression would facilitate analysis of gene products that may exert distinct biological effects at different concentrations. For example, the growth factors activin A and TGF-β affect gene expression and cell differentiation during embryoid body development in a concentration-dependent manner ***(13)***.
4. An induction system that does not compromise ES pluripotentiality: The power of the ES cell system resides in the wide variety of cellular events that can be monitored in vitro. It is important that effects on pluripotential cells or their differentiated derivatives can be attributed to expression of the exogenous gene rather than to the induction system itself.

A gene expression system exhibiting these features would allow temporal and quantitative control of heterologous gene expression in ES cells, making possible the generation of gain-of-function mutations by overexpression of the gene of interest, or loss of function mutations by overexpression of antisense transcripts. The use of a generic induction signal might provide a system that could be used further to analyze gene function in differentiated cell types derived from the three primary germ layers. These can be generated from ES cells cultured as embryoid bodies ***(1)***. Inducible gene expression systems that can direct tightly regulable gene expression in the mouse might find additional use as a tool for the investigation of gene function in vivo, and may also be suitable for the precisely controlled delivery of therapeutics in genetically altered somatic cells generated for gene therapy regimes. These applications will not discussed further in this chapter.

1.2.3. Inducible ES Cell Gene Expression Systems

In general, two approaches have been used to generate inducible gene expression systems suitable for use in mammalian cells. Firstly, inducible eukaryotic promoters that are heatshock ***(14)***, heavy metal (A. Smith personal

communication; ***15,16***), steroid ***(17–19)***, interferon (IFN) ***(20,21)*** and xenobiotic or dioxin ***(22,23)*** inducible have been used to construct gene expression vectors (*see* **Tables 1** and **2**). In general, these systems display high levels of induced expression in a range of mammalian cell types, but most have not been characterized in stable cell lines or in ES cells. Inducible systems based on eukaryotic promoters generally require the use of inducing agents that are not specific for the expression system and can exert pleiotropic effects on mammalian cells. Furthermore, some induction regimes that can be used in somatic mammalian cell types may not be appropriate for use in ES cells, which are relatively sensitive to environmental perturbations in culture. The major limitation of the reported systems is high-level basal expression in the absence of induction, precluding their use for expression of molecules, which exert biological effects at low concentrations. An exception to this is the IFN-inducible expression system based on the promoter of the 6-16 gene, which has been shown to direct high levels of induced expression in stable ES cell lines in the absence of detectable basal expression ***(21)***.

In a second approach, engineered promoters and/or receptor/transactivator molecules have been used to generate artificial inducible mammalian expression systems (*see* **Table 1**). In general, these consist of a minimal promoter that is activated/repressed by heterologous transcriptional activators or repressors. Systems based on bacterial operons such as the lactose ***(24,25)*** or tetracycline ***(26,27)*** operons of *Escherischia coli*, mammalian hormone-inducible genes ***(28,29)***, the *Drosophila* ecdysone gene ***(30)***, and the yeast GAL4 gene ***(31)*** have been described. In most cases, these promoters are induced by treatment with agents specific for the expression system such as tetracycline, IPTG, or synthetic steroids. Although this overcomes limitations associated with use of nonspecific inducers, agents such as IPTG act slowly and inefficiently in eukaryotic cells ***(26)***, which may limit the use of these systems for analysis of genes that affect rapid cellular processes.

Some of these systems, such as the doxycycline and muristerone inducible systems (*see* **Table 1**) direct little or no basal expression in stably transfected cells, whereas high levels of induced expression can be acheived. In some cases, e.g., the doxycycline-, muristerone- and RU486-inducible systems (*see* **Table 1**), the levels of induced expression are also responsive to the dose of inducing agent. The major limitation of these systems is the general requirement for high-level coexpression of heterologous receptor/transactivator molecules. An initial round of transfection, selection, and characterization is generally required to generate stable cell lines that direct high-level expression of the heterologous receptor/transactivator molecule before stable cell lines expressing the exogenous gene can be generated. This can be both labor-intensive and time consuming. In addition, continuous, high-level expression of active heterologous receptor/transactivator molecules can be deleterious to

Table 1
Inducible Promoter Systems

Promoter	Other genes	Inducing agent	Cell line	Transfection	Assay	Induction	Comments	Reference
D. melanogaster hsp70	—	42°C	H9-T	Transient	CAT indirect	ND	No basal expression Transient expression	***(14)***
Mouse MMTV-LTR	Rat AR	R1881	CV-1	Stable	CAT	50-fold	Basal expression Dose responsive	***(19)***
Mouse GST Ya	—	Xenobiotic	Hep G2 Hepa 1c1c7 Hepa BP^{r}c1	Transient	CAT	5-fold	Basal expression Low induction levels	***(22)***
Rat CYP1A1	—	Xenobiotic/dioxin	Hepa-1 Transgenic mice	Transient	β-Galactosidase	30-fold >1000-fold	Basal expression Tissue specific expression	***(23)***
Mouse MT-1	—	Cadmium	C127	Autonomously replicating vector	Immunopre--cipitation	ND	Basal expression	***(15)***
Mouse *Mx*	—	Type I-IFN	NIH 3T3	Stable	Human PLC-γ^2 Northern/Western	25-fold	Basal expression	***(20)***
Human 6-16	—	Type I-IFN	MBL-5 ES EFC-1 ES	Transient Stable	CAT Northern	50-fold 100-fold	Basal expression (Tr^a) No basal expression (St^b)	***(21)***

^{a}Tr = transient.
^{b}St = stable.

Table 2
Engineered Promoter/Transactivator Systems

Promoter	Other genes	Inducing agent	Cell line	Transfection	Assay	Induction	Comments	Reference
AdMLP/GRE	Rat GR	Dexamethasone	CHO	Transient Stable	CAT t-PA assay/CSF	>1000-fold 2–5-fold	No basal expression Low induction levels (St^b)	***(28)***
AdMLP/GRE	—	Dexamethasone	HeLa	Transient Stable	CAT	ND 10–50-fold	Basal expression (Tr^a)	***(29)***
mouse MT-I/rFL/IRE	—	Zn^{2+}, Fe^{3+}/transferrin	C127	Autonomously replicating vector	Immuno-precipitation	90-fold	Requirement for low serum levels	***(16)***
SV40 early/ *E. coli lacO*	*lac* Repressor/ VP16 ts	Shift to 32°C from 39.5°C or IPTG	HeLa, L*tk*$^-$ L*tk*$^-$	Transient Stable	CAT	>200-fold 1200-fold	Basal expression Basal expression @ 37°C Dose responsive	***(24)***
mouse *PGK1*/ *E. coli lacO*	NLS/lac repressor	IPTG	CV1	Transient	CAT	9-fold	Basal expression	***(25)***
CMV tk/tetO	tetR/VP16	Removal of tetracycline	HeLa	Stable	Luciferase	>50,000-fold	Dose responsive Continuous presence of tetracycline	***(26)***
CMV/TetO	rtTA	Doxycycline	HeLa	Stable	Luciferase and	>5000-fold	Basal expression	***(27)***
TK/TetO	rtTA	Doxycycline	HeLa	Stable	β-galactosidase	400-fold	Low basal expression Dose responsive	
E/GRE/EcRE	VP16/EcR and RXR	Muristerone	CV1 293 Transgenic mice	Transient Stable	Luciferse and β-galactosidase	212-fold 20,000-fold	Basal expression No basal expression (St^a) Dose responsive Suitable in vivo	***(30)***
D. melanogaster hsp70/ GAL4 RE	VP16tPR	RU486	HeLa R1 ES	Transient Stable	CAT	120-fold 2–23-fold	Basal expression (Tr^B) No basal (St^a) Dose responsive Low induction levels (St^b)	***(31)***

St^a = Stable.
Tr^b = Transient.

mammalian cells ***(19,28)***, presumably via generalized "squelching" effects on cellular transcription by sequestration of essential transcriptional components ***(32)***. In some systems, continuous presence of an exogenous agent such as tetracycline ***(26)*** is also required to maintain the uninduced promoter state.

In general, greater success has been achieved with systems based on engineered inducible promoters, although many of these systems have not been fully characterized in stably transfected ES cells. The reversible tetracycline system ***(27)*** can be used in ES cells (A. Smith, personal communication), the UAS/TAXI system ***(31)*** is able to direct dose-dependent, low-level, inducible expression in stable ES cell lines (*see* **Table 1**) in the absence of basal expression, and the ecdysone inducible promoter ***(30)*** has been shown to function in transgenic mice produced by pronuclear injection (*see* **Table 1**).

1.3. An Interferon (IFN)-Inducible ES Cell Gene Expression System

1.3.1. The 6-16–Based Expression Vectors

The human 6-16 gene is an IFN-stimulated gene (ISG) responsive to induction by type I IFNs ***(33,34)***. The 6-16 promoter contains two interferon-stimulated responsive elements (ISREs) ***(35,36)*** that are thought to bind activated STAT 1 and 2 proteins following IFN-induced signal transduction ***(37,38)***. The human 6-16 gene is induced preferentially and to high level in response to IFN treatment while the levels of uninduced expression are extremely low ***(39)***. These characteristics make the promoter of the 6-16 gene an attractive candidate for construction of inducible mammalian expression vectors.

Two promoter fragments of the human 6-16 gene ***(35)*** (promoter 1 [1.04 kb] and promoter 3 [0.65 kb]; **Fig. 2A**; ***21***) have been used in construction of IFN-inducible expression vectors. Both promoters contain a 0.6-kb sequence that includes the ISREs 5' of the 6-16 RNA initiation site. Promoter 1 terminates within the first intron of the 6-16 gene, whereas promoter 3 terminates within the first exon (*see* **Fig. 2A**). Results in stably transfected L*tk*- cells showed that promoter 1 directed higher levels of IFN-induced reporter gene expression than promoter 3, but also displayed higher levels of basal expression ***(35)***.

Four IFN-inducible expression vectors were constructed by insertion of promoters 1 and 3 into the promoterless backbone of pXMT2 (p1OX, p3OX; ***21***) and pOT (p1OT and p3OT; **Fig. 2B**; ***21***). Promoter pXMT2 is a modified version of pMT2 ***(40)*** that contains the mouse dihydrofolate reductase gene and the polyadenylation addition sequence from simian virus 40 (SV40) positioned 3' of the cDNA expression site. The vector also contains the adenovirus VAI gene, which improves the translational efficiency of plasmid-derived transcripts by inhibition of PKR activation ***(41)***. If the coding region inserted at the polylinker of pXMT2-based 6-16 expression vectors lacks a polyadenylation addition sequence, transcripts derived from these vectors carry the DHFR

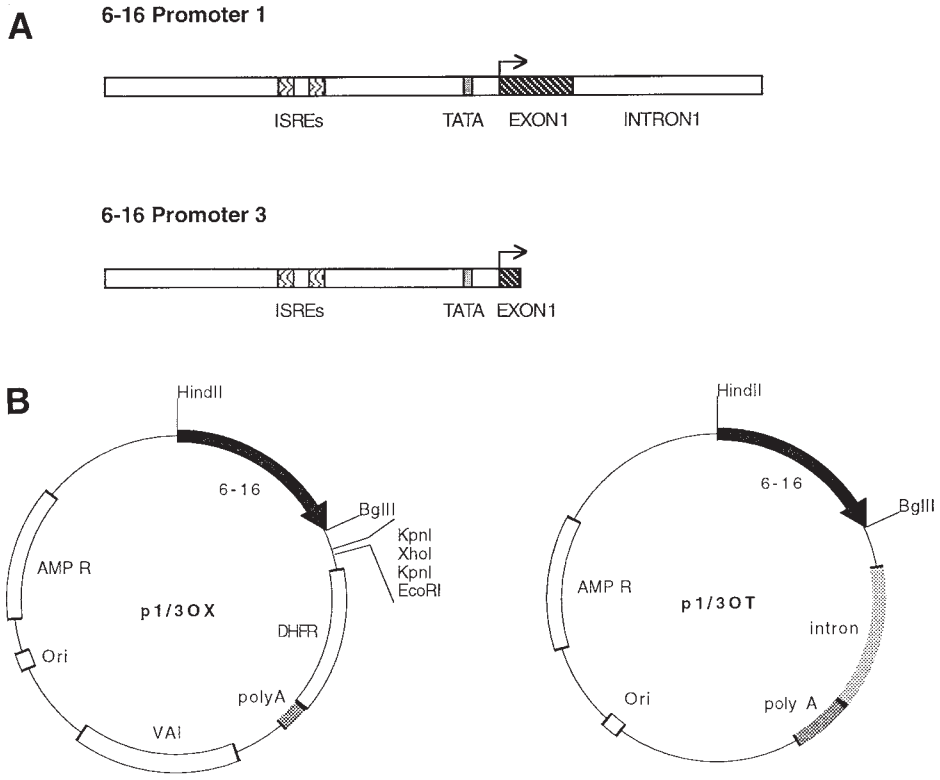

Fig. 2. Diagram illustrating the 6-16 promoters 1 and 3 **(A)**, and the 6-16–based ES cell expression vectors constructed using the pXMT2 (p1/3OX)- or pOT (p1/3OT)-derived backbones **(B)**. The transcription initiation site is indicated with an arrow. ISRE, interferon-stimulated response element; Poly A, polyadenylation addition sequence; Ori, bacterial origin of replication; AMP R, β-lacatamase gene; DHFR, dihydrofolate reductase gene; VAI, VAI gene.

gene, which is thought to enhance message stability. Promoter pOT ***(21)*** contains the small-t antigen intron and polyadenylation addition sequence from SV40 positioned 3' of the cDNA expression site.

IFN-inducible expression directed by these vectors has been demonstrated in both transiently and stably transfected ES cells. Initial results using a CAT reporter gene ***(21)*** indicated as follows.

1.3.1.1. IFN-Inducible Expression

Each-expression-vector-directed IFN-inducible expression is transiently transfected in ES cells with induction ratios ranging from approx 3- to 50-fold. In stably transfected cells, induction ratios of approx 100-fold could be

achieved. Promoter 1 directed the highest levels of induced expression and induction ratios as has been seen in other cell types ***(35)***. This suggests that sequence(s) within the first intron of the 6-16 gene have a positive effect on the level of IFN-induced expression.

1.3.1.2. Uninduced Expression

Transiently transfected cells displayed variable but low levels of basal expression. This contrasts with the situation in stably transfected cells, in which basal expression was undetectable in 18/18 independent clones (*see* **Fig. 3A**).

1.3.1.3. Dose Responsiveness

The levels of IFN-induced expression increased linearly with increasing doses of IFN up to 1000 U/mL in transiently and stably transfected cells. In cells stably transfected with the p1OX-derived vector, expression was detected within 2 h of IFN treatment, whereas maximal levels of expression were seen by 6 h and were maintained for at least 42 h. This suggests that the 6-16–based vectors will be suitable for the analysis of genes that require an extended period of expression in order to exert biological effects.

1.3.1.4. Effect of Plasmid Backbone

The highest levels of induced expression and induction ratios were directed by the promoters in the pXMT2-based vectors in both transiently and stably transfected ES cells. Higher levels of basal expression were also directed by the pXMT2-based vectors. This indicates that vector context can affect the level of both basal and IFN-induced expression directed by the 6-16 promoters. Variable expression levels may be attributable to the positive effect of the VAI gene contained on the pXMT2-based vectors and/or aberrant processing of the SV40 small-t antigen intron ***(42)*** contained within transcripts derived from the pOT-based vectors.

1.3.1.5. Effect of Inducing Agent

IFNs can induce a range of cellular effects including inhibition of viral infection, reduction in cellular proliferation, and modulation of differentiation (reviewed in ***[43]***). Treatment of a range of ES cell lines with concentrations of IFN up to 5000 U/mL had no discernible effect on colony number, colony size, or cell differentiation in vitro. Importantly, IFN treatment did not compromise the developmental potential of ES cells following return to the blastocyst.

The 6-16-based vectors have been shown to direct inducible gene expression in a range of feeder-independent ES cell lines including MBL-5 ***(44)***, E14 and E14TG2a ***(45)***, EFC-1 ***(46)***, and CP1 ***(47)***. A range of genes have been expressed in transiently and stably transfected ES cell lines using these vectors

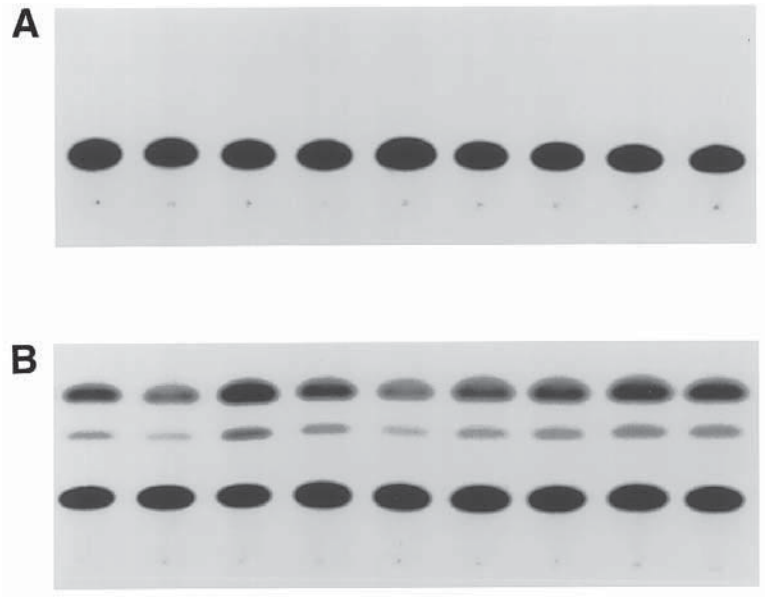

Fig. 3. CAT assay carried out on cell extracts (150 μg protein) from uninduced **(A)** or induced (48 h, 500 U/mL IFN), **(B)** independent stable ES cell lines carrying the CAT reporter gene in vector p3OT.

including the *Hesx1b* transcript of the homeobox gene *Hesx1* ***(48)***, and variant transcripts of the cytokine gene LIF (B. Haines, personal communication). The level of transcription in uninduced lines, as assessed by RNAse protection, varied according to the vector copy number, although lines with very low or absent basal expression were obtained in each case, suggesting that the vectors will be generally suitable for generation of gain of function ES cell mutants. The demonstration of IFN-inducible expression of an antisense Jak1 transcript in ES cells directed by the 6-16 vector p1OX ***(49)*** suggests that the 6-16–based vectors may also be suitable for the generation of loss-of-function ES cell mutants. Although inducible expression of proteins including β-galactosidase ***(50)*** and FLP recombinase ***(51,52)*** has also been achieved in ES cells, the 6-16–based vectors have not been optimized for RNA processing or translation, suggesting that further improvements to the levels of inducible expression may be achievable.

The ubiquitous nature of the IFN response ***(53)***, coupled with the demonstrated ability of the 6-16–based vectors to function in primitive ectoderm-like cell types, generated by the in vitro differentiation of ES cells (J. Rathjen, J. Lake, M. D. Bettess, J. M. Washington, and P. D. Rathjen, manuscript in preparation), and a range of differentiated mammalian cell types ***(39,50)*** suggests that the vectors will be suitable for use in the cell types that are produced as a

normal part of the differentiation pathway and may also provide a generic system suitable for the analysis of gene function in vitro.

1.4. Limitations of the 6-16–Based Expression System

Use of an inducing agent such as type I IFNs, which are active in mammalian cells, obviates the need for coexpression of heterologous receptor/transactivator molecules. This reduces the resources required for production of stable ES cell lines, reduces the effects caused by clonal variation, and prevents the potentially deleterious effects of continuous overexpression of transactivator/receptor molecules, as it overcomes the need for two rounds of stable transfection. However, use of IFN as an inducing agent may modulate cellular effects caused by expression of the gene of interest. Although this problem can potentially be controlled in vitro by treatment of mock transfected lines with IFN, this cannot circumvent problems associated with synergy between the expressed gene and IFN treatment. In addition to this, endogenous expression of IFN in vivo may limit use of the 6-16–based vectors in the mouse.

The effect of vector backbone on the levels of expression directed by the 6-16 promoter suggests that it may be possible to improve the expression characteristics of the vectors. Possible modifications would include removal of the splice donor site present on the 6-16 promoter 1, and replacement of the small-t antigen intron in the pOT-based vectors, which may reduce the translational efficiency of transcripts derived from these vectors ***(42)***. The removal of candidate translation initiation sites upstream of the cDNA expression site may also improve the translation efficiency of heterologous cDNAs. Alternatively, effective expression vectors may be generated by cloning the 6-16 promoters into other expression vector backbones that have been optimized for gene expression at the level of transcription and/or translation.

Due to variation caused by the site of integration and plasmid copy number, a range of stable cell lines carrying the 6-16 vectors may be required in order to produce lines that express appropriate levels of both induced and uninduced expression. Alternatively, development of an episomal 6-16 expression vector, potentially based on a polyoma virus vector suitable for use in ES cells ***(54)***, may overcome some limitations associated with integration into the ES cell genome.

2. Materials

1. ES cell lines: ES cell lines were obtained from the following sources. MBL-5, Dr. Lindsay Williams (Ludwig Institute, Melbourne, AU); E14, Dr. Anna Michelska (Murdoch Institute, Melbourne); E14TG2a, Dr. Austin Smith (Centre for Genome Research, Edinburgh, Scotland). Work with the ES cell lines EFC-1 and CP1 was carried out in the laboratory of Dr. Austin Smith (Centre for Genome Research, Edinburgh).

2. ES cell growth medium: To Hams F12 medium add 14 m*M* $NaHCO_3$, 1.176 g/L, and 80 μg/mL gentamycin (Schering). Adjust the pH to 7.2, filter through 0.22 μm and store at 4°C. Make complete ES cell medium from Dulbecco's modified essential medium (DMEM), high glucose, with L-glutamine and sodium pyruvate, without sodium bicarbonate) with 44.5 m*M* $NaHCO_3$, 3.7 g/L, 50 μg/mL gentamycin (adjust pH to 7.3, filter through 0.22 μm and store at 4°C) by the addition of 15% fetal bovine serum (Gibco-BRL), 1 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, and 1000 U/mL LIF or equivalent (*see* **Note 1**).
3. 2X BES- buffered saline (BBS): 50 m*M* *N,N-bis*(2-hydroxyethyl)-2-aminoethane-sulphonic acid (BES, Calbiochem), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4. Adjust pH to 6.99, filter through 0.22 μm, and store at 4°C.
4. 0.25 *M* $CaCl_2$: 27.8 g/L. Filter through 0.22 μm and store at 4°C.
5. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 1.47 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4. Filter through 0.22 μm.
6. Trypsin/EDTA: 1:250 trypsin, 1.0 g/L (Gibco-BRL); 0.54 m*M* EDTA, 137 m*M* NaCl, 2.7 m*M* KCl, 1.47 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4. Filter through 0.22 μm.
7. Gelatin: 0.1% gelatin (Sigma) in PBS. Filter through 0.22 μm.
8. L-glutamine: 100 m*M* L-glutamine (Sigma), 14.6 g/L. Filter through 0.22 μm and store at 4°C.
9. β-Mercaptoethanol: 0.1 *M* β-mercaptoethanol (Sigma) in PBS. Store at 4°C.
10. IFN (mouse α and β fibroblast IFN, Sigma). Store at –80°C.
11. Tissue culture plasticware: Falcon, Becton Dickinson Labware.
12. Filters: Millipore-Steritop-GV (0.22 μm).
13. Tissue culture incubators with 10% (growth of ES cells) and 3% (transfection) CO_2.
14. Biological safety cabinet.
15. Tissue culture laminar flow cabinet.
16. Benchtop centrifuge.
17. Vortex.

3. Methods

3.1. Growth of ES Cells

Grow feeder-independent ES cell lines at 37°C in 10% CO_2 on plastic culture vessels that have been coated with 0.1% gelatin and passage every 3–4 d as required.

3.2. Transient Transfection of ES Cells by Calcium Phosphate Coprecipitation (see Note 2)

1. Rinse exponentially growing ES cells in 100 mm diameter plates with PBS and incubate in 1 mL of trypsin/EDTA for 1 min at 37°C.
2. Add 1 mL of complete ES cell medium and remove cells from plate by pipetting. Harvest by centrifugation at 1200 g in a total volume of 20 mL complete ES cell medium.

3. Aspirate medium, resuspend cells in complete ES cell medium, and perform a cell count. Seed 5×10^5 ES cells into 35 mm diameter wells of a multiwell tray and incubate for 24 h at 37°C in 10% CO_2.
4. Mix 5 μg of 6-16–based expression vector DNA (purified through a CsCl density gradient) with 125 μL 0.25 *M* $CaCl_2$ and to this add 125 μL 2X BBS dropwise with mixing. Vortex the mixture and incubate at room temperature for 20 min.
5. Wash cells in a 1:1 mixture of complete ES cell and Hams F12 media with 10% foetal calf serum and leave the cells in a volume of 2.25 mL of this medium.
6. Add the DNA mixture dropwise to the cells while gently swirling the plate.
7. Transfect the cells for 16 h at 37°C in 3% CO_2. Remove the precipitate from the cells by rinsing twice in complete ES cell medium and incubate for 2 h at 37°C in 10% CO_2.
8. Add the appropriate amount of IFN (for high induction levels 1000 U/mL is sufficient) and incubate for an appropriate amount of time at 37°C in 10% CO_2.

3.3. Stable Transfection of ES Cells by Calcium Phosphate Coprecipitation (see Notes 3,4)

1. Seed 2×10^6 ES cells into 100 mm diameter dishes and form precipitate using 500 μL each of $CaCl_2$ and 2X BBS and a total of 20 μg of DNA.
2. Cotransfect the 6-16–based expression vector DNA and resistance expression vector DNA (e.g., neomycin resistance expression vector) at a ratio of 1:5.
3. One day after the transfection mix is washed from the cells, select for transfected cells in complete ES cell medium containing selective agent (200 μg/mL G 418 (Gibco-BRL) if selecting for neomycin resistance) and replace the medium every 48 h.
4. Pick resistant colonies after 7 d of selection by rinsing the plate twice in PBS and removing individual colonies. Pipet 30 μL of trypsin onto each colony, agitate the colony from the plate, and transfer to 200 μL selective medium contained in 14 mm diameter wells. Disrupt the colonies by vigorous pipetting, add 2 mL of complete ES cell medium, and expand under selection.
5. Screen resistant colonies by Southern blot for the presence of the 6-16–based expression vector DNA.
6. Seed 5×10^5 exponentially growing stably transfected ES cell lines into duplicate 35 mm diameter wells and grow overnight at 37°C in 10% CO_2.
7. Induce expression in half of the seeded ES cells 2 h after replacing the medium with fresh complete ES cell medium by treatment with the appropriate amount of IFN (1000 U/mL IFN for high-level induction) (*see* **Note 5**).
8. Measure expression from the 6-16–based expression vector using an appropriate assay. The results of an experiment using a chloramphenicol reporter are shown in **Fig. 3**.

4. Notes

1. For routine maintenance of pluripotential ES cells unpurified, LIF overexpressed by transfected Cos cells ***(5)*** or recombinant LIF (ESGRO, Amrad) can be used.

Complete ES medium is supplemented with L-glutamine and β-mercaptoethanol as it is used, as these components are relatively unstable during storage.

2. The calcium phosphate coprecipitation transfection technique is dependent on high-quality DNA. Highest efficiencies are achieved with CsCl density gradient purified DNA preparations ***(56)***. High transfection efficiencies using BES-buffered DNA–calcium phosphate precipitates are dependent on optimal pH ***(55)***. It was found that a pH of 6.99 was optimal for transfection under the conditions we describe and that thorough vortexing of the DNA calcium phosphate mixture assisted in precipitate formation. A fine, dark-colored precipitate should become visible within the 16-h incubation at 3% CO_2.
3. Generation of stably transfected cell lines can be achieved efficiently using the calcium phosphate coprecipitation method of transfection ***(55)***. Transfection using this technique requires formation of a DNA-calcium phosphate precipitate, although the mechanism of DNA uptake by this method is not understood ***(56)***. This method transfects cells with a high plasmid copy number, and generally leads to integration of concatamers into the genome of stably transfected cell lines. We used a modification of this technique ***(21)*** to generate transiently and stably transfected ES cells in the technique described.
4. Cells with high plasmid copy number are generated by calcium phosphate coprecipitation method of transfection, and this may produce stable cell lines with unacceptably high levels of basal expression. Transfection of ES cells by electroporation would provide an alternative method for the generation of stable ES cell lines. This has been achieved with a derivative of p1OT containing a LIF cDNA at the expression site and a Pgk-neo resistance cassette ***(21)***.
5. High levels of induced expression directed by the 6-16–based expression vectors can be maintained for at least 42 h in the continuous presence of IFN. Removal of IFN from the cell culture medium of a stable cell line carrying the CAT reporter in p1OX after 8 or 10 h caused a reduction in the level of expression after 48 h from 95% of maximum to 74% and 65% of maximum, respectively. The maximal expression at earlier time points was unaffected ***(21)***. This suggests that it may be possible to induce a short "pulse" of heterologous gene expression by treatment of stably transfected cultures with IFN for short periods.
6. Assessment of reporter gene expression directed by the 6-16–based vectors was carried out using CAT assays (as described by Gorman and colleagues, ***57***). When nonreporter genes are cloned at the cDNA expression site of the vectors, expression at the RNA level can be assessed by RNAse protection ***(58)*** or Northern ***(59)*** analysis or at the protein level by Western ***(59)*** analysis.

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20

Transgenic Studies in the Mouse

Improving the Technology Towards a Conditional Temporal and Spatial Approach

Sika Ristevski

1. Introduction

The biological consequences of altered gene expression as a result of gain of function mutations or gene dosage remains an important question in molecular biology and molecular medicine. This is of particular interest in medical research due to the many clinically relevant diseases that involve altered gene expression. The biology of cancer is one such example whereby a spectrum of genes may be inappropriately activated or inactivated contributing to disease progression. These genes provide ideal and direct targets for the development of cancer therapeutics and emphasize the requirement for transgenic mice as in vivo disease models. Furthermore, the inappropriate expression of transcription factor encoding genes may lead to dysregulation of gene expression and significant biological consequences. These questions can only be addressed by using technical strategies that allow the introduction or activation of a test protein in a given spatial and temporal manner. New technologies are continually being sought in order to generate sophisticated animal models that appropriately reflect the genetic basis of the disease of interest. These animals have the added benefit of being invaluable in drug discovery ***(1)***.

Here we review the current literature on transgenic mouse models and the important experimental factors to consider in the design of such experiments. Some of these factors, such as position effect and methylation, are discussed in more detail in Chapter 22.

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2. Transgenic Mouse Models of Gene Overexpression

Traditionally, gene overexpression or gain of function experiments were performed in tissue culture using cell lines infected or transfected with viral vectors. These early experiments helped to define the enhancer/promoter elements of viruses such as simianvirus 40 (SV40) ***(2)***, herpes simplex virus (HSV) ***(3)***, human cytomegalovirus (HCMV) ***(4)***, and mouse mammary tumor virus (MMTV) ***(5)*** and the transcription regulatory proteins that regulate gene expression through these enhancer/promoters, thus establishing the path for the characterisation of eukaryotic gene expression. These cell culture models were highly informative and transfection experiments continue to be used today employing a spectrum of homologous and heterologous promoter vectors. The transfection of fibroblasts with the activated oncogenes *RAS* and *MYC* not only established a role for cellular and viral genes in the transformation process but demonstrated that oncogenesis is a multistep process requiring oncogene cooperativity ***(6,7)***. Value was added to these experiments by the establishment of mouse models by organ reconstitution ***(8)*** and retrovirus infection ***(9)***.

Development of transgenic strategies for the germline transmission of transgenes in the mouse (reviewed in ***10***) rapidly facilitated an in vivo model of oncogenesis and the further elucidation of cellular pathways, which, when aberrant, contribute to the transformation process (***11***,***12***, reviewed in ***13***). It also became clear from these studies that the cell type and tissue context of activated oncogene expression was a major determinant in malignant progression.

Since their inception, transgenic mice have been used to further our understanding of gene function. For example, Alzheimer-type neuropathology was observed in mice overexpressing a mutant form (V717F) of the beta-amyloid precursor protein (APP) ***(14)***, supporting a primary role for APP in this debilitating disease and providing an ideal model for the testing of therapeutic drugs.

2.1. Ectopic Gene Expression

Many mammalian expression vectors are based on viral enhancer/promoters, such as SV40, HCMV, HSV, Rous sarcoma virus (RSV), and MMTV, primarily due to their high transcription enhancing activity and little cell type or species preference. These features are desirable in the transfection of cell lines where the objective is to express constant and high levels of protein. However, these vectors do not facilitate a control of the timing or level of expression, making it difficult to study the function of genes which are lethal when expressed inappropriately and at high level, such as those involved in cellular apoptosis.

Viral enhancer promoter-based vectors have been used in the generation of transgenic mice. The HCMV major immediate early promoter is active in fetal ***(15)*** as well as adult tissues ***(16)*** and has been used to drive the expression of a variety of genes including those that are involved in embryonic development.

However, the high-level, predominantly ectopic, expression during embryogenesis may be lethal. In addition, HCMV is not a pan-specific promoter but displays both organ and cell type specificity that correlates with the tissues naturally infected by the virus in the human host ***(17)***. This may be a limitation if the desired organ or cell type is not expressing the transgene.

In our experience, the use of the HCMV promoter to drive the overexpression of the transcription factor Ets2 was inappropriate as no transgenic founders were obtained for the generation of transgenic mice, presumably due to lethal expression levels *in utero*.

The use of endogenous housekeeping gene promoters, such as the translation elongation factor-1 alpha (EF-1α) promoter ***(18)***, and the ubiquitin C promoter ***(19)*** have been used as alternatives to viral promoters. Although transcriptional activity from these promoters may be reduced relative to viral promoters, this may be advantageous for the generation of transgenic mice.

2.2. Tissue-Specific Gene Expression

The characterization of genes and their regulatory elements has identified a growing number of tissue specific and tissue predominant promoters. These promoters are useful in the generation of tissue specific transgene expression. Examples of tissue-specific promoters include *TIE2*, specific to vascular endothelium ***(20)***, the neuron-specific enolase promoter ***(21)***, a minimal *Msx-1* promoter specific for craniofacial tissues ***(22)*** and the cardiac specific alpha-myosin heavy chain ***(23)***. Currently, the only limitation to the use of a tissue-specific promoter is that its characterization may not be complete or that a promoter for a specific cell type may not as yet have been available.

2.3. Inducible Gene Expression

The temporal control of transgene expression by the use of inducible systems has many advantages over continuous transgene expression. This is most important if a gene functions at many developmental stages whereby an early phenotype may mask a later function.

A number of systems have been developed and include the metallothionein gene promoter, which is induced by heavy metals, the MMTV LTR, which is hormone responsive, the interferon-inducible *6-16* and *Mx1* promoters, as well as a number of bipartite systems induced by the antibiotics teracycline/doxycycline, the insect hormone 20-OH ecdysone, or the synthetic estrogen analog 4-hydroxytamoxifen.

2.3.1. Metallothionein Promoter

The mouse metallothionein-1 gene promoter ***(24)*** is induced by heavy metals such as zinc and has been used in the generation of transgenic mice that

overexpress cell surface β1,4-galactosyltransferase ***(25,26)***, the cytokine IL-6 ***(27)***, and the transcription factor A-myb ***(28)***. In our laboratory, *Ets2* overexpression transgenic mice were successfully generated using either the mouse or sheep metallothionein promoters. These mice display neurocranial, viscerocranial, and skeletal abnormalities that resemble the skeletal abnormalities found in humans with Down syndrome ***(29)***. These studies support the role for *Ets2* in skeletal development and as *ETS2* is trisomic in Down syndrome, a role for its overexpression in the genesis of the abnormalities observed in this syndrome. Disadvantages of this system include the heavy-metal toxicity to cells, that induction does not give rise to high level expression, and that transgene expression has been observed without heavy-metal induction ***(29)***.

2.3.2. MMTV

The MMTV-LTR (mouse mammary tumor virus long terminal repeat) promoter is a glucocorticoid hormone inducible promoter that functions in responsive cells and tissues ***(30,31)***. Due to its inducible and high activity in breast and urogenital tissues, it has been used as an ideal system for the generation of transgenic mice that model tumor development in these tissues ***(32–34)***.

2.3.3. Interferon-Inducible Gene Expression

A number of interferon inducible genes have been identified including the human genes *6-16* and *OAS* (2'5'oligoadenylate synthetase) (reviewed in ***34***) and the murine *Mx1* ***(35)***. This property has been exploited in the design of interferon-inducible expression vectors. Expression from these promoters is absent or minimal in the noninduced state and can be activated up to 100-fold on interferon stimulation. A major advantage of this system is that all cells are interferon responsive. The *6-16* promoter has been used to drive interferon-inducible gene expression in embryonic stem cells, providing an ideal system for the functional analysis of developmental control genes (***36***, *see* also Chapter 19). The *Mx1* promoter has been used to control expression of the Cre recombinase in transgenic mice ***(37)***.

2.3.4. Hormone-Inducible Gene Expression

Hormone-inducible expression systems are primarily based on a bipartite model with the expression of a hormone responsive receptor combined with a receptor-inducible promoter to drive expression of the transgene. Three systems in current use are described as follows.

2.3.4.1. Ecdysone Receptor

Morphogenesis in the fruitfly *Drosophila melanogaster* is triggered by the steroid hormone 20-OH ecdysone, which functions via its nuclear receptor.

This natural steroid-inducible system has been manipulated to produce an ecdysone-inducible mammalian expression system that does not appear to affect mammalian physiology and is not toxic or teratogenic ***(38)***. In transient transfections, the ecdysone-inducible system has been shown to have a lower basal activity and higher inducibility compared to the tetracycline-inducible system (*see* below). This system has been tested in transgenic mice with significant transgene induction observed after intraperitoneal hormone injection, providing an ideal system for transient inducible expression.

Generation of transgenic animals requires the initial generation of an ecdysone receptor transgenic line with confirmed expression in all tissues of interest. Second, several transgenic lines carrying an ecdysone inducible gene of interest are generated and intercrossed with the transgenic receptor line. Upon administration of ecdysone, mouse tissues are examined for induction of gene expression. This bipartite system can be adapted for tissue-specific transgene expression by the generation of tissue-specific ecdysone receptor expression.

2.3.4.2. Nuclear Steroid Hormone Receptor

The steroid hormone receptors for estrogen, progesterone, glucocorticoid, mineralocorticoid, and androgen, have generated interest in their use as inducible expression systems. These receptors are activated by the binding of ligand whereby they are released from an inactive state, in complex with a number of cellular polypeptides including Hsp90 ***(39)***. Ligand binding results in nuclear translocation of the receptor and transcriptional regulation through binding to respective hormone response elements. This feature has been used in the design of steroid-inducible expression systems such as the dexamethasone-induced activity of the glucocorticoid receptor used to drive expression of genes under the control of glucocorticoid response elements ***(40)***. An alternative approach involves a fusion of the heterologous protein and the hormone-binding domain (reviewed in ***41*** and ***42***). Activity is regulated post-translationally by the addition of hormone, which releases the heterologous fusion protein from its inactive state. However, these systems have had a limited use due to the high levels of unstimulated activity (no hormone added) and only 10–20-fold induction on stimulation. In addition, they have not been commonly used in the generation of transgenic animals due to endogenous circulating hormones.

The mutant murine estrogen receptor G525R has been used to overcome the problems associated with this system ***(43,44)***. The mutant receptor no longer binds 17β-oestradiol, has no ligand-induced transcriptional activation activity, and is only responsive to the synthetic steroid 4-hydroxytamoxifen (OHT), making this an ideal receptor-inducible expression system ***(45)***. Like estrogen, OHT binds the estrogen receptor, which in turn regulates the expression of

estrogen-responsive genes. However, the G525R mutation of the estrogen receptor (ER™) renders it responsive to OHT rather than estrogen, thus abolishing the problems associated with unstimulated expression and endogenous estrogens. Furthermore, a fusion protein between the gene of interest (this system is designed to work best with nuclear-localized or nuclear-functional gene products, although not restricted to these molecules) and the mutant hormone-binding domain of ER, which is under the control of a highly active viral promoter (such as CMV), results in expression of a cytoplasmic localized fusion protein. Upon stimulation with OHT, there is a rapid translocation to the nucleus and hence transcriptional regulation by the gene of interest. This system has been used successfully for the induced expression of cellular transcription factors ***(45)***, as well as for protein kinases involved in cellular signalling pathways ***(46)*** and Cre recombinase ***(47)***.

An alternative bipartite approach involves the use of a potent estrogen responsive transcriptional activator comprising the hormone binding domain of ER fused to the DNA-binding domain of the yeast Gal4 protein and the VP16 transcription activation domain to create the fusion protein Gal4-ER-VP16. The gene of interest is placed under the transcriptional regulation of a Gal4-responsive promoter. Addition of ligand (estrogen) results in the translocation of Gal4-ER-VP16 to the nucleus and subsequent activation of gene expression ***(48)***. The induction kinetics of this system are not as rapid as the ER heterologous fusion system and it would benefit with modification of ER to ER™ in order to render the hormone-responsive transcriptional activator responsive to OHT.

2.3.4.3. The Tetracycline/Doxycycline-Inducible Expression System

The tetracycline (tc) induced gene expression system is a bifunctional system that has been modified to act either as an ''on'' or ''off'' switch for gene expression. Control elements of the Tn*10* tetracycline resistance operon of *Escherichia coli* have been used in a bipartite system along with the tetracycline-repressor (tetR) to create an efficient tetracycline-regulated switch for gene expression in mammalian cells ***(49,50)*** and for the temporal control of gene expression in transgenic mice ***(51–53)***. The system is based on the constitutive expression of a tc-controlled transactivator fusion protein, tTA, and consists of the DNA-binding tc repressor (TetR) fused to the C-terminal domain of the herpes simplex virus transactivator protein VP16. In the absence of tc, tTA binds and transactivates minimal promoters carrying multiple tet-operator (*tetO*) sites whereas in the presence of tc DNA binding does not occur and the transgene is silent. This 'off' switch is known as the tet-off system.

A mutant of TetR referred to as rTetR (compared to the transactivator protein rtTA) has a reversed function whereby doxycycline (dox, a tc derivative)

induces binding of rtTA to *tetO* and subsequent promoter activity ***(54)***. In the absence of dox, the transgene is silent, whereas in the presence of dox, a conformational change in rtRA results in its ability to bind DNA. This ''on'' switch is known as the tet-on system. Due to the rapid induction kinetics of the tet-on system relative to the much slower gene silencing kinetics of the tet-off system, it has become a popular tool for the induction of gene expression, particularly as tc and its derivatives are not toxic to mammalian cells at the low doses required in these experiments. The tet-on system is the method of choice for transgenic animals as breeding of animals is required and long-term administration of tc may result in suboptimal fertility and reproductive abnormalities.

This system has been improved with the development of a tc-induced autoregulatory tTA (rather than constitutive tTA). This eliminates problems associated with toxicity of the tTA protein as well as facilitating higher expression levels of the transgenic protein ***(55)***. In addition, a tc antagonist has been identified that has a reduced half-life and thus facilitates accelerated gene switching ***(56)***.

The ease of use of the tet-on and tet-off systems has resulted in a wide variety of studies, including the conditional expression of CaMKII ***(55)***, Epo ***(56)***, C/EBPβ ***(57)***, N-Myc ***(58)***, IRF-1 ***(59)***, Ins(1,4,5)P_3 ***(60)***, $p21^{Waf1/Cip1/Sdi1}$ ***(61)***, IκBα repressor mutants ***(62)***, the temporal control of Cre recombinase expression in transgenic mice ***(63)***, and its use as a potential vector system for gene therapy ***(64)***.

Transgenic lines carrying tTA or rtTA with confirmed expression in all tissues of interest must be established. Second, several transgenic lines carrying a *tetO*-inducible gene of interest are generated and intercrossed with the transgenic receptor lines. Upon administration of tc or dox, mouse tissues are examined for the reduction or induction of gene expression.

3. Important Considerations for the Design of Transgenic Experiments

3.1. Tagging Transgenic Protein

It is useful to design transgenic constructs such that a molecular tag is incorporated allowing the transgenic protein to be distinguished from its endogenous counterpart. In some cases, only moderate induction may be desired or achieved and it is imperative that induction at the protein level is achieved and can be confirmed. An added advantage for tagging the transgenic protein facilitates determination of its subcellular localization.

Tagging of the protein is best achieved by the fusion of an epitope to which there is a reliable antibody. The nucleotide sequence for the tag is incorporated into the open reading frame of the desired gene during construction of the expression vector. It is important to avoid disruption of functional domains and for this reason tags are often incorporated at the extreme N- or C-terminus.

Tagging systems include the human c-*myc* 9E10 epitope ***(65)*** and the FLAG epitope, for both of which monoclonal antibodies are commercially available. A variety of tags have been incorporated into expression vectors including a six-histidine, as well as tags derived from influenza virus haemaglutin, vesicular stomatitis virus G protein, and bacteriophage genes. The myc epitope has been used extensively and in a broad range of studies ***(66–70)*** as has the FLAG epitope ***(71–75)***.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used as a marker for gene expression in a number of organisms ***(76–78)*** as it is readily detected with high sensitivity, does not appear to be toxic to cells, and does not interfere with embryonic development. Interestingly, GFP fusion proteins have been used to monitor heterologous proteins in live zebrafish ***(77)***. Furthermore, a recent report demonstrates that GFP is a more-sensitive reporter system for transgenic mice than *lacZ* ***(79)***.

3.2. Enhancing Transcription

Intervening sequences (introns) have been shown to play an important role in heterologous gene expression. A number of independent studies have demonstrated that introns derived from the homologous gene ***(80)*** or a heterologous gene ***(81–84)***, increase transcriptional efficiency in transgenic mice. The splicing and 3' polyadenylation/transport pathways of the cell are coupled. Therefore, the inclusion of intronic sequences into expression constructs results in enhanced RNA 3' processing and accumulation of mature cytoplasmic RNA ***(85)***. In addition, regulatory sequences have been identified within introns that enhance tissue-specific transcription ***(86)***.

In the case of generating a minigene construct, the homologous promoter and introns (usually intron I and in certain cases intron II) ***(80)*** or a heterologous promoter and its corresponding introns ***(86)*** are used to drive expression in transgenic animals. This system is the method of choice in experiments where gene dosage and tissue-specific expression are important criteria.

The transcriptional activity of transgenes is unpredictable primarily due to uncontrolled random integration, such as integration into silent chromatin. Matrix or scaffold attachment regions can impart position-independent regulation of transgenes ***(87–89)*** by maintaining an open chromatin configuration. The locus control region sequences of human CD2 have been shown to be essential for the establishment and maintenance of an open chromatin domain in transgenic mice, and activity is detected even on integration into centromeric positions that would usually result in transgene silencing ***(90)***. Furthermore, a locus control region (LCR) from the TCRα locus has been identified and constitutes a widely active chromatin-opening function allowing for ubiquitous expression of linked transgenes ***(91)***.

3.3. YAC Transgenics

Yeast artificial chromosome (YAC) technologies, due to their capacity to stably propagate >2 Mb fragments of genomic DNA, provide an ideal basis for the generation of transgenic mice as models for human genetic disease as well as for the functional analysis of higher-order genomic structure (reviewed in ***92*** and ***93***). YAC transgenics provide the potential for functional screening of chromosomal DNA, which has not been fully characterized, and the identification of functional genes of interest. For example, a panel of transgenic mice containing a contiguous 2-Mb set of YAC/P1 clones from human chromosome 21q22.2 has been established (***94*** and refer to Chapter 21). Functional screening of this panel implicates the gene *minibrain* in learning defects associated with Down syndrome ***(95)***.

A number of problems have been associated with the generation of YAC transgenics. Large DNA fragments are highly viscous, making them difficult to microinject and a low integration efficiency requires multiple oocyte injections. These problems have been partly overcome by incorporation of antibiotic resistance markers such as *neomycin* into YACs followed by transfection into ES cells.

3.4. Enhancing Translation

The expression of heterologous proteins may be enhanced, by incorporation into the expression construct, of viral sequences that function to increase translation efficiency. A 60-bp 5'-leader sequence derived from tobacco mosaic virus RNA has been shown to enhance the translation of heterologous genes ***(96)***.

4. Common Problems Associated with the Generation of Transgenic Mice

Transgenic mouse lines are generated by microinjection of the linear DNA of interest into the nucleus of an oocyte or transfected into ES cells, which then randomly integrates into the genome. The site of integration is uncontrolled and yet is critical due to the possibility of integration into a silent locus. The incorporation of locus control region sequences may facilitate expression (as discussed earlier). However, random integration may result in the insertional inactivation (insertional mutagenesis) of a gene at the site of integration, resulting in a loss of function that may be mistakenly attributed to overexpression of the transgene. In addition, insertional mutagenesis of a gene may not be immediately apparent if a recessive gene has been inactivated, as phenotypic abnormalities will not be evident until homozygous transgenic lines have been established.

Furthermore, the site of integration may result in altered tissue specificity, although the promoter used behaves differently at its normal chromosomal localization. Neighboring regulatory elements, including repressors or strong activators, may influence the transcriptional activity of the transgene.

In addition to the problems associated with random transgene integration, it is difficult to control transgene copy number (usually integration is a singular event with multiple copies integrated in tandem). Copy number is important for a number of reasons. First, a high tandem copy number results in a gene-silencing effect (reviewed in ***97***). The mechanism of this is not known, but repetitive DNA sequences are thought to induce organization into heterochromatin and subsequent gene silencing ***(98)***. Furthermore, age-related transgene silencing has been observed, the severity of which correlates with increasing copy number ***(99)***. Second, a high copy number is undesirable if the effect of gene dosage is being addressed, as multiple copies will not recapitulate relevant levels of expression.

These problems will be overcome by the design of transgenic mice with controlled transgene integration. A model system for targeted integration has been proposed that utilizes mutant *loxP* sites in embryonic stem cells ***(100)***. This system will facilitate the integration of a single transgene copy that contains a mutant *loxP* site into a defined chromatin site, which is tagged by a reciprocal mutant *loxP* site, in a Cre recombinase transgenic background. Refer to Chapters 6 and 9 for a comprehensive description of the *loxP*/Cre recombinase system.

A final consideration is the genetic strain background to be used for the generation of individual transgenic mouse lines as discussed in Chapter 8. The number of examples of genetic background altering transgene effects is growing and should be considered when selecting a mouse strain.

5. The Future

The advantages of transgenic mice in the study of gene function and how its product may interact with other components of the cellular machinery are too numerous and require no justification. Currently, however, very few downstream cellular targets are known for the increasing number of DNA binding transcriptional regulatory proteins, the identification of which has been limited by the available methodology. Transgenic mice provide an ideal model for the study of gene targets by the coupling of tissue-specific inducible expression systems with advanced array chip technology, making the identification of target genes relatively straightforward.

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21

In Vivo Libraries of Large Insert Transgenic Mice for Genetic Mapping

Desmond J. Smith

1. Introduction

Genetic mapping of phenotypic traits in mammals has historically relied on meiotic recombination together with the use of polymorphic markers. Some examples of these markers, in chronological order, are coat color alleles, protein polymorphisms, restriction fragment length polymorphisms (RFLP) ***(1,2)***, microsatellite repeats (simple sequence length polymorphisms, or SSLPs) ***(3,4)***, and most recently single nucleotide polymorphisms (SNPs) arrayed on chips ***(5)***. These approaches have been employed to map both single-gene mutations and genes involved in complex traits.

The mouse has the important advantage that its genome can be engineered and this aspect of the mouse has recently been exploited to provide new approaches to genetic mapping. These approaches utilize the recent advent of new technologies to introduce large segments of cloned genomic DNA into the mouse germline. Panels of large insert transgenic mice have been called in vivo libraries ***(6)*** by analogy to previously described libraries where cloned genomic DNA is propagated in single-celled hosts. However, in vivo libraries depart from the usual notion of a library in that the in vivo libraries are propagated in a metazoan host, the mouse. This provides the opportunity to examine the function of the genes propagated by the library in the context of the mammalian host. Furthermore, because these large segments of DNA often contain many genes (and can encompass large genes), it is possible to examine in parallel the effects of many genes at once on the phenotype of the host. This hence constitutes a multiplex genotype/phenotype analysis, providing a valuable increase in throughput.

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Yeast artificial chromosomes (YACs) have formed the cornerstone for much of the work in which large DNA segments have been introduced into the mouse genome ***(7)***. YACs have been introduced into the germline either by transfection ***(8–11)*** into totipotent mouse embryonic stem cells (ES cells) or by microinjection into the pronucleus of the fertilized mouse egg ***(12,13)***. Other clones that can be employed in the creation of large insert transgenic mice include bacterial artificial chromosomes (BACs) ***(14,15)***, P1 phage artificial chromosomes (PACs) ***(16)***, and P1 phage ***(17,18)***.

The novel phenotypes displayed by large insert transgenic animals can be of potential practical utility, and one striking example is the creation of mice that can be used to furnish human monoclonal antibodies ***(10,19–22)***. However, it is likely that large insert transgenics will be more widely employed for the fine mapping of interesting genetic traits. This will be accomplished either by seeking phenotypes caused by overexpression of genes resident on the large insert transgene or by using these transgenes in the complementation of mouse mutations.

An example of the first approach, in which an in vivo library was employed for the fine mapping of phenotypes caused by extra gene dosage, is provided by work in which genes that are involved in learning and memory were identified from human chromosome 21q22.2 ***(6,23)***. Down syndrome is usually caused by an extra copy of human chromosome 21. However, although controversial, evidence from human mapping studies, together with similar studies using mice, had suggested that an extra copy of part of human chromosome 21, the 21q22.2 region, was sufficient to cause many of the phenotypes of the syndrome ***(24,25)***. In order to identify genes from 21q22.2 that may be involved in the learning disabilities of the syndrome, transgenic mice propagating a total 2 Mb from the region were created using four overlapping and adjacent YACs. One of the YACs (570 kb) caused learning and memory deficits in the mice, and this was replicated in two separate lines of animals, showing that the phenotype was due to the extra copy of genetic information provided by the YAC and was not simply due to an insertion effect of the foreign transgene inactivating an endogenous mouse gene.

In order to map the gene on the YAC responsible for the learning deficits, advantage was taken of the fact that, in addition to mice containing the full-length YAC, animals were also created that contained fragments of the YAC. These mice are created as a by-product of the microinjection process and probably arise from mechanical shearing of the lengthy (and hence fragile) YAC DNA during the microinjection procedure. The animals with YAC fragments are a superb resource for ultrafine structure genetic mapping, as the number of breakpoints that can be obtained as a result of this fragmentation process are far more numerous than can practicably be obtained from classical meiotic genetic mapping.

Investigation of animals containing fragments of the 570-kb YAC showed that a 180-kb fragment gave rise to learning deficits indistinguishable from animals containing the full-length YAC. Sequence and transcript analysis ***(26)*** of the 180-kb region revealed that it contained a single gene—the human DYRK gene—which is the homolog of a *Drosophila* gene, *minibrain*, which had been previously shown to be involved in normal learning and memory in the fruitfly. The gene is a dual specificity tyrosine/serine–threonine kinase expressed in the brain and is probably involved in control of cell division in developing neuroblasts. Thus, in summary, the in vivo library approach was used to screen a 2-Mb region for genes affecting learning and memory and resulted in the narrowing of this critical region down to 180-kb. This permitted the identification of a gene involved in normal learning in memory in flies, mice, and humans and which may also be involved in the learning disabilities of Down syndrome.

The second use of in vivo libraries, to clone a mutation by in vivo complementation, is exemplified by a study ***(27)*** in which the mouse mutation *vibrator* was identified. This recessive neurodegenerative mutation results in the loss of neurons in the brain and the dying neurons frequently display membrane-bound vacuoles. The mutation was localized using classical meiotic mapping to an approx 500-kb interval. Standard positional cloning paradigms were then used in an attempt to identify the gene by sequencing cDNAs of wild-type and mutant animals from the critical region. These attempts, however, were unsuccessful and did not reveal the causative mutation. In order to map the mutation more finely, an in vivo library of transgenic mice was created using a P1 phage that spanned the critical region defined by meiotic mapping. By employing a breeding scheme in which each P1 phage transgene was introduced into the *vibrator* homozygous background, it was possible to test each one of the P1s for in vivo complementation of the *vibrator* phenotype. The breeding was greatly speeded by microinjecting the P1 phage DNA into fertilized eggs that had resulted from matings between vibrator heterozygotes. This meant that the majority (2/3) of the phenotypically normal transgenic founder animals were themselves vibrator heterozygotes, thereby saving a generation of breeding. The breeding process was also eased by injecting the P1s as pairs of overlapping clones.

The complementation analysis revealed that two independent lines of one of the P1 phage complemented the *vibrator* mutation. Interestingly, two lines of a substantially overlapping P1 phage failed to complement the mutation and this suggested that the *vibrator* gene most likely lay on the nonoverlapping region of the complementing phage, an interval of about 35-kb. Thus, in vivo complementation reduced the region of DNA to be interrogated from an initial region of 500-kb (roughly 50–100 genes) defined by classical meiotic genetic mapping, down to a much smaller region of 35-kb (1–2 genes).

Genomic DNA sequence analysis confirmed that the *vibrator* mutation was in the 35-kb critical region and was due to insertion of a retrotransposon (an intracisternal A particle) into the intron of the *pitpn* gene, which encodes the phospatidylinositol transfer protein α isoform (PITPα). The insertion of the retrotransposon caused decreased levels of mRNA from the gene and decreased protein levels. The gene product plays a role in intracellular membrane trafficking via shuttling of membrane-bound lipids. Because nerve cells probably need vigorous membrane trafficking to support the exocytosis required for the release of neurotransmitter into the synaptic cleft, the decreased PITPα levels in *vibrator* homozygotes could be responsible for the characteristic membrane-bound vacuoles found in the cytoplasm of dying nerve cells.

Another situation in which in vivo complementation has been used, not to reveal the location of a gene but rather to confirm its identity, was provided by the recent cloning of the gene responsible for the mouse circadian rhythm mutant, *Clock* ***(14,15)***. This example was particularly interesting in that not only was the transgene able to correct an antimorphic (dominant-negative) allele of the gene, but an extra dose of the *Clock* gene provided by the transgene in the wild-type background also gave rise to a circadian rhythm phenotype. This satisfyingly bridges the concepts illustrated by the use of in vivo libraries in the *vibrator* and the 21q22.2 studies.

2. Materials

All solutions are prepared from analytical-grade reagents and deionized double-distilled water or water ultrapurified by Millipore (Bedford, MA) filtration. All chemicals and reagents are from Sigma (St. Louis, MO), unless otherwise indicated.

2.1. High-Density High-Molecular-Weight Yeast DNA Plugs

1. AHC: dissolve the following in water such that the final volume is 900 mL and autoclave 6.7 g yeast nitrogen base without amino acids (Difco, Detroit, MI) (or 1.7 g yeast nitrogen base without amino acids and without ammonium sulfate (Difco) supplemented with 5 g ammonium sulfate); 10 g acid casein hydrolysate (United States Biochemical Corporation, Cleveland, OH); 40 mg adenine hemisulfate. Add 100 mL autoclaved 20% glucose.
2. Kanamycin.
3. 0.125 *M* EDTA, pH 8.0.
4. SCE: 1 *M* sorbitol, 0.1 *M* Na citrate, 5 m*M* EDTA, pH 7.0.
5. InCert GTG (FMC, Rockland, ME) or SeaPlaque GTG (FMC) low-melting-point agarose (3.5–4%) in 63 m*M* EDTA, pH 8.0.
6. Yeast plug-forming molds (Bio-Rad, Hercules, CA).
7. Yellow Scotch 3M pressure-sensitive electrical tape.
8. 5 $^{3}/_{4}$ in. Pasteur pipet (Baxter, McGaw Park, IL).

9. Lyticase.
10. ES: 0.5 *M* EDTA, pH 8.0, 1% sarkosyl (N-laurylsarcosine).
11. Proteinase K (10 mg/mL solution) (Boehringer-Mannheim, Indianapolis, IN).
12. ES-proteinase K: ES supplemented with proteinase K (1 mg/mL) by adding $^1/_{10}$ th volume concentrated proteinase K solution.
13. $T_{10}E_{50:}$ 10 m*M* Tris-HCl pH 8.0, 50 m*M* EDTA, pH 8.0.

2.2. Purification and Concentration of YAC DNA for Microinjection

1. 1X TAE: 40 m*M* Tris-acetate, pH 8.0, 1 m*M* EDTA, pH 8.0.
2. 1% SeaPlaque GTG agarose (FMC) in $^1/_2$ X TAE.
3. Yellow Scotch 3M pressure sensitive electrical tape.
4. 5 $^3/_4$ in. Pasteur pipet with its narrow end sealed by holding in Bunsen burner flame.
5. Glass microscope cover slips (22 mm × 30 mm) (Fisher, San Francisco, CA).
6. Disposable razor blades.
7. CHEF mapper pulsed-field gel system (Bio-Rad).
8. Ethidium bromide: 1 μg mL^{-1} Ethidium bromide in $^1/_2$ X TAE. Conveniently prepared from a 10 mg mL^{-1} stock solution of Ethidium Bromide in water by diluting stock 10,000 fold in the buffer ($^1/_2$ X TAE) used to run the pulsed-field gel.
9. Shortwave UV transilluminator.
10. UV protective goggles.
11. UV protective face shield.
12. Toothpick ends: made by breaking toothpicks off at roughly a $^1/_4$ to $^1/_2$ of their length from the ends of the toothpicks.
13. Disposable scalpels.
14. Transparent plastic ruler.
15. 4% NuSieve GTG agarose (FMC) in $^1/_2$ X TAE.
16. β-Agarase (New England Biolabs, Beverly, MA).
17. β-Agarase buffer: either supplied by New England Biolabs (50 m*M* Bis-Tris.Cl pH 6.5, 1 m*M* EDTA) supplemented with 60 m*M* NaCl or 100 m*M* NaCl, 10 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA, pH 8.0 (*see* **Note 1**).
18. Minitan-S polysulfone membrane filter 100,000 NMWL (Millipore).
19. 150 mm diameter × 15 mm deep Petri dish (Falcon, Becton Dickinson, Lincoln Park, NJ).
20. Microinjection buffer: 100 m*M* NaCl, 10 m*M* Tris-HCl pH 7.5, 0.1 m*M* EDTA, pH 8.0.
21. 1% SeaKem GTG agarose (FMC) in $^1/_2$ X TAE.

2.3. Preparation of P1, PAC, and BAC DNA for Microinjection

1. TB: To 900 mL deionized water add 12 g Bacto-tryptone (Difco), 24 g Bacto-yeast extract (Difco), 4 mL glycerol and autoclave. Prepare a solution of 0.17 *M* KH_2PO_4, 0.72 *M* K_2HPO_4 by dissolving 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in water, adjust final volume to 100 mL and autoclave. Mix the two sterile solutions together.

2. Chloramphenicol stock: 34 mg/mL in ethanol. Store at –20°C.
3. Solution I: 10 m*M* EDTA, pH 8.0.
4. Solution II: 0.2 *M* NaOH, 1% sodium dodecyl sulfate (SDS).
5. Solution III: To prepare 100 mL add 37.5 mL 8 *M* potassium acetate to 51 mL water. To this mixture add 11.5 mL glacial acetic acid and mix. The resulting solution III is 3 *M* potassium and 5 *M* acetate, pH approx 4.8.
6. Cheesecloth.
7. Isopropanol.
8. $T_{50}E_{50}$: 50 m*M* Tris-HCl pH 8.0, 50 m*M* EDTA, pH 8.0.
9. 8 *M* Potassium acetate.
10. Ethanol.
11. DNase-free RNase A: 10 mg/mL.
12. Phenol/chloroform: 1:1 vol:vol.
13. Sodium acetate: 3 *M* Sodium acetate, pH 6.0.
14. TE: 10 m*M* Tris-HCl pH 8.0; 0.1 m*M* EDTA, pH 8.0.
15. Millipore filter: Millipore 0.025 μm VS filter (Millipore).
16. Low-range pulsed-field gel marker (New England Biolabs).

3. Methods

3.1. Preparation of High-Density High-Molecular Weight Yeast DNA Plugs

1. Ahead of time, seal the bottom of the yeast plug-forming molds with yellow Scotch 3M pressure-sensitive electrical tape.
2. For high-density (5–10X) yeast plugs you grow 500–1000 mL of yeast culture to saturation in AHC supplemented with 50 μg mL^{-1} kanamycin to inhibit bacterial growth.
3. For normal density (1X) yeast plugs to be used in analytical pulsed field gel electrophoresis (PFGE), use 100 mL saturated yeast culture.
4. Spin yeast down in a centrifuge at 2000*g* for 5 to 10 min.
5. Wash once in 50 mL 0.125 *M* EDTA pH 8.0, spin down and remove supernatant.
6. Resuspend yeast (approx 4 mL) with 4 mL SCE. Bring to 45°C in a water bath.
7. Prepare molten low melting point agarose (3.5–4 %) in 63 m*M* EDTA, pH 8.0, and bring to 45°C in water bath (*see* **Note 2**).
8. Mix 5 mL of the agarose with yeast cells (so that the final agarose concentration is roughly 1.5%), keeping at 45°C.
9. Dispense mixture into sealed yeast plug-forming molds at room temperature by rapidly pipetting the mixture into the wells of the mold using a Pasteur pipet. Allow plugs to solidify at 4°C. If yeast are *ade*$^-$, plugs will be orangey/red in color. There will be plenty of plugs (about 100).
10. After the plugs have set, remove yellow tape from bottom of mold.
11. Expel plugs by blowing them into 10 mL SCE using a pipet bulb placed in turn over each plug.
12. Add approx 10 mg lyticase to the SCE and dissolve. Incubate plugs at room temp overnight to digest cell wall.

13. Take off SCE-lyticase and add 10 mL ES-proteinase K. Incubate at 50°C overnight.
14. Next day, remove ES/proteinase K and rinse plugs with gentle shaking in roughly 30 mL $T_{10}E_{50}$. Rinse plugs about five times using fresh $T_{10}E_{50}$ with 1/2 h incubations. Plugs will become white.
15. Store plugs in $T_{10}E_{50}$ at 4°C.

3.2. Purification and Concentration of YAC DNA for Microinjection

1. For preparative PFGE, the CHEF mapper system is used. The gels are cast using 1% SeaPlaque GTG agarose in $^1/_2$ X TAE and are run in the same buffer. A gel contains two long preparative wells (about 6–8 cm each) in which are loaded DNA plugs made from yeast containing the YAC. These wells are created by using a conventional well former with 3-mm-wide teeth in which the teeth are taped together with yellow electrical tape. A few conventional-width wells may be left both between the two large wells and flanking these wells. These conventional wells are useful for loading marker plugs. Illustrative markers are the parental wild-type yeast strain (e.g., AB1380) that lacks the YAC. The YAC can then be seen as an extra band in the preparative lanes, provided the YAC does not co-migrate with the endogenous yeast chromosomes.
2. The high-density plugs are bisected before insertion into the gel wells (*see* **Fig. 1G**). The plugs are maneuvered onto a glass microscope coverslip using a Pasteur pipet with its end sealed. The plugs will adhere to the coverslip due to surface tension, and they are then bisected using a razor blade. Both halves of the bisected plugs may be loaded into the gel. The bisection is performed so that the bands in the gel will be narrowed as measured along the direction of electrophoresis, thus counteracting the band smearing that will occur because of the high DNA density of these plugs. Thus, resolution of the preparative gel is largely undiminished, but the bands in the gel are of very high DNA density.
3. The pulsed-field gel is run under the appropriate conditions for separation of the YAC DNA from the endogenous yeast chromosomes. Typical conditions for a 570-kb YAC are 120° included angle, 6 V/cm, switch time 12.55–54.17 s (linear ramp), run time 29 h 24 min, 14°C.
4. Cut out the left and right edges of the gel and a strip from the middle of the gel and stain in ethidium bromide for 1 h. Use a disposable scalpel guided with the aid of a transparent plastic ruler.
5. View under short-wave-length UV illumination (using UV protective goggles and a UV protective face shield) (*see* **Fig. 1A**) and mark the position of the YAC bands in each of the strips, adjacent to the preparative sections of the gel, by placing the sharp end of a broken toothpick into the bands.
6. Reassemble the entire gel by placing the stained strips marked with toothpicks back next to the unstained parts of the gel containing the preparative YAC bands.
7. Using a fresh disposable scalpel, cut out the unstained YAC bands that will be strips about 6–8 cm long (the width of the preparative gel wells). Use a transparent plastic ruler to steady the incisions. Try to estimate the thickness of the band so that the strip nearly encompasses the YAC DNA without also including agarose devoid of the DNA (*see* **Notes 3** and **4**).

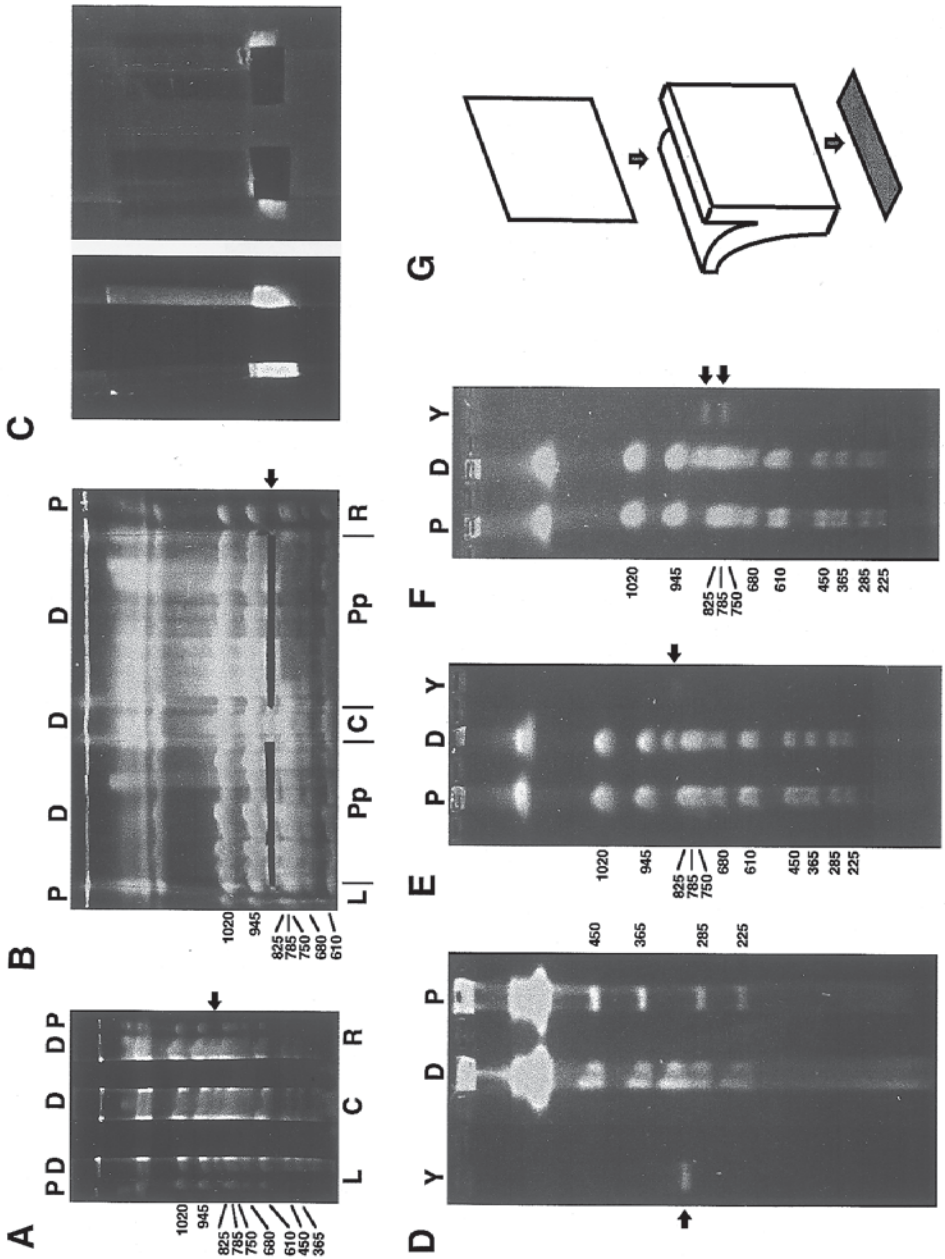

Fig. 1. Purification and concentration of YAC DNA for microinjection. Lanes P show the parental YAC strain (AB1380) that lacks a YAC, lanes D show the derivative strain bearing a YAC and lanes Y show purified YACs (20-25 ml), indicated by arrows. **(A)** The left (L), right (R), and central (C) strips from preparative pulsed-field

8. After the YAC bands have been cut out, the gel may be reassembled, viewed under UV light, and photographed in order to assess the accuracy of excision (*see* **Fig. 1B**).
9. Cut the 6–8 cm strip of agarose containing the YAC DNA into roughly three equal sections roughly 2–2.5 cm long.
10. Place the three substrips alongside each other in a pulsed-field gel apparatus casting tray, so that the substrips are pointed along the direction of electrophoresis defined by the tray. (More than one set of three substrips may be fitted into one casting tray.)
11. Pour molten 4% NuSieve GTG in $^1/_2$X TAE around the three substrips and allow to gel.
12. Concentrate YAC DNA by running a pulsed-field gel in which the YAC DNA is electrophoresed from the 1% SeaPlaque agarose substrips into the 4% NuSieve. The YAC DNA piles up at the interface between the two different concentration agaroses and is hence concentrated. Conditions may be varied to optimize this step, but conditions that are satisfactory for nearly all YACs are a switching time of 50 s, an included angle of 90°, a run time of 10 h, at 14°C.
13. A strip either from the left or the right of the concentrating gel is cut away and stained in ethidium bromide. As before, the position of the YAC DNA is determined by viewing the strip under shortwave UV light (*see* **Fig. 1C**), marking the YAC DNA with toothpick ends, reassembling the gel, and cutting the concentrated YAC DNA band out of the NuSieve using a scalpel and clear plastic ruler. The band will be about 1 cm long. The excision is carefully directed to nearly encompass the extent of the YAC DNA band, without including agarose devoid of DNA.

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gel. The sizes of the yeast chromosomes (kb) are indicated. The YAC is 900 kb and typical running conditions for this YAC were 120° included angle, 6 V/cm, switch time 57.78 s to 2 min 05.11 s (linear ramp), run time 35 h 52 min, 14°C. **(C)** Preparative pulsed-field gel after YAC band has been excised. The preparative lanes are indicated by Pp. **(C)** Concentration gel. The gel on the left shows the strip from the concentrating gel used to locate the position of the concentrated YAC DNA band. The gel on the right shows the concentrating gel after YAC band has been excised. **(D)** An analytical pulsed-field gel showing a purified 325 kb YAC. The typical running conditions for this YAC were 120° included angle, 6 V/cm, switch time 12.55–54.17 s (linear ramp), run time 29 h 24 min, 14°C. **(E)** An analytical pulsed-field gel showing the purified 900 kb YAC (*see* **A–C**). Although the YAC band was faint, and was visible on direct UV illumination, but not on this reproduction, DNA of this concentration has been used to successfully create transgenic animals. **(F)** An analytical pulsed-field gel showing a different purified preparation of the same 900 kb YAC (upper arrow). This preparation is contaminated with an adjacent yeast chromosome (825 kb) (lower arrow) but would be perfectly acceptable for microinjection. **(G)** A schematic diagram showing a razor blade bisecting a yeast DNA plug for loading into the well of a pulsed-field gel.

14. After the YAC band has been cut out, the concentrating gel may be reassembled, viewed under UV light, and photographed in order to assess the accuracy of excision (*see* **Fig. 1C**).
15. Place the NuSieve band in an microfuge tube and equilibrate in β-agarase buffer by filling the tube with buffer and incubating at room temperature for 30 min, removing the old buffer and repeating for another 30 min. Remove the second batch of buffer.
16. Weigh the gel slice in the microfuge tube and using an empty tube for comparison. Assume a density of 1 g/mL to estimate the volume of the slice.
17. Melt the slice by immersing tube in a 72°C water bath for 12 to 15 min.
18. While slice is melting, prewarm an aliquot of β-agarase at 72°C for about 1 min.
19. Add warmed enzyme to melted gel slice. Use 4 U of enzyme per 100 μL of gel slice. Gently mix enzyme and agarose by gently stirring with a yellow pipet tip roughly five or six times. Once the YAC DNA has been liquefied it must be handled gently: it is very susceptible to mechanical shearing and degradation. Also endeavor to keep cooling the agarose during these maneuvers to a minimum: any resolidification of the agarose appears (somehow) to trap all of the YAC DNA and dooms the purification attempt to failure.
20. Immediately put tube into 45°C water bath. Incubate for 2–4 h.
21. Make a boat out of an approx 2.5 cm square section of Minitan filter by folding up a roughly 3 mm section on each of the four sides to make the walls of the boat. Handle the filter with gloves. The inside of the boat is the shiny surface and the outside is the dull surface.
22. Float the boat (shiny side up) on a Petri dish (150 mm diameter × 15 mm deep) filled with approx 100 mL of microinjection buffer as the dialysate. Gently pipet the liquefied YAC DNA onto the center of the boat. The Petri dish lid is placed onto the dish to decrease evaporation. Allow floating drop dialysis to proceed for 2 h to overnight (best).
23. After dialysis, gently pipet YAC DNA into microfuge tube for storage at 4°C (*see* **Note 5**).
24. Assess the integrity of the purified DNA by running 20–25 μL on an analytical 1% SeaKem GTG agarose pulsed field gel cast and run in $^1/_2$ X TAE (*see* **Fig. 1D–F**). The concentration of the YAC DNA can be assessed on a 1% SeaKem GTG agarose minigel by running marker lanes containing known amounts of DNA alongside the YAC DNA. For confirmatory purposes, the concentration of the YAC DNA can also be assessed using a fluorometer.
25. Microinjection is performed using the usual procedures ***(1)***. The DNA is best microinjected at a concentration of approx 1 ng/mL. Because it is difficult to accurately assess the YAC DNA concentration, it is wise to inject a dilution series of the DNA using microinjection buffer for the dilution. One convenient series is to perform 1 d of microinjection with undiluted YAC DNA, the next with DNA diluted twofold by adding an equal volume of microinjection buffer to the DNA, and another day with DNA diluted threefold by adding 2 vol of microinjection buffer to 1 vol of the YAC DNA (*see* also **Notes 6–8**).

3.3. Preparation of P1, PAC, and BAC DNA for Microinjection

This protocol is essentially a standard alkaline/SDS lysis preparation of bacterial plasmid DNA, followed by a floating drop dialysis purification scheme.

1. Grow 500 mL of saturated bacterial culture in TB supplemented with 50 μg mL^{-1} kanamycin (for P1s and PACs) or 12.5 μg/μL chloramphenicol (for BACs) prepared by diluting chloramphenicol stock into the TB.
2. Spin down the bacteria in a centrifuge at 2000*g* for 5 to 10 min.
3. Resuspend bacteria in 40 mL solution I.
4. Add 80 mL solution II, mix by inversion. The mixture should clear.
5. Add 60 mL solution III, mix by inversion. A heavy white precipitate will appear.
6. Centrifuge in a medium speed centrifuge at 2000*g* for 10 min to spin down precipitate (*see* **Note 9**).
7. Decant supernatant while filtering through cheesecloth.
8. Add 90 mL isopropanol to the supernatant and mix. Spin at 2000*g* for 10 min.
9. Pour off the supernatant and redissolve the pellet in 18 mL $T_{50}E_{50}$. Add 8.5 mL 8.0 *M* potassium acetate and chill at 4°C for 15 min.
10. Spin at 2000*g* for 10 min. Decant the supernatant and add 54 mL 100% ethanol.
11. Spin at 2000*g* for 10 min. Redissolve precipitate in 1.4 mL $T_{50}E_{50}$ and transfer to microfuge tubes. Add 50 μL of 10 mg/mL DNase free RNase A and incubate at 37°C for 1 h.
12. Extract three times with phenol/chloroform, add 140 μL 3 *M* sodium acetate, pH 6.0, and 3.5 mL ethanol. Spin down precipitate, allow pellet to dry, and redissolve in TE (roughly 50 μL). Measure DNA concentration using a fluorometer. Expect about 50–100 μg DNA from a 500-mL prep.
13. Floating drop dialysis is performed by pipetting 18 μg of DNA in about 100 μL TE onto a Millipore filter floating on roughly 100 mL microinjection buffer in a Petri dish. The filter should be floating with its shiny side up. The Petri dish lid is placed onto the dish to decrease evaporation. The incubation is allowed to proceed for 2 h to (preferably) overnight. The recovery of DNA from the dialysis procedure is about 30–60 %.
14. Assessment of DNA concentration and integrity (*see* **Fig. 2**) and the procedures for microinjection are the same as described for YAC DNA (*see* **Note 10**). As for YACs, a dilution series can be beneficial. If it is desired to inject two P1s together, it is suggested that the DNA solutions should be mixed to aim for a final total concentration of roughly 2 ng/μL and a partial concentration of 1 ng/μL for each P1 (*see* **Note 11**).

4. Notes

1. Salt stabilizes the lengthy and somewhat fragile YAC DNA when it is in solution ***(28)***.
2. Because the purification process is fraught with considerable losses, it is advantageous to begin with plugs that boast as high a DNA density as possible. The protocol described here differs from previously published methods in providing a considerably higher DNA concentration in the plugs by resuspending a larger

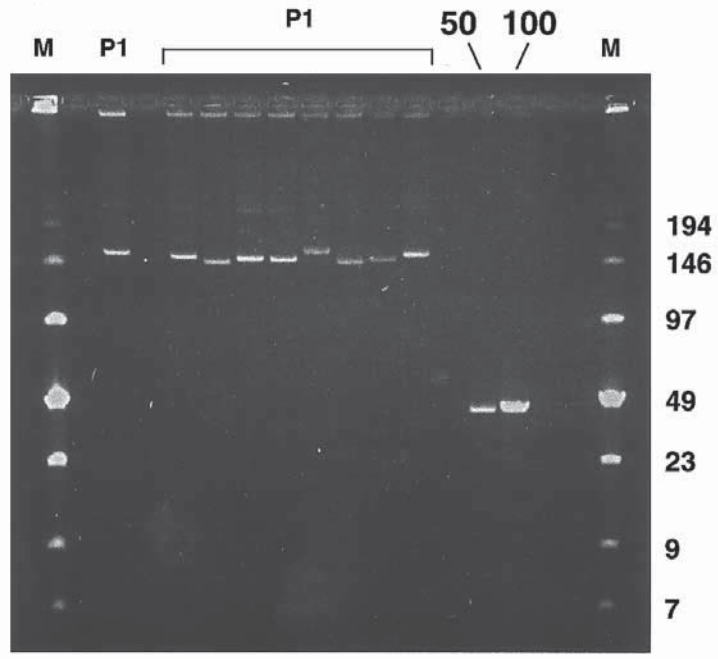

Fig. 2. Analytical pulsed-field gel showing P1 DNA purified for microinjection. The conditions of the gel were 120° included angle, 6 V/cm, switch time 1–11 s (linear ramp), run time 14 h, 14°C. Lanes M show marker DNA (low-range pulsed-field gel marker, New England Biolabs), with the size indicated in kb, lanes P1 show purified P1 DNA (1–2 μL), lanes 50 and 100 show 50 ng and 100 ng, respectively of λ DNA for estimation of P1 DNA concentration.

volume of yeast in the same volume of agarose. The yeast, therefore, takes up a proportionately larger share of the plug volume, and the mechanical strength of the plugs would be greatly reduced were it not for the fact that the protocol described here calls for 3.5–4% agarose, as opposed to the 1% agarose usually employed.

3. Often, the YAC will migrate at the same speed or very close to an endogenous yeast chromosome and it will be impossible to purify the YAC completely away from the yeast chromosome (*see* **Fig. 1F**). This is of little concern, as the entire yeast genome has been introduced into the mouse genome with no obvious effect on the animal ***(8)***.
4. Another method that has been successfully employed to liberate YAC DNA from a pulsed-field gel is electroelution (DJS, unpublished results). This approach results in efficient recovery of the YAC DNA, but the DNA has yet to tested in microinjection.
5. For long-term storage or transportation, the YAC DNA can be frozen at –70°C. Perhaps surprisingly, this does not shear the DNA and once solidified in this way it is easy to transport by express mail.

6. Transgenic animals are conveniently identified by performing STS content analysis using a PCR of tail biopsy DNA.
7. The proportion of transgenic pups from microinjection of YAC DNA is about the same as standard microinjection experiments, i.e., 10–30%. The proportion of transgenic animals that contain YAC fragments, as opposed to the full-length YAC, varies depending on the particular YAC preparation. For some preparations the majority of the transgenic animals are full length (80%), whereas for other preparations only some of the animals are full length transgenics (20%).
8. Interestingly, the transgenic G0 animals do not give rise to the expected Mendelian ratio of 50% transgenic G1s. Rather the G0 animals give rise, on average, to 25% transgenic G1s ***(6,29)*** whereas the transgenic G1 and subsequent generations produce the expected 50% frequency of transgenic offspring. This is consistent with the idea that the transgenic G0 mouse germline must be chimeric for the transgene. These observations suggest that the YAC DNA must integrate into the mouse genome after the S phase of the first mitotic cycle of the fertilized zygote. Hence the transgene would be present in just one sister chromatid of a chromosome rather than both sister chromatids as would be expected if the foreign DNA integrated into the mouse genome before the S phase. Consequently, the transgenic G0s would be 50% mosaics, with, on average, half the germline being transgenic and the other half being nontransgenic. The transgenic half of the germline will give rise to transgenic offspring with the expected 50% Mendelian ratio, whereas the nontransgenic half will not give rise to any transgenic offspring. Thus, on average, 25% transgenic offspring are expected (0.5 [Mendelian ratio] × 0.5 [average proportion of germline that is transgenic]).
9. BACs, PACs, and P1 phage are less fragile than one might expect from their fairly large sizes (90 kb for P1s up to 300 kb for PACs and BACs). This relative toughness, compared to YACs, may be due to the fact that the bacterial clones are supercoiled, whereas the YACs are linear.
10. There is no need to linearize bacterial large insert clones prior to microinjection. Even when the gene of interest takes up most of the clone insert, the majority of the transgenic animals appear to correctly express the authentic gene product ***(17)***, perhaps because of recombinational repair of the microinjected DNA after it is linearized by the fertilized mouse egg.
11. If the gene of interest is larger than the bacterial large insert clone size, it is possible to microinject two overlapping clones as a mixture into the mouse egg. Recombination between the two clones can then regenerate the entire gene ***(21)***.

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Epigenetic Effects on Transgene Expression

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1. Introduction

Gene expression in eukaryotes is regulated primarily at the level of transcription. The genomes of higher eukaryotes contain many more genes than are used in any single differentiated cell type, and cell differentiation can be viewed as the result of decisions regarding which genes will be expressed. The mechanisms of transcriptional control have been intensively studied for more than a decade, resulting in considerable understanding of the organization of DNA elements that control the transcription of individual genes (promoters and enhancers), the factors that bind these elements, and the basal transcription apparatus. These studies have been primarily concerned with mechanisms regulating the rate of transcription of an active gene, rather than mechanisms that determine whether a gene will be transcriptionally active at all. However, it is arguable that the decision to transcribe a gene is the critical determinant; work done over several decades points to complex systems regulating the on/off switch in transcription, but the workings of these systems are still relatively obscure. In general, these systems involve what are termed epigenetic processes, i.e., processes whereby genes are rendered inactive that do not involve changes in the actual DNA sequence. At present there are two known forms of epigenetic modification: DNA methylation and chromatin packaging. Changes in either one or both of these are frequently associated with the silencing of genes.

Evidence of the importance of epigenetics in the regulation of gene expression comes in part from the study of transgenes. When DNA is introduced into whole animals by microinjection of the zygote, it is found to integrate at only one, or rarely at a few, chromosomal sites. When several transgenic mouse

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lines are produced with the same DNA fragment, the outcome in terms of gene expression is not always the same. Some of the mice show no expression at all, and in others the level of expression varies from line to line. In most cases the level of expression does not correlate with the copy number of the transgene. Even the tissue specificity of expression may vary. It is generally thought that these differences are caused by differences in the site of integration, the so-called position effect, and that these differences are, at least in part, the result of differences in the nature of the surrounding chromatin. More recent studies from this and other laboratories has shown that part of this variation in level of expression from line to line is due to differences in the proportion of cells expressing the transgene ***(2–10)***. A line that expresses a transgene at a low level is frequently found to express the transgene in only a small percentage of the cells in the relevant tissue (*see* **Fig. 1**).

This variegated, heterocellular or mosaic expression has been generally overlooked until recently, because it can only be detected if transgene expression is assayed at a single cell level. Most transgenes are assayed for activity using cell lysates. Thus, the study of transgene expression, provided one uses a single cell assay, presents an opportunity to study the characteristics of silencing of gene expression in general. Various methods can be used to investigate these phenomena; use of cell autonomous reporter genes that produce a product that can be detected intrinsically (e.g., green fluorescent protein [GFP]) or histochemically (e.g., *lacZ*, GUS), fluorescence-activated cell sorter (FACS) sorting or immunocytochemistry of tissue sections with a specific antibody, or *in situ* hybridization to RNA again using tissue sections.

In this chapter, we first review what is known about gene silencing from studies using other organisms: yeast, *Drosophila*, and plants. We also describe similar phenomena reported for both transgenes and endogenous genes in mammals. We then outline what is known about the factors governing this process in transgenic mice and finally discuss the relevance of variegated gene expression to gene knockout experiments.

2. Gene Silencing in Other Organisms

The study over some decades of position-effect variegation (PEV) in *Drosophila* has provided the largest body of evidence relating to components of gene-silencing mechanisms. PEV was first described as the stochastic and clonally heritable inactivation of pigment genes translocated into or near centromeric heterochromatin in *Drosophila* (for review, *see* **ref. *11***), resulting in eyes with variegated patterns of coloring. This is a position effect on gene expression rather than a permanent alteration in an affected gene, as in several cases it has been possible to obtain full expression by moving a position-affected gene away from heterochromatin. Cytological analysis of position-effect rearrange-

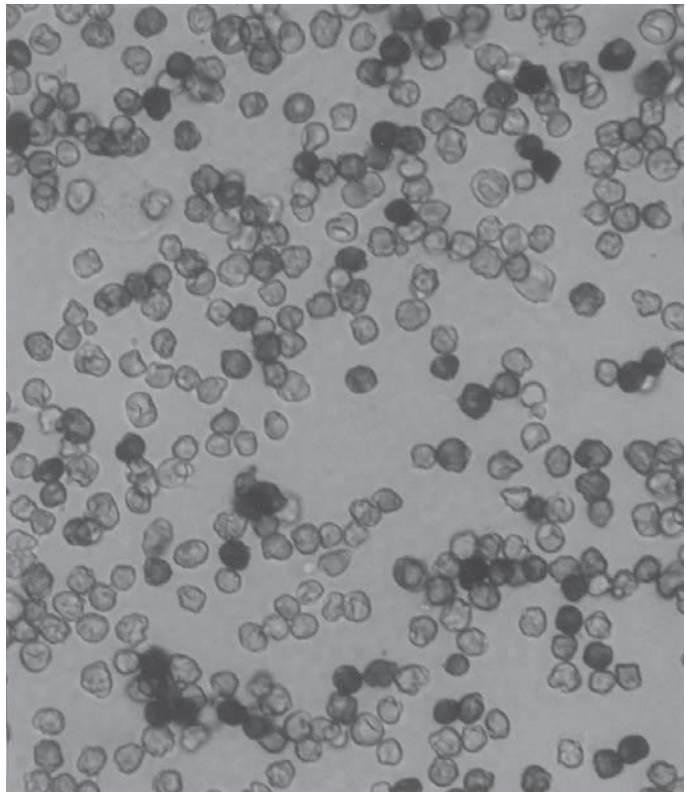

Fig. 1. Mosaic expression of a transgene in mouse erythrocytes. The transgene contains human globin promoter and enhancer elements linked to the *lacZ* reporter gene. Primitive erythroid cells were purified from germline transgenic embryos (12.5 dpc) and stained with X-gal to detect β-galactosidase activity. Transgene expression (as indicated by blue staining with X-gal) was observed in some erythroid cells but in others the transgene was inactive.

ments in salivary gland giant chromosomes demonstrated that the affected, normally euchromatic, DNA segments become compacted (like heterochromatin) ***(12,13)***. Therefore, it has been concluded that the gene inactivation observed in PEV results from an alteration in chromatin structure. Furthermore, dominant mutations that suppress or enhance PEV have been isolated by a number of research groups ***(14–18)***. Many of the genes identified in these screens encode structural components of chromatin or proteins that regulate chromatin conformation (for review, *see* ***19***). It is hoped that the molecular analysis of these genes, called modifiers of PEV, will prove useful in characterizing fundamental aspects of chromosome structure and function.

In the yeast *Saccharomyces cerevisiae*, epigenetics plays a critical role in determining the proper pattern of mating-type gene expression and in determination of cell type. Yeast cells contain three copies of the mating-type genes. The copy at the mating-type (MAT) locus is actively transcribed, whereas the copies at HML and HMR, the silent loci, are transcriptionally repressed and must remain repressed in order for the cells to display the appropriate cell type ***(20)***. There is good evidence that repression of the yeast silent loci is analogous to PEV in *Drosophila*; recessive mutations that affect the highly conserved amino-terminus of histone H4 cause derepression of the silent loci and a number of other proteins, including the four silent information regulator (SIR) proteins, involved in silencing can influence chromatin structure (for review, *see* ***21***).

The expression of several genes has been shown to be subject to reversible position effects when placed next to a telomere in the budding yeast *S. cerevisiae* and mutations in the SIR genes suppress effects imposed by the telomeres implicating the same proteins in the process ***(22,23)***. Genes placed in or near centromeres of the fission yeast *Schizosaccharomyces pombe*, are expressed in a variegated manner and inactivity of the transgene correlates with the formation of heterochromatin ***(24)***.

In the last few years, gene silencing in transgenic plants has become an area of intense interest for both applied and basic plant scientists; unwanted transgene silencing that results in variegated expression is a problem that many scientists are trying hard to understand ***(25,26)***. Gene silencing can also affect endogenous genes in plants, the best-known example being paramutation (***27***; for review, *see* ***28***). Paramutation involves the silencing of one allele following exposure to a homologous allele that is already silenced. Examples are known in many plant species, but only in snapdragon and maize have molecular structures for endogenous alleles been determined. In addition, an example of endogenous gene silencing in *Arabidopsis* resembles some aspects of paramutation, although in this case nonallelic interactions are involved ***(29)***. Silencing in plants is often associated with methylation of the gene ***(30–32)***.

3. Transgene Silencing in Mice

There is, by now, a large body of work on the silencing of transgenes in higher eukaryotes, particularly mice, and these systems present an opportunity to study the characteristics of silencing of gene expression in general. The factors governing this silencing of transgenes, which frequently results in variegated expression, include the components of the transgene in the integrated array, the number of copies of the transgene in the integrated array, and the integration site; we have discussed some of these in a recent review ***(33)***. In our studies we have used a system in which the *Escherichia coli lacZ* gene is driven by transcriptional control elements derived from the human globin

genes. Staining of erythrocytes for β-galactosidase expression permits convenient assessment of the proportion of erythrocytes that express the transgene (*see* **Fig. 1**). We have found that in many transgenic lines made with this system, expression is variegated and that each line has a characteristic degree of variegation ***(7)*** (*see* also **Table 1**). It is likely that, as in the case of heterochromatin-associated variegation in *Drosophila*, the expression status of the variegating locus in these mammalian systems is also clonally heritable; clonal stability of the expression status for several variegating transgenes has now been proven in mice ***(34–37)***.

DNA methylation has been implicated in transcriptional silencing in eukaryotes ***(38,39)***, and like chromatin structure, methylation state can be inherited through cell division. The pattern of methylation across the genome is passed to subsequent cell generations by the maintenance methylation activity of the methyltransferase enzyme. Inactive or silenced genes are frequently found to be methylated, but it remains unclear whether this methylation leads to the transcriptional inactivity, or vice versa; some species including *Drosophila* do not use DNA methylation, but are nevertheless able to epigenetically silence genes. Those working on gene silencing in *Drosophila* and yeast have focused on chromatin packaging as the epigenetic modification associated with differential gene expression ***(11)***. On the other hand, scientists working on gene silencing in mammals, e.g., X-inactivation and transgene silencing in mice, have tended to focus their attention on DNA methylation ***(40,41)***.

Studies into the molecular nature of the epigenetic modification associated with silencing in our system have found that there is a difference in the chromatin conformation of the transgene locus between active and inactive states ***(42)***. Relative to active transgenes, transgene loci that have been silenced exhibit a reduced sensitivity to general digestion by DNase I, as well as a failure to establish a transgene-specific DNase I hypersensitive site, suggesting that silenced transgenes are packaged into less accessible chromatin structures. Surprisingly, the restrictive chromatin structure observed at silenced transgene loci did not correlate with increased methylation, with transgenes from both active and inactive loci appearing largely unmethylated following analysis with methylation-sensitive restriction enzymes and by sequencing PCR products derived from bisulphite-converted DNA.

3.1. Effects of the Site of Integration

The degree of variegation is measured as the percentage of expressing erythrocytes and this is a measure of the probability that the gene will be active. **Table 1** shows the percentage of expressing erythrocytes in seven lines made with a transgene in which the mouse metallothionein promoter and a strong erythroid-specific enhancer drive expression of the *lacZ* reporter gene. In all

Table 1
Copy Number and Transgene Expression of MTα Lines

Transgenic Line	Copy Number	Percentage of Expressing Erythroid Cells at 12.5 dpc
MTα#1	>200	0
MTα#2	~15	24.7 ± 6.7
MTα#3	~70	0.82 ± 0.33
MTα#4	~10	4.6 ± 2.9
MTα#5	~60	1.1 ± 0.73
MTα#6	~110	0.14 ± 0.01
MTα#8	~5	78.7 ± 3.5

The MTα transgene contains the mouse metallothionien promoter and a strong erythroid enhancer driving the *lacZ* reporter gene (unpublished data from H. Sutherland and E. Whitelaw)

lines transgene expression is heterocellular. Because the transgene construct is the same in all cases we believe these results imply that the site of integration plays an important role in determining the degree of variegation. Presumably those lines integrated near to heterochromatic regions express the transgene in fewer cells. Variegated expression of transgenes integrated close to heterochromatic regions have been reported by others ***(8,37)***. Festenstein and colleagues ***(8)*** found that in a number of lines exhibiting variegated expression of a human CD2 transgene in mouse T cells the transgene had become integrated into regions which, from fluorescent *in situ* hybridization (FISH) analysis, appeared to be pericentric heterochromatin.

3.2. Effects of the Tissue in which the Transgene is Expressed

It is interesting to note that the frequency with which variegation is observed in globin/*lacZ* lines, where expression of the transgene is directed to the erythroid tissue ***(7,9–10,43)***, seems higher than that found with transgenes expressed in other cell lines, e.g., T cells ***(8,34)***. This may be a reflection of the high degree of chromatin compaction in the differentiating erythrocyte ***(44)***. Other cell types may be less heterochromatic; the extent of facultative heterochromatinization varies widely between lineages ***(45)*** and it may turn out that the extent of variegation is influenced by the tissue type in which the transgene is expressed.

3.3. Effects of Copy Number

It is also noteworthy from **Table 1** that there appears to be a tendency for high-copy lines to show greater variegation. We have found this to be the case

in many different studies ***(9,10)***. It has long been recognized that high-copy lines have a tendency to be transcriptionally inactive ***(31,46,47)***. This is consistent with findings of transgene expression in *Drosophila* ***(48,49)*** where there is evidence that repeat arrays become incorporated into heterochromatin, and in plants where silencing of repeat arrays correlates with repressive chromatin structure ***(32,50)***. Repeat induced silencing of transgene expression was recently shown directly in mice ***(51)***. In this study site-specific recombination (*lox*/Cre system) was used to generate a series of allelic transgenic mouse lines in which different numbers of a globin/*lacZ* construct were integrated at a constant location in the mouse genome (*see* **Fig. 2**). Reducing the copy number resulted in a significant increase in transgene expression that was observed as a suppression of variegation (more cells express the transgene). This increased expression from lower-copy arrays was found to be associated with decreased methylation and greater chromatin accessibility at the locus suggesting that, as in plants and *Drosophila*, repeat-induced gene silencing in mammals is the result of an epigenetic modification of the repetitive transgene array.

This clearly poses a problem for scientists who want to express foreign genes at high levels in whole animals; introducing more copies may be counterproductive. The existence of a small number of transgenes that show copy-number-dependent expression ***(52–57)*** does suggest that some regulatory elements exist that may prevent this repeat induced silencing, although at this stage the precise nature of such elements remains unclear. Alternatively, the sizes of transgene arrays may be controlled with site-specific recombinases. If a transgene construct contains a single copy of the recombinase target site, e.g., *lox*, then successful recombination between like oriented sites will reduce the copy number of the array (*see* **Fig. 2**). Recombination can be achieved by introducing the gene for the recombinase enzyme into fertilized mouse eggs either by breeding or by direct microinjection ***(58)***. Recently, an alternative method has become available for introducing transgenes into the mouse germ line using homologous recombination in embryonic stem (ES) cells to generate mice having a single copy of a transgene integrated into a chosen location in the genome ***(59)***. Targeting a single-copy transgene to a chosen location has many advantages. These include the ability to control the copy number, and therefore to avoid the silencing influences associated with multicopy arrays, and the ability to insert the transgene into regions of chromatin compatible with the desired developmental and tissue-specific expression ***(60)*** and therefore to avoid the influence of variable position effects. Targeted transgenes provide a more efficient and informative means of securing and comparing the expression of various sequences than has been available to date.

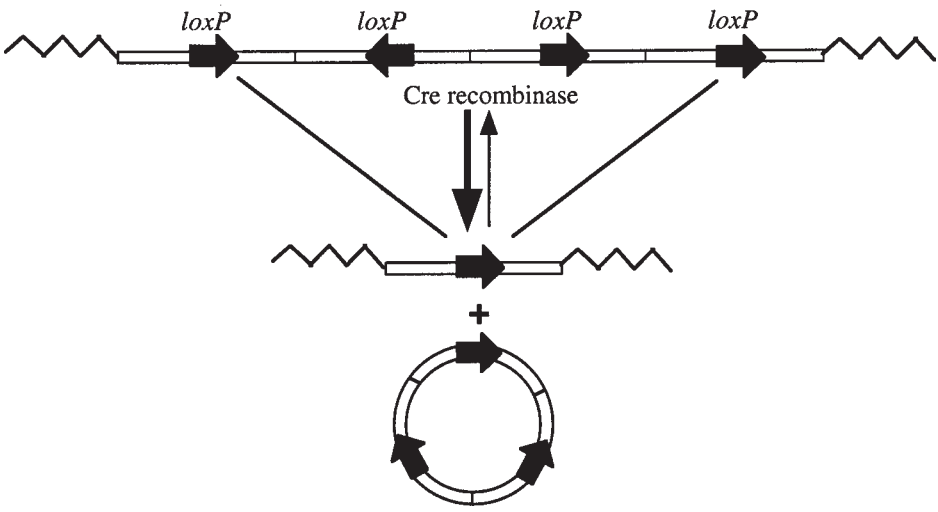

Fig. 2. Schematic representation of multiple copies of a transgene inserted as a tandem array within the mouse genome. The transgene contains a single copy of the 34 bp *loxP* site recognised by the Cre site-specific recombinase of bacteriophage P1. Cre-mediated recombination between like-oriented *loxP* sites on independent transgenes will generate a reduced-copy array which remains at the original genomic location. While this reaction is reversible, the forward (excision) reaction is heavily favoured over the reverse (integration) reaction, which requires recognition of *loxP* sites on separate molecules. The circular episome produced will be rapidly lost as the cells divide unless it is capable of autonomous replication.

3.4. Effects of Homologous Elements within the Genome

As mentioned previously, there are several examples in plants where transgene expression is modified by the presence of homologous genetic elements, either endogenous genes or other transgenes, at unlinked locations within the genome ***(61)***. These *trans*-sensing modifications of expression have been observed for homologous genetic elements situated at both allelic (i.e., homologous chromosomes) and nonallelic locations. As well as instances like paramutation, where an active allele is converted to an inactive form as a result of a nonreciprocal interaction with an inactive allele, homology-dependent silencing can also involve the reciprocal inactivation of two previously active genetic elements when brought together in the same genome (cosuppression). Although the mechanism by which transgenes can become silenced due to the presence of homologous sequences within the genome is poorly understood, in plants a correlation has frequently been observed between the inactive state and hypermethylation within the repeated sequence ***(62)***.

Although homology-dependent silencing has been most extensively characterized in plants, cosuppression of multiple homologous transgenes inserted at random genomic locations was recently reported in *Drosophila* ***(63)***, and it is likely that similar phenomena will be found to occur in mammals. Interestingly, a recent study of susceptibility to diabetes has found that IDDM2 type 1 diabetes susceptibility, previously mapped to allelic variation at the insulin gene VNTR polymorphism, depends on the identity of the untransmitted paternal allele ***(64)***, suggesting that an epigenetic mark is placed on the transmitted gene during a homologous interaction prior to meiotic separation of the chromosomes.

3.5. Effects of cis-Acting Elements in the Transgene

The likelihood that a transgene will be expressed in a heterocellular manner is greatly affected by the nature of the transgene itself. Experiments in a number of laboratories have found that the presence of enhancers and LCRs (locus control regions are defined as those that confer copy-number-dependent expression on a linked transgene) in the transgene construct increases the probability that the transgene will be expressed in a pancellular (i.e., in all cells) fashion. Variegated expression of a human CD2 transgene in murine T cells is suppressed by inclusion of a flanking element containing an LCR ***(8)***. Furthermore, in globin/*lacZ* transgenes, inclusion of the HS-40 element from the α-globin locus increases the proportion of expressing cells by two orders of magnitude ***(10)***; αHS-40 is a well-characterized transcriptional enhancer, and lacks properties normally associated with LCRs. These experiments, along with others carried out in cell lines ***(65–69)*** suggest that enhancers function to increase transcription not by increasing the rate of transcription of every template but by increasing the number of active templates. In other words, enhancers act to suppress silencing due to position effect.

Because we now know that enhancer elements are scattered throughout a particular locus, sometimes long distances from the promoter itself (e.g., the β-globin locus), this finding helps us to understand why small transgenes have generally been found to be unable to guarantee high levels of expression at all integration sites. It seems that the larger the transgene the more enhancer elements and therefore the higher the probability of pancellular expression (*see* **Fig. 3** for a schematic diagram). This is consistent with recent gene transfer experiments in embryonic stem cells; suppression of a variegated expression pattern of a transgene required a full complement of distant *cis* elements ***(70)***. Indeed these authors found that 130 kb YAC containing 60 and 30 kb of 5' and 3' flanking sequences, respectively, was required for fully appropriate regulation. This is rather a daunting finding for those of us trying to recapitulate expression in transgenic organisms.

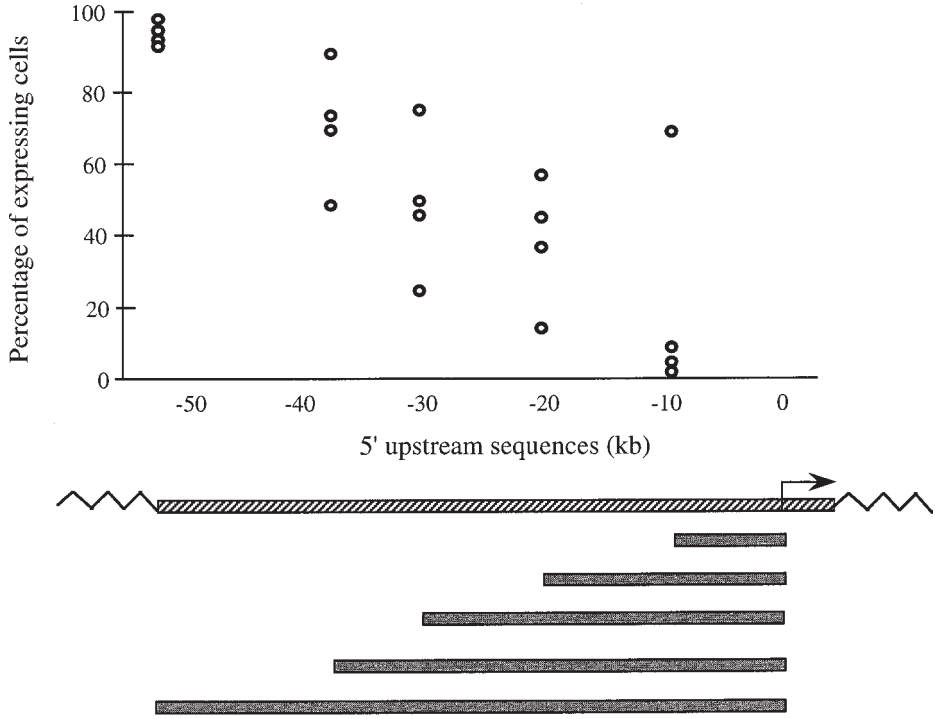

Fig. 3. Schematic representation showing the effect on expression of reducing the *cis*-regulatory elements contained within a transgene construct (adapted from 70). Constructs with smaller amounts of 5' regulatory sequence display greater variegation of expression (reduced percentages of expressing cells) and a wider variation in the size of the expressing population between different transgenic lines bearing the same construct (individual circles), indicating a greater susceptibility to position effects

3.6. Effects of the Age of the Mouse

Transgenics experiments designed to confer new traits on plants or animals generally require continued expression of the gene as the organism ages. Unfortunately, this is frequently not achieved; silencing of the transgene may occur during development within one generation ***(71–73)***, in response to environmental changes ***(74,75)*** or may occur from generation to generation with expression gradually being lost from the line ***(41,76)***.

We have found that expression of our globin/*lacZ* transgenes decreases with increasing age of the mouse ***(9,10)***. Single-cell analysis shows that this is associated with a decrease in the proportion of cells expressing the transgene rather than a decrease in the amount of expression in every active cell ***(10)***. The rate of decline varies from line to line, suggesting an influence from the site of

integration. This is consistent with experiments carried out in cell lines in which single-copy integrants of a transgene were found to silence at different rates, also suggesting a role for position effect ***(69)***. Other studies have shown that specific sequences present in transgenic constructs can promote silencing. For example, viral long terminal repeats (LTRs) are often used in constructs as they allow efficient integration, but it has also been shown that the presence of these sequences severely inhibits expression ***(77)***. There have also been a number of reports of inhibitory effects of plasmid vector sequences on transgene expression ***(78–81)***, and there is some evidence that the bacterial *lacZ* gene has an inhibitory effect on the expression of a linked reporter in transgenic mice ***(82)***.

3.7. Effects of Genetic Background

Finally, the genetic background in which the transgene resides may influence the extent of variegation. For example, backcrossing into CBA/Ca and C57BL/10 strains resulted in a threefold difference in silencing of a human CD2 transgene ***(34)***. Similarly, a mouse hsp68/*lacZ* transgene backcrossed onto C57BL/6 was expressed in all cells, whereas the same transgene locus backcrossed onto BALB/C showed variegated expression ***(4)***. It has been suggested that this effect may mean that inbred strains of mice differ from each other in the expression or function of chromatin factors that modify position effects ***(4,33,41)***.

Many gene-targeted mutations show great variation in phenotype on different genetic backgrounds ***(83)***. For example, although the keratin 8 "knockout" phenotype includes embryonic lethality, 1.6% of mice are viable in a C57BL/6 background, whereas 55% are viable in FVB/N ***(84)***. Although in many cases the modifier genes involved may exert their effect via interactions between their product and the protein of the target gene or by altering its transcription rate or mRNA stability, some, as shown by the transgenic mouse examples cited, appear to influence epigenetic modifications. This may involve modifying the degree of DNA methylation or heterochromatin formation.

4. Relevance to Systems Involving Homologous Recombination

The findings on silencing of transgenes has some important consequences for experiments aimed at producing gene knockouts in mice. Experiments such as those described in **Subheading 3.5.** make it clear that elements found at great distances from the gene in question can be playing a profound effect on the expression of that gene. The most widely used strategy for gene disruption in the mouse involves the deletion of part or all of the target gene together with concomitant insertion of a drug selection cassette, the goal being efficient creation of definitive null alleles. First, depending on the size of the deletion, such manipulations may disrupt the expression of other genes located nearby and confound the interpretation of phenotypes. Recently, a very striking case

of such neighborhood effects emerged from the targeted inactivation of the myogenic basic-helix-loop-helix (bHLH) gene, MRF4/herculin/myf-6 ***(85–87)***. The phenotypes of three slightly different knockout alleles varied widely, ranging from complete viability of homozygotes to complete lethality. The evidence suggests that differing effects of these mutations on expression of the adjacent Myf5 gene account for much of the phenotypic variation ***(88)***. Second, the presence of the PGKneo cassette at the targeted locus can influence phenotype. Fiering and co-workers ***(89)*** designed a targeted deletion of the 5' DNase hypersensitive site 2 of the LCR of the β-globin locus. They did observe significant effects on globin gene expression, but when they removed the PGKneo selection cassette from the site, they found that globin gene expression became essentially normal. This directly demonstrated that the transcriptional effects were due to the presence of the selection cassette and not to the deletion. Presumably the cassette itself can confer some epigenetic modification on the entire locus. It remains unclear whether in any of these cases disruption of the locus resulted in variegated expression of neighbouring genes. Until substantially more is understood about *cis* regulation over large regions of the genome, it is prudent to consider these effects in both design and interpretation. Alternatively, to avoid deleting sequences that may have regulatory effects on adjacent genes, one can introduce positioned stop codons to inactivate the gene ***(90)*** and this can be coupled with removal of the selection cassette by site-specific recombinases such as yeast Flp and phage P1 Cre.

DNA sequences and genes can no longer be considered in isolation when introduced into the complex milieu of the eukaryotic nucleus. There they will interact with other DNA sequences, but also with the epigenetic component of the genome, which is embedded in the structure and composition of the DNA and proteins that compose chromatin and chromosomes. Transgenesis has proved a powerful technique for the delineation and analysis of *cis*- and *trans*-acting regulatory elements in eukaryotes, but is now showing itself to be equally useful in the study of epigenetic influences in gene regulation.

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23

Positional-Candidate Cloning of Genes from Mouse Mutants

Karen B. Avraham

1. Introduction

The overall goal in human genetic disease is to understand the biological function of normal genes, and how, once perterbed, they lead to a diseased state. The limitations of using human material for experiments, the ease with which one can work with mice, and the similarities between the two organisms has led to a vast area of research using mouse models to study human disease [reviewed in ***(1)***]. Furthermore, genes and linkage organization are well conserved between human and mouse, as demonstrated by comparative genome mapping ***(2)***. Over 1000 mouse mutations, created by serendipity and genetics, have been maintained over the years, most of whose genes have yet to be cloned. Phenotypes range from autoimmune and neurological disorders to skeletal, hearing, and visual defects. Technological advances in positional and/or the candidate gene approach to cloning enable the genes encoded by the mutant loci to be identified.

One may wonder why a review on cloning of mouse mutations is in a book dedicated to describing gene-targeting protocols. This review will attempt to describe the steps one should take once cloning a gene, prior to "knocking it out," in order to determine if a mouse mutant with an alteration in your gene already exists. Alternatively, if one is interested in a particular disease or phenotype, one should investigate whether a mutant phenotype already exists, before attempting to create this phenotype experimentally. Interested in skeletal morphogenesis? Examine the phenotypes manifested in osteosclerosis (*oc*), short ear (*se*), and osteochondystrophy (*ocd*). Interested in deafness? Examine the mutants Dancer (*Dc*), Jackson circler (*jc*), or Bronx waltzer (*bv*).

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So you've identified and begun to characterize a gene. What's the first step, before isolating a clone to use in constructing a gene targeting construct? One should first determine the chromosomal location of the gene in the mouse genome. Mapping your gene may lead to several results: the identification of mouse mutations already existing for the locus in question and the identification of the homologous region in humans, both for predicting the human chromosomal location of the gene, as well as identifying a potential human disease associated with the gene.

This chapter covers two areas: (1) how to go about determining if your gene is a candidate for an existing mouse mutation, circumventing the need to create a knockout (the candidate gene approach); and (2) how to go about cloning the gene for a mutation with a phenotype of interest (by positional cloning).

2. The Candidate Gene Approach: Does a Mouse Mutation for Your Gene Already Exist?

2.1. Determining the Chromosomal Location of Gene of Interest

The ability to detect polymorphisms between mouse strains or species forms the basis for genetic linkage analysis. Inheritance of alterations in a DNA sequence can be followed by restriction fragment length polymorphisms (RFLPs; using Southern blotting) or by microsatellites (using the polymerase chain reaction, PCR). Microsatellites consist of around 10–50 copies of motifs from 1–6 bp that often occur in tandem repetition ***(3)***. The most commonly used microsatellites, CA repeats, occur on average every 30 kb and are highly polymorphic. These microsatellites can be assayed by PCR under uniform conditions when surrounded by unique DNA. Both types of polymorphisms form the basis for the mouse genetic map, in the form of cloned genes and highly polymorphic anonymous DNA segments ***(4)***.

Genetic mapping in the mouse can be performed either by using crosses between inbred laboratatory strains (intraspecific crosses) or between a laboratory strain (*Mus musculus*) and distantly related species of *Mus*, such as *Mus spretus* (interspecific crosses) ***(5,6)***. The farther the evolutionary distance between the parents of the cross, the higher the degree of genetic polymorphism. The first step in mapping a gene is to identify a polymorphism with a probe (for detecting RFLPs by Southern blot analysis) or primers (for PCR analysis) derived from your gene on the two strains or species of mice used (*see* **Fig. 1**). For detecting RFLPs, each DNA derived from each strain or species is digested with a number of restriction enzymes and hybridized with a probe derived from the gene of interest. In choosing unique primers from your gene for PCR amplification, intronic regions or untranslated regions (UTR) provide a greater chance for detecting a polymorphism. Primers spanning a microsatellite are especially polymorphic. PCR followed by restriction enzyme

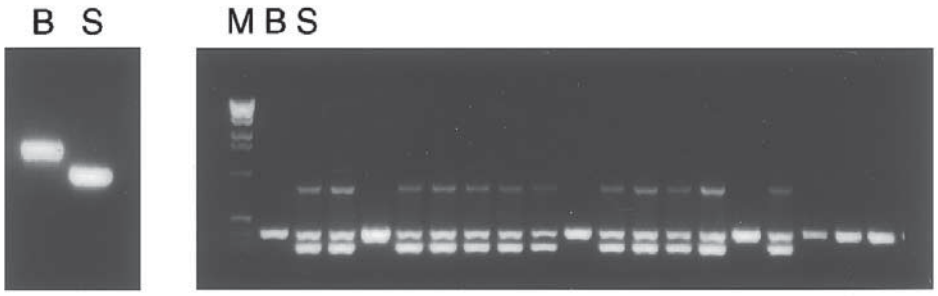

Fig. 1. Genetic mapping using the Jackson Laboratory Interspecific Backcross Mapping Panel ***(33)***. In the left panel, a polymorphism between the two parental species, *Mus musculus* (C57BL/6J; "B" allele) and *Mus spretus* (*M. spretus*; "S" allele), is detected in DNA amplified with primers spanning a $(CA)_n$ repeat. In the right panel, a portion of the backcross animals are typed with the same set of primers, showing segregation of either the "B" or "S" allele in each animal. The BSB panel [(C57BL/6J × *M. spretus*) × C57BL/6J] is shown here; therefore a "B" allele will always be present in the backcross animal and the segregation of the "S" allele is followed. The PCR reaction was run on a 2% agarose gel with 1 kb markers (M) (Gibco-BRL).

digestion (PCR-RFLP) can optimize the search for polymorphisms (*see* **Fig. 2**). If a polymorphism is not readily detected by PCR, sequencing the PCR product may detect the presence of an altered restriction enzyme site. For both DNA hybridization and PCR, care should be taken not to amplify too many fragments, as this will make following the segregation difficult; in addition, this may lead to mapping of multiple related loci.

After identifying a polymorphism between the mouse strains or species, the segregation pattern of the polymorphism is assayed on DNA derived from a panel of mice generated from the interspecific or intraspecific. Once a segregation pattern is obtained, the data are submitted to multipoint linkage analysis, where the new segregation pattern is compared to known patterns from previously mapped genes and the number of crossovers is minimized.

2.2. Sources for Mapping a Gene in the Mouse

Several laboratories have constructed interspecific backcross maps, where genes can be mapped on a collaborative basis. Two publicly available maps, requiring the investigators to perform the PCR or Southern blot analysis themselves, are available as well. Information about these maps is shown in **Table 1**. Linkage maps derived from these laboratories are accessible through the World Wide Web (WWW) (http://www.informatics.jax.org). A map based on simple sequence length polymorphisms (SSLP) or microsatellite markers has been developed at the Whitehead Institute/MIT Center for Genome Research

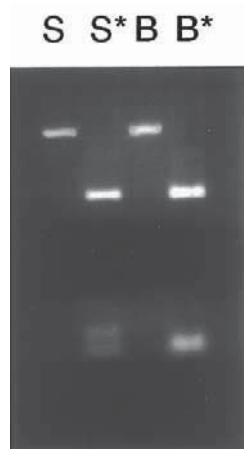

Fig. 2. PCR-RFLP for optimizing polymorphisms. No polymorphism was detected with C57BL/6J (B) and *M. spretus* (S) template DNA following PCR. Sequencing the PCR products revealed an additional *Nla*IV restriction enzyme site in the *M. spretus* fragment. Upon digestion with *Nla*IV (B* and S*), an additional band was detected in the *M. spretus* DNA (S*), whose segregation could be followed for determining the gene's chromosomal location. The PCR reaction was run on a 5% MetaPhor agarose gel (FMC BioProducts).

(http://www-genome.wi.mit.edu/) *(7)*. A subset of these markers have been mapped on the other available linkage maps, allowing for cross-referencing between the maps. The Chromosome Committees compile the genes mapped for each chromosome to generate a consensus map (http://www.informatics.jax.org/ccr/searches/index.cgl).

2.3. Searching for Mouse Mutations in Region of Your Gene

Once your gene is mapped, one should identify mouse mutations that have been mapped to the same region. This information may be found in the Mouse Genome database (MGD) (http//www.informatics.jax.org/searches/markerform.shtml). Historically, mutant loci were mapped relative to one another, so that their location relative to mapped genes can only be inferred. In some cases, backcrosses have been set up with the mutant and refined linkage maps published. A general rule of thumb is to look 10 cM in either direction of your gene. However, mutations with apparently relevant phenotypes mapping farther should be examined as well, as direct correlations of different genetic maps cannot be made. If the genetic data suggest the mapped gene and mutant locus may be the same, a comparison of the candidate gene in wild-type and mutant mice should be made on a molecular level.

Table 1
Mapping Mouse Genes: Mapping Panels Available

Source	Backcrosses	Contact
Publicly available panels		
The Jackson Laboratory Interspecific cmdata	Two reciprocal backcrosses; 188 animals	http://www.jax.org/resources/documents/
Backcross (JLIB) Mapping Panel ***(33)***	(C57BL/6J × *M. spretus*) × C57BL/6J (BSB panel) (C57BL/6J × *M. spretus*) × *M. spretus* (BSS panel)	
The European Collaborative Interspecific Backcross EUCIB Mapping Panel ***(34)***	Two reciprocal backcrosses; 982 animals (C57BL/6J × *M. spretus*)	http://www.mgc.har. mrc.ac.uk/about-mgc.html
Collaboratively available panels		
Frederick Interspecific Backcross Mapping Panel ***(4)***	Interspecific backcross; 205 animals (C57BL/6J × *M. spretus*)	Neal Copeland (copeland@ncifcrf.gov) or Nancy Jenkins (jenkins@ncifcrf.gov)
NIH Interspecific Backcross Mapping Panel ***(35,36)***	(NFS/N or C58/J × *M.m musculus*) × *M.m musculus* (NFS/N × *M. spretus*) × *M. spretus* (NFS/N × *M. spretus*) × C58/J	Christine Kozak (ckozak@nih.gov)
Seldin Interspecific Backcross Mapping Panel ***(37)***	450 animals; C3H/HeJ-gld/gld × *M. spretus*) × C3H	Mike Seldin (mfseldin@ucdavis.edu)

The candidate gene approach to identifying mutant loci has been the most successful to date. Molecular analysis to identify the mutations have been done in the following ways: (1) the identification of altered genomic fragments by Southern blot analysis [demonstrated for protein 4.2 alternations in the pallid (*pa*) mutant ***(8);*** POU-domain gene mutations, *pit1*, in the dwarf (*dw*) locus ***(9)***; and *Pax3* mutations in splotch (*Sp*) ***(10)***]; (2) PCR amplification and sequencing of genomic DNA, which can be successfully employed in cases where there are few or no introns [mutations in the MSH receptor was found in the extension (*e*) locus using this method ***(11)***]; (3) alterations in the size (or absence) of the coding region by RT-PCR and sequence analysis (used to detect a nucleotide change in little (*lit*) mice in the GHRH receptor ***(12)***]; and (4) alterations in RNA transcript levels and/or sizes by northern blot analysis, demonstrated by lack of *Gli3* expression in extra-toes (*Xt*) mutants ***(13)***.

3. Positional Cloning: Starting with a Phenotype

3.1. Backcross Analysis

Positional cloning involves the isolating of a gene based on its map location in the genome. It assumes no functional information, nor does it consider genes already identified in the region as candidates. As a first step, a backcross is set up between the mutant and a mouse strain amenable to detecting polymorphisms. For low-resolution mapping, 50–100 meioses are examined using, on average, three markers (microsatellites) per chromosome. Progeny of backcrosses are scored for the phenotype of interest and used in segregation analysis of the polymorphic markers.

Once an initial chromosomal region is identified, high-resolution, fine-structure mapping is performed by expanding the backcross in order to obtain a resolution sufficient for physical mapping. Flanking microsatellite markers within a few centimorgans (cM) of the mutation are assayed on backcross animals to define the smallest nonrecombinant region possible. Once a critical region is defined, several strategies can be undertaken in order to identify a transcription unit. As a first step, a contig of the region, a contiguous set of overlapping cloned DNA, consisting of yeast artificial chromosome (YACs), bacterial artificial chromosomes (BACs), P1s, or cosmids should be obtained ***(14)***. Each vector has its own advantages and disadvantages, including chimerism, size, and ease of experimental manipulation. Contigs from various regions are already available, and before constructing one's own, the databases should be searched for available clones (mouse genome resources; URL, http://www.ncbi.nlm.nih.gov(genome/guide/m_musculus. html).

3.2. Alternatives to Backcross Analysis

Mutations caused by deletions, inversions, or transgene insertions facilitate cloning of the genes responsible, circumventing the need for conventional

backcross and linkage analysis. Probes obtaining by cloning deletion, inversion, or transgene breakpoints provide entry into the locus' region; transcribed sequences are then searched for in the same manner as for critical regions identified by classical backcross analysis (*see* **Subheading 3.3.**). Examples include the radiation-induced deletion, short ear (*se*), used in conjunction with exon trapping [*see* **Subheading 3.3.2.**; ***(15)***]; radiation-induced inversions such as agouti (*a*) ***(16)***, kreisler (*kr*), used in conjunction with direct cDNA selection (*see* **Subheading 3.3.3.**) ***(17)***, and Snell's waltzer (*sv*), used in conjunction with exon trapping of YAC clones ***(18)***; and transgene insertions, microphthalmia (*mi*) ***(19),*** Fused (*Fu*) ***(20),*** and reeler (*rl*), used in conjunction with exon trapping ***(21).***

3.3. Identification of Transcribed Sequences

Several techniques have been developed over the recent years for searching for transcribed sequences in a well-defined genomic segment, each with its advantages and disadvantages. Following is a partial group of the most commonly used techniques. Ideally, a combination of strategies should be employed to maximize the advantage offered by each; in reality, this may be impractical.

3.3.1. Sequencing Genomic DNA

Sequencing genomic DNA has recently become less expensive, automated, and routinely performed in laboratories and is thus increasing in popularity. Once a sequence is obtained, it is compared to known genes (deposited in databases such as Genbank) and expressed sequenced tags (ESTs), as well as examined for its protein encoding potential using programs such as GRAIL ***(22).*** The entire critical region identified for the locus must be sequenced and thus is dependent on the density of markers in the region; if reduced only to 1 Mb, sequencing is costly and laborious. As the region becomes smaller, it becomes more feasible to employ this method. Sequencing becomes even more efficient when used in conjunction with rescue of the mutant phenotype, in complementation cloning (*see* **Subheading 3.3.4.**).

3.3.2. Exon Trapping

Exon trapping is based on the identification of exons "trapped" from genomic DNA, utilizing the splice acceptor and splice donor sites of the endogenous exon. It involves inserting the genomic DNA of interest into a vector such as pSPL3 [developed by A. Buckler and distributed commercially by Life Technologies; ***(23)***]. A detailed protocol for this system and list of other vectors has recently been described ***(24).*** RNA is made from cells transfected with the vector containing genomic DNA; exons are spliced out, utilizing the ability of the cells to recognize and remove introns. Following reverse

transcription (RT) and PCR amplification of cytoplasmic RNA, trapped exons are identified by PCR. Once exons are isolated, they are sequenced and analyzed as described. This technique is relatively easy, although it does not ensure obtaining 100% of the exons in a cloned fragment. Furthermore, usually many products are obtained, requiring a labor-intensive search to analyze each potential gene. The rate-limiting factor is achieving full representation of the cloned material into the splicing vector. For this reason, some prefer to exon trap smaller clones such as cosmids; however, constructing a cosmid contig from YACs is time consuming and labor intensive in itself. Exons have been successfully trapped using YACs as a source of genomic DNA when cloning myosin VI for Snell's waltzer, *sv*, and myosin VIIA for shaker-1, *sh1* ***(18,25)***, and using smaller clones when cloning leptin for the obese (*ob*) mouse ***(26)*** and winged helix nude for the nude (*nu*) mouse ***(27)***.

3.3.3. Direct cDNA Selection

Direct cDNA selection is expression based, recovering cDNA fragments that specifically hybridize to DNA templates ***(28)***. A cDNA library is constructed from tissue where one expects expression of the gene, modified so that it can be amplified by PCR. The repetitive DNA is blocked and then hybridized to a contig of genomic DNA presumably containing the gene of interest. This technique is more tedious than exon trapping, but in a comparison performed while attempting to positionally clone the nude locus, direct cDNA selection identified more transcription units than exon trapping ***(29)***. The caveat in this strategy is that an educated guess regarding expression must be made, as a cDNA library is constructed from the appropriate tissue. In several cases, both direct cDNA selection and exon trapping was performed to maximize isolation of transcribed sequences [in cloning the leptin receptor for diabetic, *db*, mice ***(30)***].

3.3.4. Complementation Cloning/Positional Complementation

With the ability to microinject large cloned fragments into mouse embryos, such as YACs, BACs, or P1s, the mutant locus may be "rescued" by a defined cloned unit. Nonrecombinant markers might be difficult to find in a small region due to clustering of crossovers, and this method is useful in further refining the critical region prior to sequencing. Clones isolated during construction of the physical map of a region are injected to create transgenic founders. The founders are crossed to mutant mice, and the loss of the phenotype in progeny is assayed. During a positional cloning search for the vibrator (*vb*) locus, a 500-kb region defined by nonrecombinant markers was reduced to 76 kb by the creation of a P1 transgenic able to complement the mutant phenotype ***(31)***.

4. Positional-Candidate Cloning

As more genes are mapped in the mouse, increasing the gene density, the two cloning approaches will converge to the positional-candidate gene approach of cloning genes responsible for mouse mutations. This approach uses information about the known location of the mutant and knowledge of previously isolated genes in that region. Today, it is difficult to localize a gene to a chromosomal location that does not already contain several candidate genes and ESTs, minimizing the tedious search for transcribed sequences. There is much work yet to be done, however. Only a small portion of the classical mouse mutations have been cloned, and the remaining mutants offer a wealth of biological information waiting to be tapped. Once a gene is cloned for a mutant phenotype, the real challenge begins — understanding the biological function of the protein and how when altered, it leads to a diseased state.

Acknowledgments

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24

Genetically Engineered Mice

Husbandry and Resources

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1. Introduction

Random and selective techniques to alter the mouse genome are now providing powerful tools for biomedical research. These strains provide experimental systems for understanding gene function, for studying defects in specific human genetic diseases, for preclinical testing of therapeutic agents and for developing new therapeutic interventions such as gene therapy ***(1,2)***. Even for diseases such as AIDS, which are not normally considered to be of genetic origin, these model systems are important for understanding the underlying pathophysiology of the disease process. In addition, such model systems permit studies that are inappropriate or impossible in human beings. There now exist valuable mouse models for virtually every major category of human health with new strains being generated daily. The use of these strains is rapidly spreading to scientists who have never previously used mice for investigative purposes. The Induced Mutant Resource (IMR) was established at The Jackson Laboratory (TJL), Bar Harbor, ME, in 1993 with the purpose of selecting, importing, archiving, and distributing these genetically engineered mouse strains to the scientific community ***(3)***. Adding this resource was considered to be a natural extension of ongoing activities at The Laboratory (over 2500 stocks are currently available from TJL as breeding mice, frozen embryos, or DNA samples). Even so, in 1993 there was uncertainty about the extent to which such a resource would be utilized. In over 5 yr of operation the IMR has experienced rapid growth in both the selection and distribution of these strains, a testament to their great utility.

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The care, breeding, health maintenance, genetic quality control, and information dissemination for genetically engineered strains presents unique problems not often encountered with other strains, with the possible exception of those carrying spontaneous mutations. We discuss these issues drawn from our experience with the IMR and present a prospectus for the future.

2. Genetically Engineered Mice

2.1. Overview

Three broad areas of technology — transgenesis, homologous recombination, and random mutagenesis — are currently being used to create new genetically engineered strains of mice and all are represented in the IMR.

Transgenic mice have genetic material randomly added to their genomes *(4)*. These strains have been used to study gene function and expression and have resulted in important disease models. Because the transgene insertion is a random event, it may occur within a gene, most often resulting in a recessive mutation not related to transgene expression. Thus transgenics also provide a vehicle for gene discovery through the mapping and subsequent cloning of a disrupted gene.

Strains created by homologous recombination (gene targeting) have a specific locus or gene altered or replaced *(5,6)*. Currently, the majority of strains created by gene targeting carry a null mutation for the gene in question. More recently, conditional targeted mutations have been created that allow control of both the onset (temporal control) and the tissue specificity of the mutation *(7,8)*. Gene targeting produces strains that are used to study gene function and to create models for human genetic diseases for which the offending gene is known.

Random mutagenesis protocols such as treating mouse gametes or embryonic stem (ES) cells with chemical mutagens *(9)* and gene trapping with retroviral vectors *(10)* also are producing valuable new models. These random approaches to mutagenesis produce both dominant and recessive mutations, although the majority of effort to date has concentrated on identifying the technically simpler dominant mutations. For such approaches to obtain their maximum value, rapid and systematized protocols for phenotypic screening must be available as well as the resources for mapping and cloning these new genes. Several large scale N-ethyl-N-nitrosourea (ENU) mutagenesis projects are currently underway, including the MRC Mammalian Genetics Unit, SmithKline Beecham, Imperial College consortium (http://www.mgu.har.mrc.ac.uk/mutabase/) and the Max Planck Institute program (http://www.gsf.de/isg/ENU.index.html).

2.2. Husbandry

2.2.1. Handling

Because genetically engineered mice are carrying a mutation whose effect on their overall well-being may not be completely understood, they should be handled with specialized care. The cost to generate such mice is considerable and every effort to ensure the establishment and health of the colony should be made. Animal care technicians responsible for the routine maintenance of the colony—facility managers, veterinarians, and research personnel working with mice—should be well informed about the nature of the mutation, distinguishing characteristics and any necessary precautions. All personnel handling the mice should be trained to observe and record any deviations in phenotype, behavior, or reproductive performance.

2.2.2. Housing

Special housing considerations may be important depending on the nature of the mutation. Genetically engineered mice that are immunocompromised need to be maintained under strict specific pathogen-free conditions in a barrier facility.

2.2.3. Breeding

The breeding of mice carrying induced mutations presents several challenges not found in the care and maintenance of standard inbred strains. If possible, strains carrying induced mutations are maintained by homozygous matings, but special breeding schemes are often necessary to propagate the mutation. Transgenic strains are most often propagated by breeding a hemizygote (transgenes incorporated on only one member of the homologous chromosome pair) with a wildtype animal (either an F_1, inbred strain, or littermate). This prevents the phenotypic expression of a possible recessive insertional mutation. Some targeted mutations must also be maintained by heterozygous sibling matings because homozygotes die *in utero* or perinatally or one or both sexes are not fertile. These strains are often maintained by breeding heterozygotes to wild-type controls for maintenance and distribution, thus producing only two genotypes.

The breeding performance of a strain may be adversely affected by the induced mutation, creating special husbandry problems for colony managers. For example, overexpression of the promoter region and exon 1 of the human Huntington's disease gene (TgN(HDexon1)62Gpb) causes disease symptoms beginning at 9–11 wk of age in mice that mimic the human ***(11)***. Hemizygous females are not fertile and hemizygous males have only a 3–4 wk breeding window of which about 50% will breed. The breeding scheme utilized is two

B6CBAF1 females crossed with one hemizygous TgN(HDexon1)62 male. IMR colony personnel have also successfully propagated this strain by transplanting ovaries from hemizygous females into histocompatible females and mating to B6CBAF1 males, greatly simplifying colony maintenance.

2.2.4. Special Diets

Some mice strains may have specific diet requirements necessitated by the elimination of a gene product. For example, breeders and preweanlings of *Cyp7a1*-deficient mice must be fed a cholic acid supplement to compensate for the loss of cholesterol 7 alpha hydroxylase ***(12)***. It is also important to note that some investigators resort to feeding mutant mice exotic diet supplements such as squash or peanut butter to improve breeding performance without carrying out controlled experiments. In the IMR, care is taken to analyze diet recommendations and standardize diets as much as possible.

2.3. Factors Affecting Phenotype

The expression of a phenotype in mice carrying an induced mutation may depend on a number of factors not readily apparent to the initial researcher or those using the model in subsequent studies. Colony health and genetic background are important contributors to the observed phenotype and transgene instability, although relatively rare, can significantly alter the expression level of the exogenous gene.

2.3.1. Colony Health Status

The health status of a colony may alter the observable phenotypes, especially for immunocompromised strains. For example, T-cell receptor alpha- and T-cell receptor beta- deficient mice were reported to develop severe ulcerative colitis ***(13)***, but these symptoms virtually disappeared following rederivation into the IMR colony. Similar findings on the effect of health status on the display of disease symptoms were reported for both *Il2*- and *Il10*- deficient mice ***(14–17)***.

2.3.2. Genetic Background

There are many well-characterized instances of the influence of inbred background on the expressed phenotype. For example, an activated HRAS transgene under the control of the whey acidic protein promoter is reported to have mammary and salivary carcinomas that frequently metastasize to the lung on a mixed C57BL/6J-SJL inbred background. On the FVB inbred background this transgene causes anaplastic carcinomas that do not metastasize ***(18)***.

Targeted mutant strains are often generated, characterized, and reported to be a mixed C57BL/6-129 genetic background in order to introduce hybrid vigor

for the initial studies and to easily verify germline transmission of the mutation. Further inbreeding or backcrossing to other inbred strains may significantly alter the initial phenotype. Such background influences on the phenotype of a mutant are the result of modifier genes in the host genome. These modifiers that represent important contributors to the observed phenotype subsequently may be mapped and cloned ***(19)***.

2.3.3. Unstable Transgene

Although infrequent, transgenes may lose copy numbers or may be completely eliminated from the host genome. We observed a delayed onset of the ALS (amyotropic lateral sclerosis) phenotype in SOD1 transgenic mice ***(20)*** and traced it to a reduction in transgene copy number. Both the original and reduced copy number strains are available from the IMR.

2.4. Genetic Quality Control

Genetic quality control for genetically engineered strains consists of verifying the presence of a transgene or mutated allele and, if inbred, verifying the strain background. The necessity of the former is obvious but the latter, which is a safeguard against accidental genetic contamination, is also important because the genetic background of a strain may have a major effect on the expressed phenotype, as discussed above.

Prior to the production of genetically engineered strains, the majority of genetic quality control consisted of verifying the genetic background of inbred strains. Biochemical and immunological markers were primarily used for this purpose. The development of PCR to amplify simple sequence length polymorphic (SSLP) markers is now being used for genetic background verification ***(21)***. The large number of SSLP markers available for the mouse ***(22)*** make this methodology more sensitive in identifying genetic contamination than by the use of biochemical markers alone.

The mating schemes used for many induced mutant strains result in litters containing a combination of wild type, and heterozygous and/or homozygous mutant mice (discussed above). The genotype of mice from such matings must be determined prior to their experimental use or distribution. Within individual research laboratories Southern blotting has largely been used for this purpose. Southern blotting is, however, too slow and expensive when distributing large numbers of mice from a resource such as the IMR. We chose to utilize the polymerase chain reaction (PCR) for the majority of our allele specific genotyping because it is rapid, the reaction conditions may be standardized, it does not require the use of radioisotopes, and it is adaptable to automation. The IMR exclusively uses PCR to genotype strains for distribution, when required.

2.5. Strain Nomenclature

Strain and gene nomenclature is often confusing to the uninitiated, but a standardized nomenclature system is critical to establish the identity or nonidentity of strains made in different laboratories or available from different sources. Without a standardized nomenclature system, access to the vast amount of information being generated by the human, mouse, and other genome projects would be impossible. Even with a system in place, not everyone is compliant, which often creates difficulty understanding what gene or strain is being discussed, especially for investigators outside a particular area of expertise. The amount of gene expression data currently being generated will certainly create unsuspected bridges between research areas, and understanding and use of standardized nomenclature will greatly facilitate the retrieval and analysis of information. We only briefly discuss nomenclature here as the rules set forth by the International Committee on Standardized Genetic Nomenclature for Mice are available through Mouse Genome Database ***(23)***.

2.5.1. Transgenic Nomenclature

Transgenes are named according to the mode of incorporation (TgH for homologous recombination, TgR for insertion via infection with a retroviral vector, and TgN for nonhomologous insertion), followed by a designation for the DNA insert in parentheses (preferably the gene symbol with an indication of the promoter), then a number indicating the founder line, and finally a laboratory registration code. For example, TgN(Alb1HBV)44Bri represents the gene for the large envelope polypeptide of the hepatitis B virus driven by the mouse albumin *(Alb1)* promoter. It was the 44th founder in the laboratory of Dr. Ralph Brinster (Bri) ***(24)***.

2.5.2. Targeted Mutant Nomenclature

Targeted alleles of genes are designated by the correct gene symbol (for the mouse, both upper and lowercase italicized letters are used) followed by (all superscripted) tm (for targeted mutation), an allele number, and the laboratory registration code. For example, $Trp53^{tm1Tyj}$ represents the first targeted mutation in the transformation-related protein 53 gene (*Trp53*) made in the laboratory of Dr. Tyler Jacks (*Tyj*) ***(25)***.

2.5.3. Genetic Background

The genetic background of mice with induced mutations is given prior to the transgene designation or allele symbol. Currently, many strains are maintained on a mixture of C57BL/6 and 129 (e.g., B6;129- $Trp53^{tm1Tyj}$) because 129-derived ES cell lines are most often used for targeting and chimeric mice are mated to C57BL/6J to determine germline transmission. Mutations transferred by backcrossing from a mixed background to an inbred background follow standard nomenclature.

3. The Induced Mutant Resource

3.1. Formation

In the early 1990s, the scientific community raised concerns over the distribution of genetically engineered mice, especially the newly created targeted mutant strains ***(26,27)***. Scientists recognized the need to protect the health and genetic purity of these strains and desired to have them distributed with as few restrictions as possible, in order to maximize the opportunities for major advances in understanding and treating human disease. There was also concern that, for an individual investigator, numerous requests to distribute a strain would utilize resources that would better be spent on research activities. At the urging from the scientific community, TJL established the Induced Mutant Resource. Initial funding to support this effort came from The March of Dimes Birth Defect Foundation and subsequently from several other voluntary health care agencies (*see* Acknowledgments) and the Howard Hughes Medical Institute. Current funding is provided by the National Center for Research Resources, the National Institute for Allergy and Infectious Disease (NIAID), and the Howard Hughes Medical Institute.

3.2. Functions

The functions of the IMR are to (1) identify, (2) select, and (3) import important genetically engineered strains of mice, (4) cryopreserve embryos or gametes from these strains, (5) transfer mutations onto defined genetic backgrounds when appropriate, (6) maintain and distribute these strains, and (7) provide information on them to the scientific community.

3.2.1. Identification of Mutants

Potential IMR mouse strains are identified, in large part, by submissions from the creators of the mice. Investigators submitting strains are asked to fill out a form (accessible electronically through the IMR's Web page) that collects basic information about the strain and also to submit a reference or preprint describing its generation and phenotype. New strains also are identified from suggestions made by the IMR and NIH advisory boards, the TJL staff and outside investigators, and through literature searches. Candidate strains are reviewed by TJL's Genetic Resources Committee.

3.2.2. Selection of Mutants

The large number of strains being generated (*see* **Fig. 1**) make it impossible to collect and distribute all strains being generated. Strains are selected for inclusion in the IMR using the following criteria: (1) their immediate need for use in biomedical research, (2) the number of requests for animals received by

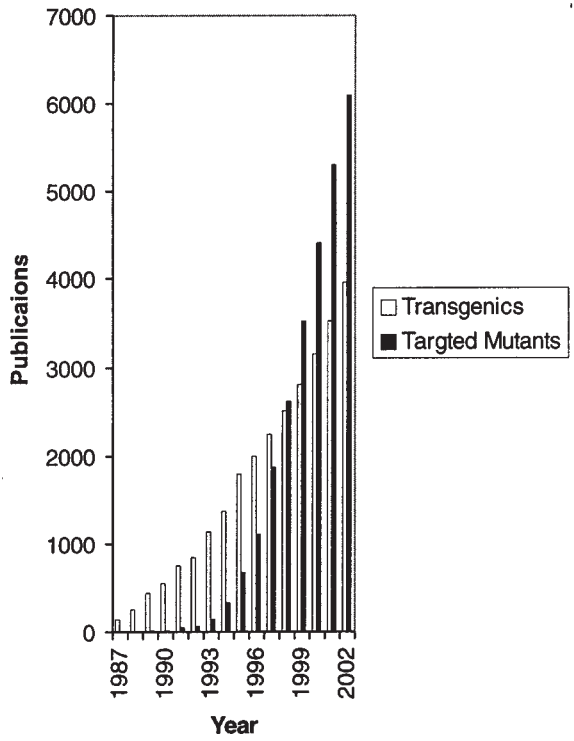

Fig. 1. The number of publications citing either transgenic mice or targeted mice. Current (1987–1997) values were obtained using SilverPlatter to search MEDLINE. The 1998–2002 values for transgenic strains were projected utilizing an annual 12% growth rate (the growth was 11% for 1996 and 12% for 1997). The potential growth in targeted mutations was calculated using historical transgenic data and assuming a growth rate 50% greater than that for transgenics at the corresponding time after the first publication.

the investigators who created them, (3) their potential for future research, (4) the time and effort needed to replace or recreate the mutation or strain, and (5) the uniqueness of the mutation or strain. The Genetic Resources Committee at TJL makes the final decision for each strain, but recommendations are sought from research area experts both inside and outside TJL. The IMR has an Advisory Board and advisory committees that assist in these decisions and individual National Institutes of Health (NIH) institutes (NIAID and NIAMSD as of this writing) have formed committees to assist with strains of particular interest to them. The IMR is a comprehensive genetic resource, offering a broad range of strains, both those in high demand as well as those of importance to only a few laboratories. The majority of strains held by the IMR are not commercially viable, but are critically important in specific research areas.

3.2.3. Importation

All animals are imported into TJL following an established procedure of re-derivation, quarantine, and testing, designed to free incoming mouse strains from any pathogens they might carry. This is done to achieve a high health status for animals of the strain and to avoid endangering resident stocks. The necessity of this procedure is demonstrated by the fact that for the past 3 yr active infection with a pathogen has been demonstrated in 51% of the mice brought to TJL. A large percentage of these infections are attributable to mouse hepatitis virus (MHV).

Animals arriving at TJL are immediately placed in isolators located in a dedicated facility. Breeder animals are mated and their progeny re-derived by hysterectomy derivation or embryo transfer, whereas additional animals of the same strain, if any, are subjected to microbial and viral evaluations. The derived pups are raised by specific pathogen-free foster mothers in a quarantine facility until after weaning when the foster mothers can be tested for acquired specific pathogens. Once negative tests have been obtained, the weaned animals can be released for transfer to an appropriate colony.

The importation process is a costly and time-consuming procedure. A strain that breeds well will usually require 10–12 wk before the first litter can be released from quarantine. A strain that does not breed well may take 6 mo or longer before a litter can be released. Mice cleared from quarantine are used to establish a colony of the strain, which is expanded to a size sufficient to support distribution. Thus, it may require 6 mo–1 yr from the time of arrival of a new strain at TJL before a colony is of sufficient size and adequate health status to permit limited distribution of breeding pairs.

3.2.4. Cryopreservation

Cryopreservation of germplasm has proven to be the best means to insure against loss of valuable strains and efficiently maintain strains when they are not in immediate demand. Frozen germplasm is unaffected by diseases, sudden reproductive failure, certain environmental accidents, and genetic "contamination" that can threaten breeding colonies. There is no evidence to indicate that genetic mutations accumulate during storage of frozen embryos and, likewise, there is no evidence to indicate that the length of time of storage in liquid nitrogen impairs the ability to recover embryos. Mice have been recovered after 25 yr in frozen storage from embryos frozen in 1972, the year in which the first mammalian embryos were successfully frozen ***(28,29)***.

Cryopreservation of mouse sperm is becoming increasingly feasible for many strains ***(30,31)***. Compared to embryo cryopreservation, sperm cryopreservation requires far fewer animals for the preservation of germplasm material and is thus more economical, but is only appropriate when the haploid

genome is sufficient for preservation. Recent advances in intracytoplasmic sperm injection (ICSI) promise to make the cryopreservation of sperm a realistic alternative in that even if damaged by freezing, thawed sperm can be used to produce viable embryos and subsequently live-born pups ***(32)***.

It is even possible now to freeze mouse ovaries ***(33)*** and spermatogonia ***(34)*** to complement embryo and sperm cryopreservation. All methods are available for cryopreserving IMR strains and the methods or combination of methods chosen for any particular strain depends on the nature of the strain and its genetic background. Targeted genes and transgenes in strains with a hybrid or mixed genetic background are normally preserved by freezing sperm, sometimes along with a few embryos, whereas those strains with unique inbred genetic backgounds are generally preserved by freezing embryos.

3.2.5. Strain Development

Many transgenic strains and a majority of targeted mutant strains arrive at the IMR on a mixed genetic background. For targeted mutants the background is most often a mixture of the 129 and C57BL/6 genetic backgrounds. Maintaining a mutation on a mixed genetic background limits its usefulness for genetic analysis since the variability of the genetic background will, in many cases lead to variability in the phenotype, as previously discussed. Mutant alleles are best studied on a defined genetic background when mutant and control mice differ only by the transgene or mutated gene being studied.

The experimental advantage of placing a transgene or mutation onto an inbred background is such that we undertake generating such congenic strains. Congenic strains are created by repeated backcrossing of mice carrying the transgene or targeted mutation from the donor background to mice of the host genetic background. Typically 10–12 generations of backcrossing are carried out, at which point statistically over 99% of the genetic background is from the host strain. The genetic background chosen for most congenic strains is C57BL/6J, but other backgrounds that convey specific experimental advantages may be used.

3.2.6. Maintenance and Distribution

Distributing genetically engineered strains as rapidly as possible to the scientific community is a primary goal of the IMR. The time requirements for the importation process and colony expansion depend on the generation time of the mouse (about 7 wk from conception to weaning and 10 wk from conception to sexual maturity), and on the individual breeding and strain characteristics of each strain. On average, the distribution of a strain begins 6 mo after its arrival in the importation facility. Delays in a strain arriving at TJL are often caused by legal (licensing) negotiations or by delays in the investigator supplying mice for importation.

Distribution of animals from the IMR begins after the mutants have cleared the importation process and the colony has been expanded to a size large enough to support limited distribution of mating pairs without jeopardizing its existence. Mice are distributed in accordance with distribution from other TJL colonies, on a "first-come, first-served" basis. Initially, if requests are received for a large number of mice from one strain, mice are shipped in small groups interspersed with shipments to other researchers to ensure the equitable distribution of these resources.

Many strains in low demand are maintained in small breeding colonies, which cannot provide large numbers of mice for a single order. Although sometimes frustrating for the investigator ordering a strain, it is not financially possible to maintain large colonies of all strains. Colonies are expanded to meet sustained demand and every effort is made to make these animals available as soon as possible.

The majority of our strains are maintained homozygously and mice are supplied in lots of mice that do not require genotyping prior to distribution and can be directly used by the investigator. Approximately 35% of the genetically engineered mutants held by the IMR are homozygous lethals or not viable or fertile and must be supplied as genotyped (tested) heterozygotes. Investigators must then breed mice at their own facility to produce the desired genotype (e.g., homozygous null mice).

3.2.7. Information Dissemination

As more genetically engineered strains are generated, comprehensive database(s) that describe phenotypes are searchable by disease category, list references, and identify holders will become vitally important to researchers seeking models to assist in their research. Currently, the two most broadly based databases are TBASE and the Mouse Knockout and Mutation Database available through BioMedNet (http://biomednet.com). TJL recently assumed responsibility for TBASE, a database for transgenic and targeted mutant animals originally developed by Dr. Richard Woychik ***(35)***. TBASE contains information on, among other items, the correct nomenclature, biology, and literature for transgenic and targeted mutants and covers stocks not available at TJL. The Mouse Knockout and Mutation Database is a subscription database that also lists phenotypic information.

A searchable database is maintained at TJL for strains held by the IMR and is accessible via the World Wide Web (WWW) (http://jaxmice.org). This database provides information on strains available from the IMR, including brief descriptions, how the mutation or transgene was created, genetic background, animal husbandry, genotypes available, genotyping protocols, initial references, and price. The IMR database also links to The Mouse Genome Database (MGD) for descriptive information on genes (http://www.informatics.jax.org).

Written materials are also available both through TJL and the WWW (http://www.jax.org/jaxmice). The JAX® Mice Catalog and the *Handbook on Genetically Standardized JAX Mice* (5th ed.) together provide a complete listing of stocks and strains available, information on each strain type to assist in the selection of mouse models and information on the general care and maintenance of mice. Other publications include a quarterly newsletter, JAX Notes; special newsletters on topics such as mouse models for neural tube defects, cataracts, and neurological mutations; and a number of specialized lists highlighting relevant mouse models for research areas such as obesity, diabetes, cancer, immunology, neurology, and cardiovascular biology.

The Jackson Laboratory provides technical support and in-depth information on mice and their genetics to scientists and users of JAX mice. This includes information on mouse models for studying human disease, genetic mapping, and other scientifically related issues.

3.3. Current Status

The IMR has accepted 689 strains since its inception (as of February 1999). Of these 611 are either being distributed as live animals (450 strains), in the importation facility (45 strains), or maintained as frozen embryos or sperm (112 strains). We are currently funded to accept about 6 new strains/month.

4. Legal Issues/Licensing

The Jackson Laboratory places no restrictions on the subsequent breeding of the mice distributed, with the exception that they or their offspring may not be bred for resale, are to be used solely for research purposes, and may not be transferred outside the recipient's institution. However, every genetic resource or institution distributing live animals must deal with legal considerations concerning the transfer of tangible research materials. Approximately one-third of the strains distributed from the IMR strains have licensing requirements by the originating institution. By far, the large majority of these requirements are directed at for-profit recipients. Strains requiring licensing are identified in all IMR literature, on the Web, and in the printed JAX Mice Catalog. If required, a license is obtained from the institution at which the mutants originated and the appropriate contact person will be provided when ordered.

In addition to negotiations with individual institutions, TJL has negotiated agreements for four patented technologies: the tetracycline-mediated control of gene expression (BASF), FLP-mediated site-specific DNA recombination (The Salk Institute for Biological Studies), the Cre-medited site-specific DNA recombination (the Cre–lox system) (E. I. du Pont de Nemours) and genetically engineered strains that develop tumors (the ''Oncomouse'' patent held by E.I.

du Pont de Nemours). Distribution to researchers in academic and non-profit institutions is not restricted for mice covered by either the tetracycline or FLP patents. All institutions receiving mice that fall under the Cre–lox or Oncomouse patents are required to be licensed by du Pont.

5. Repositories in the Future

Genetically engineered strains of mice have become critical tools both for basic biomedical research and for developing new therapeutic approaches to treat human disease. The full scientific benefit of these strains will only be realized if they are readily available to the scientific community. The current pace of scientific research and the rapid production of new strains would seem to require that hundreds (soon to be thousands) of strains should be maintained as live animals in colonies large enough to support near-immediate distribution to researchers requesting them. In reality, the resources to support such an effort are not available and compromises in the availability of these strains will have to be made. Cryopreservation as an alternative to the maintenance of live breeding colonies of strains with no current demand is one such compromise. Although the recovery of animals from the cryopreserved state is relatively more costly and time consuming than provision from active breeding colonies, the cost to maintain infrequently used strains in active breeding would make the price of animals prohibitively expensive if provided on a cost recovery basis.

Central repositories like the IMR will play a key role in making animals available with standardized health and genetic quality status and in a cost-effective manner (at the present time (February 1999) the European Mouse Mutant Archive is the only other repository for genetically engineered strain of mice). In addition, central repositories are able to provide services and expertise not commonly available in individual or even institutional facilities. Nevertheless, progress in cryopreservation of germplasm technology will soon result in the ability of individual and institutional laboratories to cryopreserve many of their own strains developed locally. In the case of sperm cryopreservation, even if the freezing is not carried out optimally, it should be possible to send preserved sperm to centers where ICSI can be performed to recover live born animals when needed.

The Human and Mouse Genome projects are moving toward increasing our understanding of gene function, gene interactions and other functional regions of the genome. Genetically engineered mice will be vitally important to this understanding and the development of new technologies will certainly fuel the continued exponential production of new strains. The challenge for the future will be to manage the vastly increasing number of resources that will be generated from these endeavors

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25

Embryo Cryopreservation for Transgenic Mouse Lines

Jillian M. Shaw and Magosaburo Kasai

1. Introduction

Embryo cryopreservation can greatly assist in the management of transgenic mice by providing a low cost means of storing mouse lines or strains while they are not needed ***(3,12,13,19,27,31)***. There are several cryopreservation protocols which are very effective for mouse embryos. The cryopreservation protocol described in this paper is a vitrification method which requires no specialised equipment other than a liquid nitrogen tank for long term storage and the plastic straws in which the embryos are cryopreserved ***(8,9)***. It is one of the simplest and most effective protocols for mouse morulae. It only takes 1 min before the embryos can be placed in liquid nitrogen for long term storage. Cryopreserved mouse lines can be reestablished as required by warming the embryos and transferring them to recipient mice (*see* **Note 1**). Transfers can be carried out as little as 10 min after removing the embryos from liquid nitrogen. While the embryos are in storage, they are cheap to maintain (cost of liquid nitrogen), do not display genetic drift, and cannot be lost due to infection or disease ***(12,31)***.

2. Materials

2.1. Mice

2.1.1. Embryos for Storage

We recommend the use of in vivo fertilized embryos at the morula stage for mouse cryobanking, as they are robust and tolerate handling, cryopreservation, and transfer relatively well (for other stages, *see* **Note 2**). Morula stage embryos can be flushed from the oviduct and uterus of the mouse on the 3rd day after mating (d 1 is the day that the copulation plug is found).

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Superovulation can be used to try to increase the number of embryos per donor mouse. A standard protocol is an intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (e.g., Sigma G 4877 or Intervet Folligon) followed 48 h later by an intraperitoneal injection of 5 IU human chorionic gonadotrophin (hCG; e.g., Sigma CG-5 or Intervet Chorulon). After the hCG injection, the females should be placed with fertile male mice. Injections are scheduled so that embryos can be collected 78 h after the hCG injection. For mouse strains which respond poorly or unpredictably to superovulation, natural mating may prove more productive.

2.1.2. Pseudopregnant Recipients for Embryo Transfer

For embryo transfer, recipient mice must be mated to vasectomized males. The females should be d 3 pseudopregnant (plugs on d 1) at the planned time of embryo transfer. The recipients must show evidence of successful pseudopregnancy at the time of surgery (ovaries with large red corpora lutea on d 3).

2.2. Media and Solutions

All solutions should be made up with high-quality water (e.g., Milli-Q water, Millipore Corp.).

1. HEPES-buffered medium M2 for embryo manipulation ***(15)***: Dissolve 5.533 g NaCl, 0.356 g KCl, 0.162 g KH_2PO_4, 0.293 g $MgSO_4 \cdot 7H_2O$, 0.349g $NaHCO_3$, 4.969g HEPES (sodium salt), 4.349 g sodium lactate syrup (60% DL syrup), 0.036 g sodium pyruvate, 1.0 g glucose, 0.06 g penicillin G, 0.05 g streptomycin sulphate, and 0.01 g phenol red (optional) in approximately 700 mL water. Add 0.252 g $CaCl_2$ $2H_2O$ predissolved in 100 mL water. Add 4.00 g bovine serum albumin (BSA). The BSA can take 1 h to dissolve. Do not shake or stir vigorously after adding the BSA, as it froths. Finish by making it up to 1 L with water. The osmolarity should be 280–300 mOsmol, and the pH 7.2–7.4. Filter sterilise through a 0.2-μm filter. This sterile M2 can be kept refrigerated at +4 to +6°C for 2 wk or kept frozen at –20°C for several months (*see* **Notes 3** and **4**). If necessary the BSA can be omitted.
2. Cryopreservation solution EFS40: This is M2 containing 40% v/v ethylene glycol, 18% w/v Ficoll 70, and 0.3 *M* sucrose. First, prepare FS solution, which is M2 containing 30% w/v Ficoll and 0.5 *M* sucrose (**Notes 3–5**). To prepare the FS solution, add 35.1 mL of M2 (omitting the BSA) and 15.0 g Ficoll 70 (MW ~70,000; Sigma F 2878), to a 200-mL flask. Leave it for a few hours until Ficoll dissolves thoroughly. Add 8.56 g sucrose and dissolve it thoroughly. Then add 105 mg BSA and dissolve. To prepare the EFS40, add 4 mL ethylene glycol and 6 mL FS solution together in a 10-mL test tube with a tight stopper (*see* **Note 5**). Use two 1-mL syringes (with an 18-gauge needle) to measure solution volumes, as the viscosity of the liquids will make accurate pipetting difficult. Mix completely by repeated tilting. The 10-mL EFS40 can be filtered through a

0.45-μm filter; gently warming the solution makes the solution less viscous and easier to filter. The EFS solution can be stored at –20°C for at least 1 mo. If it is made up correctly, the EFS40 should remain liquid (not freeze) at –20°C.
3. Dilution solution: M2 with 0.5 *M* sucrose. Weigh out 1.71 g sucrose in a graduated tube and make it up to 10 mL line with M2 buffer. Filter through a 0.2-μm filter.
4. Anesthetic solution: 0.5 mL Ketamine (100 mg/mL ketamine hydrochloride), 0.5 mL Rompun (20 mg/mL xylazine hydrochloride, Bayer AG) and 9 mL PBS. Filter sterilize through a 0.2-μm filter.
5. Reversing agent for anesthetic: Reverzine 0.5 mL (1.25 mg/mL yohimbine hydrochloride, 2 mg/mL 4-aminopyridine) and 2 mL PBS. Filter sterilize through a 0.2-μm filter.
6. 70% Alcohol.

2.3. General Equipment

2.3.1. For Embryology and Embryo Transfer

A stereo dissecting microscope with transmitted light (light from underneath) and good-quality lenses is most suitable for work with embryos. A zoom microscope is best, as relatively high magnification is needed to accurately evaluate embryo quality/embryo damage after warming (e.g., 40-fold magnification and 20× eyepieces). If the dissecting microscope is also to be used for embryo transfers, one with a long working distance is best (e.g., 6–10 cm at the lowest magnification).

1. *Light source:* Good lighting is needed for embryo transfers. Although any type of lamp can be used, it is best to have a fiberoptic light source, as the light it provides is cold and does not dry out the operation site. A fiberoptic unit with two or more flexible arms is ideal.
2. *Minor equipment for embryology:* (a) One milliliter syringes and hypodermic needles (26 and 30 gage); (b) glass Pasteur pipets for handling embryos can be finely drawn over a flame to give an opening only slightly larger than the embryos (**Fig. 1.**) (*see* **Notes 6–8**); (c) a rubber bulb or mouth pipet, to which the glass pipet is attached to collect and move embryos; (d) Petri dishes and/or watch glasses for embryo collection and culture; (e) a warm plate at 37°C.
3. *Minor equipment for embryo transfer:* Metal clips e.g., 7.5 mm "Michel" clips (cat. no. 1400 Medicon, Tuttlingen, Germany) or 9 mm "Autoclip" (MikRon, Becton Dickinson MD), to close the skin wound. Clip applicator. Two pairs of very fine no. 5 forceps (e.g., Sigma T 4662 or T 4537). Two pairs of fine sharp scissors. Needles and syringes for anaesthetic. Warming tray/plate at 37°C.

2.3.2. For Cryopreservation/Storage (see ***Notes 9–19***)

To store embryos efficiently for long periods of time, the embryos must not be warmed above –140°C at any time during storage. Embryos are most commonly stored in liquid nitrogen (–196°C) or in liquid nitrogen vapor.

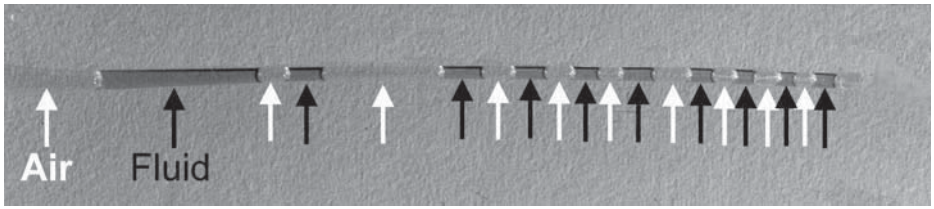

Fig. 1. Embryos should be injected into the cryoprotectant with as little fluid accompanying them as possible.

Ultralow electrically powered freezers (those with liquid nitrogen backup are preferable) that hold a temperature of –140°C or below can also be used (*see* **Notes 9–14** on the safety issues associated with the use of liquid nitrogen). If you have to purchase a storage container, **Notes 15–19** may be of assistance.

1. *Liquid nitrogen containers for benchtop work:* Small, 1 L, wide-necked containers for liquid nitrogen are used for benchtop work and for transporting samples short distances, such as between the bench and the storage tank. Use stainless steel dewars, or other insulated containers (e.g., thick-walled styrofoam boxes), not evacuated glass-walled flasks (*see* **Note 14**). Containers which can accommodate 133 mm straws horizontally are ideal.
2. *Plastic straws:* Embryos are most commonly cryopreserved in 0.25 mL plastic insemination straws (e.g., IMV, AA 201 L'Aigle, France). These straws are specifically made for cryopreservation and can be obtained from suppliers of veterinary products or products for artificial breeding of domestic animals (*see* **Note 14**). Although straws are available in a range of colors, clear straws have the advantage in that they allow you to see the pipet and cryoprotectant solution during loading. We use straws as supplied by the manufacturer. Sterilization of plastic straws by irradiation is not recommended, as it can have detrimental effects on the freezing properties of some types of straws ***(20)***. Straws may, if required, be sterilised using ethylene oxide, or purchased presterilized from the manufacturer.
3. *Heat sealer:* Heat sealers, such as those used to seal plastic bags or autoclave bags, can be used to seal plastic insemination straws. If such equipment is not available, the straws can be sealed with a hydrophilic powder, e.g., polyvinyl alcohol (PVA) (cold-water soluble Sigma P-8136), or polyvinylpyrrolidone (PVP) (Sigma PVP-40).
4. *Storage goblets and canes:* Straws are commonly placed in small (e.g., 16 mm wide) plastic goblets and clipped onto canes (Nunc) before being placed in storage.
5. *Water bath:* Stirred water baths with accurate temperature control can be obtained from scientific suppliers. Alternatively, any container filled with tap water at the correct temperature is adequate as long as it is big enough to fit a straw.
6. *Minor equipment used for most freezing protocols:* (a) Water-resistant marker pens with fine tips. These are used to label dishes, straws, and tubes. Do not use

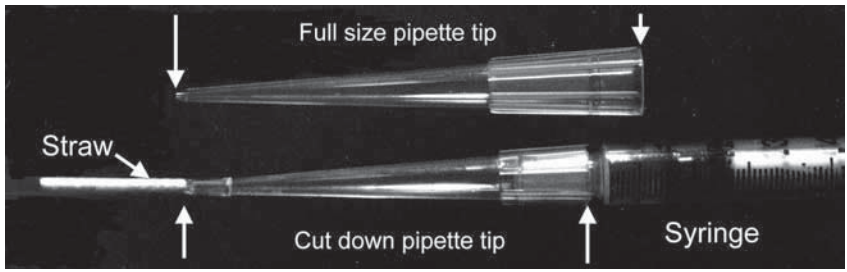

Fig. 2. To increase control over the flow of solutions and embryos in a mouth pipet, load the pipet with alternating air (white arrows) and fluid (dark arrows) as this reduces the effect of capillary action (pipet opening on the right).

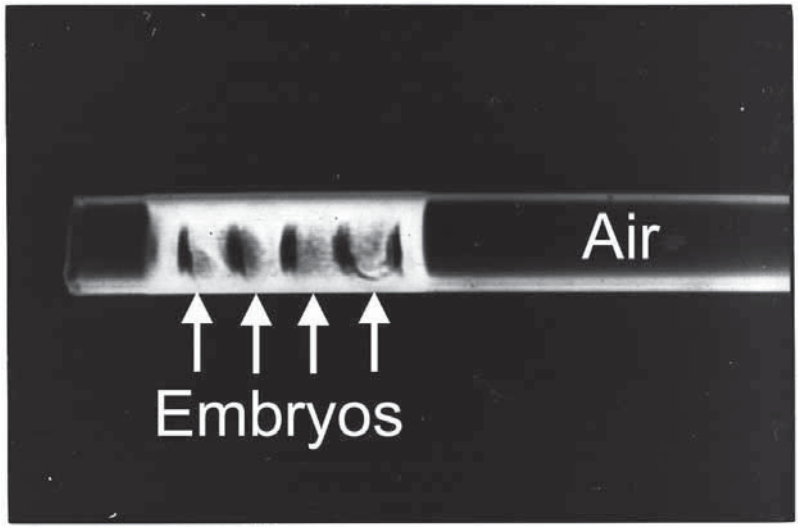

Fig. 3. A yellow tip which has been trimmed and pushed onto a 1 mL syringe can be used to load or expel solution(s) from the straws. The unit must be airtight.

pens with erasable or water soluble ink. (b) Equipment for handling embryos (glass pipet and rubber bulb or mouthpiece, **Fig. 2**). (c) Sharp scissors. They are needed to give a clean cut when removing the end of straws. (d) A syringe (0.5–1 mL) with pipet tip or flexible tubing to connect it to the straw (the join must be airtight, *see* **Fig. 3**). This is needed to move the solutions in and out of the straws. (e) Accurate timers; those with a count-down alarm are ideal. (f) A styrofoam (polystyrene) boat to hold the straws in the liquid nitrogen vapor. **(g)** Gloves and goggles to prevent frost bite when handling liquid nitrogen. (h) Large artery forceps or the equivalent are useful to manipulate straws and tubes within liquid nitrogen and nitrogen vapor.

(i) Tongs with very long handles or blades to hold cold items. (j) A flashlight is helpful for searching for straws or goblets lost in the liquid nitrogen.

3. Methods

3.1. Embryo Collection and Cryopreservation (see Notes 12–32)

1. Set up naturally cycling or superovulated mice to generate the embryos. As embryos are collected when the mice are d 3 pregnant, the mice should have had a mating plug on the morning of d 1.
2. Before killing the mice, prepare all the equipment and solutions needed for embryo collection and cryopreservation. If more than one strain is to be collected or cryopreserved, then fresh pipets, dishes, and solutions must be used for each strain to prevent mixups.
3. Prepare the following for embryo collection. Sharp scissors and fine forceps to remove the reproductive tract from the mouse, Petri dishes (35 mm) with 2–3 mL buffer (M2) to place the tract in, and fine-drawn pipet or a syringe with a blunted 30-gage needle and buffer (M2) to flush the embryos from reproductive tract.
4. Prepare the following for cryopreservation: A wide dewar or thick-walled polystyrene box with liquid nitrogen for the benchtop work. This should be precooled with liquid nitrogen at least 30 min before starting, and must contain at least 5 cm of liquid nitrogen at the time the freezing starts. The solutions for cryoprotectant should be made up or brought out of the freezer to warm up to room temperature. Straws can be labeled and loaded with cryoprotectant at this time or once all the embryos are ready to be cryopreserved (**steps 7** and **8**, below). There should be one or more timers set for 1 min and a preheated heat sealer (or equivalent as discussed).
5. Once all the preparations are complete, kill the mated mice by cervical dislocation or by a method acceptable to the local governing ethics committees.
6. Place the oviducts and uterine horns in the dish with M2 buffer. Use a syringe with a 30-gage needle to push buffer through the tract. This should flush the morula into the dish. Collect the embryos in the M2 under a dissecting microscope and then wash them to remove debris (e.g., two to three changes of buffer). Pool the washed embryos in fresh M2. Select embryos with normal morphology for cryopreservation.
7. Label the straws with a fine-tipped indelible marker pen with the necessary information (date, strain number per straw, and so on). Do not use pens with water-soluble ink, as it will wash off during warming.
8. Load the straw by connecting a 1-mL syringe, in which the plunger is fully depressed, to the straw **(Fig. 3)**. The simplest loading pattern is to draw up 30 μL EFS40 solution (a 15-mm-long column), and a small amount of air (e.g., a 5-mm-long space) into the straw. This leaves a small air space between the opening and the EFS **(Fig. 4)**. More proficient users can use the more complicated loading patterns to help expel the embryos after warming (*see* **Note 20**). These contain 65 mm of 0.5 *M* sucrose solution (approx 125 μL); 15 mm air; 3 mm EFS40; 4 mm air; 13 mm EFS40. The dilution solution should enter the powder in the

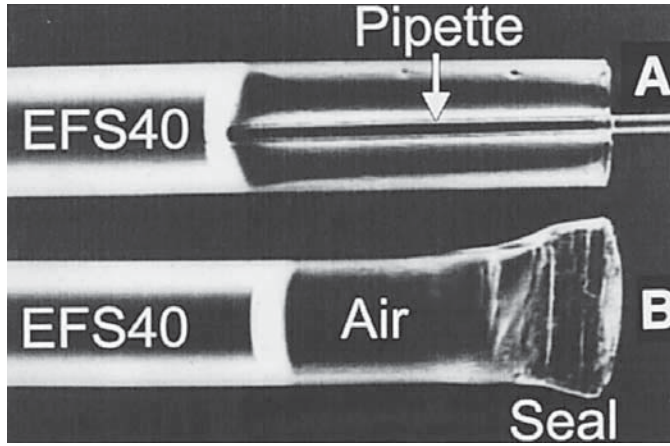

Fig. 4. There should be a small air space between the straw opening and the EFS40 **(A)**. This helps prevent embryo losses and damage while the straw is sealed **(B)**.

middle of the cotton plug at the top of the straw. This makes the powder swell and seals the top end of the straw. The air column in the open end will be about 10–18 mm. Place loaded straws horizontally. Straws are easier to pick up if they are placed so that the cotton plug just overhangs the edge of the bench.

9. Aspirate small groups of embryos (e.g., 8–12) into a finely drawn glass pipet with a minimal volume of accompanying buffer (*see* **Notes 20** and **21** and **Fig. 1**). Ideally, a pipet only marginally wider than the embryos should be used, and the embryos loaded as closely packed as possible **(Fig. 3)**. This is to minimize the amount of buffer injected with the embryos into the cryoprotectant solution. If too much buffer is injected with the embryos, the cryoprotectant's capacity to protect the embryos from injury will be compromised. Restriction bubbles **(Fig. 1)** help control the flow of fluid and embryos in the pipet.
10. The straw should be placed/held horizontally at all times. Do not touch the region of the straw containing the EFS40 (so that the solution stays at room temperature).
11. Insert the tip of the pipet deeply into the cryoprotectant in the straw (**Figs. 4** and **5**). Gently, but rapidly, expel the embryos into the EFS40 as the pipet is smoothly withdrawn, and then start the timer (1 min). It is useful to have an air bubble immediately behind the embryos to act as a marker/plunger. Try to get the embryos uniformly spaced through the cryoprotectant column (*see* **Notes 21** and **22**).
12. Seal the straw firmly at both ends (**Figs. 4** and **6**). If a heat sealer is used, the temperature of the sealer should be approximately 95°C. If straws are to be sealed with PVA or PVP powder, an extra-small (e.g., 5 mm long) bead of cryoprotectant needs to be present at the open end of the straw. The straw is then pushed into the powder to form a firm plug inside the end. The small extra bead of cryoprotectant should cause the powder to swell slightly and form a tight plug (*see* **Note 23**).

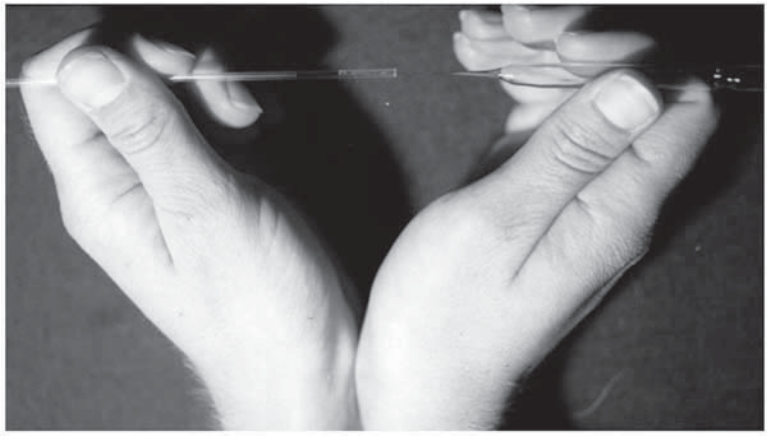

Fig. 5. Some users find it easier to load straws when they press their wrists/heels of their hands together while inserting the pipet into the straw. The figure depicts a right-handed worker; left-handed workers may prefer to hold the pipet in their left hand.

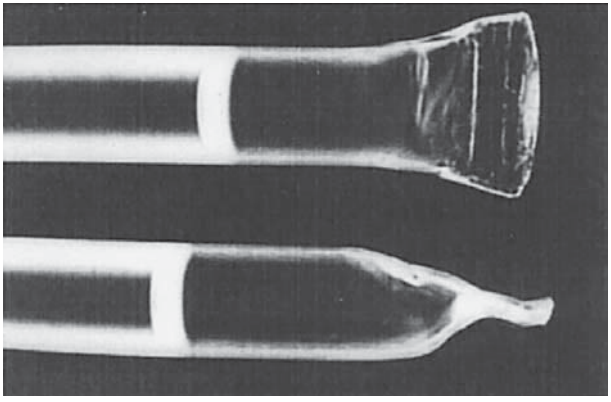

Fig. 6. Example of a straw sealed with a autoclave-bag sealer (top and side view).

13. Following a total exposure period of 1 min (*see* **Note 24**) to the EFS40 solution, place the straw on the styrofoam boat (in the vapor phase of the dewar) to cool (*see* **Note 25** and **Fig. 7**). The straw will cool more uniformLy if it does not rest directly on the foam **(Fig. 7)**. Once the straws have cooled in the vapor phase for 3–5 min, they can be moved into the liquid nitrogen. Straws can be plunged directly into the liquid nitrogen, but the proportion of intact embryos tends to be slightly lower, and if the diluent is included in the straw they will tend to crack open. If you have to cool the straw directly in liquid nitrogen, immerse the straw very slowly into the liquid.

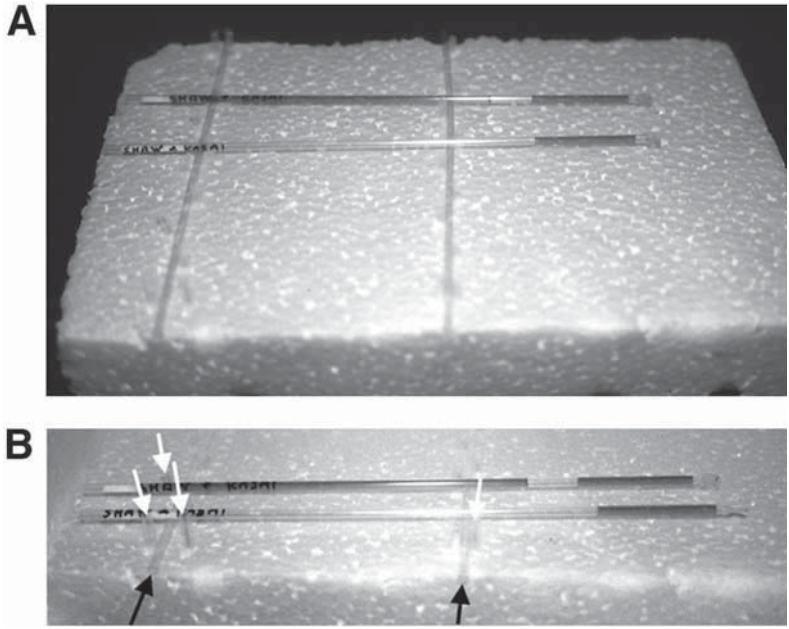

Fig. 7. **(A)** A polystyrene boat, floating on the surface of the liquid nitrogen, is used to support straws in the vapor phase during the cooling and warming steps. **(B)** Detail showing how pieces of straws, inserted at 1 cm intervals (white arrows), serve as dividers, preventing the straws from falling over the edge or coming into contact with each other. Two other empty straws (black arrows) are placed across the polystyrene float to hold the straws above the surface of the polystyrene.

14. For storage, label goblet(s) and cane(s) with an indelible marker. Each should record the date, strain, and, if possible, a unique label/number, which can be cross-referenced to paper/computer records.
15. Place the labeled goblet(s), cane(s), and forceps in the liquid nitrogen to cool. Use these precooled forceps for all handling steps.
16. Using the cold forceps, rapidly but gently move the straws from the liquid nitrogen into the goblets. Do not bend the straw, and keep the portion of the straw containing the embryos as cool as possible at all times. If the straw is brought above the lip of the dewar, the temperature may rise by as much as 100°C in 5 s and may compromise the viability of the embryos. If the goblet is to be stored below the surface of liquid nitrogen, the straws should be wedged tightly into the goblet with, a wedge of tissue paper, e.g. (*see* **Note 26** and **Fig. 8**).
17. Move the cane with attached goblet(s) to the storage tank, and complete your records (e.g., cards/books/sheets/ computer database).
18. During storage, ensure that the liquid nitrogen level in the storage tanks are maintained.

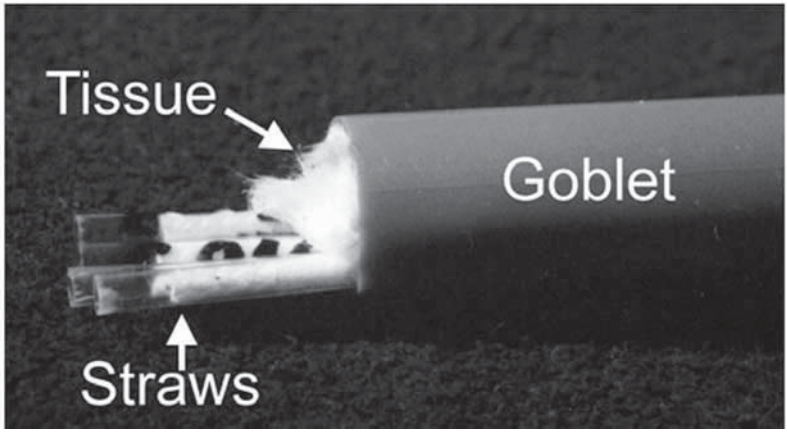

Fig. 8. Wedge the straws in place with a piece of tissue paper or cotton wool.

3.2. Warming

1. Before starting to warm straws (*see* **Note 1**), prepare all the equipment and solutions that will be needed: A dewar/thick-walled polystyrene box should be precooled with liquid nitrogen at least 30 min before starting and should contain at least 5 cm of liquid nitrogen at the time the warming starts. A polystyrene boat should be placed on the surface of the liquid nitrogen (*see* **Note 25**). Set up a waterbath at 20°C. Label three 35 mm Petri dishes for each straw that is to be warmed; the first should contain 3 mL dilution solution (0.5 *M* sucrose in M2), and the other two should contain 3 mL of M2. Other items include: two or more timers, sharp scissors, a 1-mL syringe attached to a pipet tip/tubing to expel the cryoprotectant from the straw, a dissecting stereo microscope, and pipets to move embryos.
2. Check that you have all equipment, solutions, and animals needed for transfer if embryos are to be transferred (*see* **Note 2**).
3. Move straws from the storage tank to the dewar containing liquid nitrogen. Handle with care, as liquid nitrogen may be trapped inside the straw (*see* **Note 13**). If nitrogen is trapped in a straw, keep it at a low subzero temperature (deep in the nitrogen vapor just above the level of the liquid nitrogen) until all liquid nitrogen has evaporated from inside. This minimizes the risk of the straw exploding. Do not touch cold straws with your fingers; use long tongs or forceps.
4. Start the warming procedure by placing the straws on the polystyrene support (*see* **Note 25**) floating on the surface of the liquid nitrogen (*see* **Note 27**).
5. Leave the straw in the vapor phase, on the support, for 3–5 min.
6. Rapidly, but gently, lift the straw from the support into room temperature air **(Fig. 9)**.
7. Hold the straw in air for 5–10 s (hold them steady), and then immerse the straw fully into 20°C water (*see* **Note 27**).

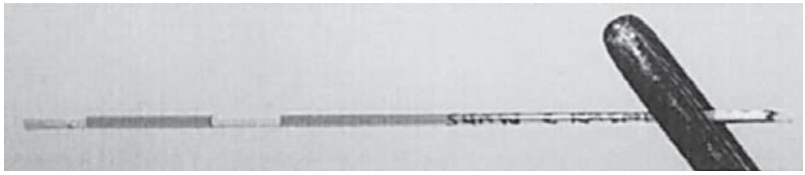

Fig. 9. To thaw straws, grab them near the plug with large forceps (or equivalent) and hold them horizontally in air for 10 s.

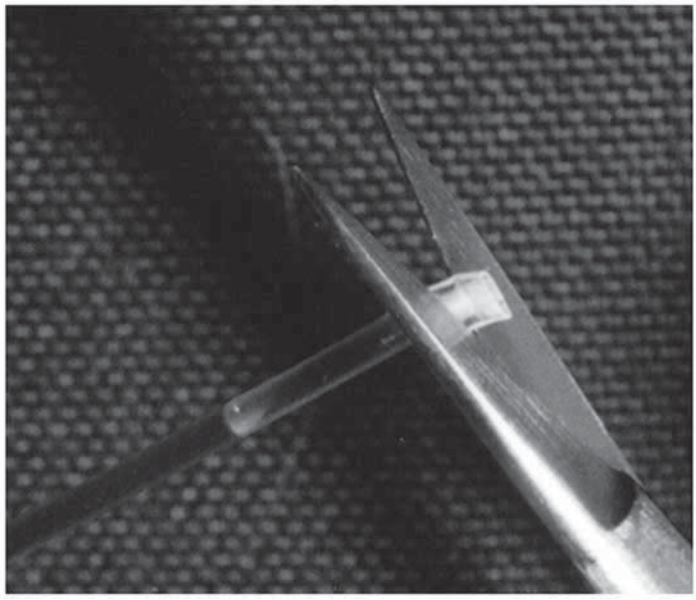

Fig. 10. Cut off the seal with sharp scissors.

8. Remove the straw from the waterbath as soon as the solution(s) have liquefied (approx 5 s).
9. Hold the straw firmly, but be careful to not warm the EFS column with your fingers, then quickly wipe the straw dry and remove the seals from both ends with sharp scissors **(Fig. 10)**. The straws must be held horizontally to reduce the likelihood of accidental embryo losses as the seals are removed. Remove the upper seal first, cut it off immediately below the plug (*see* **Note 28**). It is safest to hold the straw opening over the dilution solution while removing the lower seal (the solution should not be allowed to drip out). Do not bend or flex the straw while the seals are being removed. A bent straw will recoil (flick) as soon as the end is removed, and this tends to uncontrollably catapult the solutions and embryos out of the straw.

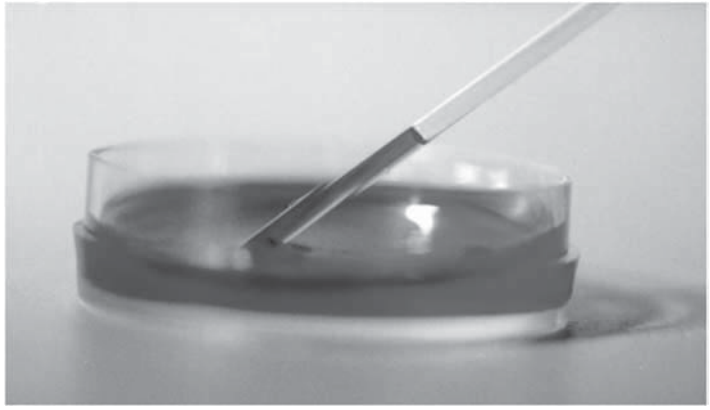

Fig. 11. Place the straw into the dish containing the dilution solution and slowly expel the straw contents. The straw should be touching the bottom of the dish.

10. Once the seals have been removed, very gently insert the tip of the syringe (with the plunger pulled out) into the straw opening which is furthest away from the EFS solution. Tilt the straw slightly (EFS end down) and lower the straw opening until it is touching the bottom of the dish containing the 0.5-*M* sucrose solution **(Fig. 11)**.
11. Gently push the syringe plunger to expel all the contents of the straw into the dish. Start the timer. Occasionally, embryos remain stuck to the sides of the straw, to prevent these from dehydrating, very gently, aspirate some solution back into the straw (from a region of the dish devoid of embryos), then leave the straw lying horizontally (until **step 13**).
12. Dishes must be handled gently to prevent excessively rapid mixing of the EFS and the diluent.
13. Gently shake the dish after 3 min, to thoroughly mix the diluent and cryoprotectant solution. Assemble the embryos to count them and assess their appearance. If all the embryos are not recovered, decant the residual sucrose solution remaining in the straw into the culture dish and examine for the lost embryos.
14. After a total of 5 min in the first dilution solution, assemble the embryos with a pipet and transfer them to the M2 buffer.
15. After 5 min in M2, wash the embryos in two more changes of fresh M2 buffer. Reassess the appearance of the embryos and select embryos for transfer or for further in vitro culture (*see* **Notes 29** and **30**).

3.3. Embryo Transfer

Mature female recipient mice should be 3 d pseudopregnant (mated by a vasectomized male 3 d earlier) at the planned time of warming/embryo transfer.

1. Anaesthetize a mature female recipient with an injection of anesthetic into the peritoneal cavity (e.g., 0.35 mL/25 g mouse). The mouse should not be operated

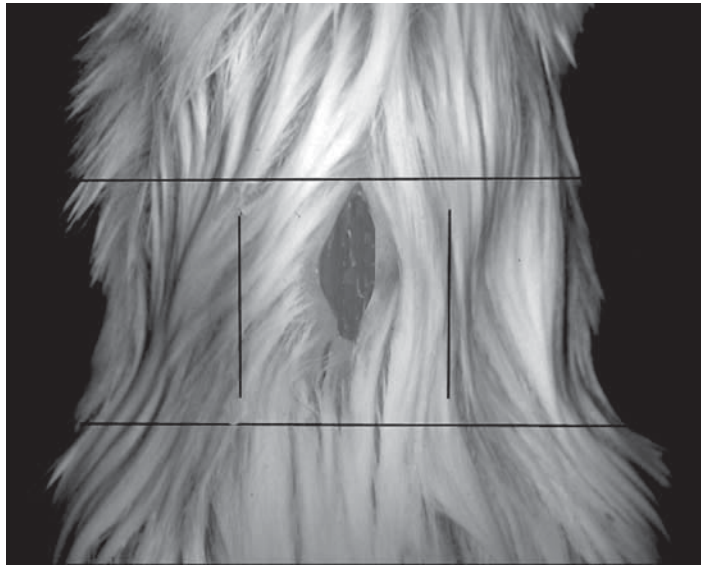

Fig. 12. Surgical embryo transfers are made through a middorsal approach. The 1 to 1.5 cm skin incision should start from a line level with the top of the hips to a line level with the bottom of the ribs.

on until after it ceases to respond to a toe pinch. While the mouse is under anesthetic, it should be monitored regularly. If at any time the breathing becomes very slow or irregular, the anesthetic may be too deep; stop the operation and try to restore the body temperature and breathing rate before surgery resumes. If the mouse starts to move before the operation is over, it should be given more anesthetic (e.g., 0.05 mL). Take care not to give an overdose of anesthetic.

2. Swab the back of the recipient with 70% alcohol, shave or clip the fur at the operation site, and then reswab the area with alcohol to remove loose fur.
3. With a pair of sharp scissors, cut a single 1–5-cm middorsal incision through the skin running from a line level with the top of the hips to a line level with the bottom of the ribs **(Fig. 12)**.
4. Pull the skin sideways over the flank until the abdominal wall can be seen. It should be possible to see the mouse ovary just inside the abdominal wall.
5. Make a 1-cm cut through the abdominal wall, parallel to the backbone. To minimize the risk that the intestines are cut during this step, it is safest to start by getting a pinch (small amount) of the abdominal wall and lifting the wall up as far as possible. Then, make a very small hole immediately under your forceps to let air into the abdominal cavity. As soon as the air enters, the intestines should slip down, away from the abdominal wall.
6. Locate the ovarian fat pad through the hole. Using blunt forceps, gently grasp the fat pad. Exteriorize the upper portion of the uterine horn with the fat pad through

the incision. Stop the reproductive tract from falling back into the abdomen (e.g., by clipping or pinning the fatpad to the skin or by enveloping it with dry, sterile filter paper).

7. Make sure that the ovary has well-developed (red rounded) corpora lutea (CL). If there are no CL on the first ovary, do not insert any embryos until you have extracted the opposite ovary to determine whether it has CL. If neither ovary has CL, or the CL are at an inappropriate stage (small or not red), do not perform the transfer.
8. Aspirate the number of embryos that you intend to transfer to the first uterine horn into a finely drawn but firm (not too long or too flexible) glass pipet (*see* **Note 8**). Try to limit the amount of M2 accompanying the embryos in the pipet.
9. Using fine forceps, gently grab the uterus approximately 0.5 cm from the oviduct.
10. Without letting go, pierce the uterus very close to the forceps with a fine needle (e.g. 26–30 gage). The needle should pass through to the lumen of the tract and then be withdrawn. This is easiest to perform under a stereo-dissecting microscope.
11. Insert the pipet with the embryos through the hole made by the needle. Expel the embryos once the pipet has been inserted into the lumen, then withdraw the pipet. It is useful to have an air bubble behind the embryos to act as a marker/plunger.
12. Release the fat pad (remove the clip/pin/paper), then lower the tract very gently into the abdominal cavity.
13. Repeat the procedure for the opposite side. The same middorsal skin incision can be used, but a new incision needs to be made in the abdominal wall on the opposite side of the mouse.
14. Close the skin wound with firmly applied clips (usually three Michel or two autoclip clips **[Fig. 13]**). To ensure that the skin on both sides of the wound are clipped together, pull the sides of the wound together by taking a large pinch of skin above and below the wound (i.e., the skin along the backbone at the level of the hips and the ribs). This should stretch the skin on either side of the wound and bring the skin on both sides together **(Fig. 13)**. Once the skin on both sides of the wound is aligned, the clip can be applied. The two skin edges should be together near the top (highest point) of the clip. Properly applied clips do not fall off.
15. Keep the mice warm until they recover from the anesthetic by having their box on a warming plate (check the temperature regularly). If Rompun ketamine has been used, they can be given reverzine (into the tail vein) to speed their recovery. Administration of antibiotics should not be necessary.
16. Monitor the recipients daily to ensure that the wounds heal.
17. If the recipients become pregnant, young will be born on d 19–21 of pregnancy (*see* **Notes 30–32**).

4. Notes

1. In this chapter we use the term “warming”, not “thawing”. Solutions like EFS40 are stiff (vitrified) at low temperatures and soften when they are warmed, but they do not change state (from a *crystalline* state to a *liquid* state) during warming. The term “thawing” should be restricted to solutions that change state during warming; this includes all solutions which contain ice **(Fig. 14)**.

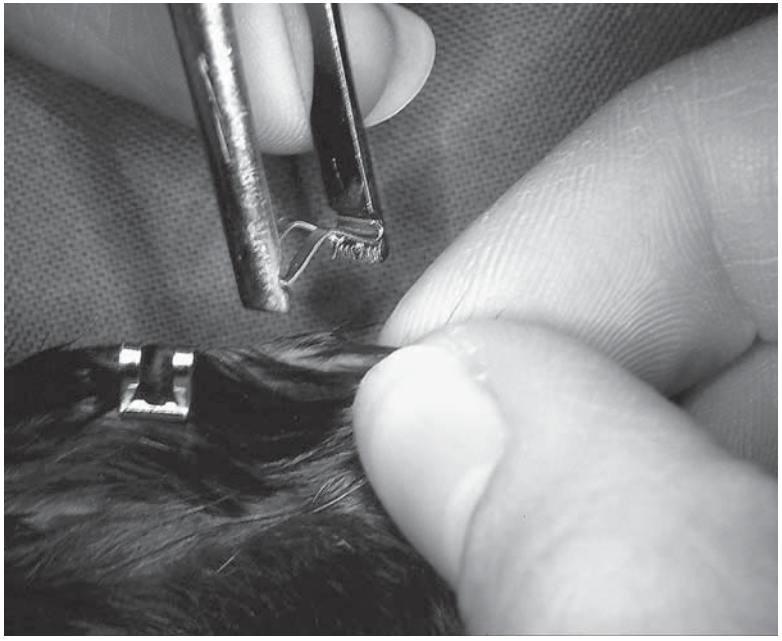

Fig. 13. The skin can be closed with clips (these are Becton & Dickinson 9 mm autoclips).

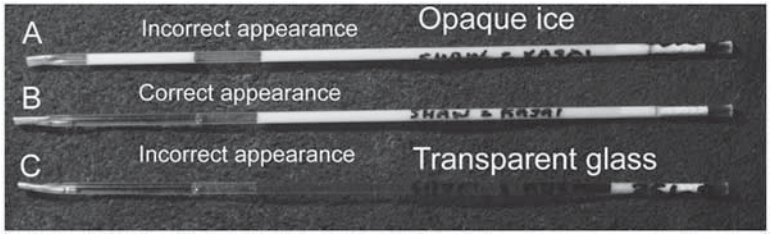

Fig. 14. The EFS40 solution should remain in a clear, transparent glass throughout the cooling and warming steps. The dilution solution should turn milky (with ice). If the EFS turns milky **(A)** or the diluent solutions remains transparent **(C)**, the solutions or cooling or warming rates are incorrect.

2. Mouse embryos at stages other than the morula can be cryopreserved by the protocol described here. Excellent results have been achieved with 8-cell embryos. The survival for other stages may be lower ***(11,32)***. For earlier stages of embryos, e.g., 2-cell embryos, a two-step treatment at 25°C improves the postwarming survival. First, suspend the embryos in EFS20 (which is a mixture of 2 mL ethylene glycol, 2.4 g Ficoll, and 1.37 g sucrose made up to 10 mL) for

2 min to promote prepermeation of ethylene glycol. Then load the embryos into the EFS40 solution in the straw, and cool in liquid nitrogen vapor after 1 min of exposure to EFS40 ***(11)***. Later stages of embryos, e.g., blastocysts with a large blastocoel, should also be treated in two steps at 25°C before cooling ***(32)***: Initially, suspend the embryos in 10% (v/v) ethylene glycol in PB1 medium at 25°C for 5 min for permeation of ethylene glycol into the blastocoel, then load them into the EFS40 in the straw with a minimal volume of the solution, and cool in liquid nitrogen vapor after 30-s exposure to EFS40 ***(32)***. Unfortunately, the protocol using EFS40 is unsuitable for unfertilized mouse oocytes ***(11)***. If embryos at the 1- to 4-cell stage are to be transferred, they must be transferred to the oviduct of d 1 recipients (d 1 oviducts even accept embryos at the morula stage).

3. Originally, EFS40 was composed with a modified phosphate-buffered saline ***(8)***. Although these give excellent results, phosphate-buffered solutions, unlike M2, tend to precipitate if they are frozen. The M2 described in this chapter can be replaced by any other HEPES -buffered media which is suitable for embryo handling on the bench at room temperature. Buffers may be made or purchased.
4. The M2 buffer described in this chapter contains 4 mg/mL BSA; this can be replaced by 1–10% serum. These additives or other polymers, such as Ficoll, Dextran, or polyvinyl alcohol (1%), help by preventing the embryos from sticking to dishes and pipets. If embryos are to be exported, polymers may have to replace BSA/serum in all solutions. One problem with BSA is that there are differences between types of BSA, suppliers, and batches.
5. This is an alternative method for making the cryoprotectant solution EFS40: Add 4 mL ethylene glycol (Sigma E 9129), to 1.8 g Ficoll (70,000 MW, Sigma F 2878), in a 10-mL graduated tube with a tight lid. Let this mixture stand until the Ficoll has dissolved (this may take 1 h or more). Add 1.026 g sucrose (Sigma S 9378) and finally add M2 buffer to make a total volume of 10 mL. Although protein or serum can be included in the EFS40, protein and serum-free EFS40 has the advantage of being wholly defined, which avoids or reduces any potential problems when exporting embryos to other countries. For a small volume of sample, one-tenth of the FS solution can be made in a 10-mL test tube with a tight stopper.
6. Pipets are usually made by holding a glass Pasteur pipet over a flame, rotating the pipet until the glass is softened all round, and then pulling the two ends of the pipet apart outside the flame. The temperature of the glass, the time delay between removing the pipet from the flames, and the speed at which the pipet is pulled apart will dictate the outcome.
7. To control the movement of fluid in and out of the straw, do not have a single column of air or fluid; instead load the pipet with alternating air/fluid/air/fluid, e.g., **(Fig. 2)**. Each alternating bubble slows the flow of fluid in and out of the pipet. If the number of bubbles is judged accurately, the fluid in the pipet will not move on its own but only in response to gentle aspiration/ inspiration. If there are too many alternating bubbles, the fluid will be almost impossible to move.
8. Pipets for a uterine transfer should be relatively robust (thick-walled) and do not need to be very long. Once the pipet is pulled, it should be broken off at a point

where the inner diameter is slightly larger than the embryos. If you have problems with the edges catching on the uterine lining, one way of solving this is to make a larger-bore pipet. Then bring the pipet opening towards the flame to cause the edges to round up and the opening to shrink. It is important to remove the pipet before the opening becomes too small for the embryos.

9. Nitrogen at high concentrations can displace oxygen and cause asphyxia. Rooms in which liquid nitrogen is to be used or in which tanks are to be kept must be well ventilated.
10. No liquid nitrogen should accompany embryos which are to be transported by car or plane. Specialized vapor shippers which are precooled with liquid nitrogen but are emptied before the embryos are placed in them are highly effective. Most brands stay below –190°C for 2–3 wk.
11. Contact with liquid nitrogen or any conductive material (e.g., metal) which is/has been in contact with liquid nitrogen can cause severe frost-bite. Eye and hand protection should be worn. If liquid nitrogen saturates a person's clothing/shoes, these items should be removed as quickly as possible, or rapidly doused with a large amount of water.
12. Liquid nitrogen behaves like water and can splash and spill. Spills will cause most floor coverings to crack, and splashes can cause personal injury.
13. Any situation in which liquid nitrogen is trapped within an enclosed space can lead to an explosion. To reduce the risk of serious injury, (a) seal straws correctly to prevent liquid nitrogen getting in and (b) if liquid nitrogen does get into a container, hold it in the vapor phase until the trapped gas has evaporated.
14. Use only materials designed for use with liquid nitrogen. Eppendorf tubes and silvered glass liners (thermos flasks) must be avoided. Although such evacuated glass flasks appear to be highly suitable as they hold their temperature well, they are dangerous because at –196°C they are very fragile and can (and do) explode without warning, causing glass fragments to shoot in all directions. A thick walled polystyrene box is suitable for the cooling step (**Fig. 15**).
15. Liquid nitrogen evaporates from storage tanks at a rate determined by the efficiency of the storage tank. When purchasing a tank for liquid nitrogen, the required storage capacity should be considered as well as its weight (when full), the neck width, insulation properties, and whether the materials are going to be stored in goblets or on canes. Wide-necked tanks are more convenient as storage containers, but tend to allow faster evaporation of the liquid nitrogen. The insulation properties of a storage tank can decline very markedly with age with the result that old or second-hand tanks may be uneconomical.
16. The volume of liquid nitrogen needed will depend on the freezing procedure chosen, the type of biological freezer, the size and specifications of the storage tanks, and the frequency of use of the equipment. It is usual for large laboratories to use 20–80 L each week.
17. At the present time, embryos are most commonly stored in the liquid phase of liquid nitrogen. If there is any risk that the samples could contain infectious agents, such as viruses, they should be stored in the vapor phase of liquid nitro-

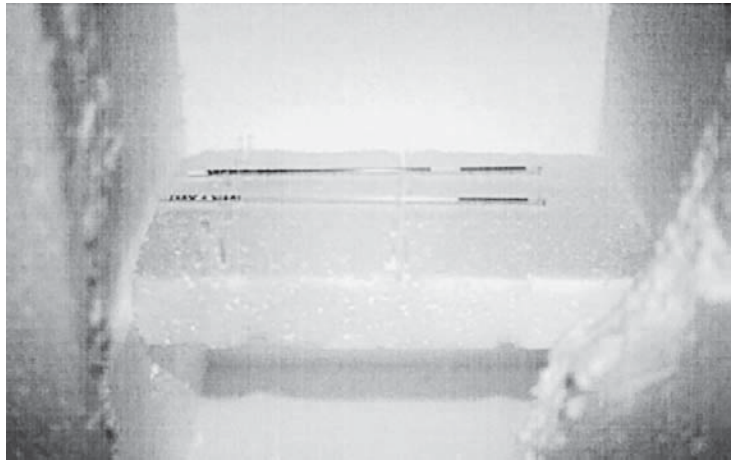

Fig. 15. Photo of a polystyrene box (with one side removed), big enough to accommodate a polystyrene boat with straws lying horizontally. The boat should float freely on the surface of the liquid nitrogen.

gen or in a very low temperature freezer (–140 to –150°C). The reason for this is that viruses can be spread by the liquid nitrogen between samples within a tank, and there are documented cases of bags for blood transfusion acquiring hepatitis by this route ***(29)***. Liquid nitrogen tanks with only a small amount of liquid nitrogen at the bottom can serve as vapor-phase storage tanks, but the temperature at the top of the tank needs to be monitored to ensure that it does not rise too far above –150°C. The installation of copper tubes, rods, or sheets reaching from the liquid nitrogen to the top of the specimens may be necessary if the temperature gradient from the surface of the liquid nitrogen to the top of the tank is too great.

18. A spare tank can be useful when searching for lost straws or goblets. The spare tank enables the canisters with cryopreserved materials to be placed in the backup tank while the storage tank is thoroughly searched (or emptied).
19. It is advisable to install alarms in all tanks and freezers containing valuable materials. If the temperature rises above a set level, these should give out an audible alarm or ideally be connected so that security or the person(s) in charge of the laboratory are automatically contacted by phone so that the tank or freezer is attended to as soon as possible. An automatic phone alarm system must be organized to exclude telephone-answering machines from answering on the emergency contact numbers. Storage tanks may swiftly develop problems as exemplified by a 120-L tank in our department going from full to totally empty overnight. The only external evidence of a problem was some frost on the tank lid and neck. To prevent loss of stocks by such incidents, we recommend that irreplaceable stocks be stored in more than one tank, and if possible, at more than one location.

20. When straws are to be loaded with both EFS40 and diluent, considerable care is needed to prevent the EFS40 from being diluted. This can be achieved by preparing one 1 mL syringe for each solution (one for EFS40; one for diluent). Put a long (dry or dried) needle, e.g., 21G × 3 1/2 in. (0.80 × 90 mm) on each syringe. Hold the straw upright (plug end down) or at a slight angle, then insert the needle (via the upper opening) as far as possible into the straw. Gently expel around 120 μL dilution solution so that it fills the lower portion of the straw; gravity should pull it down into the plug. Withdraw the needle, taking care not to wet the sides of the straw. Then, insert the second syringe (EFS40) into the remaining, dry, portion of the straw and inject a single 30–40 μL column (**Figs. 9** and **14**), or an inner very small column (10 μL) a further small air column (10 μL) and a further column of EFS40 (approx 25 μL). The air lock(s) (air bubble) separating the solutions should stop them from mixing. If the solutions have been loaded and handled correctly, the straws should have the "correct" appearance (**Fig. 14**, straw b) upon thawing.
21. If the user is having problems loading the embryos very tightly into the loading pipet (thereby introducing too much buffer into the EFS40), an alternative strategy is to place an excess amount of cryoprotectant (1 mL) in a dish, then to pipet embryos, in as small a volume of buffer as they can, onto the surface of the cryoprotectant solution. Swirl the embryos into the cryoprotectant and then lower the straw into the dish. Push the straw as close as possible to each embryo and then suck it into the straw. This method is difficult, particularly since the embryos shrink and distort in the cryoprotectant and rapidly become difficult to identify as embryos.
22. If you find it difficult to load the straw because your hands are too shaky, try the following: either press the palms of your hands together while inserting the pipet into the straw, this should make your hands steadier **(Fig. 5)**, or if you have a stereo-dissecting microscope, place the straw, resting horizontally, on the stage and focus on the straw opening while slowly advancing the pipet towards, and into, the opening.
23. Straws can be heat sealed without a heat sealer either by **(A)** clamping the end of the straw firmly with wide forceps (e.g., artery forceps) prewarmed to over 100°C or **(B)** bringing the end of the straw towards a heat source (e.g., a bunsen flame) until the edges start to melt, then clamping the melting edges together with artery forceps at room temperature. This should be carried out with caution as the straws can burn. If the straw does catch light, do not panic; stop the fire by clamping the burning portion with the artery forceps.
24. We described the protocol assuming that the room temperature is 25°C. When the room temperature is lower, e.g., 20°C, the exposure time in EFS40 can be 0.5–2 minutes. On the other hand, at higher temperature, e.g., 30°C, the exposure time must be limited to 30 s ***(8,9)***.
25. During cooling and warming, the straws are placed on a boat, e.g., a flat piece of polystyrene floating on the surface of the liquid nitrogen. The temperature of the vapor at the surface of the "boat" should be less than –150°C. To ensure that the

temperature is this low, the boat must not be too thick (e.g., 1 cm) and the distance from the top of the boat to the lip of the container should be more than 5 cm. It is best if the boat is made so that the straws do not fall into the liquid nitrogen and has supports that keep the straws just above the surface of the polystyrene. A simple way of achieving this is to place two empty straws across the boat, and alongside both of these insert (at 1 cm intervals) short pieces of straws into the polystyrene **(Fig. 7)**. Insert the upright pieces on alternating sides of the "support" straw to hold them in place. Note that the supports are offset (not central) so that the region of the straw containing the EFS40 does not contact either the support straws or the polystyrene **(Fig. 7)**. If the work area is drafty, a loosely fitting lid should be placed over the opening of the LN_2 container.

26. To prevent liquid nitrogen from becoming trapped inside the goblets, small holes should be punched through the plastic near the base (e.g., with sharp scissors). The holes should help prevent the goblets from floating, but it is advisable to also place a heavy object (e.g., a paper clip) in the goblet. To prevent straws from floating or being tipped out of the goblets, the straws should be wedged in place with tissue paper or cotton wool **(Fig. 8)**. If the goblets are to be stored on canes, make sure that they are of appropriate size so that the goblet seats firmly onto the cane.
27. This is a vitrification procedure. The EFS40 should appear clear and transparent throughout the cooling, storage, and warming steps and the dilution solution should develop ice and become milky **(Fig. 14)**.
28. An alternative method for recovery is to cut off only the heat-sealed end(s) and to expel the contents of the straw by pushing the cotton plug with a metal rod into a culture dish containing dilution solution.
29. If you are going to culture the cryopreserved embryos after recovery, prepare a 100-μL drop of culture medium (e.g., M16, **ref.** ***15***) in a 35-mm culture dish under paraffin oil or mineral oil, and equilibrate it in a CO_2 incubator at 37°C overnight. Physical injury of blastomeres is clearly observable after a short period of in vitro culture. After 1 d of culture, transferable normal embryos can be identified by the formation of a blastocoel. Blastocysts can also be transferred to recipients at d 3 of pseudopregnancy.
30. When used correctly, the cryopreservation protocol outlined in this chapter should result in very little damage to embryos of most major strains. If embryos are not healthy after warming, it is likely that the method is being used incorrectly. If modifications do not help, then contact either author by e-mail or fax for advice (J. Shaw, fax: 61-3-9550 5554, e-mail: Jill.shaw@med.monash.edu.au. M. Kasai, fax: Japan-81-88-864-5219, e-mail mkasai@cc.kochi-u.ac.jp).
31. Cryopreservation can damage embryos, including their DNA ***(2,4,6,17,22)***, but if used correctly this problem should be negligible ***(2,4,6,22)***.
32. Always run a test run, including embryo transfer, for each strain. It is possible that your specific strain cannot be cryopreserved efficiently by this particular method. In this case, other stages of development or alternative slow cooling ***(5,7,10,18,23,24)*** rapid cooling ***(10,13,14,21,30)***, or vitrification ***(1,5,16,28)*** methods may be more effective ***(25)***.

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