Methods in Molecular Biology™

VOLUME 209

Transgenic Mouse

Methods and Protocols

Edited by

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HUMANA PRESS

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Introduction: The Use of Transgenic Mice in Biomedical Research

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1. Introduction

Recently, transgenic technologies to express cloned genes in the mouse have become fully mature. These advances have made the mouse one of the most useful animal models for biomedical research. The main strength of the mouse, compared with other model organisms, such as worms (*Caenorhabditis elegans*) and flies (*Drosophila melanogaster*), is that this mammalian model is highly comparable to the human in respect to organ systems, tissues, physiologic systems, and even behavioral traits. Moreover, the mouse carries virtually all genes that operate in the human. Hence, the mouse is unique in offering the possiblity to understand genotype-phenotype relationships that are relevant for unraveling the biologic role of these genes in the human. The advantages of the mouse over other mammalian model animals, such as the rat, rabbit, or pig are the availability of hundreds of genetically homogeneous inbred strains and the superior possibilities for gene modification in the germline. In addition, embryonic stem (ES) cells are widely available. Some of these advances originate from the fact that mice are easy to breed and have short generation times. Moreover, because of their small size, mice can be housed in large numbers, which keeps the costs of experiments within an affordable range.

In many cases, transgenic studies will start after obtaining evidence for a particular role of the gene in disease. This evidence may come from human genetic studies. Transgenic mice are generated to confirm the role of a disease mutation and will help to unravel the underlying molecular and biochemical mechanisms. In addition, the disease model will help in designing novel

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

therapeutic strategies. It should be noted that for disease models, a thorough screen through the existing resources is advised, before considering the generation of a particular mouse model. The Jackson Laboratory can provide a large number of different mouse models (*see* http://www.jax.org) that emerged from transgenic studies and were obtained after random mutation events (either spontaneous or induced).

Many other transgenic studies are initiated on the basis of the predicted gene function. Such predictions come from homology between the mouse gene and genes in other organisms. One of the most striking examples is in the field of developmental biology regarding the analysis of the homeobox genes. These genes were first discovered in *D. melanogaster*. Subsequently, their evolutionarily conserved biologic function was shown using transgenesis in the mouse.

In parallel with the elucidation of the complete genome sequences of mice, humans, and many other organisms, it becomes increasingly likely that transgenic studies will be initiated on the basis of genomic studies. Via genomics, large numbers of genes or proteins are studied in parallel. Interesting loci will emerge that require functional analysis. In many cases, the gene function is unknown. Often, however, even a homozygous null mutation in a well-conserved gene will not show a phenotype. Aside from functional redundancy, this may occur because the laboratory mouse has not been under any selective pressure since the early 1900s. The impressive history and specific characteristics of the laboratory mouse has been well documented ***(1)***. In the relatively safe lab environment, and in the absence of natural stress conditions, one might need to study different mouse phenotypes extensively and may come up with only subtle effects.

The best option for discovering gene function in such cases is to increase the stress on the system, for which there are numerous techniques. By crossing the novel mouse model onto a sensitized strain, increased genetic pressure is achieved. For instance, when the role of a gene in atherosclerosis is under study, the novel mutant mouse can be crossed with a known model for atherosclerosis. Since inbred mouse strains generally are resistant to atherosclerosis, it is important to make use of an athero-susceptible background. The apolipoprotein E-deficient mouse has been used extensively as a susceptible background to expose the role of other genes. Alternatively, changing the environmental conditions may increase the susceptibility of the mouse to atherosclerosis. High-fat diets are commonly used to induce atherosclerosis, thereby exposing the role of genetic factors.

2. Mouse-Human Differences

Differences between mouse and human are more obvious to the physician than to the molecular biologist. To the molecular biologists, it is remarkable

that mice are so different, since virtually all genes are conserved between mouse and human. Despite differences at the DNA sequence level, it is in most cases possible to substitute entire gene regions and observe no differences in gene regulation and gene function. The same holds true when examining, for example, lipoprotein metabolism. From a distance, mice and humans show very different metabolisms, rendering the mouse almost resistant to diet-induced atherosclerosis. In fact, only very few minor differences in lipid metabolism between mouse and human are known. When these differences are being "repaired" or compensated for, the lipoprotein metabolism changes into a "human-like" metabolism, and the mice are rendered highly susceptible to develop atherosclerosis.

From a medical perspective, the disease processes in the mouse are likely to be different. First of all, the mouse progresses rapidly through development and has a short life span. Therefore, diseases that emerge during a specific developmental stage have only a short window of opportunity. Also, with a life span of only 2 years, mice will age rapidly, and chronic human diseases only have months to develop in a mouse. An example is the absence of tumors in mice heterozygous for a mutation in the retinoblastoma gene, which is a tumor supressor gene in humans (*see* Chapter 17). However, in general, the short time frame is not a major problem in mouse studies on cancer and neurodegenerative and cardiovascular disease. Second, the physiologic properties of a mouse are tuned to its small size. With an average weight of only 40 g, its is clear that many diseases will have a completely different course in a mouse, when size and body mass are important. For example, a mutation in the dystrophin gene, causing Duchenne's muscular dystrophy in humans, does not have a severe outcome in the mouse. Moreover, a model for human atherosclerotic plaque rupture in the mouse is also difficult to obtain, which may be in part related to the fact that the arteries differ in size by approximately 100-fold. The main strength of the mouse models is that at the molecular and cellular levels, they correspond well to the human. However, one should not expect the mouse to copy human disease completely.

3. Transgenic Technology: "Gain of Function" Versus "Loss of Function"

Since the publication of one of the landmark papers on "conventional transgenics" in 1982 by Palmiter and colleagues ***(2)***, the technology for generating conventional transgenics using microinjection of fertilized oocytes has not altered much. Because many constructs were based on intron-less cDNA clones and not much insight was available regarding gene regulation, it has been difficult to create good mouse models. At present, however, knowledge of regulation of gene expression has dramatically improved, leading

to the generation of mice with a more predictable expression pattern of the transgene.

From a geneticist's viewpoint, these conventional transgenic mice represented gain-of-function models, because one could only add a gene. However, human genetic disease is more often characterized by loss of function and by homozygous recessive gene mutations, which were both beyond the reach of conventional transgenesis. Then, in 1988, a seminal paper was published by Mansour and colleagues ***(3)*** demonstrating the feasibility of interrupting a target gene by a neomycin gene in mouse ES cells. By choosing the site of the neomycin selection cassette at a position that would disrupt the transcription of the target gene, it was possible to generate a loss-of-function mutation. This technology became known as *gene targeting* via homologous recombination in ES cells, and was primarily used to generate knockout mice. Importantly, the strategy for successfull gene targeting did not require an in vitro selection method to detect the loss of the gene product of the targeted gene. In principle, every gene could now be silenced using gene targeting. Hence the mouse became the species of choice for biomedical research. At present, it is feasible to generate both loss-of-function and gain-of-function models and also to restrict these changes to specific cell types and developmental stages (discussed below).

4. Contents of This Book

4.1. Basic Mouse Technology

Chapter 2 is devoted to general mouse technology, including mouse husbandry and microinjections. The use of pronuclear injection of DNA in fertilized oocytes as well as the injection of embryonic stem cells in blastocysts is explained. Having access to many mouse strains is one of the most general problems of mouse facilities. In Chapter 3, protocols are provided on the preservation of mouse strains by cryopreservation of sperm. Although the technology sounds simple, it is not, and the authors were among the very few who were able to develop reliable protocols for cryopreservation. Moreover, exchange of sperm may also turn out to be more convenient than shipping live mice to other laboratories, although it is not in practice as yet. The important advantage would be that, except for (vertically transmitted) viruses, the receiving lab does not have to worry about introducing unwanted contaminants such as parasites and bacteria.

4.2. Conventional Transgenic

Chapter 4 deals with the generation of conventional transgenic mice by pronuclear injection of fertilized oocytes. A detailed overview is given on construct design and the preparation of DNA suitable for pronuclear microinjec-

tion. This chapter explains the pros and cons of short cDNA constructs versus larger genomic constructs. In the early days, cDNA-based transgenic projects did not work out well, owing to methylation and subsequent silencing of the transgene or lack of RNA stability. The exclusion of plasmid vector sequences and inclusion of introns did resolve some of these problems. Furthermore, insight into the mechanism of gene regulation has improved significantly and has allowed for a more robust construct design.

Chapters 5–7 elaborate on several different strategies for transgene design and application. The use of tetracycline-inducible transgenes is presented in Chapter 5. The tetracycline system is currently the best system for inducible gene expression available. Other systems exist as well. The application of inducible promoters (metallothionein, interferon) has been reported. However, these promoters need endogenous ligands for activation, which also leads to the simultaneous activation of other endogenous genes. An exception is the use of an insect promoter activated by ecdyson. However, the costs of ecdyson prohibit its routine laboratory use in the mouse.

Special attention is paid to the use of large bacterial artificial chromosome (BAC) clones for conventional transgenics in Chapter 6. Although they may seem somewhat more laborious to work with, based on the experience outlined in this book, their disadvantages should be outweighed by their advantage: BAC clones offer large insert clones, which usually yield highly reproducible and faithful tissue-specific gene expression patterns. In fact, the fidelity of these expression patterns can only be matched by using homologous recombination strategies (*see* below).

Essential for exploring gene expression is the appropriate use of reporter systems. The systems presented in Chapter 7 (including β-galactosidase, green fluorescent protein, and luciferase) are widely used for testing expression patterns of promoters and other regulatory elements using conventional transgenesis. In addition, these reporters can be inserted in genes via homologous recombination to follow endogenous expression patterns. These reporters are indispensable for following the effect of the tetracycline and Cre-LoxP systems, as described in subsequent chapters. Also, the use of reporters has been crucial in several research areas in which the identification of subsets of cell types is important, such as developmental biology.

4.3. Gene Targeting using ES Cells

Chapter 8 (ES technology) summarizes the most essential protocols for generating knockout mice by homologous recombination in ES cells. Since the technology has now been around for more than a decade, most of the pitfalls have been eliminated, which guarantees that mutated pluripotent ES cells will

be produced with high efficiency. One of the drawbacks of the system was the fact that the null mutation was transmitted via germline cells. Hence, all cells of the body would inevitably carry the induced mutation. Recently, however, the Cre-LoxP system (*see* Chapter 9) has been developed, allowing cell type-specific knockouts. In fact, a predetermined LoxP-mediated recombination upon induction by Cre has opened up a vast range of new possibilities, including the generation of larger chromosome rearrangements and knockin mutations replacing the mouse gene with a gene of choice. The use of tissue-specific inducible Cre expression allows control of the gene mutation in time and place. Hence, acquired somatic mutations can be reproduced at any stage of development. This potential has been crucial for the generation of highly realistic disease models, for instance in the cancer field. The full range of possiblities offered by the Cre-LoxP system are outlined in Chapters 9 and 10.

Often, when genes are studied that play a role in development or in the basal transcriptional machinery, homozygous knockouts will turn out to be lethal. Fortunately, there are many solutions for continuing with knockout mice that show embryonic lethality. One approach is the Cre-LoxP system mentioned above. Another option is to generate a homozygous knockout in ES cells and then generate a mouse that is completely ES cell-derived. Chapter 11 describes such an approach by fusing ES cells with tetraploid embryos. This technology of embryo aggregation is also highly useful for generating mice via ES cells, as an alternative to microinjection of ES cells into blastocysts. In this case the ES cells will be aggregated with cleavage stage embryos.

4.4. Alternative Strategies for Studying the Role of Genes in Mouse

Apart from transgenic technology, there are several other approaches toward studying gene function in the mouse that may gain in importance because they are compatible with the desire to combine functional research in the mouse with the demands of genome-wide high-throughput research.

One strategy makes use of techniques from the gene therapy field to generate mice expressing genes in predefined cell types. Chapter 12 provides the method of choice for generating adenovirus vectors. The advantage of adenoviruses is that the protocol is not complicated, and adenoviruses can easily be grown to high titers. Moreover, adenoviruses can infect most nondividing cells and can be used to assess gene function readily in vivo. Moreover, it should be noted that viral transduction can be used for local delivery of Cre-recombinase. Hence, there is no need to set up transgenic mouse strains expressing Cre and to spend time generating a breeding program to make the appropriate Cre-LoxP combinations. Many exciting techniques have been developed for improving the usefulness of adenoviral gene transduction,

particularly with regard to the specificity of target recognition and the duration of gene expression. These developments make the application of viral transduction highly useful as a complementary strategy to transgenesis (for a review, *see* **ref. *4***). However, a more extensive treatment of this topic falls outside the scope of this book.

A completely different approach toward developing mouse models and detecting disease-related genes makes use of random mutagenesis. There are many strategies for generating mouse mutants, ranging from the random insertion of DNA fragments to point mutations induced by *N*-ethyl-*N*-nitrosourea (ENU) as described in Chapter 13. The ENU method stands out from the others, in that it is truly random. Protocols have been generated for mice, as well as for ES cells. The only disadvantage of ENU is that the affected gene is more difficult to find, compared with mutations induced by transposons, (pro)viral insertions, or other DNA fragements. The main selling point of the random mutagenesis approach is the fact that the project starts with the phenotype at hand, which makes the correlation between the gene and the phenotype *a priori* of great interest.

4.5. Mouse Models in Biomedical Research

The five concluding chapters (Chapters 14–18) provide insight into the work that needs to be done, after the mouse model has been generated. Chapter 14 provides an overview of the pathology studies that should be completed with every new mouse strain generated. Often, transgenic mice are only examined for the presence of predicted phenotypes. It is important, however, to asses the complete mouse for abnormalities. Although one could not apply all possible phenotyping methods to every new mouse model, it is advisable to carry out pathology studies according to the protocols provided.

The remaining four chapters provide some entry points into the four fields of research addressed here, i.e., immunology (Chapter 15), atherosclerosis (Chapter 16), cancer (Chapter 17), and neurobiology (Chapter 18). Chapter 15 provides a protocol for bone marrow transplantation. At present, the use of bone marrow transplantation is not only restricted to immunologists, who are able to substitute part of the immune system of live mice. Other applications make use of the replacement of macrophages (atherosclerosis) or the introduction of somatic stem cells. Bone marrow transfer has also been used to develop the first gene therapy protocols using retroviral modified bone marrow. The most essential protocol for atherosclerosis studies is the measurement of the lesion area in the proximal aorta, which is provided here in detail. Chapters 17 and 18 review concepts regarding the use of transgenic models in cancer and neurobiology, illustrating many of the approaches that were outlined in the preceding chapters, e.g., the use of reporter genes and the Cre-LoxP system.

To conclude, I want to stress the importance of performing a broad analysis of the newly generated mouse models. For example, my own scientific background is in the field of atherosclerosis. At the time the first transgenic mouse was born in our laboratory, we had a specific desire to understand the role of a particular apolipoprotein mutant (APOE3Leiden) in lipoprotein metabolism. We initially underestimated the usefulness of this mouse model, because our attention was exclusively focused on the effects of APOE3Leiden on plasma lipid levels. It should be clear, however, that transgenic mouse models force researchers to stretch scientific horizons, because one studies the impact of the mutant allele on the entire animal, rather than on one particular aspect of metabolism. Some 10 years later, mouse models for lipoprotein metabolism are central to most studies on atherogenesis in the mouse, and these models also play a role in studying diabetes and obesity. To appreciate this potential of mouse models, it is advised to embark on these studies with a multidisciplinary team; the actual generation of the model takes less effort than the subsequent research that may originate from it.

References

1. Silver, L. M. (1995). *Mouse Genetics: Concepts and Applications*, Oxford University Press, Oxford, UK.
2. Palmiter, R. D., Brinster, R. L., Hammer, R. E., et al. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* **300,** 611–615.
3. Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336,** 348–352.
4. Somia, N. and Verma, I. M. (2000). Gene therapy: trials and tribulations. *Nat. Rev. Genet.* **1,** 91–99.

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Genetic Modification of the Mouse

General Technology; Pronuclear and Blastocyst Injection

J. Willem Voncken

1. Introduction

Transgenesis, one of the first techniques specifically aimed at germline alterations, makes use of the introduction of exogenous DNA sequences, *transgenes*, into an organism's genome. A transgenic animal is by definition an organism that has had extra, often foreign, DNA artificially introduced into its genome. Transgenesis is applicable to a wide range of mammalian species including mice, rats, rabbits, livestock such as sheep, pigs, and cattle, and recently even primates. The focus of this chapter, however, is on mice, because these are the most widely used laboratory animals for transgenic studies in experimental research. In addition to the conventional transgenic technology described in detail below, mice are presently the only mammalian species suitable for gene targeting by homologous recombination in embryonic stem (ES) cells. Gene targeting allows for genetic germline manipulation at predetermined genomic loci and is an important and powerful extension of the current molecular genetic tools to generate experimental animal models for the study of gene function and disease. This topic and some of its exciting applications are described in Chapters 8–10. In contrast to ES cell technology, there is no need for homology between the injected DNA and the host genome. Transgenic animals are generated by (retro)viral transduction of early embryos, by introduction of transgenes in ES cells, or, more commonly, by microinjection of DNA directly into one of the pronuclei of a fertilized mouse egg ***(1–5)***. Typically, microinjected DNA will integrate at one site within the genome, often as a concatamer (a multicopy insertion), arranged in a head-to-tail fashion.

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
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Transgenesis may be used to study overexpression or ectopic expression of a gene of interest. Alternatively, the effect of mutation of a gene may also be studied. In both instances the basis of study is analysis of the resulting altered phenotype. Depending on the choice of regulatory sequences directing transgene expression (*see* Chapter 4), the expression of a transgene may follow the expression pattern of its endogenous counterpart or may be limited to distinct cell types or particular developmental stages. Alternatively, transgene expression may occur in cell types in which the endogenous gene is normally inactive (ectopic gene expression). Transgenes, which hold mutant forms of genes either spontaneously occurring or genetically engineered, may exert dominant effects. Although genetic manipulation is possible in tissue culture, the interaction of transgenes with other genes, proteins, and other components of the intact organism provides a much more complete and physiologically relevant picture of the transgene's function than could be achieved in any other way. In addition to studies on gene function and pathology, transgenesis therefore represents an important and biologically relevant tool to complement in vitro gene expression studies aimed at, e.g., delineation of signal transduction pathways or identification of tissue-specific regulatory elements.

In **Fig. 1** below, an overview is presented of the different experimental aspects involved in making genetically modified animals via conventional transgenic technology (i.e., pronuclear injection of one-cell stage embryos) or gene targeting (i.e., embryonic stem cell injection into blastocysts). In the following sections, some basic guidelines for the production of transgenic mice will be provided, including superovulation, microinjection of one-cell stage zygotes, and identification of transgenic founder mice. Since the animal and the equipment used for embryonic stem cell injection into blastocysts are in essence very similar to those used for microinjection of fertilized eggs, blastocyst injection and uterine transfer are discussed in this chapter as well (*see* **Fig. 1** and **Subheadings 3.2.**, **3.3.**, and **3.4.**). For basic molecular cloning techniques and strategies and molecular detection methods such as polymerase chain reaction (PCR)-based or Southern blot analysis, we recommend *Molecular Cloning. A Laboratory Manual* by Sambrook, et al. ***(6)***. Transgenic construct design is discussed in Chapter 4.

1.1. Mouse Husbandry: Choice of Genetic Background

As transgenic technology and protocols are described in this chapter, it is assumed that the researcher has access to a professional and well-equipped laboratory animal facility with ample experience in mouse handling, breeding, and the surgical and/or dissection techniques required for the production of transgenic mice. Animals used to generate transgenic and targeted mouse lines comprise fertile donor females and fertile males for fertilized oocyte

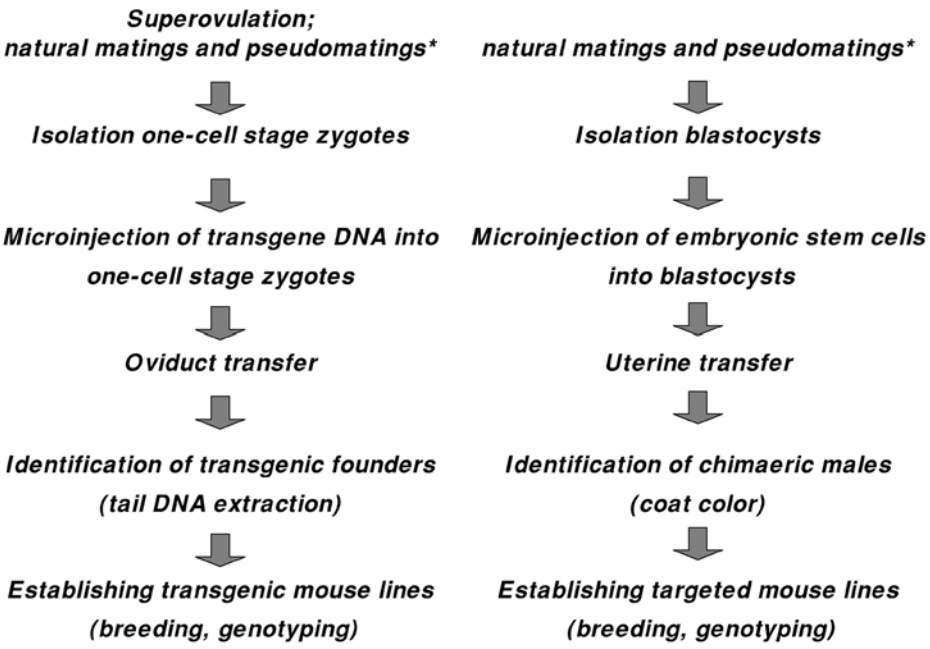


Fig. 1. Stepwise overview of the procedures for generating genetically modified mice by pronuclear injection of DNA (left column) and by gene targeting in embryonic stem (ES) cells and subsequent injection of these ES cells into blastocysts (right column). *, Matings between foster females and vasectomized males.

production, and fertile females and vasectomized males for oviduct or uterine transfer. Fertile females are either superovulated at a critical age and mated to studs, so as to obtain ample numbers of one-cell stage zygotes for pronuclear injection (*see* **Subheadings 3.1.** and **3.1.1.**), or mated naturally to studs for the production and isolation of blastocysts into which genetically modified ES cells are introduced. Normal females are mated to vasectomized males to produce pseudopregnant females, which will foster transplanted, micromanipulated embryos after birth (*see* **Subheadings 3.1.** and **3.1.2.**). Procedures and requirements concerning animals used for fertilized oocyte production and for oviduct transfer and guidelines for equipment and microsurgical techniques are described in excellent detail in *Manipulating the Mouse Embryo* by Hogan et al. ***(1)*** and in the video guide *Transgenic Techniques in Mice* by Pedersen and Rossant ***(2)***. In addition, these media provide comprehensive information on historical and genetic backgrounds of in- and outbred strains, on mouse embryology, and on dissection of specific developmental stages.

The need for genetic standardization of experimental animal models in experimental and applied research has historically been one of the reasons

why inbred strains were established. A defined genetic context is important, for instance to establish the genetics of cancer susceptibility, for studies on polygenic diseases, or for immunologic studies. In these instances, inbred mice are preferred to generate transgenic mouse models, because of their strain homogeneity. A unique collection of inbred mouse strains is available worldwide. By definition, an inbred strain is derived by 20 generations of brother-to-sister matings and is essentially homozygous at all genetic loci ***(1)***. The choice of genetic background is determined by the aim of the experimental model. Sometimes a reason for widespread application is simply a historical one (i.e., best studied strain in a given context), whereas in other instances there may be a clear advantage in using a particular strain because of a certain predisposition, although the exact underlying genetic cause (e.g., modifier loci, quantitative trait loci [QTLs]) is not always known.

Although there is considerable choice in inbred strains, the most widely used strain is C57BL/6J, also known by the acronym B6. The C57BL/6J strain, for instance, appears more sensitive to diet-induced atherosclerosis, which makes this strain particularly valuable in cardiovascular research. The same inbred mouse strain is also frequently used in immunologic and behavioral studies. A common disadvantage of inbred strains, however, is their reduced reproductive capacity and relative poor yield of one-cell stage zygotes (fertilized eggs) upon superovulation compared with F1 hybrids. Furthermore, "inbred" zygotes often have an attenuated viability in vitro, microinjection, and transplantation. An exception may be the recently introduced FVB/N inbred strain, which does superovulate well, yielding reasonable numbers of fertilized eggs ***(7)***. In most other instances, however, F1 hybrids are used to generate fertilized eggs for microinjection (sometimes up to 30 or more). A relatively large fraction of F1 hybrid-derived zygotes will develop to term. One of the most commonly used F1 hybrids is C57BL/6J × CBA (BCBA). Other F1 strain hybrids applied are C57BL/6J × SJL, C3H/HeJ × C57BL/6J, C3H/HeJ × DBA/2J, and C57BL/6J × DBA/2J ***(1)***.

2. Materials

2.1. Superovulation; Natural Matings and Pseudomatings

1. Light cycle-controlled mouse room.
2. Female mice (4–6 weeks of age).
3. Fertile male mice (8–12 weeks up to 8 months of age).
4. Foster mothers (preferably experienced mothers 3–6 months of age).
5. Vasectomized males (any strain; we use Swiss).
6. 1-mL syringes.
7. 26- or 27-gage 1/2-inch needles.

8. Sterile 0.85% (w/v) sodium chloride solution or sterile water.
9. Follicle-stimulating hormone (FSH) analog: pregnant mare serum gonadotropin (PMSG); 1000 IU.
10. Luteinizing hormone (LH) analog:; human chorionic gonadotropin (hCG); 1500 IU.

Hormones are available from Intervet, Boxmeer, The Netherlands; Folligon; Chorulon; or Sigma: PMSG, cat. no. G4877 (1000 IU); hGC, cat. no. CG-2 (2500 IU).

2.2. Isolation of One-Cell Stage Zygotes and of Blastocysts[BL]

[BL] This superscript denotes materials specifically used for blastocyst and ES cell manipulation.

1. Mineral oil (Sigma, cat. no. M8410).
2. M2 medium (Sigma, cat. no. M7167).
3. M16 medium (Sigma, cat. no. M7292).
4. Blastocyst isolation medium: 10% fetal bovine serum (FBS), 10 m*M* HEPES in Dulbecco's modified Eagle's medium (DMEM) medium (+penicillin/streptomycin).[BL]
5. Blastocyst culture medium: 10% FBS in DMEM medium (+penicillin/streptomycin).[BL]
6. Penicillin and streptomycin solution, 10,000 U/mL (GIBCO, cat. no. 15140-114).
7. Pyruvate (Sigma, cat. no. P-2256).
8. Hyaluronidase type IV-S (Sigma, cat. no. H-3884).
9. Bovine serum albumin, (BSA), fraction V (Sigma, cat. no. A9647).
10. Phosphate-buffered saline (PBS; optional).
11. Depression slides (optional).
12. 10-cm Petri dishes.
13. 35-mm Petri dishes for microdroplets.
14. Fire-polished Pasteur pipet.
15. Transfer pipet (drawn Pasteur pipet with an internal diameter of ± 200 μm.
16. Mouth pipet/tubing.
17. Syringes (1-mL)/needles (27 gage).[BL]
18. Synthetic clay.
19. Incubator at 2.5% CO_2.
20. Dissection microscope.
21. Fiberoptic illuminator.
22. Diamond pencil.
23. Small iris scissors.
24. Set of tweezers.
25. 70% ethanol.
26. Paper towels

2.3. Microinjection of One-Cell Stage Zygotes; In Vitro Culture of Injected Zygotes; Injection of Blastocysts with Embryonic Stem Cells[BL]

[BL]This superscript denotes materials specifically used for blastocyst and ES cell manipulation.

1. Mineral oil.
2. M2 medium.
3. M16 medium.
4. Blastocyst isolation medium: 10% FBS, 10 m*M* HEPES in DMEM medium (+penicillin/streptomycin).[BL]
5. Blastocyst culture medium: 10% FBS in DMEM medium (+penicillin/streptomycin).[BL]
6. 10-cm Petri dishes.
7. 35-mm Petri dishes.
8. Incubator at 2.5% CO_2.
9. Transfer pipet (drawn Pasteur or other glass pipet; internal diameter of ± 200 μm.
10. Mouth pipet/tubing.
11. Synthetic clay.
12. Glass capillary tubes (Leitz, A>520119) for holding pipets .
13. Glass capillary tubes (Narishige, G-1) for injection needles[BL] or glass capillaries with inner filament (Narishige, GD-1).
14. Sigmacote (Sigma, cat. no. SL2).
15. 96 % ethanol (analytical grade).
16. Pipet puller (e.g., Narishige, PB-7).
17. Microforge (e.g., Narishige, MF-9).
18. Microgrinder (e.g., Narishige, E-40).[BL]
19. Inverted microscope system (e.g., Nikon, Olympus, Leitz, Zeiss).
20. Micromanipulators (e.g., Narishige).
21. Dissection microscope.
22. Diamond pencil.
23. Stage micrometer.
24. Fluorinert (Sigma, cat. no. FC77).
25. Readout type,[BL] motor-driven microinjector, or large 50-mL glass injection syringe.

2.4. Oviduct Transfer; Uterine Transfer[BL]

[BL]This superscript denotes materials specifically used for blastocyst and ES cell manipulation.

1. Mineral oil.
2. Clean M2.
3. Blastocyst isolation medium: 10% FBS, 10 m*M* HEPES in DMEM medium (+penicillin/streptomycin).[BL]

4. Blastocyst culture medium: 10% FBS in DMEM medium (+penicillin/streptomycin).[BL]
5. Pair of small scissors (iris).
6. Two pairs of tweezers (size 5).
7. One pair of tweezers (size 4A).
8. Serafine clamp.
9. Suture and/or wound clip system.
10. Kimwipe tips.
11. Transfer pipets, 100–120 μm diameter.
12. Synthetic clay.
13. Inhalation anesthetic (*see* **Note 1**).
14. Small desiccator.
15. Injection sedative (*see* **Note 1**).
16. Syringes (1 mL)/needles (27 gage).[BL]
17. 96% ethanol.
18. Dissection microscope.
19. Fiberoptic illuminator.
20. Operation platform

2.5. Identification of Transgenic Founders; Tail DNA Extraction

2.5.1. Rapid Procedure Tail-Tip DNA Extraction

1. Tail mix: 100 m*M* Tris-HCl, pH 8.5, 5 m*M* EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 m*M* NaCl.
2. Proteinase K: stock solution: 20 mg/mL.
3. Isopropanol.
4. 70% ethanol.
5. TE buffer: 10 m*M* Tris-HCl, pH 7.2–7.6, 1 m*M* EDTA.
6. Pasteur pipets, flame-polished.
7. Reaction tubes, 1.5 mL.
8. 55°C oven.
9. Rotator

2.5.2. Standard Procedure Tail-Tip DNA Extraction

1. Tail mix: 50 m*M* Tris-HCl, pH 8.6, 100 m*M* EDTA, 1% SDS, 100 m*M* NaCl.
2. Protease K: stock solution: 10 mg/mL.
3. RNase: 10 mg/mL (heat-inactivated; 10 min at 100°C).
4. Phenol: Water saturated (bidest; autoclaved); adjusted to pH 7.0 with 1 *M* Tris-HCl, pH 8.0.
5. Chloroform.
6. Isoamylalcohol.
7. Phenol-chloroform-isoamylalcohol (24 : 24 : 1; Gibco, cat. no. 15593-031).
8. Isopropanol.
9. 96% ethanol.

Table 1
Timetable of Superovulation and Matings to Obtain One-Cell Stage Embryos for Microinjection

Day	Time point	Action	Week day						
1	1.00–2.00 PM	PMSG injections[a]	Sa	Su	Mo	Tu	We	Th	Fr
3	12.00–1.00 PM	hCG injections[a]	Mo	Tu	We	Th	Fr	Sa	Su
	3.00–4.00 PM	Natural matings							
4	10.00–11.00 AM	Isolation of fertilized eggs	Tu	We	Th	Fr	Sa	Su	Mo
	Afternoon	*Microinjections*							
	3.00–4.00 PM	Pseudomatings[b]							
5	Morning	Oviduct transfer of two-cell stage embryos	We	Th	Fr	Sa	Su	Mo	Tu

[a]Day/night rhythm: 6.00 AM: light, 6.00 PM: dark.
[b]Matings between foster females and vasectomized males.
hCG, human chorionic gonadotropin; PMSG, pregnant maie serum gonadotropin.

10. 70% ethanol.
11. TE buffer: 10 m*M* Tris-HCl, pH 7.2–7.6, 1 m*M* EDTA.
12. Pasteur pipets, flame-polished.
13. Reaction tubes, 1.5 mL.
14. 55°C oven.
15. Rotator.
16. Bench or wrist shaker.
17. 37°C waterbath.

3. Methods

3.1. Superovulation; Natural Matings and Pseudomatings

Animals have access to water and standard chow *ad libitum* and are housed under a 12-h day/night regimen, most often comprising a 6 AM to 6 PM light period (*see* **Note 2**). We have good experience using 4–6 week-old B6CBA/F_1 females (Jackson Laboratories) for superovulation. **Tables 1** and **2** present a summary of the different actions in the procedures.

3.1.1. Superovulation; Natural Matings[BL]

[BL]This footnote indicates methods specifically used for blastocyst and ES cell manipulation.

1. Dissolve lyophilized PMSG and hCG in 0.85% NaCl solution or sterile water to a final concentration of 50 IU/mL. PMSG may be stored frozen at –20°C or –80°C in 0.8 –1.0-mL aliquots and thawed when needed. One aliquot will be enough for injection of six females. Alternatively, concentrated stocks of PMSG

Table 2
Timetable of Natural Matings to Obtain Blastocysts for Injection

Day	Time point	Action	Week day						
1	3.00–4.00 PM	Natural matings[a]	Mo	Tu	We	Th	Fr	Sa	Su
2	3.00–4.00 PM	Pseudomatings[b]	Tu	We	Th	Fr	Sa	Su	Mo
3	3.00–4.00 PM	Pseudomatings[c]	We	Th	Fr	Sa	Su	Mo	Tu
5	Morning	Collect blastocysts	Fr	Sa	Su	Mo	Tu	We	Th
	Afternoon	Blastocyst injection Uterine transfer							
6	Morning	Uterine transfer	Sa	Su	Mo	Tu	We	Th	Fr

[a]Day/night rhythm: 6.00 AM: light, 6.00 PM: dark.
[b]Matings between foster females and vasectomized males.
[c]Optional, in case not many copulation plugs are detected on day 3.

and hCG (200 IU/ml) may be stored at 4°C for a period of 2 months, but they need to be diluted properly prior to use.

2. Thaw a vial of PMSG and inject 4–6 week-old females with 100 µL PMSG (5 IU) between 1.00 and 2.00 PM on the first day.
3. On day 3, 46–48 h after PMSG injection, thaw a vial of hCG and inject the same females with 100 µL hCG (5 IU; 12.00–1.00 PM).
4. Transfer females directly to fertile studs or in the course of the afternoon (*see* **Note 3**). Use one male per female. To ensure the maximum number of fertilized eggs, use these male mice only once a week. At 8 months of age, or when the plugging ratio drops below 70%, replace the male mice.
5. Check for copulation plugs the next morning.

BLIf natural matings are carried out for blastocyst production, use females of 2 to 4 months of age. In terms of numbers of mice used, the same guidelines apply as for pseudomatings, i.e., two to three females per male (*see* **Subheading 3.1.2.**). Average yield per plugged female (C57blk/6J) is 5–7 blastocysts. To produce reasonable amounts of blastocysts for injection, superovulation is sometimes used in different laboratories. The quality of the blastocysts obtained in this manner may, however, vary considerably.

3.1.2. Pseudopregnant Females

Mating females with vasectomized (or genetically sterile) males will generate pseudopregnant females, which are required for reimplantation of microinjected zygotes or blastocysts ***(1)***. Basically any genetic background may be used to generate pseudopregnant foster females, provided the females from this strain are known as "good mothers." Most F1 hybrids or outbred strains

can be used; for practical reasons we use females from the same background (B6CBA/F1) used for superovulation. If possible, experienced mothers are preferred. The females should be at least 2 months old (i.e., >20 g body weight) but should not weigh over 30 g: the older and heavier the females, the more problems can be expected in terms of fat accumulation, which can seriously hamper oviduct transfers. Since the females are mated in natural estrus, obtaining enough pseudopregnant females for oviduct transfer can be problematic. It is advisable to mate at least five or six females per intended oviduct transfer. To increase the chances of mating a female in estrus, several females (two to four) are put in with one vasectomized male, in contrast to 1-on-1 matings between superovulated females and fertile studs. If one has experience in judging whether females are in estrus, this can be helpful in obtaining sufficient plugged pseudopregnant females. Females are placed in a cage with a resident male, *not vice versa*. Vasectomized male have been housed separately for at least 1–2 weeks! (*see* **Note 3**). Oviduct transfers take place on the day of copulation plug detection. The surplus of plugged, pseudopregnant foster females can be reused after 2 weeks. The vasectomized male mice can be used twice a week for matings. The foster females are checked regularly for signs of pregnancy. Depending on the strain, the litter will be delivered around 19–21 days after oviduct transfer.

3.2. Isolation of One-Cell Stage Zygotes; Isolation Blastocysts[BL]

[BL]This footnote denotes methods specifically used for blastocyst and ES cell manipulation.

When microinjections are planned, the order of procedures has some (chrono)logic to it. Although the planning of a microinjection session is highly personal, some suggestions are presented in **Table 3**. Day/night rhythm is as in **Table 1**.

3.2.1. Preparation Media

M2 and M16 media can either be prepared from separate stock solutions as described in Hogan et al. ***(1)*** or purchased prefabricated (Sigma). Individual researchers should consider and test personal preferences on location, since, e.g., composition and indications for storage conditions tend to vary. For the sake of simplicity, the preparations below use prefab solutions. It is of importance to avoid collecting dust in media and other liquids. For this reason we do not wear powdered gloves, or at least we wash off the powder and dust thoroughly before use.

1. M2 medium: to 50 mL M2 medium, add 0.5 mL penicillin/streptomycin solution. If necessary, adjust the pH to 7.3–7.4 with 5 N NaOH. Add BSA to a final

Table 3
Timetable of Preparations for Pronuclear Microinjection

Action[BL]	Time
Prepare media	Day before, morning
Check plugs, sacrifice superovulated females	9.00 AM
Isolate one-cell stage zygotes	9.30 AM
Set up microinjection stage; holding pipet	10.30 AM
Injection needles	11.00 AM
Set up microinjection needle	1.00 PM
Pronuclear injection of one-cell stage zygotes	1.00 PM onward

[BL]Preparations for blastocyst isolation and injection are very similar. The microinjection system for ES cell injection is usually controlled by Fluorinert or mineral oil.

concentration of 4–5 mg/mL (*see* **Note 4**). Filter-sterilize (0.22-μm filter). Prewash filters with PBS (sterile) or discard the first few milliliters. Make 2-mL aliquots in sterile tubes and store at 4°C until use. These aliquots can be used for a month according to the manufacturer's specifications.

2. M16 medium: to 50 mL M16 medium, add 0.5 mL penicillin/streptomycin solution. Filter-sterilize (0.22-μm filter). Discard the first few milliliters. Make 2-mL aliquots in sterile tubes and store at 4°C until use. These aliquots can be used for 1 month according to the manufacturer's specifications. Incubate two small tissue culture dishes with 5–8 M16 drops (10–20 μL) under paraffin oil overnight at 100% humidity, 2.5% CO_2 (*see* **Note 4**).
3. Hyaluronidase solution: dissolve 50 mg hyaluronidase in 50 mL M2 medium (Sigma). If necessary, adjust pH to 7.3–7.4 with 5 N NaOH. Filter-sterilize (0.22-μm filter). Discard the first few milliliters. Make 0.5-mL aliquots and store at –20°C.
4. Pyruvate: dissolve 36 mg pyruvate into 10 mL of water. Filter-sterilize (0.22-μm filter). Discard the first few milliliters. Make 0.5-mL aliquots and store at –20°C.

[BL]The blastocyst isolation and culture media are prepared 1 day in advance. The culture medium is incubated overnight at 5% CO_2, at 37°C.

*3.2.2. Isolation of Fertilized Oocytes (*see ***Note 5****)*

1. On the day of isolation, add 20 μL of the pyruvate stock to the 2 mL M2 and M16 aliquots and mix by swirling gently.
2. Prepare the depression slides: one with 0.2 mL M2, two depression slides with 0.2 mL hyaluronidase solution, and three depression slides with 0.2 mL M16 medium. Place each depression slide in a 9-cm Petri dish and incubate them briefly at 37°C in the incubator. Alternatively, droplets of media may be prepared in 35-mm dishes.

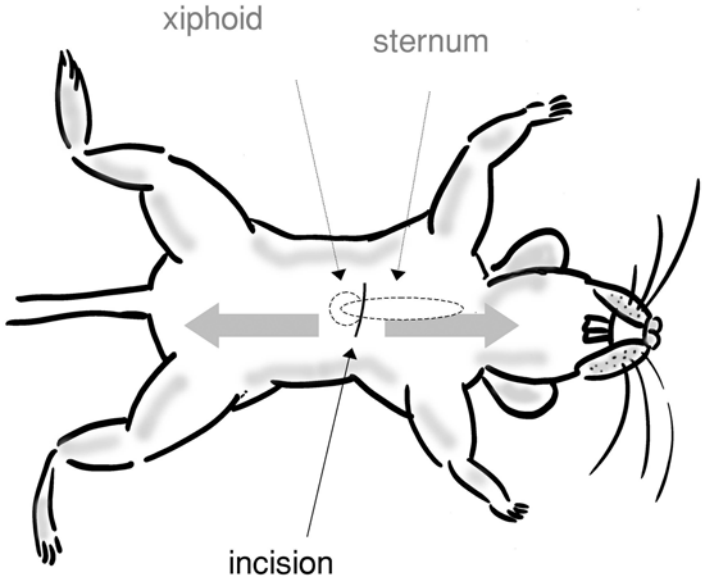


Fig. 2. Dissection donor females and isolation of fertilized eggs. An incision is made over the xiphoid, after which the skin is firmly pulled back in the indicated direction (gray arrows).

3. Collect females with a clear copulation plug. Sacrifice the mice by CO_2 asphyxiation or cervical dislocation and transfer animals to the location where zygotes are isolated.
4. Place females' abdomen upward on a paper towel, wet the abdomen with 70% ethanol, and make an incision over the xiphoid (**Fig. 2**).
5. Grasp the skin with both hands and pull back firmly in opposite directions (rostrally and caudally), essentially skinning the mouse completely (**Fig. 2**).
6. Make incisions in the body wall by lifting it with tweezers, and cutting it with scissors; expose the body cavity completely.
7. Move intestines aside gently and grab ovaries by the fat pad; remove ovaries plus oviducts by cutting through the transition to the uterus horn and transfer to a dish with PBS to wash off blood or debris.
8. Rupture the swollen ampullae ***(1)*** with sharp tweezers or a needle in M2 medium containing hyaluronidase (e.g., in a dish or on a depression slide). Cumulus masses are released into the medium and slowly fall apart by enzymatic action. Once freed of cumulus cells, the one-cell stage zygotes are washed free of hyaluronidase right away. Use pipets (ID 200 μm) and fresh M2 medium, and subsequently wash and transfer to M16 droplets under mineral oil in a 35-mm dish (37°C, 2.5% CO_2) until further use. Handling 35-mm dishes is easiest on top of the lid of a 10-cm dish, depression slides are best placed inside a 10-cm dish.

3.2.3. Isolation of Blastocysts

1. Dissect the uterus by a cross-section through the cervix and subsequent separation of the uterus from attached mesenteries, blood vessels, and fat.
2. Expose the lumen of the uterus horns by a transverse section below the transition to the oviduct. Make cross incisions at the opening to prevent constriction of the created opening.
3. Transfer one uterus to a dish with clean isolation medium. Disconnect the uterus horns form the cervix by a cut just above the bifurcation point.
4. Carefully insert a short 27-gage needle at the opening (closest to the cervix) and flush the blastocysts out using 0.5–1.0 mL medium.
5. Collect blastocysts with a wide-bore pipet (200–250 μm), transfer to a drop culture with clean medium, wash, and transfer to culture medium at 37°C, 5% CO_2.
6. Fully expanded blastocysts are easiest to use for injection; culturing blastocysts for some hours at 37°C, 5% CO_2 may increase the percentage of useable embryos.

3.3. Microinjection of One-Cell Stage Zygotes; In Vitro Culture of Injected Zygotes. Injection of Blastocysts with Embryonic Stem Cells[BL]

[BL]This footnote indicates methods specifically used for blastocyst and ES cell manipulation.

3.3.1. Preparations

The availability of an operational microinjection setup is considered a prerequisite for successfully applying transgenic technology. If no microinjection unit is available, several types of microscopes, micromanipulators, injectors, and peripheral equipment [to make injection needles and holding pipets for either pronuclear or blastocyst injection (*see* Chapter 8)] such as needle pullers, microforges, and grinders are commercially available (Leitz, Narishige, Nikon, Olympus, Sutter, Zeiss). See Hogan et al. ***(1)*** for a detailed description of a microinjection setup. Microinjection of one-cell stage zygotes and subsequent transplantation are essentially carried out as described ***(1)***. It is highly recommended to consult the video guide *Transgenic Techniques in Mice* by Pedersen and Rossant ***(2)*** for a visual reference to the protocols and procedures outlined in this chapter. In practice one will see that slight deviations from an existing protocol are possible and sometimes necessary in order to make things work for the individual user. Therefore, the protocol below only presents some of the most essential steps in the microinjection procedure.

Use dust-free gloves when preparing holding pipets and injection needles. Holding pipets are heat-polished until an opening of about 10–20 μm remains.

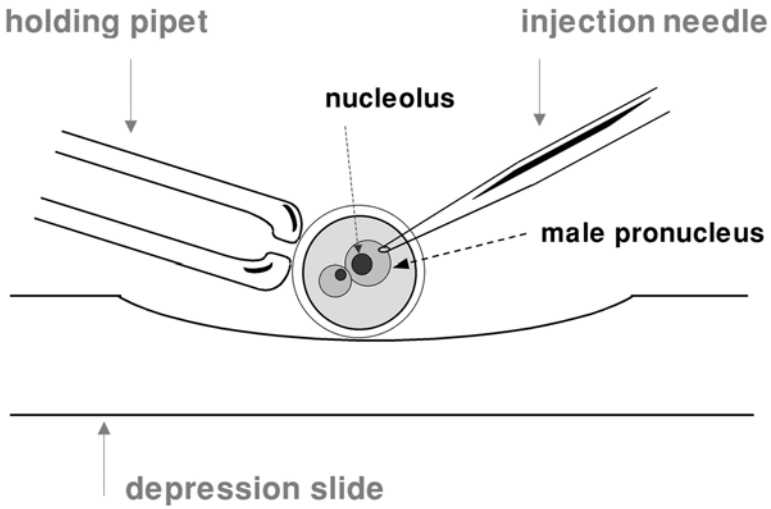


Fig. 3. Schematic representation of a microinjection chamber (side view). The basis of the injection chamber in this figure is a depression slide. Microinjection is carried out in a droplet of M2 medium.

Holding pipets and depression slides can be siliconized and rinsed extensively with clean, dust-free 100% ethanol; siliconized holding pipets can be reused. Injection needles can be treated similarly: dip the needle tip into a silicon solution and rinse it with alcohol before the needle is opened.

Several types of injection chambers may be used for microinjection. These chambers may consist of a slide and a Perspex ring (outer diameter slightly smaller than the width of the slide; 1.5 mm in height), which is fixed in position on a siliconized microscopic slide with 2% agarose. Alternatively, a depression slide can be used as an injection chamber. The injection chamber contains a droplet of 10 μL of M2 under light mineral oil. Depending on the manner in which DNA is loaded into the injection system, a 3–5 μL DNA droplet may be positioned next to the M2 droplet (also under oil), or a needle with an inner filament is back-filled. The chambers are prepared just before use and kept in a 90-mm Petri dish in the CO_2 incubator until use. Several injection chambers may be set up in parallel in this fashion and may be used alternately. **Figure 3** depicts an example of a microinjection setup.

3.3.2. Pronuclear Injection

1. Place the microinjection chamber on the microscope. Position the holding pipet. Positioning of both the holding pipet and the needle is usually carried out at a magnification of 100×.

2. Just before injection, transfer a number of one-cell stage zygotes (20–40) into the M2 droplet of the injection chamber. Transferring one-cell stage zygotes between injection chambers and culture droplets is best done under a dissection microscope. Since culture conditions inside the injection droplet are suboptimal, take no more zygotes than can be injected within 20–30 min.
3. Fill the injection needle with DNA solution from the DNA droplet and position the injection needle. In case of a back-fill system (i.e., glass capillary tubes with an inner filament), an opening is created in the injection needle by gently tapping it against the holding pipet and breaking off the very tip of the needle. Microinjection is done at 300–400× magnification.
4. Fix an egg in position with the holding pipet.
5. Gently push the injection needle through the plasma membrane and bring it close to the nuclear membrane of one of the pronuclei. In most cases the male pronucleus is the most accessible, because it is larger. If at this stage a small burst of DNA invaginates the plasma membrane further, then the membrane was not properly pierced. Reposition the zygote on the holding pipet and try again.
6. Carefully penetrate the nuclear membrane, avoid touching the clearly visible nucleoli, and inject the DNA solution into the pronucleus. Stop injecting at the slightest swelling of the pronucleus.
7. Gently withdraw the injection needle. If any material is accidentally pulled out of the nucleus, or when injected zygotes lyse frequently, the needle should be replaced.
8. Place the zygotes within the optic field strategically, so that yet to be injected, abnormal and lysed zygotes, and successfully injected zygotes are conveniently separated (**Fig. 4**).
9. Wash successfully injected zygotes free of M2 medium, transfer to a 35-mm dish containing a few CO_2-buffered M16 droplets, and keep in an incubator at 37°C, 2.5% CO_2.
10. Repeat the microinjection procedure until all one-cell stage zygotes are injected.
11. Keep successfully injected zygotes overnight at 37°C, 2.5% CO_2.

Although after a successful injection session as much as 90% of zygotes may survive the microinjection procedure, on average 60–75% of the zygotes can eventually be used for oviduct transfers. Transfer of zygotes is possible on the same day as the microinjection (one-cell stage). However, we prefer to culture the one-cell stage embryos overnight. During this time the embryos will undergo the first cleavage and develop into two-cell stage embryos. In this manner it becomes possible to make a selection of properly developed embryos before transfer into the recipient females' reproductive tract.

3.3.3. Blastocyst Injection

1. Place the microinjection chamber on the microscope. Sometimes a cooling stage (4–10°C) is used to make the blastocysts more rigid. Not all laboratories use this

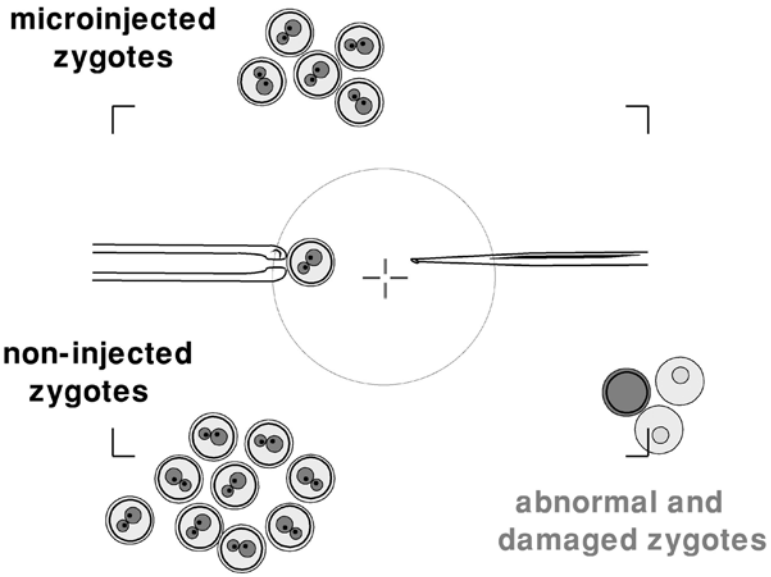


Fig. 4. Overview of the microinjection field (top view): noninjected, successfully injected, and abnormal zygotes or zygotes that were damaged during microinjection are all kept apart throughout the injection procedure

application. Positioning of the holding pipet and injection needle is carried out at a magnification of 100x. Optimal needles are bevelled to an opening of 12–14 µm at 30–35°C. Injection needles may be siliconized and reused.

2. Just before injection, transfer a number of blastocysts (20–25) into the injection droplet. Carry out injections in isolation medium or HEPES-buffered ES cell culturing medium (*see* Chapter 8).
3. Make ES cell suspensions by trypsinizing and preplating the suspension to get rid of feeder cells, which support ES cell growth in culture (*see* Chapter 8). We typically keep a few small ES cell cultures on hand to be able to repeat this procedure throughout the injection day. Wash ES culture twice with Ca^{2+}/Mg^{2+} free PBS and trypsinize the feeders plus ES cells. Sediment cells (5 min, 22–32*g* at ambient temperature) and suspend in ES cell medium; preplate on gelatinized culturing surface (*see* Chapter 8) and harvest after 15–20 min. Most feeder cells will have attached, whereas ES cells will not. If desired, the preplating procedure may be repeated. Collect ES cells and sediment; suspend well in a small volume of Ca^{2+}/Mg^{2+}-free PBS and transfer to a Petri dish: make several drop cultures in either PBS or isolation medium.
4. Transfer single ES cells into the injection chamber and load the injection needle with a fair number of ES cells (e.g., 75–120). Typically, 12–15 ES cells are injected into one blastocyst.

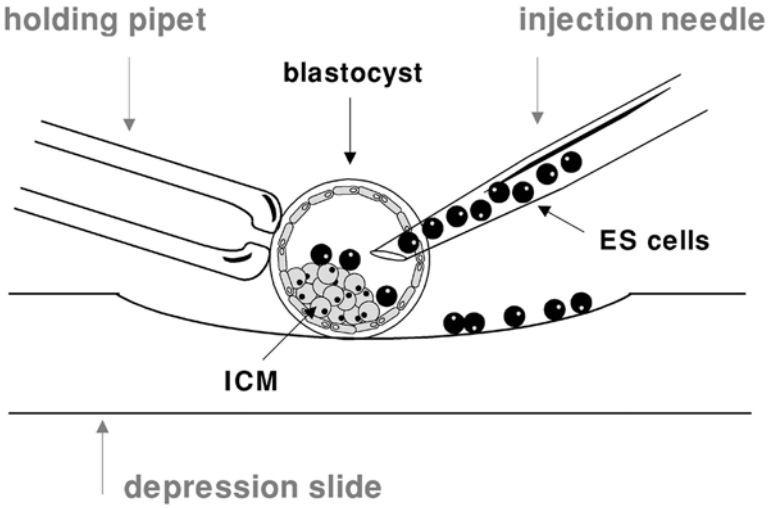


Fig. 5. Schematic representation of a blastocyst-injection chamber (side view). Genetically manipulated embryonic stem (ES) cells (black) are injected into a recipient blastocysts (gray). ES cells will adhere and become part of the inner cell mass (ICM) and partake in development. The resulting newborn will be a coat color chimera.

5. Fix a blastocyst in position with the holding pipet, with the inner cell mass (ICM) located away from the injection site (**Fig. 5**). Find a junction (window) between two trophoblast cells and insert the needle through the zona pellucida through the junction into the blastocoel; gently expel the ES cells.
6. Remove the injection needle gently from the blastocoel; the blastocyst will visibly collapse shortly afterwards. Move the injected blastocyst aside (as in **Fig. 4**) and inject the next one.
7. Transfer successfully injected blastocysts to a 35-mm dish containing a few mL of CO_2-buffered culture medium and keep in an incubator at 37°C, 5 % CO_2.
8. Repeat the injection procedure until all blastocysts are injected. ES cell suspension will tend to aggregate; repeat the preplating procedure with fresh suspension.

Either transfer successfully injected blastocysts to pseudopregnant females on the same day or culture them overnight at 37°C, 5% CO_2.

3.4. Oviduct Transfer; Uterine Transfer

3.4.1. Preparations for Oviduct Transfer

Injected zygotes are transferred on either on the same day as microinjection took place, or the next day. If oviduct transfer is carried out on the same day, about 2× 20 one-cell stage zygotes are transferred bilaterally to one recipient

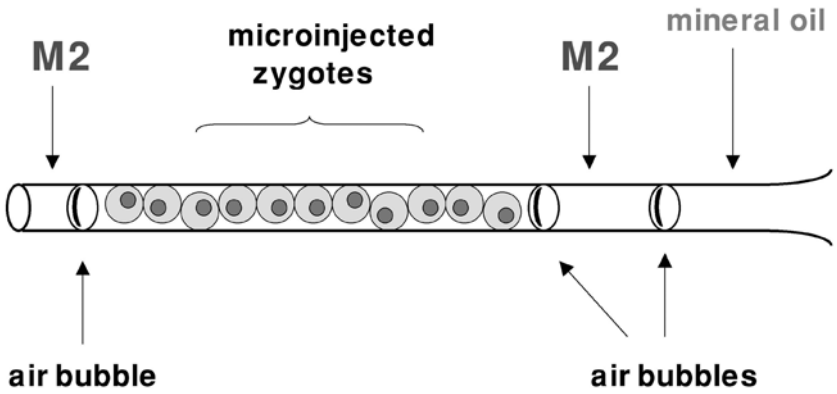


Fig. 6. Transfer pipet: microinjected zygotes are surrounded by small air bubbles, which function as markers during the oviduct transfer.

pseudopregnant female (i.e., 20 on each side). If two-cell stage embryos are transferred, 1 day later than miocroinjection, usually a smaller number of embryos suffices (2× 12–14; *see* **Notes 6** and **7**).

1. Load transfer pipets as depicted in **Fig. 6**: draw in mineral oil until it reaches the part where the pipet widens (to avoid capillary suction): M2 medium–air bubble–M2–air bubble–20x closely stacked embryos–air bubble–M2.
2. Fix the loaded transfer pipets with synthetic clay to the bench, placing their shafts containing the embryos over a wetted Kimwipe®, so as to prevent evaporation of medium and clogging of the opening. Take care not to knock over the loaded pipets.
3. Preanesthetize the mouse using an inhalation anesthetic in a small desiccator (*see* **Notes 1** and **8**) and subsequently inject the female with a sufficient dose of sedative to anesthetize the animal for at least 30 min (*see* **Notes 1** and **8**).
4. Place the mouse on an operation platform (10-cm dish with a plastic elevation positioned out of center).
5. Wet one flank with 100% ethanol and, with tweezers, clear a short horizontal "parting" in the fur of about 1 cm in length parallel to the spinal column just below the rib cage (**Fig. 7A**); grasp and lift the skin with forceps and make a small incision (with scissors); insert scissors into the incision and tear open the skin along the "parting" in the fur until an opening of approx. 6–8 mm is generated.
6. Grasp and lift the body wall with forceps and make an incision with the scissors, avoiding blood vessels; tear open in the same direction as the previous tear and attach suture to the upper "lip" of the wound (body wall).
7. Gently pull out the ovary and oviduct by the fat pad attached to the ovary; attach a serafine clamp to the fat pad, pull out the uterus a bit more and position the clamp over the flank of the mouse in such a manner that the infundibulum

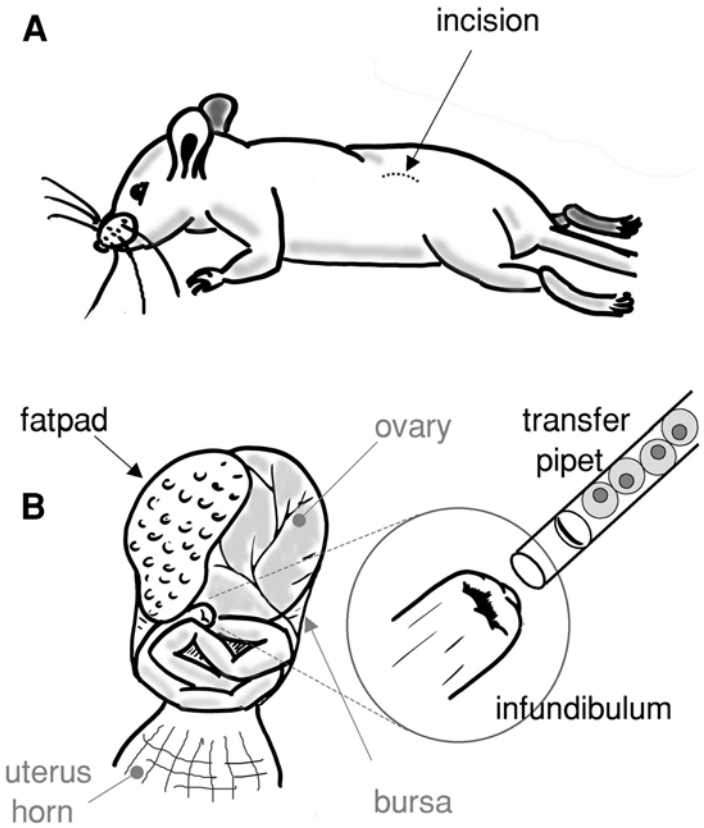


Fig. 7. Oviduct transfer. **(A)** An incision is made in the flank of the pseudopregnant female, just below the rib cage. **(B)** The ovary and oviduct are surrounded by the bursa, which needs to be ruptured for oviduct transfers. Inset: infundibulum and transfer pipet (*see* **Fig. 5**) magnified.

(i.e., the entrance to the fallopian tube), still covered by the bursa, is visible (**Fig. 7B**).

8. Transfer the mouse under a dissecting microscope, magnification 15–20×. Use fiberoptic illuminators for lighting during surgery.
9. *At this stage, take the mouthpiece of the transfer pipet in your mouth; the other end remains in synthetic clay on the microscope or table surface.*
10. Gently tear an opening into the bursa covering the ovary and infundibulum with two tweezers (number 5); avoid tearing blood vessels; if bleeding is caused inadvertently, use Kimwipe tips to soak up excessive fluids.
11. Locate the infundibulum, pick up the transfer pipet, and bring it into position (i.e., line it up with the position of the infundibulum and fallopian tube). Probe

the infundibulum carefully with the transfer pipet and, when certain of a deep enough insertion, gently expel the injected embryos by blowing.

12. While you are expelling the embryos, the air bubbles surrounding them become visible in the fallopian tube: if two or more are observed, the embryo transfer was successful. Pull out the pipet completely and transfer the mouse back under the fiberoptic illuminator.
13. Gently maneuver the uterus horn back into the body cavity using the blunt tip of the serafine clamp, close the body wall with one stitch and a tight knot, and use sutures or a wound clip to close the wound in the skin; proceed with the other side (*see* **Notes 6** and **7**).
14. When the procedure is complete, ear-mark the female, if desired (*see* **Note 9**) and return the animal to a cage.
15. *Clean all equipment used with soap and water and 70% ethanol and sterilize.*

3.4.2. Uterine Transfer[BL]

A uterine transfer is very similar to an oviduct transfer, with the exception that injected blastocysts are transferred directly into the uterus lumen. Therefore, gently pull out the uterus horn a little further than you would for an oviduct transfer (*see* **Fig. 7B**); carefully insert a sterile 27-gage needle into the uterus lumen through the uterus wall. The insertion direction should be almost parallel to the uterus horn, to avoid puncturing the uterus. It is imperative not to stretch the uterus walls too much during needle insertion, since the inner and outer muscle layers of the uterus will move in respect to each other when the needle is withdrawn and the uterus relaxes again; the consequence will be that the entrance through the hole is blocked. Remove the needle and carefully insert a glass transfer-pipet containing the injected blastocyst through into the uterus lumen. Gently aspirate blastocysts into the uterus lumen; push back the uterus into the abdominal cavity and close the animal (*see* **Subheading 4.4.1.**).

3.5. Identification of Transgenic Founders; Tail DNA Extraction

In essence, genotyping of mice, whether derived by transgenic technology or via targeted mutagenesis in ES cells, is carried out by similar procedures. Genotyping founders is much more straightforward in case of germline chimeras (ES cell manipulation; blastocyst injection) since this can be done visually. Chimeras and their offspring are discussed in Chapter 8. Transgenic founders will have to be genotyped by DNA analysis.

If expression in vivo of transgenic constructs is not embryonically lethal, litters resulting from microinjected zygotes will typically comprise 10% or more transgenic pups. Transgenic founder (F0) mice are identified through Southern blot or by polymerase chain reaction (PCR) analysis of purified mouse-tail DNA. Southern blot analysis, as opposed to PCR, offers the possibility of

actually studying the inserted transgene in terms of copy number (i.e., number of transgenes inserted; *see* **Notes 10** and **11**), rearrangements, multiple integration sites, and even genomic position. On occasion, genomic integration of a transgene occurs after the first cell division. In essence, this results in mice mosaic for transgene integration and expression. In such instances, the F0 animal will have a lower copy number than its F1 offspring. In case mosaic animals do transmit the transgene in a submendelian ratio to their F1 offspring, their F2 offspring should of course show normal mendelian segregation. Alternatively, in case of multiple chromosomal integration sites, F1 offspring within a given line may reveal a lower copy number than the F0, because multiple integration sites tend to segregate upon breeding. Unstable integrations may present sporadic similar copy number discrepancies between F0 and F1 mice.

Transgene rearrangements and subhaploid copy numbers may indicate poor or absent expression and germline transmission. Such mice would show up positive in a PCR-based identification assay but are worthless for study since no breeding lines can be established from these animals. Nevertheless, once useful transgenic founders have been identified, subsequent identification of transgenic offspring does not require Southern analysis but may be done by PCR. Southern blot and PCR analyses have been described in detail elsewhere ***(6)***. Here, a number of procedures for tail-tip DNA extraction are provided (and are described in detail elsewhere). In addition to DNA analysis, it is wise to include transgene expression analysis of several tissues of F1 animals from different founder lines. This may include both RNA and protein analysis, for instance the detection of apolipoproteins in the circulation, provided that immunologic detection reagents and/or biochemical assays are available.

3.5.1. Rapid Procedure Tail-Tip DNA Extraction

This method will allow for a rapid DNA extraction from mouse tails, which can be used for PCR-based screens without problems and have even been reported to yield DNA of sufficient quality for Southern analysis ***(8)***. However, to get rid of contaminating proteins and RNAs for an exact quantitative Southern analysis, a more thorough DNA purification might be required (*see* **Notes 10** and **11**). A relatively fast method of DNA isolation is described just below; the next section (*see* **Subheading 3.5.2.**) outlines a tail DNA purification method employing phenol extraction. Mice are genotyped by tail DNA analysis (*see* **Notes 10** and **11**). Genotyping can be done from 10 days post partum onwards. To identify the individual mice following genotyping, animals need to be marked at the time of the tail cut. Relatively young animals (i.e., 10–12 days post partum) are toe-marked; on older animals (3 weeks of age) ear-marking is an option. Consult the animal facility to adhere to the marking system used on location.

1. Collect mouse tail-tips, about 0.5 cm (10 days post partum) to 1.0 cm (3 weeks post partum), in 1.5-mL reaction tubes on ice and either store dry at –80°C or in tail mix at –20°C, if not processed immediately. It is convenient to sedate mice lightly using an inhalation sedative, especially when older mice are marked. It is possible at this time to take a small blood sample for biochemical analysis.
2. Add 660 μL tail mix and 10 μL proteinase K (20 mg/mL). Incubate overnight at 55°C, preferably while rotating. This will greatly help in dissolving and subsequently isolating the tail DNA
3. Pellet the remaining debris (hairs, bone) in a microfuge at 12,000*g* for 10–15 min at ambient temperature. Then transfer the supernatant to a fresh microfuge tube.
4. Add 0.6× vol (i.e., ± 400 μL) isopropanol, and mix by inverting the tubes *gently* (25x), allowing the DNA to precipitate in visible threads. (Vigorous shaking will hamper visible precipitation.)
5. Either pellet the DNA briefly (15–20 s, 12,000*g*) or fish the DNA clump out using a flame-polished glass Pasteur pipet.
6. Rinse the DNA that sticks to the glass tip in 70% ethanol and let the DNA air-dry briefly; transfer to 500 μL TE
7. Dissolve the DNA thoroughly by incubating it for 10–15 min at 55–65°C, followed by firm vortexing or shaking. Alternatively, we use the rotator at ambient temperature to dissolve the DNA overnight.
8. Store the DNA samples at 4°C until needed
9. 1.5 μL DNA suffices for a PCR reaction. If a Southern blot is attempted at this stage, 30–50 μL can be used.

3.5.2.Standard Procedure Tail-Tip DNA Extraction

1. Collect mouse tails and add 700 μL tail mix, or store (*see* **Subheading 3.5.1.**); add 25 μL proteinase K to each tube and incubate at 55°C overnight (in rotator).
2. Add 10 μL RNase and incubate for 1–2 h at 37°C.
3. Transfer contents of tubes to new tubes* containing 400 μL phenol-chloroform-isoamylalcohol using a cut blue tip, and shake firmly for 15 min (bench or wrist shaker) at ambient temperature.
4. Centrifuge at 12,000*g* for 30 min at ambient temperature.
5. Transfer supernatant to tubes containing 400 μL phenol-Chloroform-Isoamylalcohol, and shake (bench or wrist shaker) for 15 min at ambient temperature.
6. Centrifuge at 12,000*g* for 30 min at ambient temperature.
7. Transfer supernatant to tubes containing 400 μL chloroform to remove phenol traces, and shake 5 min at ambient temperature.
8. Centrifuge at 12,000*g* for 5–10 min at ambient temperature.
9. Transfer supernatant to tubes containing 0.6x vol (± 440 μL) isopropanol.
10. Mix by inverting the tubes *gently* (25x), allowing the DNA to precipitate visibly (*see* **Subheading 3.5.1.**).
11. Fish out DNA with a (flame-polished, slightly bent at the tip) Pasteur pipet and wash it in 1 mL 70% ethanol. If the expected amount of DNA is too low to fish

out, pellet at 12,000*g* for 10 min at ambient temperature, and wash the pellet with 70% ethanol (2x).
12. Let the DNA air-dry for a while, and then place the pipet in a tube (50–100 µL TE) until DNA comes off; dissolve th DNA by incubating for 10–15 min at 55–65°C, and subsequent incubation at 37°C, mixing firmly and repeatedly by hand until fully dissolved.
13. Determine the DNA concentration: dilute 5 µL DNA sample in 600 µL water. Concentration (in µg/µL) is: OD_{260nm} (OD_{260} DNA = 50 µg/mL) x 6; typical yield: 50–200 µg DNA
14. Perform a quantitative Southern analysis (*see* **Notes 10** and **11**).
15. DNA samples may be stored at 4°C or –20°C.

*Tubes that have been incubated overnight at 55°C with SDS inside tend to leak when phenol-chloroform-isoamylalcohol is added

3.6. Establishing Transgenic Lines; Breeding and Analysis

After identification of valuable transgenic founders and F1 mice, transgenic mouse lines will have to be established. It may be useful to genotype the animals relatively early (10 days post partum); *see* **Subheading 3.5.1.**), for instance when rapid onset of disease in a given mouse necessitates early diagnosis or compromises life span. PCR-based genotyping on 1 day postpartum mice and even on mouse blood, hair, or toes has recently been described ***(8–12)***. In addition, early genotyping will allow the researcher to sort out valuable transgenic mice from litters before weaning and to cull the mouse colony accordingly, which reduces housing expenses significantly.

If the mouse model needs to be studied in a particular genetic background, the transgenic lines need to be backcrossed to the strain of choice. Eight to 10 backcrosses of a transgenic line to an existing inbred strain, although not inbred by definition (*see* **Subheading 1.1.**) ***(1)***, produces a genetic background sufficiently homogeneous for experimental purposes. A mouse line may be bred to "homozygosity": to increase transgene expression (i.e., double copy number); such double transgenic mice are produced by transgenic male-to-transgenic female matings. If this is done within one mouse line, disease or altered behavior and even (embryonic) lethality all unrelated to the expression of the transgene may be the consequence: such conditions may be the result of recessive mutations caused by transgene integration into specific loci. X-chromosomal integrations can be particularly cumbersome in this respect, although such integrations have been known to result in unexpected and exciting discoveries ***(13)***. Y-chromosomal transgene integrations, conversely, if not silenced, make genotyping a fairly straightforward task. Sex-chromosome targeting, on the other hand, can speed up the creation of null-mutant models significantly.

Once transgenic mouse lines have been established, a typical initial screen would include a complete analysis by a qualified mouse pathologist and a full-spectrum expressional and biochemical analysis to evaluate the effect of transgene activity. Ideally, several independently derived transgenic mouse lines should be studied to validate the animal model.

The above discussion clearly shows that establishing mouse lines can be complex at times. It is therefore advisable to keep clear records of the history of all animals used in breeding programs (*see* **Note 9**). For further information, *see* **Note 12**.

4. Notes

1. To reduce stress and excessive bleeding, a brief sedation with an inhalation anesthetic may be preferred. Several inhalation and injection sedatives are approved of by animal welfare committees and are commercially available; we use ether or Metofane (methoxyflurane) as the inhalation anesthetic and 2.5% avertin (described in **ref.** ***1)*** as the sedative during microsurgery. Twenty microliters of avertin/g body weight suffices, although less is preferred and is possible if the researcher is proficient in fallopian tube transfers; on the whole, 250–300 μL per average-sized female will do. To stop bleeding, epinephrine can be used ***(1)***.
2. Although the light period is most often 6.00/7.00 AM to 6.00/7.00 PM, the day-night rhythm at which animals are kept may be adjusted to local needs/convenience. A light period from 2.00/3.00 AM to 2.00/3.00 PM is an option. The authors' experience is that fertilized oocytes, which are a bit more advanced in development at the time of microinjection, are often more successfully microinjected: slight ruffling of the plasma membrane makes it easier to penetrate it undamaged. However, since membrane ruffling signals onset of the first cleavage, this methodologic variation may mainly be an option for the experienced. Since mouse ovulation and fertilization and subsequent development of zygotes follow a preset time course, hormone injection regimens should be adjusted accordingly, as should the time of fertilized oocyte isolation.
3. For matings, female mice are always placed in with males, not vice versa. Introducing a male into an unfamiliar environment will significantly affect its performance. A surplus of plugged pseudopregnant females may be used again after 2 weeks. Unsuccessfully superovulated females (i.e., without a visible copulation plug) may be kept aside for later use as foster females.
4. Several measures are taken to reduce stickiness of the embryos and glass injection needles. Adding BSA to incubation media is one. Reducing the CO_2 concentration in the incubator is another factor that increases injection efficiency in our hands. We typically use 2.5% instead of 5% CO_2.
5. *In order to keep animals specific-pathogen free (SPF), all equipment and working surfaces should be sterilized before isolation of fertilized oocytes, microinjection, and oviduct transfer. If the afore-mentioned experimental procedures are not*

carried out within the confines of the animal facility, foster mice should be transported in filter-top cages.

6. For oviduct transfers, use transfer pipets with an inner diameter only slightly wider than that of zygotes. Too wide a transfer pipet will be relatively hard to insert into the infundibulum. We typically use transfer pipets with a shaft of 100–120 μm (OD) maximum. Ruler slides (slides with an engraved millimeter/micrometer scale) are very useful to estimate the bore size of transfer pipets.
7. It is advisable not to pull the fallopian tube out by grabbing the infundibulum: its fragility will cause it to be damaged very easily. Prying and maneuvering of the infundibulum is possible if carried out extremely gently. Some laboratories prefer unilateral oviduct transfers, and other adhere to bilateral transfers, since sometimes unilateral transfers will not result in pregnancy.
8. *An indicator for full sedation is the absence of a blinking reflex when one blows (puffs) softly on the animsl's eyes. Care should be taken to minimize perioperative stress on the animal; eyes can be covered with a paper towel wetted in 0.85% sodium chloride, or should be closed manually at regular intervals. Animals should be kept warm after surgery until fully awake.*
9. It is advisable to keep meticulous records of the history of all animals (i.e., transgenic lines, individual mice within a line, experimental and autopsy records, pedigree, and so on) at all times. We also record success rate of superovulations, natural matings, pseudomatings, oviduct transfers, litter sizes, and percent of transgenic animals obtained in a given session. A range of software is available for this purpose.
10. To carry out a quantitative Southern analysis, 15 μg (or less) DNA is fragmented by restriction endonucleases (RENs) in a 50 μL reaction volume. If REN digestion of DNA is still hampered by contaminants in solution, 1.5 μL of a 0.1 *M* spermidine solution may be added (Sigma, cat. no. S0266) to the reaction mix; alternatively, the reaction volume may be increased to 300 μL and spermidine may be added (9 μl, 0.1 *M*); however, the DNA should be precipitated before loading it onto an agarose gel. When preparing the REN digestion in the presence of spermidine, care should be taken to mix all components at room temperature; spermidine is not useful in restriction buffers without salt, for it will precipitate the DNA.
11. To estimate the copy number of a particular transgenic founder or line, 1 ng of a purified, linearized transgenic construct of 15 kb will roughly equal a haploid copy number of 1 in 15 μg control tail DNA. We typically run 0.65–0.7% agarose gels. The quality of the Southern blot after hybridization (i.e., straight bands, good separation) is greatly improved when electrophoresis is carried out at low power over a few days.
12. Several companies provide a wealth of mouse-related information via the internet: (http://www.criver.com, http://www.harlan.com, http://www.jax.org, http://www.taconic.com). A source providing a particularly extensive database on knockout and transgenic mice and related topics is "The Transgenic/Targeted Mutation Database TBASE at http://tbase.jax.org. In addition, useful web sites

and internet links can be found at http://www.bioresearchonline.com and at websites of transgenic facilities at a number of universities in Europe and the United States. Important information regarding the status of the mouse genome map, including available yeast artificial chromosome (YAC) clones, is provided by the Whitehead Institute at http://www-genome.wi.mit.edu/cgi-bin/mouse/index, the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/, and The European Collaborative Interspecific Mouse Backcross (EUCIB) consortium at http://www.hgmp.mrc.ac.uk/MBx/MBxHomepage.html.

References

1. Hogan, B., Beddington, R., Constantini, F., and Lacey, E. (1994) *Manipulating the Mouse Embryo. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Pedersen, F. and Rossant, J. (1989) *Transgenic Techniques in Mice: A Video Guide*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Pieper, F. R., de Wit, I. C., Pronck, A. C., et al. (1992) Efficient generation of functional transgenes by homologous recombination in murine zygotes. *Nucleic Acids Res.* **20,** 1259–1264.
4. Sigmund, C. D. (1993) Major approaches for generating and analyzing transgenic mice. An overview. *Hypertension* **22,** 599–607.
5. Bronson, S. K., Plaehn, E. G., Kluckman, K. D., et al. (1996) Single-copy transgenic mice with chosen-site integration. *Proc. Natl. Acad. Sci. USA* **93,** 9067–9072.
6. Sambrook, J., Fritsch E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed. (Ford, N., ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. Taketo, M., Schroeder, A. C., Mobraaten, J. E., et al. (1991) FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc. Natl. Acad. Sci. USA* **88,** 2065–2069.
8. Laird, P. W., Zijderveld, A., Linders, K., et al. (1991) Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* **19,** 4293.
9. Chen, S. Z. and Evans, G. A. (1990) A simple screening method for transgenic mice using the polymerase chain reaction. *Biotechniques* **8,** 32–33.
10. Ohhara, M., Kurosu, Y., and Esumi, M. (1994) Direct PCR of whole blood and hair shafts by microwave treatment. *Biotechniques* **17,** 726–728.
11. Malumbres, M., Mangues, R., Ferrer, N., Fu, S., Pellicer, A., et al. (1997) Isolation of high molecular weight DNA for reliable genotyping of transgenic mice. *Biotechniques* **22,** 1114–1119.
12. Henneberger, C., Grantyn, R., and Rothe, T. (2000) Rapid genotyping of newborn gene mutant mice. *J. Neurosci. Methods* **100,** 123–126.
13. Cases, O., Seif, I., Grimsby, J., et al. (1995) Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. *Science* **268,** 1763–1766.

3

In Vitro Fertilization/Cryopreservation

Susan Marschall and Martin Hrabě de Angelis

1. Introduction

The mouse has become the most profound model system for investigating the genetics and pathogenetics of human diseases. A huge number of new mouse strains has arisen, and much effort has been expended to increase the number of suitable mouse models. In nearly all animal facilities, the maintenance of breeding colonies is limited and the mouse strains have to be archived in an efficient way.

It is well recognized that cryopreservation of germ plasm provides efficient management of genetic resources, and embryo freezing is the most commonly used method to date. However, 100–500 embryos are normally required before the freezing procedure for reliable reestablishment of a breeding colony from frozen embryos ***(1)***. Thus preservation of a mutant strain may demand an expansion of the breeding colony before abolition. Compared with embryo freezing, the cryopreservation of spermatozoa offers a number of advantages. Sperm from a single male could potentially give rise to as many as 20 times more offspring than embryos from a single female. Moreover, males would not be treated with gonadotrophins, and the collection and manipulation of spermatozoa would be faster ***(2)***. Additionally, this method would facilitate the preservation of strains in which female reproductive problems are characteristic ***(3)***. However, the difference between freezing haplotypes (spermatozoa) and full genomes (embryos) has to be kept in mind. Sperm freezing is problematic when the total genome genotype is of interest, e.g., congenic or inbred strains. Recovery of frozen embryos results in animals with the genetic background of their parents. To obtain the same genetic background after recovery of frozen spermatozoa, oocyte donors from the same strain have to be used. This might

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

Table 1
Cryoprotecting Solution for Sperm Cryopreservation[a]

		Preparation		
Component	Concentration	g/40 mL	Source	Cat. no.
Raffinose	18%	7.2	Sigma, St. Louis, MO	R-7630
Skim milk	3%	1.2	DIFCO, Detroit MI	0032-17-3

1. Warm approx. 50 mL of ultrapure water up to 60°C. *See* **Note 1**.
2. Dissolve the components in 30 mL warm water and fill it up to 40 mL.
3. Centrifuge the solution at 13,792*g* (13,000 rpm; Biofuge Fresco, Hereaus, rotor #3325B) for 60 min.
4. Take the supernatant (has to be clear) and sterilize it (nonpyrogenic filter 0.45 μm; Millipore Molsheim, France, cat. no. SLH025BS).
5. Measure the osmolarity of the medium (480–500 mOsm). (*See* **Note 2**)
6. Make aliquots convenient for your usage and store them at –20°C for no more than 3 months.

[a]According to the methods described in **refs. *3*** and ***8***.

be important because several mutations lead to altered phenotypes in different genetic backgrounds. Sperm freezing of strains with maternally inherited alterations will be inappropriate if appropriate oocyte donors are not available.

The full power of the methods described will be achieved if as many laboratories as possible implement the techniques and use them.

2. Materials

2.1. Cryopreservation and Thawing of Mouse Spermatozoa

2.1.1. Freezing

1. Cryoprotecting solution (*see* **Table 1**).
2. For dissection of caudae epididymis: fine scissors, fine forceps, watchmaker's forceps; 4-well dishes (Nunc, Wiesbaden, Germany, cat. no. 176740; *see* **Note 1**); 2 wells per male, 1 filled with 200 μL 0.9% NaCl for washing, and 1 filled with 160 μL cryoprotecting solution for freezing.
3. For sperm quality evaluation: HTF medium (*see* **Note 3**; **Table 2**); 40-mm culture dishes (Nunc, cat. no. 150318); equilibrated mineral oil (**Table 2**; Sigma, St. Louis, MO, cat. no. M-8410); incubator (37°C, 5% CO_2).
4. Equipment for sample preparation: spring scissors, watchmaker's forceps; HTF medium; 0.25-mL French-type ministraws (Minitueb, Deisenhofen, Germany, cat. no. 13407/0010); 1-mL syringe; welder; labels for straw identification (e.g.,

Table 2
HTF-Medium for Sperm Quality Evaluation and In Vitro Fertilization[a]

	Preparation		
Component	mg/100 mL	Source	Cat. no.
NaCl	593.75	Sigma	S-9888
KCl	34.96	Sigma	P-5405
KH_2PO_4	5.04	Sigma	P-5655
$MgSO_4 \cdot 7H_2O$	4.93	Sigma	M-9397
Sodium lactate 60%	342 μL	Sigma	L-7900
Glucose	50.00	Sigma	G-6152
$NaHCO_3$	210.00	Sigma	S-5761
Sodium pyruvate	3.65	Sigma	P-4562
Penicillin G	7.50	Sigma	P-4687
Streptomycin	5.00	Sigma	S-1277
$CaCl_2 \cdot 2H_2O$	60.00	Sigma	C-7902
Bovine serum albumin (BSA)	400.0	Sigma	A-4378

1. Dissolve everything in 75 mL of ultrapure water (*see* **Note 1**) (except $CaCl_2 \cdot 2H_2O$ and BSA).
2. Dissolve 60 mg $CaCl_2 \cdot 2H_2O$ in 25 mL water and add it to the rest of the medium.
3. Gas the medium using a pipet immersed in the solution with a mixture of 5% CO_2, 5% O_2, and 90% N_2 for 10 min.
4. Add BSA and sterilize the solution before storage (nonpyrogenic filter 0.22 μm; Millipore, cat. no. SLGVR25KS).
5. Measure the osmolarity of the medium (260–280 mOsm) (*see* **Note 2**).
6. Store the medium at 37°C for no more than 1 week; leave the lid open.
7. Equilibrate an aliquot of the final medium with mineral oil (Sigma, cat. no. M-8410), e.g., put 25 mL HTF medium and 25 mL oil in a tube and shake it carefully; put in in the incubator overnight (open lid); use this equilibrated oil to cover drops of medium (HTF, KSOM, M2).

[a]According to the method described in **ref. *24***.

pieces of 0.5-mL French-type plastic straws (Minitueb, cat. no. 13408) and self-adhesive labels (CILS, East Sussex, UK).

5. For sample freezing: liquid nitrogen; freezing canister (insert a polystyrene block into the bottom of a syringe, heat-seal the syringe tip, and attach the syringe to an acrylic handle (**Fig. 1A**).
6. For sample storage: liquid nitrogen tank; cryopreservation cups (Sigma, cat. no. C-3928).

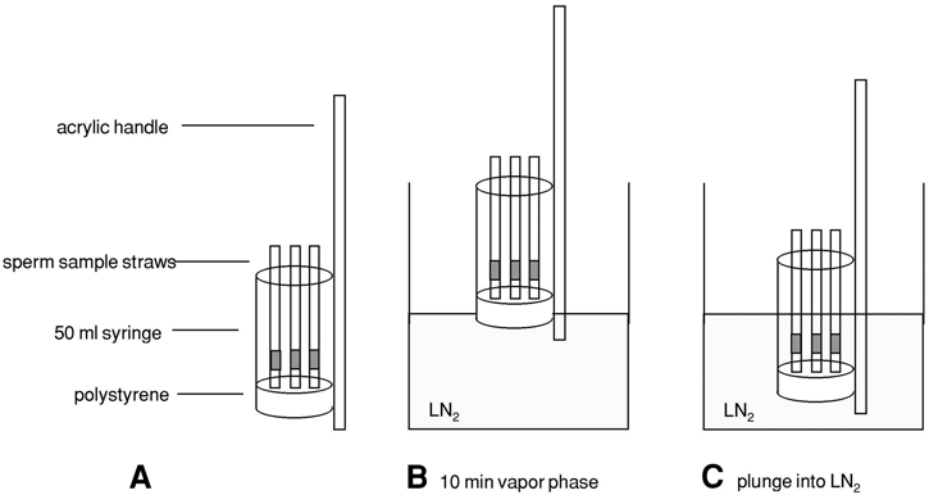


Fig. 1. Freezing of the sperm samples.

2.1.2. Thawing

1. For thawing: waterbath (37°C); 40-mm culture dishes (Nunc), HTF medium, and equilibrated mineral oil for in vitro fertilization (*see* **Subheading 2.2.**).

2.2. In Vitro Fertilization

2.2.1. Superovulation

1. Intergonan® 1000 IU (Intervet, Toenisvorst, Germany).
2. Ovogest® 1500 IU (Intervet).
3. Physiologic salt solution.
4. For injection: 1-mL syringe and 26-gage needle.

2.2.2. Oocyte Collection

1. For dissection: scissors, forceps, and preparation needles.
2. For collection: 40-mm culture dish (Nunc) with equilibrated mineral oil (Sigma; *see* **Note 1**); 58-mm culture dishes (Nunc, cat. no.150326), HTF medium (*see* **Note 3**), and equilibrated mineral oil for oocyte washing.

2.2.3. Fertilization and Embryo Handling

1. HTF medium (*see* **Note 3**).
2. KSOM medium (**Table 3**).
3. Equilibrated mineral oil.
4. 40- and 58-mm culture dishes (Nunc) (*see* **Note 1**).
5. Incubator (37°C, 5% CO_2).

Table 3
KSOM-Medium for Overnight Embryo Culture[a]

	Preparation		
Component	mg/100 mL	Source	cat. no.
NaCl	559.50	Sigma	S-9888
KCl	18.50	Sigma	P-5405
KH_2PO_4	4.75	Sigma	P-5655
$MgSO_4 \cdot 7H_2O$	4.95	Sigma	M-9397
Sodium lactate 60%	174 µL	Sigma	L-7900
Glucose	3.60	Sigma	G-6152
EDTA	0.38	Sigma	E-5134
$NaHCO_3$	210.00	Sigma	S-5761
Glutamine	14.50	Sigma	G-5763
Sodium pyruvate	2.20	Sigma	P-4562
Penicillin G	6.30	Sigma	P-4687
Streptomycin	5.00	Sigma	S-1277
Phenol red	0.10	Sigma	P-3532
Essential amino acids (50×)	1000 µL	Gibco/BRL	11130-036
Nonessential amino acids (100×)	500 µL	Gibco/BRL	11140-035
$CaCl_2 \cdot 2H_2O$	25.00	Sigma	C-7902
Bovine serum albumin BSA	100.00	Sigma	A-4378

1. Dissolve everything in 70 mL of ultrapure water (*see* **Note 1**) (except $CaCl_2 \cdot 2H_2O$ and BSA).
2. Add essential amino acids and nonessential amino acids (Gibco/BRL, Karlsruhe, Germany) and bring up to a total volume of 75 mL.
3. Dissolve 25 mg $CaCl_2 \cdot 2H_2O$ in 25 mL water and add it to the rest of the medium.
4. Gas the medium using a pipet immersed in the solution with mixture of 5% CO_2, 5% O_2, and 90% N_2 for 10 min (should have a salmon color).
5. Add BSA and sterilize the solution before storage (nonpyrogenic filter 0.22 µm; Millipore, cat. no. SLGVR25KS).
6. Measure the osmolarity of the medium (250–270 mOsm) (*see* **Note 2**).
7. Store the medium at 37°C for not more than 2 weeks; leave the lid open.

[a]According to the methods described in **refs. *28*** and ***29***.

6. For embryo handling: Positive Displacement Digital Micro Pipette (Nichiryo, Flanders, NJ, cat. no. 800-5) with fire-polished capillaries (Nichiryo, 5 μL, cap-5) or mouth pipette arranged with a yellow Eppendorf tip as a mouthpiece, silicon tubing (GLW, Wuerzburg, Germany, cat. no. SS04), connection for capillary (Becton-Dickinson, Franklin Lakes, NJ, cat. no. 5888), and capillaries (Hilgenberg, Malsfeld, Germany, cat. no. 1415025; drawn with an internal diameter of approximately 200 μm).
7. M2 medium (Sigma, cat. no. M5910): add 4.349 g/L of lactic acid (60% syrup), 0.35 g/L sodium bicarbonate, 0.06 g/L penicillin, and 0.05 g/L streptomycin; sterilize with a 0.22-μm filter (Millipore); the medium can be stored for 6 months at 4°C.
8. Hyaluronidase (Sigma, cat. no. H3884): prepare a stock solution (10 mg/10 mL M2 medium or water); sterilize with a 0.22-μm filter; make aliquots and store them at –20°C; for usage, dilute them to approximately 300 μg/mL in M2 medium.

3. Methods

3.1. Cryopreservation of Mouse Spermatozoa

Since the first reports of successful cryopreservation of mouse spermatozoa were published ***(4–6)***, numerous different methods have been described in the literature, all reporting varying degrees of success. A large number of mouse strains have been used for cryopreservation of spermatozoa and the in vitro fertilization rate, the rate of development to fetuses, and the percentage of live offspring vary among the strains (**Table 4**).

Some transgenic and wild-type mice strains have been successfully recovered using frozen-thawed spermatozoa ***(7–10)***. Before freezing valuable mouse strains, a number of prerequisites have to be met. Despite the numerous different published cryopreservation methods, the problem remains that successful methods from one laboratory often prove unsuccessful in other laboratories, indicating that underlying critical factors have not been elucidated ***(11)***. Therefore it is crucial to prove the quality and success of the chosen methods in one's own lab and to demonstrate the reliability of recovering the frozen strains.

In general, spermatozoa show extreme sensitivity to osmotic challenge, and, irrespective of the species, only a proportion of the spermatozoa survive cryopreservation ***(12)***. Owing to injuries caused by the freezing-thawing process, a decrease in fertilizing ability or quality between fresh and frozen spermatozoa is observed. Even if the sperm freezing techniques of mouse spermatozoa represent an efficient method of preserving genetically valuable strains, it is not clear whether it is really useful and effective for all known mouse strains. In particular, it has proved difficult to freeze C57BL/6 sperma-

Table 4
Overview of Mouse Strains Used for Cryopreservation

Strain	Reference
Inbred	
Balb/c	***5,13,25***
C3H/He	***13***
C3H/HeH	***7***
C3HeB/FeJ	***22***
C57BL/6N	***5,13,25***
C57BL/6J	***7,8,14***
CBA/2N	***13***
CBA/CaBlk	***26***
DBA/2N	***5,13***
kk	***25***
129/J	***14***
Outbred	
ddY	***5***
ICR	***4,5,13***
Hybrid	
B6CF1 (C57BL/6 x Balb/c)	***6***
B6C3F1(C57BL/6Nx C3H/He)	***13***
B6D2F1(C57BL/6Jx BA72N)	***3,14,27***
BDF1(C57BL/6N x xDBA/2N)	***13***
CDF1 (Balb/c x DBA/2N)	***13***
(C3H/HeH x Balb/c)F1	***7***

tozoa successfully ***(7,13,14)***. However, even for this strain cryopreservation can be used, although a partial zona dissection (PZD) has to be performed before in vitro fertilization to facilitate the penetration of the spermatozoa ***(8)***. In addition, the more advanced method of intracytoplasmic sperm injection (ICSI) may be used, involving direct injection of single spermatozoa into the oocytes ***(15,16)***. This method is also necessary when freeze-dried spermatozoa, which are completely motionless, are used ***(17)***.

It should be noted that 1) reproductive differences between mouse strains seen in vivo are also evident in vitro; 2) hybrids normally have better fertility than inbred strains; and 3) different mouse strains may have different freezing sensitivities, resulting in varying sperm quality after thawing.

This chapter gives a detailed description of the sperm freezing procedure (according to **refs. *3*** and ***8***) routinely used at the GSF Research Center. Mostly mutant males on a C3HeB/FeJ background are cryopreserved. They are usually

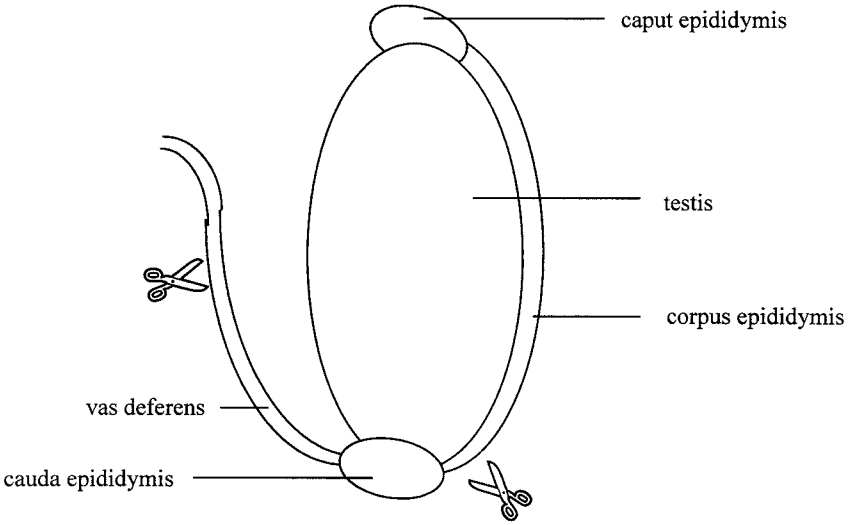


Fig. 2. Testis and epididymis from the scrotum of a mature male mouse. The cauda epididymis and vas deferens of each side are dissected because only sperm from the cauda epididymis are suitable for in vitro fertilization.

between 3 and 5 months old because spermatozoa of younger males can be less vigorous. Ideally, the males have already proved fertility. Before freezing, they should be kept single for 2 weeks and should not be mated. Since it is not always possible to meet these conditions, unseparated or old males can also be used; they can have good sperm quality.

It is recommended to establish the sperm cryopreservation techniques without changing the following protocol. If possible, it is also recommended to start with males from the same strain routinely used at the GSF. As soon as the results (cleavage rate, live offspring) are satisfactory, you can change any steps you like!

3.1.1. Collection of Spermatozoa

1. Sacrifice the male mouse by cervical dislocation and dissect the two caudae epididymides (**Fig. 2**), removing as much fat as possible. Place the tissue into 0.9% NaCl in the appropriate well of the 4-well dish (on ice, or alternatively at 37°C; *see* **ref. *3***).
2. Wash the tissue in NaCl, i.e., remove all remaining fat and large blood vessels with a watchmaker's forceps and a spring scissors.
3. Place both caudae epididymides into the cryoprotecting solution in the appropriate well of the 4-well dish (on ice). Transfer as little NaCl as possible.
4. In the cryoprotecting solution, cut each cauda epididymis and vas deferens several times with a spring scissors (under a microscope).

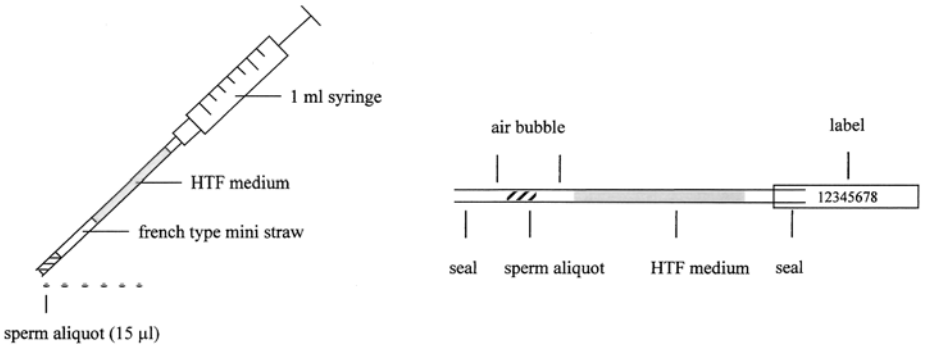


Fig. 3. Filling and configuration of the sperm sample straws.

5. Leave the 4-well dish on ice for approximately 3–5 min (on ice or at 37°C, *see* **ref. *3***). In this time the spermatozoa should disperse from the tissue (gray clouds). To get a homogeneous suspension, shake the dish carefully. (Do not pipet the suspension because this can destroy the spermatozoa!) It is a bad sign if the spermatozoa do not start to disperse by themselves. They may already be dead or immotile.

3.1.2. Freezing of Spermatozoa

1. Before preparing 10 × 15-µL aliquots per male, 2 µL of the homogeneous sperm suspension are transfered in 100 µL HTF medium covered with equilibrated oil. The sample is put in the incubator for 60 min to capacitate the spermatozoa. At the GSF Research Center the quality (defined by motility, progressive motility, and concentration) of each prefreeze and postthaw sperm sample is determined by using an IVOS Semen Analyzer (Hamilton Thorne, Beverly, MA). For each analysis, 15 µL of the diluted sperm suspension are used. The counts are carried out at 37°C. Motility is measured as any movement of a sperm head, and progressive motility reflects the count of sperm cells moving with a linear velocity greater than 50 µm/s ***(3)***. It is also possible to define sperm quality by eye. Try to evaluate 1) the ratio of living/dead spermatozoa; 2) the straightness of the swimming spermatozoa (can be seen at the border of the medium drop); and 3) the velocity of the spermatozoa. The deterioration in sperm quality caused by the freezing-thawing process, combined with bad sperm quality before freezing, can result in unsuccessful fertilization.
2. Pipet 10 × 15-µL aliquots on the lid of a dish.
3. Connect the 1-mL syringe with the straw, aspirate approx. 100 µL HTF medium, an air bubble, the sperm aliquot, and another air bubble (**Fig. 3**); the HTF medium simply acts as a weight so that the straws will not float when they are plunged into liquid nitrogen. (Don't use water because of detrimental effects to the spermatozoa!)
4. Weld both ends of the straw; be sure they are tightly closed.

5. Mark the straws by attaching the label on one end of the straw (**Fig. 2**); be sure that the label can not drop off!
6. Repeat **steps 3–5** with all aliquots.
7. Place samples in a freezing canister, put the freezing canister in the liquid vapor phase (–120°C) for 10 min (descending cooling rate of –20 to –40°C/min), and be sure that it does not sink (**Fig. 1 B**); then plunge the freezing canister directly in liquid nitrogen (–196°C) (**Fig. 1C**).
8. For storage, the samples can be collected in cryopreservation cups (previously filled with liquid nitrogen; Sigma); place five sperm sample straws from one male into each of two cryopreservation cups using a large forceps, and store them in the liquid nitrogen tank. It is strongly recommended to store the two sets of samples per male in different nitrogen tanks, ideally in two different locations to secure a backup system.

3.1.3. Thawing of Spermatozoa

1. Prepare a waterbath maintained at 37°C.
2. Place the frozen straw directly into the waterbath for 10–15 min.
3. Meanwhile prepare dishes for the in vitro fertilization (*see* **Subheading 3.2.**), 200 µL HTF medium per dish covered with equilibrated mineral oil.
4. Dry the straw with a tissue and cut both ends. (Keep straw horizontal!)
5. Remove only the sperm suspension and add 2–4 µL of the suspension (*see* **Notes 4** and **5**) to the prepared medium drops. (If by mistake medium flows out of the straw and mixes with the sperm suspension, take a greater volume of the diluted sperm suspension to get a similar sperm concentration in the final fertilization drop.)
6. Place all drops in the incubator for 1–1.5 h to capacitate the spermatozoa. (During this time they acquire their full fertilizing ability.)

3.2. Recovery of Frozen Spermatozoa with In Vitro Fertilization

In vitro fertilization is routinely used for the recovery of frozen spermatozoa. Two basic requirements have to be must be met for success: 1) capacitated spermatozoa; and 2) mature, unaged oocytes.

To capacitate spermatozoa in vitro, they must be incubated in a suitable medium (similar to the environment of the female genital tract) for 1–2 h at 37°C (*see* **Subheading 3.1.3.**).

To acquire a larger number of oocytes, superovulation has to be performed. This treatment with exogenous hormones increases the yield of naturally produced oocytes and allows specific timing of oocyte collection. Follicle growth is stimulated with pregnant mare serum gonadotropin (PMSG), and simultaneous ovulation is induced with human chorionic gonadotropin (hCG). Several parameters, e.g., age, strain, dose, and injection time of hormones, can influence the efficiency of superovulation and should be considered.

Generally F1 hybrid females are recommended for superovulation because of their high yield of oocytes in contrast to inbred strains. Nevertheless, it is often desirable to have a homogeneous genetic background for spermatozoa and oocytes, in case you must use the appropriate inbred strain even if it is a low ovulator.

The best age used for superovulation is strain-dependent. At the GSF Research Center C57BL/6 and C3H females are routinely used for in vitro fertilization. For superovulation C57BL/6 females are between 5 and 6 weeks old, and C3H females are between 6 and 8 weeks old. (They can also be used earlier.)

The amount and timing of injection are also very important. For both hormones, 5–7.5 IU per mouse are most commonly used. Nevertheless, each strain can react differently, and if nothing is known about a specific strain, a dose-response check should be performed. For C57BL/6 females we use 3.75 IU, and for C3H females we use 5 IU. hCG should be injected between 48 and 50 h after the PMSG injection. A longer or shorter interval may interfere with the response. The oocytes are then collected between 14 and 15 h after hCG injection. If they are collected earlier than 13.5 h after injection, insemination may fail. Collection after 16 h or later is also unsuitable. At this time oocytes have already begun to age and can no longer be fertilized. With this injection schedule, collection of the spermatozoa (in vitro capacitation) has to be timed as well.

Additionally, the in vitro fertilization technique opens up some opportunities to rescue endangered strains. For example, the possibility of reproducing males that are supposed to be infertile via in vitro fertilization has been demonstrated with aged males ***(18)***, and it has also been reported that spermatozoa retrieved up to 24 h after death can be used to fertilize oocytes, with the resulting zygotes developing into live offspring ***(19)***. Cryopreserving the remaining spermatozoa of the appropriate male could secure further opportunities for future research.

Successful in vitro fertilization results in two-cell embryos after overnight culture. These embryos must be transferred into pseudopregnant recipient females to develop them to term (*see* **Subheading 3.2.4.**). Alternatively, artificial insemination can be performed. This method involves direct transfer of sperm into the oviduct, for example ***(20,21)***.

3.2.1. Superovulation Procedure

1. Prepare the hormones: add 20 mL 0.9% NaCl to PMSG (Intergonan) and 30 mL 0.9% NaCl to hGG (Ovogest) and mix well, to a final concentration of 50 IU/mL.

2. Make aliquots convenient for the numbers of females you want to inject; these aliquots can be stored for 1 month at –20°C.
3. Three days before in vitro fertilization, inject the females intraperitoneally with hCG; 4 PM is the recommended injection time.
4. One day before in vitro fertilization, inject the females ip with PMSG; 6 PM is the recommended injection time.

The number of females used as oocyte donors for in vitro fertilization depends, for example, on 1) if you have high or low ovulators; 2) what cleavage rate you expect; and 3) how many pseudopregnant females you can expect for the embryo transfer. Using C3H females for the in vitro fertilization of one male, we usually take 20 animals for superovulation. On average, they produce 15 ooyctes per female. If you have a cleavage rate of 50%, for example, you will have 150 embryos. We normally transfer between 16 and 20 two-cell embryos, so one would need seven to eight pseudopregnant recipient females for the recovery of this single male. In any case, the number of oocytes you introduce depends on the number of offspring you need. With the amount of spermatozoa of one single male, it is normally no problem to fertilize several hundred oocytes.

In vitro fertilization is presently used as a sophisticated method to speed up back-, inter-, or out-crosses ***(7,22)***.

3.2.2. Oocyte Collection Procedure

1. Before oocyte collection, make sure you have prepared the spermatozoa in time! Since they need to capacitate, you should start thawing them 1–1.5 h before (*see* **Subheading 3.1.3.**). Use, for example, one fertilization dish per 5–10 females (depending on the number of ooyctes you expect), and divide the sperm suspension on these dishes (2–4 µL per dish in 200 µL HTF medium).
2. Sacrifice the females 14 h after hCG injection.
3. Dissect both oviducts of each female and transfer all oviducts in a dish with equilibrated mineral oil.
4. Transfer the oviducts in the oil of a 58-mm dish prepared for washing (approx. 400 µL HTF medium covered with oil).
5. In the oil, open the swollen ampulla with a preparation needle; the oocyte/cumulus-complexes are expelled in the mineral oil.
6. Pull the complexes in the medium drop to remove any residue of tissue or blood, which can be detrimental for the spermatozoa.

3.2.3. In Vitro Fertilization Procedure

1. Transfer the washed oocyte/cumulus complexes in the fertilization dishes (already containing capacitated spermatozoa) (*see* **Notes 4** and **5**).
2. Incubate oocytes and spermatozoa for 4 h (37°C, 5% CO_2).

3. Wash the oocytes in each fertilization dish in 3 drops (50 μL each) of KSOM medium.
4. Transfer the oocytes in 100 μL KSOM medium covered with oil for overnight culture (37°C, 5% CO_2).
5. On the next day evaluate the amount of two-cell embryos. Only embryos that have two symmetrical blastomeres are taken for the embryo transfer.
6. For embryo transfer, the two-cells are washed 3 times in drops of M2 medium (50 μL each). Add approx. 10 μL hyaluronidase in the first drop to remove remaining cumulus cells and spermatozoa on the surface.
7. Transfer the oocytes in 100 μL M2 medium covered with oil and put them on a warming plate (37°C) until you transfer them.

3.2.4. Embryo Transfer

In this chapter only the basic requirements for embryo transfer are described. Details concerning the production of pseudopregnant females and vasectomized males and the microsurgical techniques of embryo transfer are particularly described in Chapter 2 of this book and in Hogan et al. ***(23)***.

To complete their development, embryos have to be transferred into the reproductive tract of a pseudopregnant female. Pseudopregnancy is important to guarantee that implantation can occur. It is also indispensable to choose the right time of pseudopregnancy. Embyros from one cell to the morula stage are transferred into the oviduct of 0.5-day post coitum females; embryos at the blastocyst stage should be transferred in the uteri of 2.5-day post coitum females. Outbred and F1 females are particularly suitable as recipient females because they raise even small litters reliably. Pseudopregnancy is generated by mating the females (8–10 weeks, weight below 30 g) to vasectomized or genetically sterile males (for vasectomy, *see* **ref. *23***). It is recommended to mate at least 10 females per planned embryo transfer. Two females can be mated to one vasectomized male. Without the possibility of fertilization, a vaginal plug is produced, which allows precise timing of mating (has to be checked until noon on the morning after mating!). Females that have no plug or are not needed can be used again (females with plug 10–14 days after mating).

Before surgery, you can load the embryos in a mouthpipet. (Embryos should be in M2 medium.)

1. Anesthetize the female for oviduct transfer.
2. Expose the ovary, oviduct, and part of the uterus ***(23)***.
3. Open the bursa covering the oviduct and ovary with a fine spring scissors.
4. Expose the infundibulum and fix it with a small pad.
5. Carefully introduce the capillary already containing the embryos.

6. Expel the embryos in the infundibulum.
7. Carefully return the ovary and oviduct to the abdomen.
8. Close the body wall with one stitch and close the skin with a wound clip.
9. After surgery, keep the mouse warm and undisturbed until it wakes up.

3.3. Alternative Method

Depending on the liquid nitrogen storage system you have, it might be useful to freeze the sperm samples in cryovials. Therefore an alternative way of cryopreserving spermatozoa is described according to the methods of Sztein et al. ***(3)*** and Thornton et al. ***(7)***.

1. Collect caudae epididymides in 1-mL cyroprotecting solution.
2. To disperse the sperm, shake the dish gently and allow the sperm to swim out for 3 min.
3. Remove all tissue.
4. Aliquot 100 μL into cryotubes (10 × 100-μL aliquots).
5. Place samples in liquid nitrogen vapor (*see* **ref. *7*** for cooling chamber) and plunge them directly into liquid nitrogen after 10 min.
6. Thawing: put the sample in a water bath (37°C) for 2 min.
7. Take care that the tube has not been filled with liquid nitrogen before plunging it into the water bath. If nitrogen is present in the tube, wait for it to evaporate and escape first.
8. Pipet the sample into an Eppendorf tube; centrifuge at 3000 rpm for 4 min (Eppendorf 5415C microfuge; 720*g*).
9. Resuspend the pellet in MEM medium (Sigma, cat. no. M-4655); add per 100 mL: 2.5 mg NaPyruvate (Sigma, cat. no. P-2256); 0.38 mg EDTA (Sigma, cat. no. E-5134); 7 mg penicillin (Sigma, cat. no. P-4687); 5 mg streptomycin (Sigma, cat. no. S1277), 300 mg bovine serum albumin (BSA; Sigma, cat. no. A-3311).
10. Incubate for 10 min (at 37°C) and allow resuspension of the spermatozoa.
11. Use 10 μL of the sperm suspension per fertilization drop (200 μL MEM medium).

4. Notes

1. Water, plasticware, and oil can have detrimental effects. In case of failure, try another source.
2. Osmolarity of the media is important and should be checked!
3. HTF medium is not the only medium you can use. Different media may work better for other mouse strains. You can try, for example, MEM or Whittingham media.
4. Check sperm motility before coincubation with oocytes. A suspension with almost no motile cells is unlikely to be succesful!
5. A higher amount of sperm suspension introduced *may* maximize the yield of fertilized eggs.

References

1. Mobraaten, L. E. (1986) Mouse embryo cryobanking. *J. In Vitro Fertil. Embryo Transf.* **3,** 28–32.
2. Tao, J., Du, J., Kleinhans, F. W., et al. (1995) The effect of collection temperature, cooling rate and warming rate on chilling injury and cryopreservation of mouse spermatozoa. *J. Reprod. Fertil.* **104,** 231–236.
3. Sztein, J. M., Farley, J. S., Young, A. F., and Mobraaten, L. E. (1997) Motility of cryopreserved mouse spermatozoa affected by temperature of collection and rate of thawing. *Cryobiology* **35,** 46–52.
4. Okuyama, M., Isogai, S., Saga, M., Hamada, H., and Ogawa, S. (1990) In vitro fertilisation (IVF) and artificial insemination (AI) by cryopreserved spermatozoa in mouse. *J. Fertil. Implant.* **7,** 116–119.
5. Tada, N., Sato, M., Yamanoi, J., et al. (1990) Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. *J. Reprod. Fertil.* **89,** 511–516.
6. Yokoyama, M., Akiba, H., Katsuki, M., and Nomura, T. (1990) [Production of normal young following transfer of mouse embryos obtained by in vitro fertilization using cryopreserved spermatozoa]. *Jikken Dobutsu* **39,** 125–128.
7. Thornton, C. E., Brown, S. D., and Glenister, P. H. (1999) Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. *Mamm. Genome* **10,** 987–992.
8. Nakagata, N., Okamoto, M., Ueda, O., and Suzuki, H. (1997) Positive effect of partial zona-pellucida dissection on the in vitro fertilizing capacity of cryopreserved C57BL/6J transgenic mouse spermatozoa of low motility. *Biol. Reprod.* **57,** 1050–1055.
9. Nakagata, N., Ueda,S., Yamanouchi, K., et al. (1995) Cryopreservation of wild mouse spermatozoa. *Theriogenology* **48,** 635–643.
10. Nakagata, N. (1996) Use of cryopreservation techniques of embryos and spermatozoa for production of transgenic (Tg) mice and for maintenance of Tg mouse lines. *Lab. Anim. Sci.* **46,** 236–238.
11. Katkov, II and Mazur, P. (1998) Influence of centrifugation regimes on motility, yield, and cell associations of mouse spermatozoa. *J. Androl.* **19,** 232–241.
12. Watson, P. F. (1995) Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.* **7,** 871–891.
13. Nakagata, N. and Takeshima, T. (1993) Cryopreservation of mouse spermatozoa from inbred and F1 hybrid strains. *Jikken Dobutsu* **42,** 317–320.
14. Songsasen, N. and Leibo, S. P. (1997) Cryopreservation of mouse spermatozoa. II. Relationship between survival after cryopreservation and osmotic tolerance of spermatozoa from three strains of mice. *Cryobiology* **35,** 255–269.
15. Kimura, Y. and Yanagimachi, R. (1995) Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* **52,** 709–720.

16. Yanagimachi, R. (1998) Intracytoplasmic sperm injection experiments using the mouse as a model. *Hum. Reprod.* **13 (suppl 1),** 87–98.
17. Wakayama, T. and Yanagimachi, R. (1998) Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nat. Biotechnol.* **16,** 639–641.
18. Parkening, T. A. (1989) Fertilizing ability of spermatozoa from aged C57BL/6NNia mice. *J. Reprod. Fertil.* **87,** 727–733.
19. Songsasen, N., Tong, J., and Leibo, S. P. (1998) Birth of live mice derived by in vitro fertilization with spermatozoa retrieved up to twenty-four hours after death. *J. Exp. Zool.* **280,** 189–196.
20. Nakagata, N. (1992) Production of normal young following insemination of frozen-thawed mouse spermatozoa into fallopian tubes of pseudopregnant females. *Jikken Dobutsu* **41,** 519–522.
21. De Repentigny, Y. and Kothary, R. (1996) An improved method for artificial insemination of mice—oviduct transfer of spermatozoa. *Trends Genet.* **12,** 44–45.
22. Marschall, S., Huffstadt, U., Balling, R., and Hrabe de Angelis, M. (1999) Reliable recovery of inbred mouse lines using cryopreserved spermatozoa. *Mamm. Genome* **10,** 773–776.
23. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Quinn, P., Kerin, J. F., and Warnes, G. M. (1985) Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil. Steril.* **44,** 493–498.
25. Tada, N., Sato, M., Amann, E., and Ogawa, S. (1993) Effect of pre-freezing equilibration and post-thawing centrifugation on the fertilizing capacity of frozen mouse epididymal spermatozoa. *Cryoletters* **14,** 195–206.
26. Penfold, L. M. and Moore, H. D. (1993) A new method for cryopreservation of mouse spermatozoa. *J. Reprod. Fertil.* **99,** 131–134.
27. Songsasen, N. and Leibo, S. P. (1997) Cryopreservation of mouse spermatozoa. I. Effect of seeding on fertilizing ability of cryopreserved spermatozoa. *Cryobiology* **35,** 240–254.
28. Lawitts, J. A. and Biggers, J. D. (1991) Optimization of mouse embryo culture media using simplex methods. *J. Reprod. Fertil.* **91,** 543–556.
29. Ho, Y., Wigglesworth, K., Eppig, J. J., and Schultz, R. M. (1995) Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol. Reprod. Dev.* **41,** 232–238.

4

Transgene Design

J. Willem Voncken

1. Introduction

The application of transgenesis has increased exponentially since its introduction in the early 1980s, and it is still one of the most powerful methods to study gene function. As approaches to solve scientific problems became more complex, transgene design evolved alongside. At this moment the versatility in strategies and applications of transgenic animal models is both staggering and exciting. An attempt to give a fully comprehensive overview of all variations in transgene design described in the scientific press would be an illusion and would exceed the aim of this chapter. Nevertheless, the strategy for generating a transgenic animal model (i.e., design of a transgene) warrants special attention to ensure the highest chances for success. Therefore, this chapter provides a concise overview of the elementary requirements of a transgenic construct and some considerations in transgene design. In addition, a number of aspects are discussed that may influence choices early on in the process of designing and generating a transgenic mouse model.

The first choice in transgene design concerns the donor species (origin) of the transgene DNA and the biologic properties of the transgene (*see* **Subheading 1.1.**). Second, transgenes, including elements that control their expression, may either be fully derived from a native genomic locus or be assembled from genomic DNA or copy DNA (cDNA) and (heterologous) regulatory elements (*see* **Subheading 1.2.**). In addition, a range of regulatory systems offers a certain degree of control over transgene expression (*see* **Subheadings 1.3.** and **1.4.**). Size constraints, inherent to particular cloning systems, may limit the use of native regulatory elements: if a transgene becomes too large for regular plasmid- or cosmid-based vectors, or when genetic complementation is desired

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen © Humana Press Inc., Totowa, NJ

(e.g., with DNA fragments spanning large genomic deletions), one can switch to systems that allow cloning of very large DNA segments (*see* **Subheading 1.3.** and Chapter 6). A number of frequently encountered drawbacks are worth paying extra attention to; these are summarized in the notes (*see* **Notes 1–5**).

Since the purity of the microinjected DNA is the very first determinant of success, detailed protocols are provided for DNA purification methods of conventionally sized (i.e., at maximum 20 kb) transgenes (*see* **Subheading 2.**). For purification of large DNA segments, the reader is referred to Chapter 6.

1.1. Origin of the Transgene

In considering transgenic technology, the choice of origin of the transgene, i.e., the species from which the transgene originates, is an important one. The origin of a transgene may range from prokaryotes (e.g., reporter genes such as β-galactosidase; *see* Chapter 7) to worms or flies and higher eukaryotes like humans. Human DNA is applied most widely to generate transgenes in experimental biomedical research. This choice offers several advantages.

First, from a biomedical viewpoint, many known genetic disorders in humans have been mapped and extensively characterized at the molecular level: mutations or deletions have often been identified, and mutant alleles are readily available ***(1–7)***. This makes it possible to generate transgenic models with "diseased" alleles and to study structure-function relationships in the context of common (mouse) alleles.

Second, despite structural divergence, most genes have been well conserved between mouse and human. This may become an important issue when, for instance, the biologic activity of the (trans)gene product is dependent on protein-protein interactions or homodimerization. Such interactions may no longer occur between proteins originating from species that have diverged too much during evolution. It is possible that the (trans)gene product alters expression of the mouse homologe, either at a transcriptional, translational, or posttranslational (stability) level. Needless to say, these aspects are important, since they may all affect the outcome of experiments.

Third, at a practical level, screening for founder mice generated by pronuclear microinjection may be difficult when the transgene is also derived from the mouse; the same holds true for expressional analysis; such analyses would require quantitation at the DNA and mRNA levels, respectively. Structural differences between human and mouse genes make it relatively easy to screen for transgeneity. Primary sequence differences, often concentrated in noncoding regions (introns), provide a convenient way to distinguish between the transgene and the endogenous mouse gene by simple restriction endonucleases analysis. Sequence differences are not necessarily confined to noncoding

regions but may also occur in coding regions (exons): the mRNA transcribed from the human transgene and that from the endogenous murine counterpart may differ in size and/or nucleotide composition. The latter may become useful in case size similarity hampers straightforward interpretation. If gene products differ at the amino acid level, Western analysis also presents a means to discriminate between endogenous and transgene-related expression, provided antisera are available that specifically detect the (trans)gene product.

In summary, there is a wide choice of origin of the DNA used to construct a transgene. As holds for the choice of expressional control (*see* **Subheading 1.3.**), the choice of transgene origin is mostly determined by the aim of the experimental model itself. For biomedical studies, the use of human transgenes may be preferred, if not obligatory. If the study of gene function is the aim and overexpression is the experimental approach, a human gene may simply offer a practical solution for screening purposes.

1.2. Intron-Exon Boundaries

Whereas cDNA-based expression vectors on average work fine in vitro and designing a transgenic construct using cDNA may seem straightforward, cDNA–based transgenes often function in vivo, but expression levels are frequently low and such transgenes are often silenced. It appears important to preserve the intron-exon boundaries at least to some extent in a transgene. The native intron-exon structure of a gene does not need to be preserved in its entirety, however. If the size of a genomic DNA transgene is too large for conventional cloning techniques, combining cDNA sequences with a few genomic intron-exon boundaries may circumvent this problem (**Fig. 1**). Inclusion of only one generic intron in a transgene has been shown to augment transgene expression significantly ***(8,9)***. It appears that the origin of the intron need not necessarily be the same as that of the (trans)gene of interest, but may in fact be heterologous or even a hybrid of sequences from different origins. Often, (part of) the first (*noncoding*) exon attached to a promoter, is used in combination with coding sequences within a transgene; care should be taken that transgene translation starts at the intended ATG, and not in upstream heterologous exon sequences (*see* **Subheading 1.3.**). Moreover, the effect of including introns in a transgene seems independent of its position within the transcriptional unit, although 3′-positioned splice acceptor and donor sequences have been known to result in aberrant splicing products. These observations suggest that recognition and processing by splicosomes is instrumental in the observed upregulation of transgene expression. In addition, some endogenous introns appear to harbor regulatory elements with structural and functional similarities to enhancers, locus control regions (LCRs), or matrix attachment

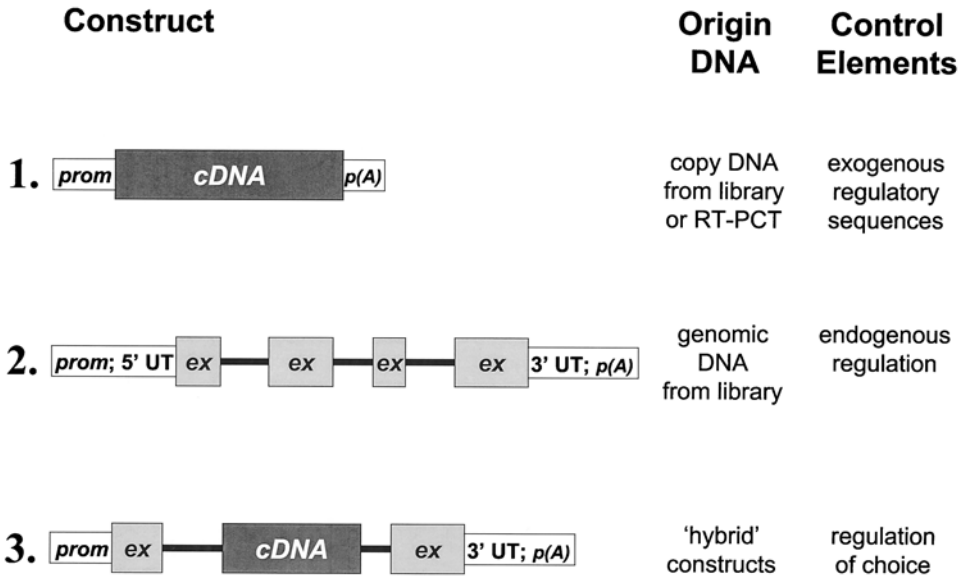


Fig. 1. Schematic overview of basic construct design. The basis of the above scheme is the use of eukaryotic coding sequences. The origin of the coding sequences is indicated in the figure. Since regulatory regions are usually not cloned along with cDNA, these have to be provided "separately" and are most often not endogenous (1). Endogenous regulatory elements may be included when the DNA originated from a genomic clone (2). The experimenter has a certain degree of freedom to tailor transgene design to specific requirements (3; *see* also **Fig. 3**). The example depicts a transgene constructed in part of genomic and cDNA sequences. Choice of cDNA and or genomic DNA-based transgenes is discussed in **Subheading 1.2.** *Prom*, promoter sequences; *ex*, exon; *p(A)*, poly(A$^+$) signal; 5′ and 3′ UT, 5′ and 3′ untranslated regions; thin black lines, introns (2, 3); RT-PCR, reverse transcriptase polymerase chain reaction.

regions (MARs) (*see* **Subheading 2.2.**) which direct transgene expression in a position-independent or cell type-specific fashion ***(9–17)***.

1.3. Endogenous Regulatory Elements; Transgene Size

The choice of regulatory elements that drive transgene expression is broad (**Fig. 2**) and is primarily determined by the aim of the model. However, in all instances, a number of indispensable elements that control gene expression need to be included in a transgene. The promoter, the region of DNA at which gene expression is initiated by binding of the RNA polymerase transcriptional machinery, is the most basic and essential element controlling gene expression. The promoter region should comprise a Kozak/ATG sequence at which transgene translation commences (*see* **Subheading 1.2.**) ***(18)***. If the expression

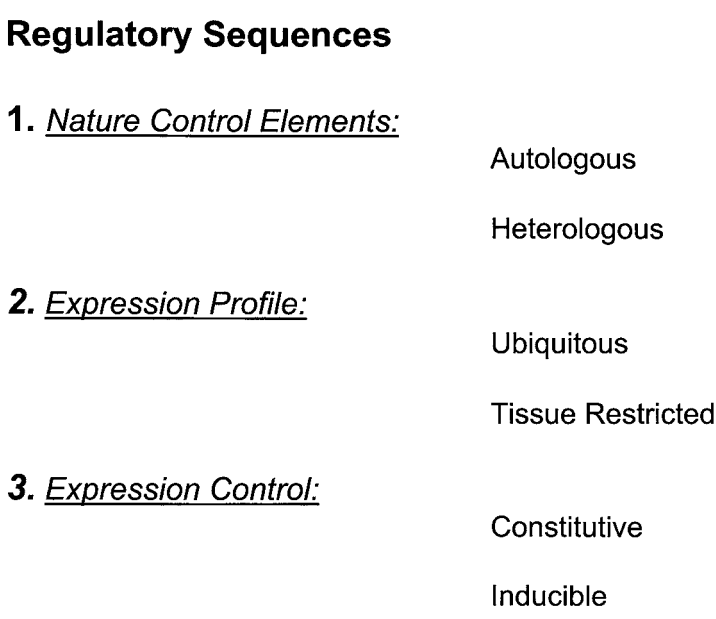


Fig. 2. Regulatory sequences in transgene design. Depending on the nature of the animal model and its specific application, there are numerous choices so far as regulation of transgene expression is concerned; such regulatory control may comprise more than a promoter only (*see* **Subheading 1.3.**). 1, Eukaryotic regulatory sequences may be derived from the gene of interest, i.e., autologous (*see* **Subheading 1.3.**) or from a different gene; 2, the required expression profile may be systemic or tissue specific (*see* **Subheadings 1.3.** and **1.4.**); alternatively, (over)expression in all tissues may be achieved with more general promoters; 3, finally, specific animal models or embryonic lethality may dictate the need for an inducible expression system (*see* **Subheading 1.4.**). Regulatory sequences of viral origin are widely used to drive transgene expression and frequently confer tissue-specific expression characteristics to a transgene. Reporter genes are often derived from prokaryotic coding sequences (*see* Chapter 7), as are the (heterologous) regulatory elements in inducible expression systems (*see* Chapters 5 and 9).

pattern of a transgene needs to parallel that of the endogenous mouse gene, one needs to include native regulatory elements. Regulatory elements can be included that augment transgene expression, such as enhancers, which typically act in an orientation-independent manner. MARs, scaffold attachment regions (SARs), and chromosomal insulators are believed to insulate (trans)gene expression from influences of surrounding chromatin ***(15)***. LCRs confer position-independent and copy number-dependent expressional characteristics to a transgene. In addition, LCRs provide transgene expression at physiologic

levels, and often with cell lineage-specific enhancer activity. The application of LCRs in transgenesis is discussed in detail elsewhere (reviewed in **ref. *15***). The advantage of including such elements in transgenes is obvious: whereas transgenes with "minimal" promoters may become inactive by insertion into transcriptionally silent chromatin, transgenes carrying, for instance, LCRs will not. However, not all endogenous loci contain such elements and most often their position relative to coding regions within the locus is not known. If faithful reproduction of the endogenous expression profile is required (*see* also **Subheading 1.4.**), without actual knowledge of the position of the regulatory element within a transgene, there is an obvious advantage to using large DNA segments as transgenes (*see* **Subheading 1.3.**).

In the early days of transgenesis, it was often difficult to obtain faithful transgene expression patterns, i.e., those that parallel expression of their endogenous counterparts, for a number of reasons (e.g., lack of knowledge in regard to nature and location of regulatory sequences of a locus; size restrictions of cloning systems). The use of a full-length relatively small mammalian gene (i.e.,15–20 kb), including 5′ and 3′ and internal regulatory regions, may yield faithful transgene expression patterns. In such a fortunate situation not only coding sequences, but also cell lineage-specific and other regulatory elements are located within or close to the intron-exon structure of a locus. However, the exact location of elements that exert transcriptional control over a (trans)gene of interest need not always be known and often these may be many kilobases removed from the actual transcriptional start site.

For a number of applications, like genetic complementation of large deletions and gene therapy, it is imperative to include such regulatory features in a transgene ***(19–21)***. Fortunately, when transgenes become too large (i.e., up to 100–150 kb) for "conventional" plasmid-based cloning, there are a number of modern cloning techniques that have overcome this hurdle: one needs to resort to cloning systems employing P1 artificial chromosomes (PACs), yeast artificial chromosomes (YACs), or bacterial artificial chromosomes (BACs) (*see* Chapter 6). In principle, any gene can be cloned into these systems. Exceedingly large YACs (>500 kb) are transferred into embryonic stem cells first and via this route are used to generate transgenic mice (*see* Chapter 6). An obvious and important advantage of using large stretches of genomic DNA is that with these systems the chances of obtaining cell lineage-specific, integration site-independent, and copy number-dependent expression characteristics are greatly improved ***(22,23)***.

1.4. Heterologous Regulatory Elements

If overexpression or ectopic expression is required, general-type heterologous promoters (e.g., such as widely applied viral promoters) and/or enhancers

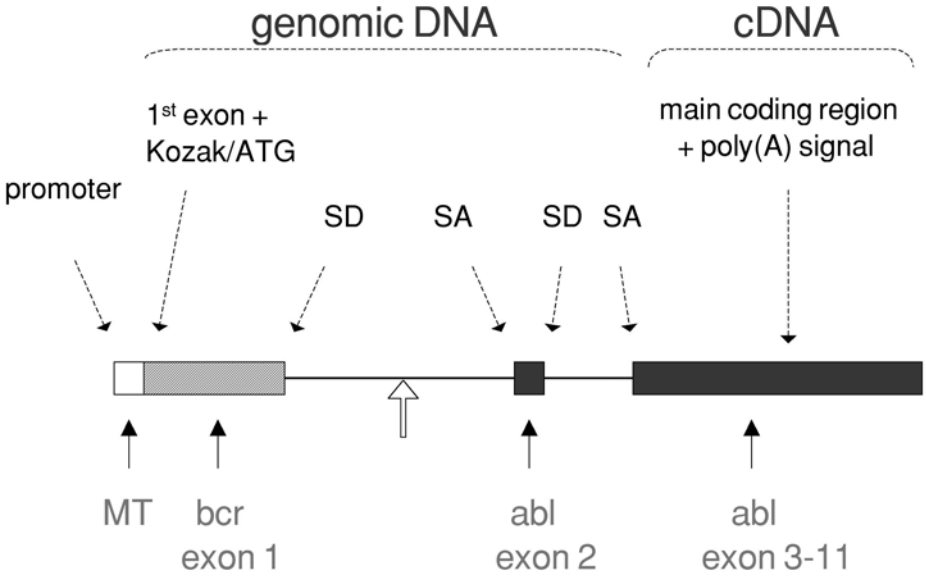


Fig. 3. A textbook example of transgene design. The transgene depicted was constructed to study the development of Philadelphia-positive childhood leukemia in a mouse model. The Philadelphia chromosome, the hallmark of this clinical type of leukemia, results from a reciprocal translocation between chromosomes 9 and 22. As a result, the *BCR* locus and the *ABL* locus become joined. The genomic *ABL* locus itself is more than 200 kb in size, the first intron spanning approximately 175 kb. Breakpoints within the *ABL* locus are known to occur relatively 5′ within the first intron. The transgene harbors a heterologous (*see* **Subheading 1.4.**) general-type metallothioneine promoter ***(24)***. The first *BCR* exon plus part of the first intron, which is fused (open arrow) to a short part of the first *ABL* introns and the second *ABL* exon, provides a splice donor (SD) and a splice acceptor (SA) site and preserves a simple but truthful mammalian intron-exon structure (*see* **Subheading 1.2.**); an additional SD/SA pair comes from the *ABL* exon 2/intron 2 and intron 2/exon 3 boundaries. The main body of *ABL* exons, which spans about 32 kb, was cloned into the transgene as a cDNA segment. In this configuration, the transgene spans a mere 10 kb. (Adapted from **ref. *46***.)

are widely used. The use of heterologous and autologous (i.e., endogenous to the gene in interest) regulatory elements is often combined (*see*, for example, **Figs. 1** and **3**). The very first transgenic mouse models generated made use of the general-type metallothioneine promoter (pMT) ***(24–27)***. This promoter was used to control expression of human, rat, or viral transgenes and, although showing a relatively high level of basal expression, proved to be further inducible with glucocorticoids, heavy metals, or a bacterial endotoxin (lipopolysac-

charide; LPS) ***(28,29)***. The use of heterologous promoters and other regulatory elements has become widespread and as indicated before, is determined primarily by the aim of the animal model (**Fig. 2**).

To ensure global and ubiquitous expression of a particular transgene, general-type promoters, such as those derived from histones, β-actin, or housekeeping genes [e.g., phosphoglycerate kinase (PGK); *see* Chapter 8], but also viral promoters are often applied. Needless to say, if a heterologous promoter is chosen to drive transgene expression, one should adhere to those promoters that have been proved to function in vivo, or should test the novel system thoroughly first. In the latter case, the experimenter should realize that in vitro expression characteristics of novel promoters may be very different than those in vivo. It is therefore strongly recommended to test novel promoters in vivo before they are used in a transgenic animal model.

If tissue-restricted expression patterns are required, specific promoters are chosen that confer this selectivity. To determine the minimal requirements for tissue-restricted expression of a particular promoter, the promoter needs to be dissected at the molecular level. Classical promoter studies can be applied in vitro and in vivo to map the elements present within and surrounding a gene of interest. The promoter is tested in vivo by fusing it to reporter genes such as β-galactosidase, CAT, luciferase or green-fluorescent protein (GFP; *see* Chapter 7). This approach is standard for examining spatiotemporal expression patterns and tissue specificity of native promoter sequences in vivo.

To bypass embryonic lethality of a transgene or to study the effects of tissue-restricted transgene (over)expression, tissue-specific, inducible, or combinations of these regulatory systems (binary transgenic systems) may be employed. Several binary transgenic systems have been developed, often employing prokaryotic expression control systems ***(30–37)***. A number of exciting applications of transgenic technology are described in Chapters 5, 6, *7*, 9, and 12.

2. Materials

2.1. Transgene Release and DNA Preparation

2.1.1. Transgene Release

1. Restriction endonuclease(s) of choice.
2. Spermidine (Sigma, cat. no. S0266).
3. Ethidium bromide (10,000X stock = 5 mg/mL in sterile water).
4. Phenol-chloroform-isoamylalcohol (24 : 24 : 1; Gibco, cat. no. 15593-031).
5. 2 *M* Sodium acetate, pH 5.0.
6. 96% Ethanol.
7. 70% Ethanol.
8. TE buffer: 10 m*M* Tris-HCl, pH 7.2–7.6, 0.1 m*M* EDTA.

2.1.2. Low Melting Point Agarose and Acid-Phenol Extraction

1. Low melting point agarose (e.g., Seaplaque GTG; FMC, cat. no. 50110 or Bio-Rad, cat. no. 162-0100).
2. Electrophoresis buffer: TAE (0.04 *M* Tris-acetate,1 m*M* EDTA) or TBE (0.09 *M* Tris-borate, pH 8.0, 2 m*M* EDTA).
3. Acid phenol : Phenol saturated with 0.3 *M* sodium acetate, pH 5.0; store at –20°C in the dark.
4. Phenol-chloroform-isoamylalcohol (24 : 24 : 1).
5. 2 *M* Sodium acetate, pH 5.0.
6. 96% Ethanol.
7. 70% Ethanol.
8. TE buffer: 10 m*M* Tris-HCl, pH 7.2–7.6, 1 m*M* EDTA.

2.1.3. Electroelution

1. Electrophoresis buffer: TBE (0.09 *M* Tris-borate, pH 8.0, 2 m*M* EDTA).
2. Electro-elution buffer: TBE (0.09 *M* Tris-borate, pH 8.0, 2 m*M* EDTA).
3. Dialysis membrane (Micropore; MWCO: 3500).
4. Microtrap cups ("Little Blue Tank" elctrophoresis system; ISCO, Lincoln, Nebraska); instructions provided by the manufacturer.
5. Phenol-chloroform-isoamylalcohol (24 : 24 : 1).
6. 2 *M* Sodium acetate, pH 5.0.
7. 96% Ethanol.
8. 70% Ethanol.
9. TE buffer: 10 m*M* Tris-HCl, pH 7.2–7.6, 1 m*M* EDTA.

2.2. DNA Purification

1. Elutip-D minicolumns (Schleicher & Schuell, cat. no. 27370).
2. 3- and 5-mL polypropylene syringes.
3. Disposable filters (Schleicher & Schuell, Uniflo 0.45 μm, cat. no. 02340).
4. 1 *M* Tris-HCl, pH 7.4.
5. 0.5 *M* EDTA, pH 8.0.
6. 5 *M* Sodium chloride, TC (tissue culture) grade.
7. Elutip buff. I: 20 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 0.2 *M* NaCl TC grade.
8. Elutip buff. II: 20 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 1.0 *M* NaCl TC grade.
9. TE buffer for microinjection: 10 m*M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA.

3. Methods

3.1. Transgene Release, DNA Preparation

The transgene is released from vector sequences by restriction endonuclease (REN) digestion. Removal of prokaryotic sequences should be carried out as completely as possible (*see* **Note 1**). Depending on the materials available, several methods can be pursued to extract the linearized transgene from

agarose gels: acid phenol extraction (*see* **Subheading 3.1.2.**), agarose treatment (*see* **Note 6**), and electroelution (*see* **Subheading 3.1.3.**). Electroelution is performed by trapping DNA onto a dialysis membrane. In practice, DNA is electroeluted from gel slices inside dialysis tubing or by using a system commercially marketed by ISCO. Since the latter is simpler to use, this approach is described in this subheading. For standard electroelution using dialysis tubing, we refer the reader elsewhere ***(38)***. For subsequent purification of DNA for microinjection, we usually use Elutip-D columns, with highly satisfactory and consistent results (*see* **Subheading 3.1.4.** and **Note 7**).

3.1.1. Transgene Release

1. For regular sized transgenes, 15 μg of transgene DNA is sufficient to purify for microinjection. For practical reasons, we usually isolate up to 50 μg DNA. Vector sequences are typically removed in a relatively large reaction volume (i.e., 400–600 μL), in the presence of spermidine to ensure full digestion at all restriction sites.
2. Run a REN digestion check (10–15 μL digested DNA) and stop the reaction by adding 2 μL of 0.5 *M* EDTA. At this stage the DNA may be mixed with loading buffer and directly loaded onto a preparative agarose gel, or, alternatively, extracted by phenol-chloroform-isoamylalcohol extracted first.
3. Separate the transgene and vector sequences by electrophoresis overnight at low power. On the next day, cut out fragments (*see* **Note 6**). There are several alternative methods to work up transgene DNA subsequently by electrophoresis (*see* **Subheadings 3.1.2.** and **3.1.3.**).

3.1.2. Low Melting Point Agarose and Acid-Phenol Extraction

See **Note 6** and **ref. *39***.

1. Low melting point agarose (LMA) gels are cast and left to polymerize for about 2 h at 4°C. To separate DNA fragments on LMA gels, a somewhat higher percentage of agarose should be used than for regular agarose gels.
2. After electrophoresis, gel slices are collected in 1.5-mL Eppendorf tubes (*see* **Note 9**) and briefly spun down to collect at the bottom of the tubes (max. 500 μL agarose clumps/tube).
3. Incubate tubes at 65°C for 15 min; place acid phenol at 65°C as well.
4. Add 1 vol warm phenol and mix thoroughly (keep the individual tubes at 65°C while handling); spin at full speed for 8 min at ambient temperature (Eppendorf centrifuge).
5. Transfer as much as possible of the supernatant to 500 μL acid phenol, mix, and spin at full speed for 5 min.
6. Collect the supernatant (avoid interphase), add 500 μL phenol-chloroform-isoamylalcohol, mix well, and spin at full speed for 5 min at ambient room temperature.

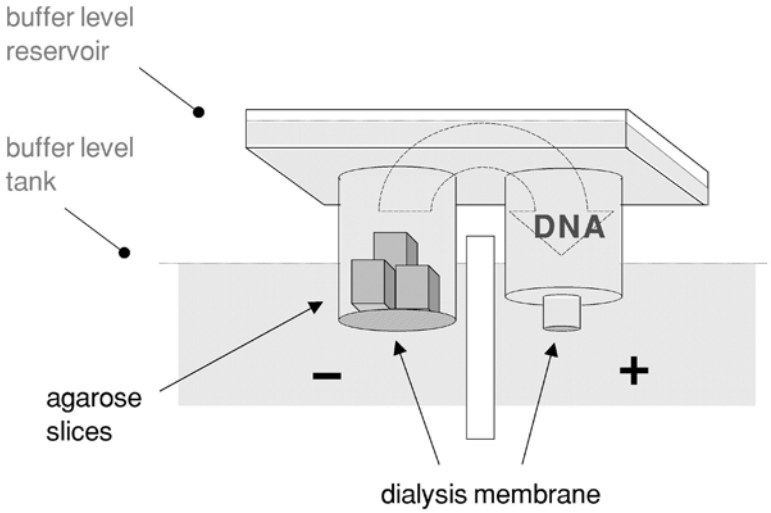


Fig. 4. Schematic representation of the "Little Blue Tank" electroelution system. The two cylindrical reservoirs communicate through a buffer compartment on top; in an electric field, DNA migrates (open arrow) from the agarose slices (in large reservoir, left) onto the dialysis membrane (in small reservoir, right) (*see* **Subheading 3.1.3.**).

7. Estimate volume of supernatant; add 1/10th vol 2 *M* sodium acetate, pH 5.0 (*see* **Note 8**).
8. Fill the tube using 96% ethanol and precipitate DNA at –20°C overnight or for 15–20 min on dry ice.
9. Spin down the precipitated DNA at full speed for 20 min at 4°C and wash twice with ice-cold 70% ethanol.
10. Remove ethanol with a drawn-out Pasteur pipet and air-dry briefly; pool samples in 100 µL TE buffer.

3.1.3. Electroelution

1. Load digested DNA onto an agarose gel; carry out electrophoresis overnight in TBE buffer.
2. Boil dialysis tubing for 10 min in dH_2O/1 m*M* EDTA; use immediately or store at 4°C under water; rinse before use: 2× dH_2O, 1× buffer. Slice dialysis tubing open carefully, and fix dialysis membranes (inside facing up) to both the large and small reservoir (**Fig. 4**).
3. Collect gel sections containing the desired DNA fragment; place these in the largest of the cups, next to each other. Fill reservoirs with TBE buffer and place in electrophoresis unit (**Fig. 4**).
4. Run at high current for 2–3 h.

5. Reverse current for 60 s to release DNA from dialysis membrane. At this point it is advisable to verify over a UV light (*see* **Note 9**) that all DNA has moved out of the gel slices and has migrated onto the dialysis membrane in the small reservoir.
6. Carefully remove most buffer from the small reservoir, leaving approximately 200 μL. Rinse the dialysis membrane thoroughly by pipeting up and down repeatedly; wash the membrane with a small volume of clean TBE buffer.
7. Transfer DNA to clean Eppendorf tube and clean DNA by phenol-chloroform-isoamylalcohol extraction.
8. Precipitate DNA by adding 1/10th vol 2 *M* sodium acetate, pH 5.0.
9. Add 2.5x vol 96% ethanol and precipitate DNA at –20°C, overnight or 15–20 min on dry ice.
10. Spin down the precipitated DNA at full speed for 20 min at 4°C and wash twice with ice-cold 70% ethanol.
11. Remove ethanol with a drawn-out Pasteur pipet and air-dry briefly; pool samples in 100 μL TE buffer.

3.2. DNA Purification

See **Note 10**.

1. Adjust DNA solution to solute concentrations of Elutip buffer I: to 100 μL DNA in TE buffer add 9.0 μL 1 *M* Tris-HCl, pH 7.4, 0.8 μL 0.5 *M* EDTA, 20 μL 5 *M* NaCl, 370 μL dH_2O.
2. Cut off Elutip-D column just below the white matrix and activate column with 1–2 mL Elutip buffer II (5-mL syringe).
3. Wash column with 5 mL Elutip buffer I.
4. Attach filter between syringe and Elutip.
5. Load column with 400 μL DNA slowly (dropwise).
6. Wash both filter and column with 2–3 mL buffer I; remove buffer completely (i.e., flush air through) and discard filter.
7. Rinse sterile Eppendorf tube with sterile water to remove dust.
8. Elute DNA with 400 μL Elutip buffer II, very slowly.
9. Add 1 mL 96% ethanol; *no extra sodium acetate is added, and no coprecipitants are added* (*see* **Note 8**)
10. Precipitate at –20°C overnight, or 15–20 min on dry ice.
11. Pellet precipitate DNA at maximum speed for 30 min, 4°C.
12. Wash pellet twice with 70% ethanol.
13. Remove ethanol with a drawn-out Pasteur pipet and air dry briefly.
14. Dissolve pellet in 50 μL filtered (Uniflo, 0.45 μm) *special TE buffer for microinjection:* 10 m*M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA.
15. Run concentration check; make a microinjection solution of 1–5 DNA ng/μL. (Keep stock at –20°C.) Freezing aliquots of the working solution is recommended (i.e., this prevents repeated freezing and thawing).

4. Notes

1. Aside from promoter choice, the overall structure of a transgene has considerable influence on its activity. In eukaryotes, foreign DNA sequences (e.g., viral DNA, transgenes) are often methylated and inactivated as part of a host defense system against transcription of potentially harmful genes ***(40–42)***. Although all aspects of the exact mechanisms by which (trans)genes are transcriptionally silenced are not clear the presence of prokaryotic sequences (i.e., plasmid) should be minimized in a transgenic construct. [The strategy (i.e., restriction sites) by which the transgene is released from plasmid sequences is a crucial part of the transgene design.] In addition, it appears that the presence of *bona fide* intron-exon structures in a transgene circumvents some of these problems (*see* **Subheading 1.2.**).
2. Expression of the transgene may be low in several independent animal lines or not detectable at all. Although this may indicate a flaw in the design of the construct, it is possible, that the (trans)gene product causes embryonic death: transgene (over)expression interferes with normal embryogenesis. In the latter case, the use of different regulatory sequences should be considered (*see* **Subheadings 1.3.** and **1.4.**). To rule out poor construct design, it is strongly recommended to evaluate the biologic activity (i.e., basic expression) of a transgene and the size of the transgenic mRNA in cultured cells (e.g., by transient transfection or electroporation and subsequent Northern analysis).
3. High copy number insertions are often associated with a significant decrease in transgene expression as a result of silencing ***(44–45)***, most likely because these are perceived as repeats in the mammalian genome. The presence of regulatory elements that confer position-independent and copy number-dependent transgene expression may circumvent this problem. Occasionally insertion as inverted repeats may also affect transgene activity ***(28)***.
4. An inserted transgene may affect the expression of (nearby) endogenous genes, which influences the phenotype in an unforeseen manner. Transgene insertion may cause haplo-insufficiency, or when integrated in an imprinted locus or in a gene on the X chromosome, it may even cause a null-mutant phenotype, entirely unrelated to the intended model. To ascribe a certain phenotype to transgene activity, it is therefore imperative to include more than one independent transgenic line in the studies, as is standard for embryonic stem cell-mediated genetic manipulation in mice (*see* Chapters 8 and 9).
5. Although without doubt more trouble than conventional transgenesis, it is possible to study the behavior of recessive mutations in mice, by overexpression at supraphysiological levels ***(18)***. Alternatively, a (conditional) knockin for the mutation may be generated, or the transgenic lines may be backcrossed to a (conditional) knockout (*see* Chapters 8–10) for the endogenous gene ***(19)***.
6. All work with phenol should be carried out in a fume hood or otherwise properly ventilated space. A suitable alternative to acid phenol extraction is enzymatic agarose removal by agarase (Boehringer Mannheim, cat. no. 1417215). Care should be taken to adhere to the manufacturers instructions.

7. Elutip-D purification is a convenient method to purify DNA for pronuclear injection (*see* Chapter 2). Yields are not very high (loading capacity column), but the DNA obtained is ultrapure. As an alternative to Elutip-D purification, dialysis against TE buffer for microinjection is often used. However, care should be taken that the materials used are absolutely free of soap and other contaminants, since these substances have a strongly negative effect on the survival of injected fertilized eggs.
8. Since DNA for microinjection needs to be extremely pure, it is not precipitated with coprecipitants. Moreover, some coprecipitants, like Dextran T-500 (Pharmacia) for instance, are toxic to zygotes.
9. Manipulation of DNA that is to be used for generation of transgenic animals via pronuclear injection should be done with utmost care: shield the DNA as much as possible from UV light (i.e., daylight, UV light box) when in the presence of ethidium bromide, since DNA can be damaged and mutated as a result. Working surfaces and equipment (UV tray, scalpels) should be clean when one is isolating fragments from agarose gels.
10. In our experience, the slightest impurities will have a serious impact on the efficiency with which transgenic founders are generated. Often residual ethidium bromide is a source of trouble. Traces of ethidium bromide in a DNA preparation are easily detected on an agarose gel from which ethidium bromide has been omitted. If DNA is detectable in this fashion, the preparation should be reextracted with phenol-chloroform-isoamylalcohol a number of times.

References

1. Bates, G. P., Mangiarini, L., Mahal, A., and Davies, S. W. (1997) Transgenic models of Huntington's disease. *Hum. Mol. Genet.* **6,** 1633–1637.
2. Beal, M. F. (1998) Mitochondrial dysfunction in neurodegenerative diseases. *Biochim. Biophys. Acta* **1366,** 211–223.
3. Gramolini, A. O. and Jasmin, B. J. (1997) Duchenne muscular dystrophy and the neuromuscular junction: the utrophin link. *Bioessays* **19,** 747–750.
4. Erickson, R. P. (1996) Mouse models of human genetic disease: which mouse is more like a man? *Bioessays* **18,** 993–998.
5. Hsiao, K. and Prusiner, S. B. (1991) Molecular genetics and transgenic model of Gertsmann-Straussler-Scheinker disease. *Alzheimer Dis. Assoc. Disord.* **5,** 155–162.
6. Leavitt, B. R., Wellington, C. L., and Hayden, M. R. (1999) Recent insights into the molecular pathogenesis of Huntington disease. *Semin. Neurol.* **19,** 385–95.
7. Mehler, M. F. (2000) Brain dystrophin, neurogenetics and mental retardation. *Brain Res. Brain Res. Rev.* **32,** 277–307.
8. Choi, T., Huang, M., Gorman, C., and Jaenisch, R. (1991) A generic intron increases gene expression in transgenic mice. *Mol. Cell. Biol.* **11,** 3070–3074.
9. Aronow, B. J., Silbiger, R. N., Dusing, M. R., et al. (1992) Functional analysis of the human adenosine deaminase gene thymic regulatory region and its ability

to generate position-independent transgene expression. *Mol. Cell. Biol.* **12,** 4170–4185.
10. Betz, A. G., Milstein, C., Gonzalez-Fernandez, A., et al. (1994) Elements regulating somatic hypermutation of an immunoglobulin kappa gene: critical role for the intron enhancer/matrix attachment region. *Cell* **77,** 239–248.
11. Brooks, A. R., Nagy, B. P., Taylor, S., et al. (1994) Sequences containing the second-intron enhancer are essential for transcription of the human apolipoprotein B gene in the livers of transgenic mice. *Mol. Cell. Biol.* **14,** 2243–2256.
12. Charron, G., Guy, L. G., Bazinet, M., and Julien, J. P. (1995) Multiple neuron-specific enhancers in the gene coding for the human neurofilament light chain. *J. Biol. Chem.* **270,** 30604–30610.
13. Dale, T. C., Krnacik, M. J., Schmidhauser, C., et al. (1992) High-level expression of the rat whey acidic protein gene is mediated by elements in the promoter and 3′ untranslated region. *Mol. Cell. Biol.* **12,** 905–914.
14. Krnacik, M. J., Li, S., Liao, J., and Rosen, J. M. (1995) Position-independent expression of whey acidic protein transgenes. *J. Biol. Chem.* **270,** 11119–11129.
15. Li, Q., Harju, S., and Peterson, K. R. (1999) Locus control regions: coming of age at a decade plus. *Trends Genet.* **15,** 403–408.
16. Liska, D. J., Reed, M. J., Sage, E. H., and Bornstein, P. (1994) Cell-specific expression of alpha 1(I) collagen-hGH minigenes in transgenic mice. *J. Cell Biol.* **125,** 695–704.
17. Zimmerman, L., Parr, B., Lendahl, U., et al. (1994) Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. *Neuron* **12,** 11–24.
18. Kozak, M. (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* **196,** 947–950.
19. Manson, A. L., Trezise, A. E., MacVinish, L. J., et al. (1997) Complementation of null CF mice with a human CFTR YAC transgene. *EMBO J.* **16,** 4238–4249.
20. Sun, T., Jayatilake, D., Afink, G. B., et al. (2000) A human YAC transgene rescues craniofacial and neural tube development in PDGFRalpha knockout mice and uncovers a role for PDGFRalpha in prenatal lung growth. *Development* **127,** 4519–4529.
21. Hodgson, J. G., Agopyan, N., Gutekunst, C. A., et al. (1999) A YAC mouse model for Huntington's disease with full-length mutant huntington, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron* **23,** 181–192.
22. Schedl, A., Montoliu, L., Kelsey, G., and Schutz, G. (1993) A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice. *Nature* **362,** 258–261.
23. Vassaux, G., Manson, A. L., and Huxley, C. (1997) Copy number-dependent expression of a YAC-cloned human CFTR gene in a human epithelial cell line. *Gene Ther.* **4,** 618–623.
24. Palmiter, R. D., Norstedt, G., Gelinas, R. E., et al. (1983) Metallothionein-human GH fusion genes stimulate growth of mice. *Science* **222,** 809–814.
25. Palmiter, R. D., Brinster, R. L., Hammer, R. E., et al. (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* **300,** 611–615.

26. Brinster, R. L. and Palmiter, R. D. (1984) Transgenic mice containing growth hormone fusion genes. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **307,** 309–312.
27. Brinster, R. L., Chen, H. Y., Trumbauer, M., et al. (1981) Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* **27,** 223–31.
28. Palmiter, R. D., Wilkie, T. M., Chen, H. Y., and Brinster, R. L. (1984) Transmission distortion and mosaicism in an unusual transgenic mouse pedigree. *Cell* **36,** 869–77.
29. Ghoshal, K. and Jacob, S. T. (2000) Regulation of metallothionein gene expression. *Prog. Nucleic Acid Res. Mol. Biol.* **66,** 357–384.
30. Fishman, G. I. (1998) Timing is everything in life: conditional transgene expression in the cardiovascular system. *Circ. Res.* **82,** 837–844.
31. Chin, L. and DePinho, R. A. (2000) Flipping the oncogene switch: illumination of tumor maintenance and regression. *Trends Genet.* **16,** 147–50.
32. Gardner, D. P., Byrne, G. W., Ruddle, F. H., and Kappen, C. (1996) Spatial and temporal regulation of a lacZ reporter transgene in a binary transgenic mouse system. *Transgenic Res.* **5,** 37–48.
33. Furth, P. A., St Onge, L., Boger, H., et al. (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306.
34. Jaisser, F. (2000) Inducible gene expression and gene modification in transgenic mice. *J. Am. Soc. Nephrol.* **11 (Suppl 16),** S95–S100.
35. St-Onge, L., Furth, P. A., and Gruss, P. (1996) Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res.* **24,** 3875–3877.
36. Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. G. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **92,** 6522–6526.
37. Zhao, B., Magdaleno, S., Chua, S., et al. (2000) Transgenic mouse models for lung cancer. *Exp. Lung Res.* **26,** 567–579.
38. Sambrook, J., Fritsch E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed. (Ford, N., ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. Heisterkamp, N., Groffen, J., and Stephenson, J. R. (1983) The human v-abl cellular homologue. *J. Mol. Appl. Genet.* **2,** 57–68.
40. Scrable, H. and Stambrook, P. J. (1999) A genetic program for deletion of foreign DNA from the mammalian genome. *Mutat. Res.* **429,** 225–237.
41. Doerfler, W. (1991) Patterns of DNA methylation—evolutionary vestiges of foreign DNA inactivation as a host defense mechanism. A proposal. *Biol. Chem. Hoppe Seyler* **372,** 557–564.
42. Clark, A. J., Harold, G., and Yull, F. E. (1997) Mammalian cDNA and prokaryotic reporter sequences silence adjacent transgenes in transgenic mice. *Nucleic Acids Res.* **25,** 1009–1014.

43. Martin, D. I. and Whitelaw, E. (1996) The vagaries of variegating transgenes. *Bioessays* **18,** 919–923.
44. Henikoff, S. (1998) Conspiracy of silence among repeated transgenes. *Bioessays* **20,** 532–535.
45. Garrick, D., Fiering, S., Martin, D. I., and Whitelaw, E. (1998) Repeat-induced gene silencing in mammals. *Nat. Genet.* **18,** 56–59.
46. Heisterkamp, N., Jenster, G., ten Hoeve, J., et al. (1990) Acute leukaemia in bcr/abl transgenic mice. *Nature* **344,** 251–253.

5

Generating Conditional Mouse Mutants via Tetracycline-Controlled Gene Expression

Kai Schönig and Hermann Bujard

1. Introduction

With the tetracycline (Tc)-controlled gene expression system (Tet system), an experimental approach has been developed that permits alteration of individual gene activities in complex genetic systems in a truly conditional fashion, i.e., it is temporally defined, quantitative, and reversible. Monitoring the phenotypic changes that accompany such highly defined alterations has provided new insights into numerous biologic processes hereto not amenable to genetic dissection. The various versions of the Tet system ***(1–3)*** were successfully incorporated into a variety of model organisms such as *Streptomyces cerevisiae*, *Dictyostelium*, *Drosophila*, *Arabidopsis*, moss, mice, and rats (for review, *see* **refs. *3–5***).

Thus they have developed into efficient tools for the study of gene function in vivo. In particular for the mouse, which has become a widely used model for mammalian genetics, thanks to transgenesis and embryonic stem cell technology, the advent of a generally applicable conditional gene expression system adds another level of sophistication to the study of gene function. Indeed, exploiting the Tet regulatory systems in vivo is beginning to provide fundamental insights into such complex biologic processes as development, disease, and behavior ***(6–13)***.

We discuss here the basic principles underlying the Tet systems and point out essential technical and methodologic implications that should be reconciled when this regulatory system is applied to the mouse. We generally refrain from giving detailed experimental protocols, as we would merely reiterate previously described procedures ***(14–16)***, of which most are common practice in molecular and cell biology as well as in the generation of transgenic mice.

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen © Humana Press Inc., Totowa, NJ

1.1. Principles and Components

Specificity is the crucial parameter for a regulatory circuit that is superimposed from outside onto the complex regulatory networks of higher eukaryotic cells. In this regard, the Tet system indeed exhibits unusually favorable properties, which are based on three features:

1. The evolutionary distance between regulatory elements of the Tet system, which are of prokaryotic origin, appears to prevent specific interactions with essential components of the eukaryotic cell, and thus pleiotropic effects.
2. The interactions between the elements of the *Tn10*-derived tetracycline resistance operon from *E. coli*, Tet repressor (TetR), tet operator (*tetO*), on the one hand, and TetR and its inducers, particularly doxycycline (Dox), on the other hand, are unusually specific, as reflected by their respective association constants ***(17)***.
3. The effector molecules, tetracyclines, belong to a class of compounds that have been thoroughly investigated because of their importance as antibiotics widely used in animal and human medicine. There are numerous Tc derivatives to choose from, but in the past Dox has proved to be a superb effector molecule owing to its high affinity to TetR ($K_a = 3 \times 10^{12} M^{-1}$) and its excellent tissue penetration and pharmacokinetic properties ***(18)***.

The initially described and still most widely applied version of the Tet regulatory system incorporates two crucial elements ***(1)***:

1. The Tc-controlled transcription activator tTA, a fusion protein comprising the N-terminally located wild-type TetR fused to the transcription activation domain of protein 16 of the herpes simplex virus, VP16 (**Fig. 1A**).
2. A minimal (i.e., enhancerless) cytomegalovirus immediate early (IE) promoter fused to heptamerized *tetO* sequences, the cognate binding sites of TetR (**Fig. 1B**). This promoter construct is henceforth called P_{tet}-1 ***(3)*** (the variants of tTA-responsive promoters are collectively referred to as P_{tet}).

Fig. 1. *(see facing page)* Tetracycline-controlled fusion proteins and their target promoters. (**A**) Fusions between TetR/rTetR with domains capable of either activating or silencing transcription, respectively. tTA is a fusion protein between the Tet repressor of the *Tn10* Tc resistance operon of *E. coli* consisting of 207 amino acids and the 128-amino acids long carboxyterminal portion of the transactivator protein VP16 from herpes simplex virus. In tTA2, the VP16 moiety of tTA was replaced by three acidic minimal activation domains (F), each consisting of only 13 amino acids. $tTS^{Kid\text{-}1}$ is a fusion between TetR and a 61-amino acid-long KRAB domain, a transcriptional repression domain derived from the human kidney protein Kid-1. In $tTS^{Kid\text{-}1}$, the silencing moiety is connected to TetR via the nuclear localization signals (nls) derived from the SV40 Tag. (**B**) tTA/rtTA-responsive promoters. These promoters are fusions between heptamerized *tetO* sequences (indicated as gray boxes) and minimal promoters

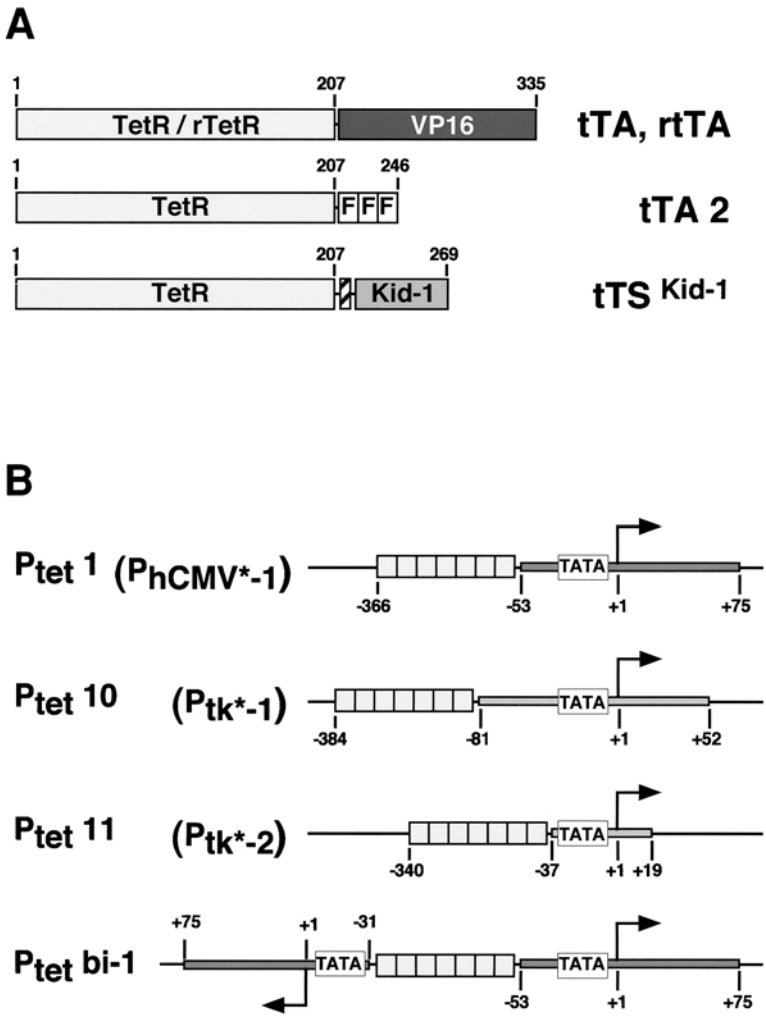


Fig. 1. *(continued)* derived from viral or cellular RNA polymerase II promoters. The original $P_{hCMV*-1}$ is derived from the human cytomegalovirus IE; the other two promoters (P_{tk*}-1 and P_{tk*}-2) are derivatives of the HSVtk promoter (1). For simplicity, all tTA/rtTA-responsive promoters from our laboratory have been renamed P_{tet}-1 to n. The HSVtk-derived promoters (P_{tet}-10, P_{tet}-11) show lower basal activity in transient expression experiments compared with P_{tet}-1. However, they cannot be activated to the level of the latter. In the bidirectional promoter P_{tet}bi-1, heptamerized *tetO* sequences are flanked by two divergently oriented hCMV-derived minimal promoters. Positions spanning promoter regions and *tet* operator sequences are indicated with respect to the start site of transcription (+1). These bidirectional promoters permit the coregulation of two genes.

When a target gene is placed under the control of P_{tet}-1, it is transcriptionally silent, provided it is inserted into a proper chromosomal locus (*see* discussion below). By contrast, in the presence of tTA, which binds to the *tetO* sequences within P_{tet}-1, this hybrid promoter can be activated to high levels. This activation is abolished or prevented by Tc or Dox, which binds to the TetR moiety of tTA, thereby preventing its association to *tetO* (**Fig. 2**). The resulting regulation factors are remarkable and reach up to five orders of magnitude in stable cell lines ***(1)*** and transgenic mice ***(16)***.

Several significant modifications and improvements of the original Tet system have been developed over the years. Most notably, the generation of the so-called reverse Tc-controlled transactivator rtTA ***(2)***. It is based on a mutant TetR, which exhibits fundamentally different binding characteristics toward *tetO*: it requires Dox or anhydrotetracycline for binding to *tetO* and thus for activating P_{tet}-1 (**Fig. 2**). Using the rtTA system, a target gene is activated by the addition of Dox. This is of particular advantage when applying Tet regulation to transgenic animals: the induction of transcription is of course greatly accelerated since saturation of a system like the body of a mouse with a small effector substance is an intrinsically more rapid process than its depletion, as is the case with the tTA system.

Despite its proven functionality ***(16)*** and usefulness ***(19,20)*** in transgenic mice (**Table 1**), the original rtTA showed some limitations:

1. At higher intracellular concentrations, rtTA can cause considerable background activity owing to a residual affinity of rtTA to *tetO* in the absence of Dox.
2. In cell culture, rtTA requires 1–2 μg/mL of Dox for full activation of rtTA. Whereas such a concentration can be readily realized in cell culture as well as in various tissues of the mouse, it is a problem for regulation in the brain once the blood-brain barrier has formed. Thus, even though Dox penetrates the blood-brain barrier fairly well, the concentrations achieved in this compartment of the mouse body are considerably lower compared with the serum level. Accordingly, rather high concentrations of Dox in the food of the animals (approx. 6 mg of Dox/g of food) are needed for full activation of rtTA ***(13,19)***.

Both limitations were abolished by the development of a new generation of rtTAs ***(21)***. They were selected by a functional screen in yeast. Although they show the same basic properties of the original rtTA, these new transactivators have lost their residual affinity to *tetO* in the absence of Dox, and—equally important—they sense Dox at considerably lower concentrations when compared with rtTA. This holds true in particular for $rtTA2^S$-M2, which is fully activated by 50–80 ng/mL of Dox (**Fig. 3**).

Several further modifications are worth mentioning. For a number of reasons, the VP16 domain of tTA/rtTA was replaced by minimal activation domains ***(22)***,

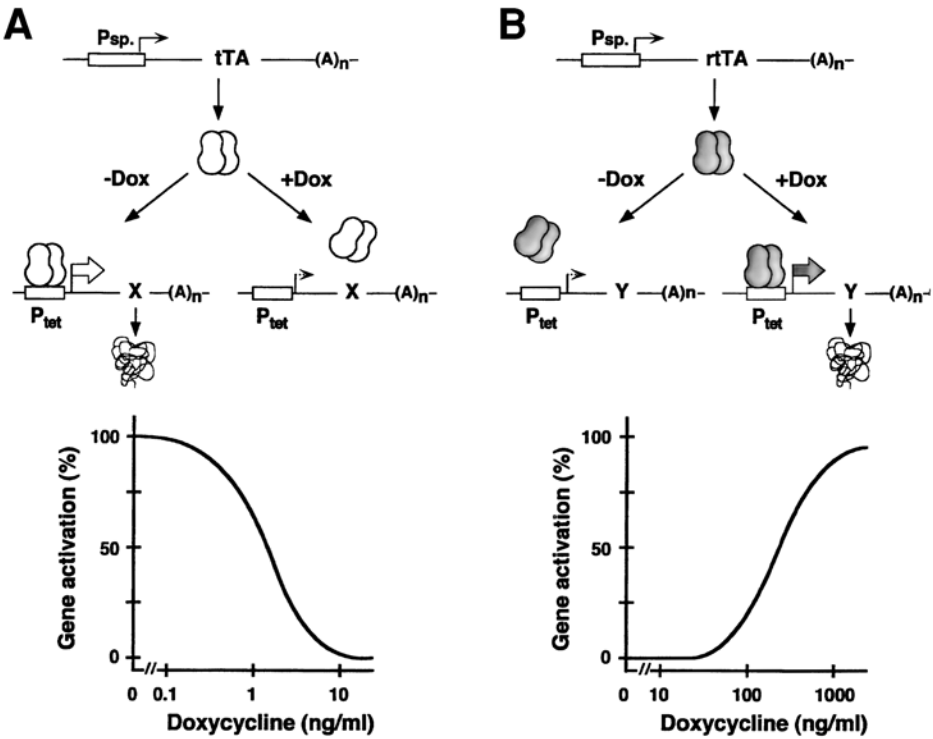


Fig. 2. Schematic outline of the Tet regulatory systems. (**A**) Top: Mode of action of the Tc-controlled transactivator (tTA). tTA binds in the absence of the effector molecule Dox to the *tetO* sequences within P_{tet} and activates transcription of gene x. Addition of Dox prevents tTA from binding and thus prevents initiation of transcription. Bottom: Dose response of Dox on tTA-dependent gene expression. Gene activity is maximal in the absence of the antibiotic, whereas increasing effector concentrations gradually decrease expression. At concentrations of 1–10 ng/mL of Dox, tTA-dependent transcription is shut off. (**B**) Top: Mechanism of action of reverse Tc-controlled transactivators (rtTA). The original rtTA is identical to tTA with the exception of four amino acid substitutions in the TetR moiety, of which three are needed to convey a reverse phenotype to the binding properties of TetR: rtTA requires Dox for binding to *tetO* sequences within P_{tet} in order to activate transcription of gene y. Bottom: Dose response of Dox on the rtTA-dependent transcription activation in HeLa cells. By increasing the effector concentration beyond 50 ng/mL Dox, rtTA-dependent gene expression is gradually stimulated and reaches maximum between 1 and 2 μg/mL of Dox (Note: dose response of novel rtTAs; **Fig. 3**).

Table 1
Mouse Lines Expressing tTA or rtTA Genes[a]

Promoter controlling trans-activator genes	Tissue specificity	tTA	rtTA	Reference
Rat α-myosin heavy chain (MHCα)[b]	Heart muscle	+		***(45)***
Mouse mammary tumor virus (MMTV) long terminal repeat[b]	Salivery gland, epithelial cells of secretory organs, skin, bone marrow B-cells	+		***(46)***
Rat insulin (RIP)	Pancreatic β- cells	+		***(33)***
Human cytomegalovirus (hCMV)	Various tissues	+	+	***(16)***
Liver-enriched activator protein (LAP)[b]	Liver	+	+	***(16,24)***
Albumin	Liver	+		***(47)***
Mouse major urinary protein (MUP)	Liver	+		***(47)***
CaMKII-α[b]	Brain	+	+	***(7,12,19)***
Prion	Brain	+		***(8)***
Neuron-specific enolase (NSE)[b]	Brain	+		***(48)***
Tek	Endothelial cells	+		***(49)***
Tie	Endothelial cells	+		***(49)***
Immunoglobulin heavy chain enhancer and SRα promoter	Hematopoietic system	+		***(9)***
Bovine keratin 6 (K6)	Keratinocytes	+		***(50)***
Bovine keratin 5 (K5)	Epidermis, hair follicle	+	+	***(51)***
Human keratin 14 (K14)	Epidermis, squamous Epithelia		+	***(11)***
Endogenous endothelin receptor B (Ednrb)	Melanocytes, neural crest	+		***(6)***
Ick proximal promotor	T-cell lineage	+		***(52)***
MHC class II Eα	Thymic, epithelial, dentritic, B cells, macrophages	+		***(53)***
Muscle creatine kinase (MCK)	Muscle	+		***(54)***
Tyrosinase	Melanocytes		+	***(10)***
Retinoblastoma gene (Rb)	Brain, lung, spleen	+		***(55)***
Intronic IgH enhancer, minimal promoter	Thymus, bone marrow	+		***(56)***
Fatty acid binding protein (Fabp)	Small intestine, cecum, Colon, bladder		+	***(57)***
Ednrb	Melanocytes, neural crest		+	***(6)***
Opsin	Photoreceptor cells		+	***(58)***
Interphotoreceptor retinoid-binding protein (IRBP)	Photoreceptor cells		+	***(58)***

Table 1 ***(Continued)***

Promoter controlling trans-activator genes	Tissue specificity	tTA	rtTA	Reference
Human CD2 regulatory region	T-cell lineage		+	***(34)***
P2 receptor locus	Olfactory sensory neurons		+	***(35)***
Clara cell (CCSP)	Respiratory epithelial cells		+	***(59)***
Human surfactant protein (SP) C	Respiratory epithelial cells		+	***(59)***
Peripheral myelin protein 22 (PMP22)	Schwann cells	+		***(37)***
Human cytokeratin 18 (K18)	Trachea, upper bronchi, submucosal glands		+	***(60)***
Smooth muscle 22α (SM22α)	Smooth muscle cells	+		***(61)***
Clara cell (CCSP)	Lung/airway	tTS		***(41)***

[a]Compilation of mouse lines expressing tTA or rtTA under the control of various promoters. Some mouse lines show an astonishing cell type specificity despite the ectopic position of the respective promoters driving the transactivator genes. Only the rTA^{LAP}-1 mouse line contains one of the new synthetic transactivators ($rtTA2^{S}$-S2). All other lines express the "classical" VP16 TetR/rTetR fusions. The compilation is probably not complete and will be outdated rapidly.

[b]Available from The Jackson Laboratory.

yielding a set of tTA/rtTAs with a graded activation potential (to be discussed below). Moreover, all tTA/rtTAs of the new generation are encoded in synthetic sequences optimized in various ways for expression in human cells ***(21)***.

Finally, a development that had considerable impact was the construction of bidirectional promoters, P_{tet}bi ***(23)***. Here, the heptamerized *tetO* sequences are flanked on both sides by minimal promoters (**Fig. 1**), which allows for the simultaneous regulation of two target genes, whereby the respective transcription units face in opposite directions. These constructs enjoy special popularity when one gene is used as reporter gene. Thus, the initial characterization of functional transgenic animals is facilitated, particularly when expression of the actual gene of interest is difficult to detect. By using the luciferase gene as the reporter, the range of regulation and the tightness of control can be readily assessed, whereas, e.g., the *lacZ* or GFP gene allows monitoring of expression unit activity *in situ*. As will be shown below, in this context the luciferase gene appears particularly useful since it permits regulated gene activities to be monitored in transgenic animals in a noninvasive way ***(24)***.

1.2. Tet Regulation in the Mouse

The prospects of possibly generating truly conditional mutants at the level of higher organisms has sparked numerous attempts to transfer the Tet system

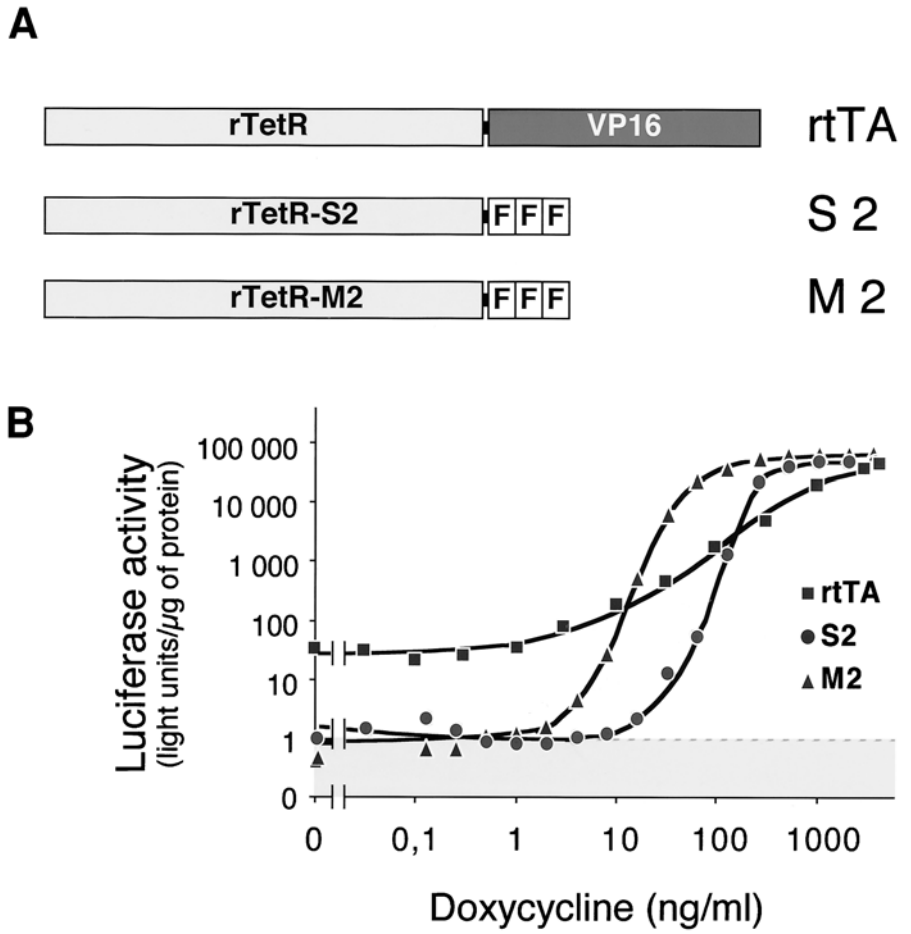


Fig. 3. The rtTA family. (**A**) Schematic outline of rtTA, rtTA2^S-S2, and rtTA2^S-M2. The S2 and M2 versions of rtTA, obtained by random mutagenesis of tTA and screening in a yeast system, contain a totally different set of mutations responsible for the reverse phenotype compared with the original rtTA. In contrast to rtTA, both versions can be fused to minimal activation domains (F) without loss of their properties as reverse transactivators. Both the S2 and the M2 versions are encoded in synthetic genes that are optimized for expression in mammalian cells. (**B**) Dose response of the three rtTAs in HeLa cells. HeLa X1-6 cells containing a stably integrated P_{tet}-1/luciferase reporter unit were stably transfected with the respective rtTA gene controlled by P_{hCMV}. In contrast to rtTA, the new versions, S2 and M2, do not show any background activity under these standard conditions (2 relative light units [rlu]/µg protein is the instrumental background). Both S2 and M2 are fully induced at lower Dox concentrations; M2 in particular, which is fully active at 50–80 ng/mL of Dox, appears suitable for transcription regulation in the brain.

also into the mouse. Obviously, the use of animals in which the activity of a gene of interest may be controlled reversibly in a temporal, spatial, and quantitative manner would open up new perspectives for the analysis of gene function in vivo. There are three experimental strategies for transferring target genes under Tet control into animals: transgenesis via DNA injection into fertilized eggs, homologous recombination via embryonic stem (ES) cell technology, and direct transfer of DNA, of recombinant viruses, or of ex vivo modified cells to defined compartments of the animal. Here we focus primarily on the first approach, which has been used most frequently in recent years.

Early studies have shown that individual genes can indeed be tightly and cell type specifically controlled in the mouse and that such gene activities can be quantitatively regulated in a noninvasive way by simply supplying the animals with Dox in the drinking water ***(16,25)***. Following the strategy most generally applied, two classes of mouse lines are generated: one controls a tTA or rtTA gene by a tissue-specific promoter, thereby directing controlled gene expression to a subset of cells, the other contains a gene of interest driven by P_{tet}-1, or preferably by a P_{tet}bi-1 construct. Crossing individuals of respective lines leads to double transgenic animals, with the activity of the target gene depending on the presence or absence of Dox in the water supply of the animal. The number of mouse lines of the two classes is rapidly increasing, and a compilation of published strains is shown in **Tables 1** and **2**. As these mouse lines will become generally available, in vivo studies will be greatly facilitated and each new and well characterized mouse strain of either class will be a precious addition to the system.

The long experience with tetracyclines in veterinary and human medicine was of great advantage for the application of the Tet system in the mouse. Thus, it was not unexpected that Tet regulation would even function in the developing embryo ***(6,26)***. Actually, since Dox not only readily penetrates the placenta but is also present in the milk of feeding mothers, tTA-controlled genes can be kept inactive all through the development of an animal until adulthood before they may be turned on by Dox depletion ***(26)***. Moreover, some tetracyclines cross the blood-brain barrier sufficiently well and thus allow regulation in the brain ***(12,13)***. Nevertheless, for regulating genes in different organs, the fact that the tissue distribution of tetracyclines can vary, as does the biologic half-life time, must be reconciled. This is particularly important if partial induction in specific tissues is attempted ***(16)***. Moreover, it should be pointed out that the kinetics of induction of tTA and rtTA differ dramatically, since in one case (tTA) the system has to be depleted of Dox, whereas in the other (rtTA) it has to be saturated with Dox. The latter process is of course intrinsically more rapid. Therefore, in studies in which genes should be kept silent during development, the rtTA principle is the preferred one. Nevertheless,

Table 2
tTA/rtTA-Responsive Mouse Lines[a]

Genes under Tet control	tTA/rtTA-responsive promoter		Reference
	P_{tet}-1	P_{tet}bi-1	
luc (L7)[b]	+		***(16)***
lacZ, nls/*luc* (nZL-2)[b,c]		+	***(27)***
lacZ	+		***(12)***
lacZ nls	+		***(49)***
GFP and *lacZ*		+	***(62)***
luc/Cre recombinase (LC-1)		+	***(24,39)***
Cre recombinase	+		***(57)***
CaMKII-Asp^{286}	+		***(12)***
Calcineurin dominant active	+		***(63)***
NR1 subunit of *N*-methyl-D-aspartate (NMDA) receptor/*lacZ*	+		***(64)***
SV40 Tag	+		***(33,47)***
Ras^{V12G}	+		***(10)***
MYC	+		***(9)***
BCR-ABL1 oncogene	+		***(65)***
Id1 transcription protein	+		***(45)***
Activated bovine protein kinase C-β	+		***(66)***
Human κ opiod receptor/*lacZ*[b]		+	***(67)***
Endothelin receptor B (EDNRB)	+		***(6)***
ErbB2 receptor tyrosine kinase	+		***(11)***
MHC II Eα	+		***(53)***
Diphtheria toxin A chain (DTA)	+		***(26)***
d-Diphtheria toxin A	+		***(35)***
Cre- IRES-tau/*lacZ*	+		***(35)***
ΔFosB[b]	+		***(48,68)***
Prion (PrP^{C})	+		***(8)***
Lymphocyte-specific protein tyrosine kinase $p56^{lck}$	+		***(34)***
cAMP response protein (CREB)	+		***(48)***
Ornithine decarboxylase (ODC)	+		***(48)***
Truncated forkhead transcription factor (FKHR)	+		***(52)***
Murine dystrophin minigene	+		***(54)***
Huntingtin fragment and *lacZ*		+	***(7)***

Table 2 *(Continued)*

Genes under Tet control	tTA/rtTA-responsive promoter		Reference
	P_{tet}-1	P_{tet}bi-1	
AML1-ETO fusion	+		***(38)***
FGF-10	+		***(69)***
BOB.1/OBF.1 transcription factor/*luc*		+	***(56)***
Glycogen synthase kinase-3β/*lacZ*		+	***(70)***
Retinoblastoma	+		***(55)***
Calcineurin autoinhibitory domain	+		***(13)***

[a]Compilation of mouse lines containing various target genes under the control of P_{tet}-1 or P_{tet}bi-1. Particularly tight control must exist for the lines containing the diphtheria toxin A chain. Very tight control has also been demonstrated for the L7 and LC-1 mouse lines. Again, this compilation is probably not complete; new lines are being described at an increasing rate. *luc*, luciferase gene; nls, nuclear localization sequence.

[b]Available from The Jackson Laboratory

[c]This mouse line expresses *lacZ* and *luc* rather asymmetrically; accordingly, relatively low luciferase activities are monitored.

cycle times of 2–5 days can be achieved, e.g., in the liver for both systems (**Fig. 4**; Schönig and Bujard, unpublished data), and partial induction in various organs is also feasible by proper adjustment of Dox in the drinking water ***(16)***. It should be noted that even prolonged exposure of the animals to Dox, including breeding of offspring, at 2 mg/mL (in the drinking water) has not yielded detectable adverse effects in the animals ***(27)***.

An impressive example for achieving highly cell type-restricted regulation by targeting the tTA/rtTA genes to a defined locus via homologous recombination has been described by Shin et al. ***(6)***. To study the role of the endothelin receptor B (EDNRB) during embryonic development, the tTA and rtTA genes, respectively, were placed under the control of the Ednrb promoter, with the P_{tet}-1 controlled Ednrb minigene replacing the endogenous Ednrb gene. By properly administering Dox to females pregnant with the respective double transgenic embryos, it was possible to determine a critical time window of 2 days within the embryonic development at which Ednrb signalling is required for melanocytes and enteric neurons to colonize their respective sites properly.

In the context of preclinical studies for gene therapy, numerous regimens were developed for the transfer of Tet-regulated genes into animals via naked DNA ***(28)***, different viral vectors ***(29)*** or ex vivo modified cells ***(30)***. Here it may suffice to point out that long-term expression and stringent Tet regulation has been demonstrated by numerous experimental approaches, for example,

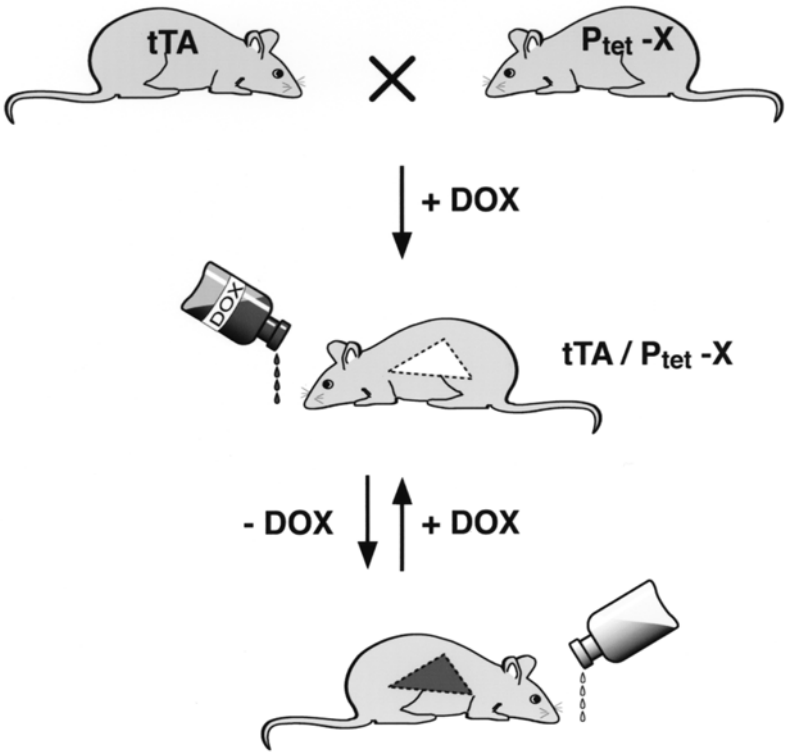


Fig. 4. Establishing Tet regulation in the mouse. The outline shows the generation of double transgenic animals from single transgenic mouse lines of which one expresses the tTA (or rtTA) gene under control of a tissue-specific promoter, whereas the other contains the target gene (x) under control of P_{tet} inserted in a transcriptionally silent but activatable (s/a) locus. Gene regulation is controlled by administration of Dox in the water supply of the animals.

by the transfer of adenoviral vectors to the brain of rats, to control tyrosine hydroxylase ***(29)***, or by injecting ex vivo engineered myoblasts into skeletal muscle of mice, to control erythropoietin and thus the hematocrit ***(30)***.

In the following discussion, we consider general points to be considered when setting up Tet regulation in vivo.

1.2.1. Generating tTA/rtTA Mouse Lines for Tissue- or Cell Type-Specific Tet Regulation

Cell type-specific Tet regulation is achieved by placing the gene encoding tTA or rtTA under appropriate control. Two obvious strategies come to mind: placing the transactivator gene directly under control of the promoter of interest

by homologous recombination via ES cell technology, or fusing the respective promoter to the transactivator gene at the DNA level followed by DNA transfer into fertilized eggs and random integration of the transcription unit into the genome. Both principles have been successfully applied.

The "precision approach" via homologous recombination appears to be the most attractive and, as discussed above, has yielded impressive results (*see* also **ref. *31***). Three points to be considered should nevertheless be discussed here:

1. In general, we have little information on the in vivo strength of the promoter targeted, which may be too strong, too weak, or even altered by the recombination event. In the first case, tTA or rtTA will be overexpressed and may cause squelching ***(32)***, a phenomenon generally observed when transcription factors are expressed out of proportion. Pleiotropic effects and even cell death may be the consequences. In the second scenario, the synthesis of tTA/rtTA may be too low even though we know that, e.g., in HeLa cells, less than 10,000 tTA molecules are sufficient for full activation of P_{tet} ***(1)***. One way to minimize the risk of the recombination approach is to use transactivators with graded activation potentials. Baron et al. ***(22)*** have described a panel of transactivators that differ in their activation strength up to 10,000-fold depending on the activation domains fused to TetR. It may therefore be wise to generate *a priori* several mouse lines with transactivators that differ in their activation potential.
2. There is increasing evidence that even small changes within a chromosomal locus may perturb the overall fidelity of the expression unit. Thus, the site of integration has to be carefully chosen and in case there is no information for an educated guess, one might again consider several different integration sites.
3. Many, if not all, promoters have rather defined windows of activity. For most questions, this is exactly what one is looking for: to superimpose Tet regulation onto the natural expression pattern of a gene. However, there are scenarios in which one would like to probe the effect of a gene product outside its natural expression profile, e.g., in developmental and differentiation processes. For this type of question, one would like to be independent of the control of the gene under study. Placing the transactivator gene under a different, ectopic regulation would be the solution.

The second approach, in which a tTA/rtTA gene is linked to a promoter and randomly integrated into the mouse genome by pronucleus injection of fertilized eggs, has been widely and successfully applied. High-fidelity cell type-restricted expression of the transactivator genes has been achieved for a number of promoters such as the promoter driving the insulin ***(33)***, the heart muscle-specific α-myosin heavy chain ***(25)***, the T-cell-specific receptor ***(34)***, and the olfactory epithelial receptor gene ***(35)***, to name just a few.

In numerous cases, however, the specificity and activity of promoters driving tTA/rtTA genes is affected by the chromosomal locus where the transcription

unit has been inserted. In general, this is a disadvantage, and as long as we do not know more about promoters in the context of various chromatin settings, we will not be able to circumvent this problem predictably. However, there are positive aspects of this phenomenon. Since the insertion site affects not only the specificity of a promoter but also its activity, there will automatically be a selection for animals in which tTA/rtTA is not overexpressed. Thus even strong promoters like the cytomegalovirus IE promoter have been used successfully to drive tTA and rtTA in transgenic mice ***(16)***. The second advantage is that, depending on the insertion site, promoters can gain artificial specificities, which sometimes restrict Tet regulation to just a subgroup of cells for which the promoter is specific. An example is the highly hepatocyte-specific tTA mouse line, TA^{LAP}-2, ***(16)*** generated with the liver-enriched activator protein (LAP) promoter ***(36)***. Thus, it can be foreseen that a number of quite useful mouse lines with highly specific, although artificial, expression patterns will be generated as "byproducts," and the main question will be how the scientific community learns about their existence.

A combination of the two approaches discussed above is the transfer of tTA/rtTA genes controlled by a promoter of interest and embedded in large fragments of chromosomal DNA, as they can be generated via BAC or YAC technology. The goal of this strategy is to reassemble the natural chromatin environment around the promoter driving the tTA/rtTA transcription unit and to achieve thereby the genuine activity profile of the respective promoters. An excellent example of this approach is described by Perea et al. ***(37)***, who succeeded in controlling via Dox a Schwann cell-specific expression of peripheral myelin protein 22 (PMP22) and showed its effect on myelination of axons. They demonstrated that demyelination caused by overexpression of PMP22 is reversible when expression is reduced to normal levels. These mouse lines appear to be interesting models for studying Charcot-Marie-Tooth disease.

How to identify a useful transactivator mouse line? The only reliable way is to cross respective DNA-positive founder animals to "indicator" mouse lines. Our laboratory has generated two such strains, the L7 line ***(16)***, in which the luciferase gene is under P_{tet}-1 control, and the nZL-2 line ***(27)***, in which both the luciferase and the *lacZ* gene are under control of P_{tet}-bi-1. Both lines are available from The Jackson Laboratories (**Table 2**). Use of the L7 line allows one to test transactivator strains for function. In many cases, this can even be done in a noninvasive way (*see* **Subheading 3.3.**) or by quantitatively assaying luciferase activity in the extracts of the respective tissues. Using the nZL-2 line, double transgenic animals can be examined for cell type-specific Tet regulation

by *in situ* X-gal staining. The two analyses allow one to select transactivator lines with respect to three important parameters:

1. Range of regulation. This is revealed by comparing luciferase activity in the presence and absence of Dox. This range is dependent on the intracellular concentration of tTA/rtTA, which in turn depends on properties of the integration site. In this regard, we have observed vast differences among different founders, of which 10–30% show nevertheless a good regulatory potential.
2. Cell type specificity of regulated expression. This parameter becomes obvious through *in situ* analysis of double transgenic animals in which the *LacZ* (e.g., in the nZL-2 line) or the GFP genes ***(38)*** are under P_{tet} control.
3. Position effect variegation (PEV). In transgenesis, in which transcription units are randomly integrated into the mouse genome, one frequently observes that, despite cell type-specific expression, the target gene is not active in every cell within an apparently homogeneous population, resulting in a mosaic-like expression pattern. Such patterns are considered to be a function of the particular insertion site. **Figure 5** examplifies this phenomenon. Two hepatocyte-specific mouse lines, TA^{LAP}-1 and TA^{LAP}-2 ***(16)***, were generated with the identical DNA construct in which the LAP promoter drives transcription of the tTA gene. When individuals of these lines are crossed with animals of the nZL-2 indicator line, only TA^{LAP}-2/nZL-2 (but not TA^{LAP}-1/nZL-2) double transgenic animals show PEV. Corresponding results were obtained with other mouse lines, demonstrating that PEV or mosaicism is not an intrinsic property of the Tet system but the result of a particular arrangement of the transferred transcription unit within the genome. PEV is, however, also observed with certain promoters apparently independently of the chromosomal insertion site. The human cytomegalovirus IE appears to be such an example.

In summary, using appropriate indicator mouse lines, tTA/rtTA strains can be thoroughly characterized with respect to the most important parameters, including kinetics of induction and longevity of expression (*see* **Subheading 1.4.**) before they are crossed to animals carrying the target gene under P_{tet} control. Such well-characterized transactivator strains are valuable, as they can be utilized in the study of a variety of target genes in defined cell populations (**Fig. 6**).

1.2.2. Generating Mouse Lines Containing the Target Gene under P_{tet} Control

As for transactivator mouse lines, two approaches are feasible. Transfer of the P_{tet}-controlled transcription unit is accomplished via homologous recombination into defined loci or by DNA injection into fertilized eggs and

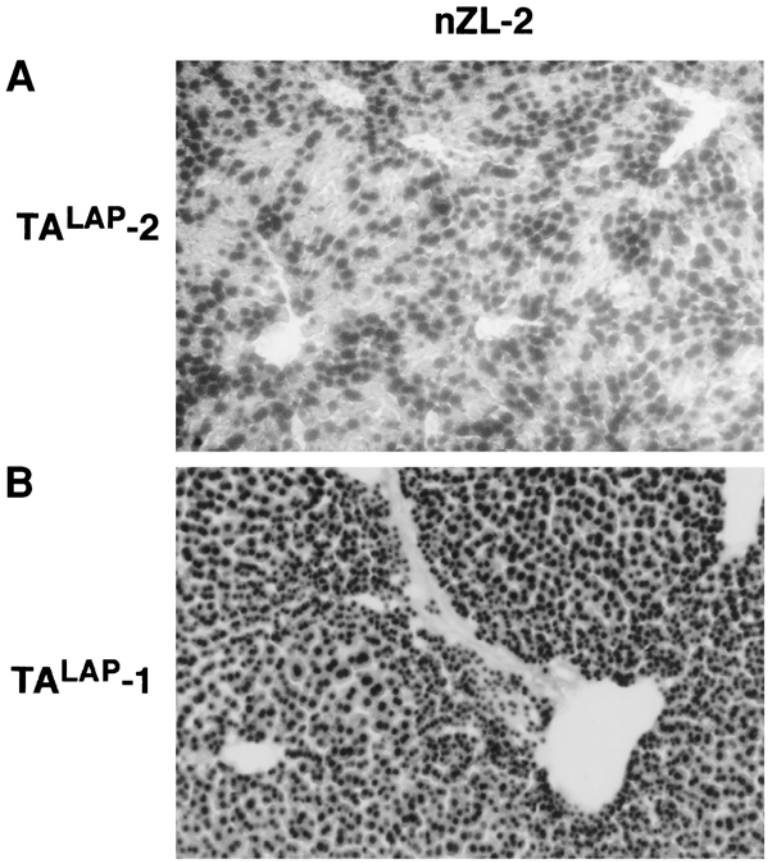


Fig. 5. Locus of integration influences expression pattern. Animals of two mouse lines in which tTA is under the control of P_{LAP} (TA^{LAP}-1, TA^{LAP}-2) were crossed with individuals of the indicator line nZL-2 (controlling the *lacZ* and the luciferase genes via P_{tet}bi-1). *lacZ* expression in liver sections was examined via X-gal staining. A clear position effect variegation for tTA expression is revealed in the TA^{LAP}-2 (**A**) but not in the TA^{LAP}-1 line (**B**).

random integration into the mouse genome. For both approaches, it is sensible to consider a few parameters that influence the quality of regulation in the resulting animals. All P_{tet} hybrid promoters consist of a minimal promoter fused to an array of *tetO* sequences. In principle, these promoters should be solely responsive to tTA or rtTA. However, the minimal promoter moiety is also susceptible to outside activation from nearby enhancers. Therefore, the "tightness" of these promoters depends strongly on the integration site. Moreover, the analysis of a set of founder lines containing the same P_{tet} target

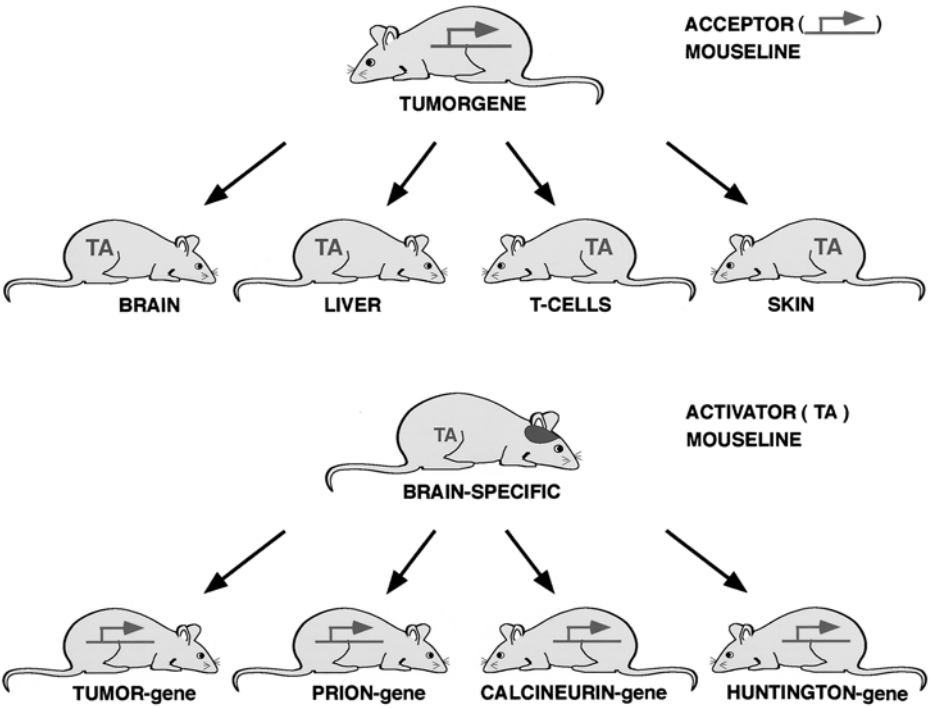


Fig. 6. Making use of the "zoo" of Tet mouse lines. The rapidly increasing number of transgenic mouse lines expressing either the tTA or rtTA gene tissue specifically or containing various target genes under P_{tet} control will allow for a large variety of combinations by which genes of interest can be studied in different cell types, as indicated in the sketch. To exploit the potential of the system fully, the various mouse lines must be made available to the scientific community in a nonbureaucratic way, a problem that remains to be solved.

gene construct integrated in different chromosomal locations has revealed that not only the tightness of P_{tet}, but also the maximal level of activation in response to tTA or rtTA are influenced by the respective integration site ***(27)***. Interestingly, we have found all possible combinations: loci where P_{tet} exhibits no measurable background activity and at the same time a very high activation potential (regulation factors $\geq 10^5$), loci with high background and low activation via tTA/rtTA, and so on.

The take-home lesson from these results is that, using the random integration approach, mouse lines can be identified in which (owing to the site where the P_{tet}-controlled target gene is inserted) very stringent control and a wide window of regulation of a gene of interest can be achieved. Examples are the

L7 mouse line ***(16)***, in which the luciferase gene is under P_{tet} control, and the LC-1 strain, in which luciferase and Cre recombinase are controlled by P_{tet}bi-1 ***(24,39)***. Thus, efficient control of a target gene can, in principle, be achieved by random insertion and screening for the proper line. Using site-specific integration for placing a tTA/rtTA-responsive expression unit into a defined chromosomal locus, it will certainly be advantageous to consider distances between known and suspected enhancer elements of the locus when chosing the exact insertion site, even though, given our knowledge of the dynamics and function of chromatin, such decisions are based on guesses for the time being. In any case, encouraging examples in which the tTA/rtTA gene has been "knocked in" along with the P_{tet}-controlled cDNA encoding the target gene while "knocking out" the endogeneous gene copy have been described ***(6,31)***.

How does one generate an tTA/rtTA-responsive mouse line? We strongly recommend placing any target gene under the control of P_{tet}bi-1 and coregulating with the gene of interest a gene encoding a reporter function like luciferase, β-galactosidase, or GFP. Our lab prefers luciferase for the reporter function for several reasons:

1. Luciferase is a highly sensitive indicator for quantitatively assessing tightness of control and regulation factors.
2. Luciferase activity can be detected noninvasively.
3. Adverse effects of luciferase expression, even over long periods, have never been observed.

Using the reporter genes in the context of the bidirectional promoter, P_{tet}bi founders can be analyzed for function by setting up primary fibroblast (*see* **Subheading 3.1.**) cultures, which can be transfected with tTA encoding DNA in the presence and absence of Dox. This assay will reveal the animals in which P_{tet}bi is accessible for tTA. Positive founders are then crossed to well-characterized tTA or rtTA individuals, and in many cases functionality of regulation can be monitored noninvasively by measuring luciferase bioluminescence (**Subheading 3.2.** and **Fig. 7** [*see* Color Plate 1 following p. 144]).

Whether regulation is tight and covers a sufficiently wide range is subsequently assessed by determining luciferase activity in extracts of respective tissues. At this level, it is also important to examine whether the target gene is properly coregulated with the luciferase gene (*see* **Note 6**).

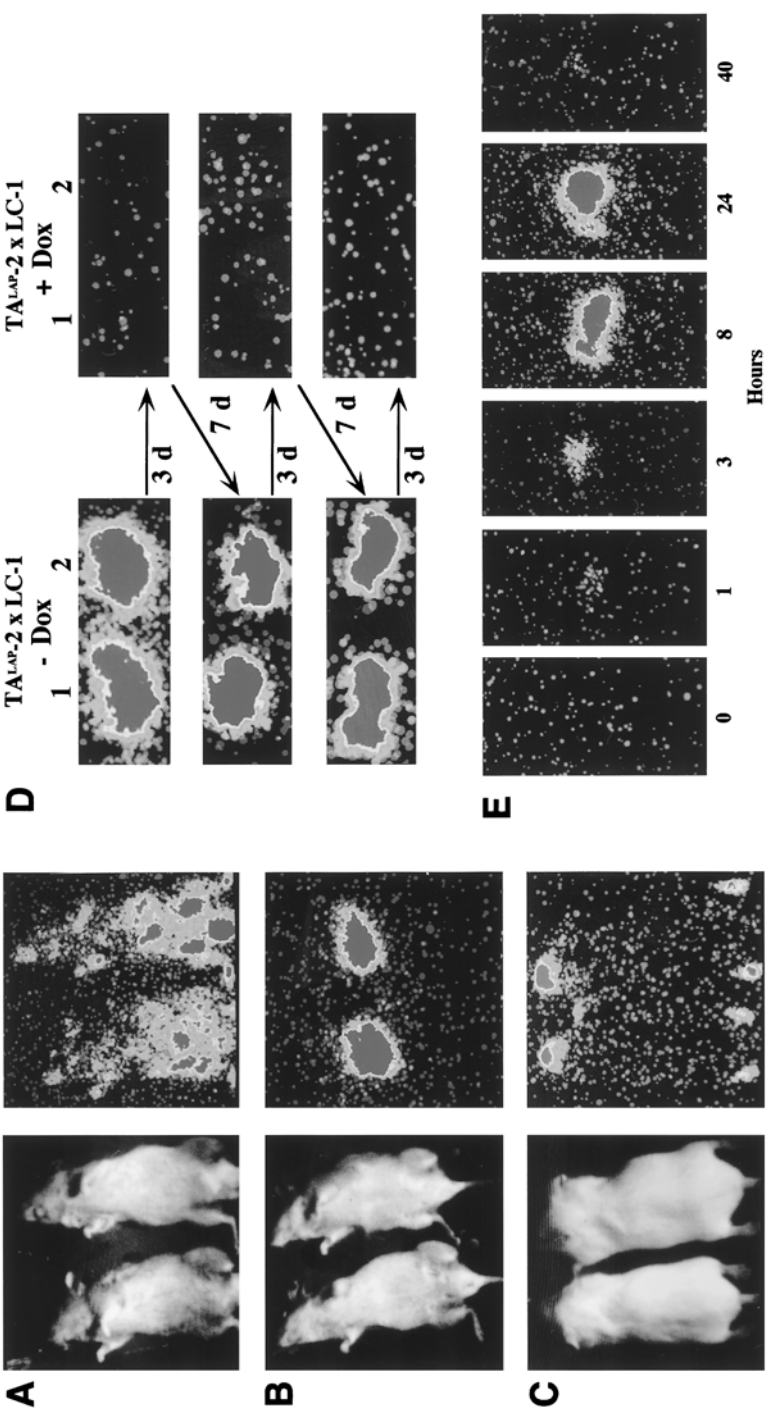
In analyzing our indicator mouse lines L7, nZL-2, and LC-1, we realized that the P_{tet}-controlled loci are accessible for tTA and rtTA in all tissue/cell types examined including hepatocytes, skeletal muscle cells, T-cells, neurons, pancreatic cells, and so on. Even though the extent of regulation and the background activities in the noninduced state ***(16)*** may not be strictly comparable

at a quantitative level (to which, of course, the tTA/rtTA mouse line contributes as well), these data suggest that genomic loci exist (or arise as a result of the integration process) that are particularly suited for tTA/rtTA-controlled regulation and that are functional in many—possibly all—states of cellular differentiation.

1.3. Tightness of Tet Control

As discussed above and in more detail in **refs. *3***, ***14***, and ***40***, tightness of Tet regulation depends almost exclusively on the crosstalk between the minimal promoter moiety of P_{tet} and nearby enhancers, particularly after the residual binding affinity of the rtTAs originally described has been abolished ***(21)***. There may be cell types in which the particular composition and abundance of transcription factors invoke an intrinsic activity of the minimal CMV promoter embedded in P_{tet}-1 ***(40)***. On the other hand, it is also clear that extremely tight Tet regulation can be achieved with this very promoter after screening for suitable integration sites. Thus, assessing quantitatively the background activity of TA^{LAP}-1/L7 double transgenic animals reveals that in the OFF state, i.e., in the presence of Dox, only one molecule of luciferase exists per hundred hepatocytes, indicating that an even smaller fraction of cells produces mRNA at any time ***(16,27)***. Nevertheless, very high levels of luciferase in hepatocytes are induced by Dox withdrawal in these animals. A mouse line of particular interest in this context was generated by G. Fishman and colleagues ***(26)*** in which the gene encoding the diphtheria toxin A chain (DTA) was placed under Tet control. Interestingly, not only would the single transgenic animal propagate normally, demonstrating a virtual silence of the DTA gene, but also double transgenic animals producing tTA in cardiac muscle would happily live as long as Dox was present in the drinking water. Lethal heart muscle-specific DTA activity was induced by Dox withdrawal. These results demonstrate that extremely tight control can be achieved considering that one molecule of DTA is assumed to kill a cell. At the same time it is obvious that the way to generate and identify such mouse lines is time-consuming and expensive. Therefore, more efficient solutions to this problem are of great interest.

One seemingly elegant approach has been repeatedly proposed: to flank P_{tet}-controlled expression units with so-called insulators. So far, we are, however, not aware of any example in which this strategy has unequivocally been shown to function ectopically in a context that would be relevant for Tet regulation. By contrast, a highly efficient and, as it appears, rather generally applicable way to shield P_{tet} from outside activation is the use of Tc-controlled transcriptional silencer (tTS) proteins, which are fusions between TetR and domains capable of suppressing the onset of transcription. When properly set up, tTS and rtTA


A
B
C
D
TA^LAP-2 x LC-1
- Dox
1
2
3 d
7 d
3 d
7 d
3 d
TA^LAP-2 x LC-1
+ Dox
1
2
E
0
1
3
8
24
40
Hours

bind mutually exclusively to *tetO* sequences within P_{tet}, depending on the presence or absence of Dox, as outlined in **Fig. 8**. This approach was shown to reduce substantially the basal activity of "high-background" loci in cell lines ***(40)*** and similarly, Zhu et al. ***(41)*** succeeded in efficiently reducing background activity via tTS in animals that control interleukin-12 (IL-12) specifically in the lung while maintaining full inducibility. Thus, even though the rtTA/tTS approach is experimentally more demanding, as one more component is required, it increases the probability of identifying tightly regulatable animals. Moreover, tTS also appears to be suited to reducing background activity of P_{tet}-controlled transcription units integrated in a predetermined locus via homologous recombination.

To reduce the experimental complexity of transferring three DNA constructs (encoding rtTA, tTS, and the target gene) under P_{tet} control into mice, Zhu et al. ***(41)*** recommend coinjection of all three DNAs into the pronucleus of fertilized eggs. Indeed, the various DNA constructs will with high probability integrate into the same locus and thus not segregate during breeding, seemingly a considerable advantage. However, even though this approach may occasionally yield good results, one should proceed with caution for the following reasons. During the integration events, usually more than one copy of a DNA construct is inserted. Cointegration of two transcription units encoding rtTA and the target gene under P_{tet} control, respectively, will with high probability place enhancer elements of the promoter driving the rtTA gene in close proximity to the minimal promoter within P_{tet}-1, resulting in elevated basal activity, as

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Fig. 7. *(See previous page)* Monitoring Tet-controlled gene expression in live animals. **(A–C)** Tissue-restricted expression of the luciferase gene. Animals of different tTA-expressing mouse lines were crossed with individuals of the LC-1 line, in which the luciferase and the Cre genes are coregulated by P_{tet}bi-1. The synthesis of tTA was driven by P_{hCMV} (**A**), P_{LAP} (**B**), and P_{CamKII} (**C**), respectively. Whereas P_{hCMV} is active in a variety of tissues, the activity of P_{LAP} (TA^{LAP}-2 mouse line) is restricted to hepatocytes. Brain (neuron) specific expression is seen with a mouse line generated with the αCamKII promoter ***(12)***. Interestingly, the footpads of the animals light up as well. (**D**) Switching luciferase expression in the liver. After exposure of the double transgenic animals shown in **B** to Dox for 3 days, luciferase activity has vanished. It can be reestablished by Dox withdrawal (in the experiment shown, after 7 days). Using a recently generated rTA^{LAP-1} mouse line, cycle times of only 2–3 days were determined. (**E**) Kinetics of hepatocyte-specific induction of luciferase activity. Using rTA^{LAP-1}/LC-1 double transgenic animals, the time course of induction can be monitored noninvasively by ip injection of 2 mg of Dox at time zero. Monitoring luciferase activity at the times given shows that induction has occurred within 1 h. For experimental details, *see* **Subheading 3.3.** and **ref. *24***.

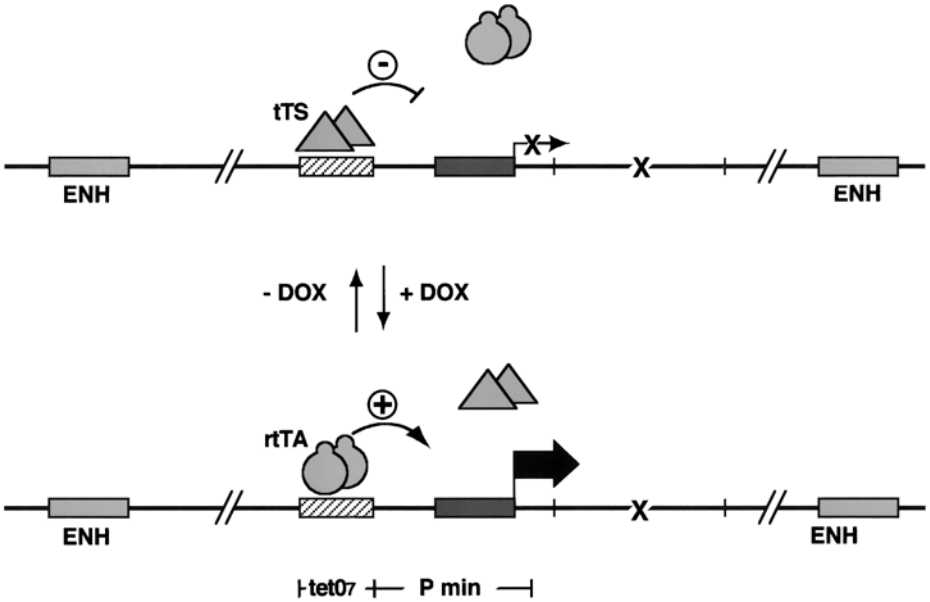

Fig. 8. Shielding minimal promoters within P_{tet} via tTS. Using a Tc-controlled transcriptional silencer (**Fig. 1**), P_{min} within P_{tet} can be protected from outside activation by nearby enhancers and other positively acting transcriptional factors that may interact and generate a background activity. As tTS is a fusion between TetR and a silencing domain, it will bind to *tetO* in the absence but not the presence of Dox. Upon Dox administration, tTS will dissociate from P_{tet}, and activation via rtTA will occur.

has been observed in numerous cases. In addition, placing the transcription unit encoding tTS into the same locus via cointegration positions further promoter/enhancer elements into the neighbourhood of P_{tet}. Even though P_{tet} might be shielded by tTS from outside enhancers, this may be a transient situation since transcriptional silencers are also known to work over a distance. It thus is likely that tTS, bound to P_{tet}, does not only shield this promoter from outside activation but also suppresses its own promoter integrated nearby. A homeostatic situation will be the result, and whether the expected regulation will be achieved will depend on quantitative parameters that are determined by the topography of the various integrates and their numbers in the overall context of the chromosomal locus.

Considering the many futile (and therefore unpublished) attempts to shortcut the generation of Tet-regulated transgenic mice by coinjecting both transcription units, we cannot recommend this approach, even though several functional mouse lines obtained by this approach have been generated (**Table 3**). Moreover, it should be emphasized that well-characterized mouse lines express-

Table 3
Mouse Lines with Combined P_{sp} tTA/rtTA and P_{tet}/Target Gene Expression Units

Mouse line	Tissue specificity	Reference
CC10-rtTA and P_{tet}-1-IL11	Lung parenchyma/airway	***(20)***
Whey acidic protein (WAP)-rtTA and P_{tet-1}-Cre	Mammary gland	***(44)***
Retinoblastoma (RB)-rtTA and P_{tet-1}-Cre	Thalamus, muscle, cerebellum, eye	***(44)***
Ovine β-lacto-globulin-rtTA and P_{tet1}-α-lactoglobulin	Mammary gland	***(71)***
Site-specific insertion of tTA and P_{tet-1} in endogenous SK3 locus	Brain	***(31)***
CC10 promoter-rtTA and P_{tet-1}-human regulated upon activation, normal T-cell expressed and secreted (hRANTES)	Lung parenchyma/airway	***(72)***
Type II collagen promoter-tTA and P_{tet-1}-MMP-13*	Chondrocytes	***(73)***

Compilation of mouse lines that were obtained by coinjection of two expression units either as separate DNAs or positioned on one DNA construct. In general, regulation in these mouse lines is not as tight as in tTA/rtTA responsive lines selected for low or no background. On the other hand, a sufficient regulation factor was found in most of the mice which would lead to a change in the phenotype and thus to insights into the function of the gene of interest.

ing tTA or rtTA in a highly cell type-specific way or mouse lines containing interesting target genes in loci where tight control and high induction levels can be achieved are particularly valuable, as they can be exploited in numerous projects, in which cell-specific expression of the transactivator can be combined freely with any target gene (**Fig. 6**). With respect to tTS, it would certainly be worthwhile to develop a mouse line in which tTS is expressed ubiquitously, which would then allow one to cross the silencer trait into another line whenever required.

1.4. Kinetics of Induction and Longevity of Expression

The tTA system has been used to measure half-life times of short-lived RNAs in cell culture, as tTA-dependent transcription can be readily abolished

by the addition of Dox to the medium ***(42)***. These experiments revealed that transcription of the P_{tet}-controlled gene is turned off in less than 5 min, which is in good agreement with in vitro dissociation times of the TetR/*tetO*/Tc complex ($t_{1/2}$ approx. 2 min). These data suggest that in mice the limiting parameter for turning off (tTA) or turning on (rtTA) transcription from P_{tet} by the addition of Dox is the delivery of the effector molecule to the target sites within the specific compartment of the animal. To probe the kinetics of induction in the mouse, we examined the hepatocyte-specific induction of luciferase in rTA^{LAP}-1/LC-1 double transgenic animals in a noninvasive way ***(24)***. As can be seen in **Fig. 7**, upon ip injection of 2 mg of Dox into these animals, bioluminescence can be detected within 1 h, reaches a maximum around 8 h, and then declines, owing to the clearance of Dox from the system. Similar time courses of induction and repression would be expected for other organs, with the exception of the brain. In the brain, it seems that the required Dox concentrations are reached more slowly owing to the gradient that forms across the blood-brain barrier, and, indeed, using the TA^{CAM}-1 (line B in **ref. *12***) and the LC-1 mouse lines, cycle times of around 5 days were determined ***(24)***.

Obviously, in studying embryonic development, a more rapidly reacting system may sometimes be appropriate. In such studies, the route of Dox delivery, e.g., by injection, which provides an initial high local concentration, will shorten the lag phase. An important improvement in this context would of course be a Dox antagonist that could rapidly terminate Dox action. Unfortunately, such a compound is not yet available. On the other hand, one should be aware that in most cases the half-life of the gene product under study is considerably longer than that of luciferase (2.5–3 h in various organs of the mouse; ***27***). The functional cycle times will therefore often be limited by this very parameter.

The activity of a transcription unit randomly integrated into the mouse genome will depend on the local chromatin arrangement, and there is plenty of evidence that insertion into heterochromatin can prevent transcription from the respective promoters. Although such silent integrates can be readily identified at an early stage when founder lines are characterized, a more serious question concerns long-term transcription and its regulation in established mouse lines, as transcriptional silencing may occur at later times, particularly when sequences susceptible to methylation are contained within the construct transferred. To date, however, there is no indication that the Tet regulatory elements are particular targets for transcriptional silencing. Actually, several P_{tet}/target gene mouse lines that have been around for up to 10 years display no change in the expression characteristics. The same holds true for numerous tTA/rtTA lines from our and other laboratories. This reasoning is supported by

an extension of the experiment shown in **Fig. 7**, in which luciferase in TA^{LAP}-1/LC-1 animals was repeatedly turned on and off and monitored noninvasively. The very same individuals were examined again after the luciferase gene had been switched off for over 3 months, and identical luciferase intensities and cycle times were observed (Schönig and Bujard, unpublished observations). Thus, if transcriptional silencing over time will be observed in some mouse lines, the present data suggest that such silencing is caused by sequences cointroduced with the Tet regulatory elements or to the particular site of integration.

1.5. Concluding Remarks

The Tet systems have been broadly applied to control gene activities at the cellular and organismal levels. When properly set up, they allow for tight transcriptional control over a wide range of regulation. Importantly, high-fidelity tissue-specific regulation has been achieved in transgenic mice following various strategies of gene transfer. Moreover, there is good evidence that homogeneous Tet-controlled expression can be established and that position effect variegation, whenever observed, is not owing to intrinsic properties of the Tet system. The main problem that remains in setting up highly specific regulation in a predictable and efficient way in vivo is our limited knowledge of chromatin structure and dynamics and how this is affected by a local perturbance, which always accompanies transgenesis independent of whether random integration or targeted insertion via homologous recombination is used as the experimental strategy. This problem, however, is not a specific one for the Tet system; it is common to all approaches that modify a local chromatin structure. Thus, applying the strategies presently available, one may often have to generate a set of transgenic variants, as discussed above, and to select for the proper transgenic lines.

Nevertheless, the straightforward approaches described here have already provided a wealth of information and in recent years have yielded some spectacular results. Thus, applying Tet regulation to the central nervous system, E. Kandel's group has carried out pioneering work. By controlling the expression of mutant forms of the Ca^{2+}/calmodulin-dependent protein kinase II ***(12)*** and calcineurin ***(13)***, they could correlate conditionally altered synaptic plasticity with spatial memory storage and retrieval.

Furthermore, modeling of human neuropathologies in the rodent as a prerequisite for developing therapeutic treatments has recently also led to most remarkable results. Thus, the Tet systems were exploited to create mouse models for prion ***(8)*** and Huntington's disease ***(7)***. In these models, the power to turn a gene's transcription on or off with Dox has allowed researchers to

assess not only the role of specific proteins thought to be involved in the etiology of the disorder, but also the consequence of their elimination when the respective gene is turned off after the manifestation of the pathologic symptoms. For example, in the Huntington's disease model, a mutated huntingtin gene was placed under Tet control, and its expression led not only to the neuroanatomic abnormalities characteristic of Huntington's disease, but also to pathologies resulting in motor dysfunction. Strikingly, in symptomatic adult animals, the suppression of transgene expression by Dox led to full reversal of pathologic symptoms and to normal behavior.

Equally remarkable are recently described tumor models in which the genes encoding, e.g., c-myc ***(9)***, K-ras^{12} ***(10)***, and ErbB2 ***(11)*** were placed under cell type-specific Tet control. In these animals, it was not only possible to study the onset and progression of tumor development, but, most excitingly, in all three models full tumor regression was observed when expression of the tumor-inducing gene was switched off. These results strongly indicate that a major fraction of tumors requires the continued activity of the tumor-initiating oncogene for its maintenance. Thus, these animal models do not only open up new ways for the study of tumor development and regression, they may also be useful for target validation and drug efficacy studies.

Finally, the numerous Tet mouse lines generated (compiled in **Tables 1–3**) have not only provided a wealth of new information on gene functions in vivo previously not accessible, they will also continue to reveal new insights as they become generally available and are used in various combinations as schematically outlined in **Fig. 6**.

2. Materials

2.1. Probing Primary Mouse Ear Fibroblasts of Transgenic Founder Animals for Functional Integration of P_{tet}bi-1-Controlled Target Genes

1. Phosphate-buffered saline (PBS).
2. RPMI-1640 medium.
3. Fetal calf serum (FCS; *see* **Note 1**)
4. Penicillin/streptomycin.
5. Fungizone® (GIBCO BRL) or nystatin (GIBCO BRL).
6. Sterile glass slide.
7. Razor blade.
8. Sterile culture/centrifuge tubes.
9. Sterile 35-mm culture dishes.
10. Collagenase type IA (Sigma, cat. no. C-2674).
11. LIPOFECTAMINE Reagent (GIBCO BRL).

2.2. Administration of Dox

2.2.1. Preparation of Dox

1. Doxycycline hydrochloride (Sigma, cat. no. D-9891).
2. Sucrose.
3. Dark drinking bottles.

2.2.2. Intraperitoneal Injection of Dox

1. Doxycycline hydrochloride (Sigma, cat. no. D-9891).
2. 0.9% NaCl solution.

2.3. Monitoring Luciferase Activity in Live Animals

1. Avertin (*see* Chapter 2).
2. D-luciferin (*see* **Note 2**).
3. Photon counting camera, e.g., two-stage ICCD C2400-47 (Hamamatsu) fitted with a Nikon lens (35-mm/f1.2).
4. Image analysis system.
5. Image processor, e.g., Argus 20 (Hamamatsu).

3. Methods

3.1. Probing Primary Mouse Ear Fibroblasts of Transgenic Founder Animals for Functional Integration of P_{tet}bi-1-Controlled Target Genes

1. Collect a small piece of mouse ear (approx 3 × 3 mm) and place it immediately into a sterile tube containing 1 ml of RPMI-1640 medium with 30 % FCS and 300 IU/mL penicillin/streptomycin. To avoid fungal contamination, one may add amphotericin B (Fungizone) or nystatin to a final concentration of 2.5 μg/mL or 250 U/mL, respectively. For best results, proceed immediately. (However you may keep the material at room temperature for several hours.)
2. Cut the ear tip on a sterile glass slide with a razor blade into small pieces and transfer the material into a 3.5-cm dish containing 2 mL of RPMI-1640 with 30% FCS, pencillin/streptomycin, and 1 mg/mL collagenase; digest overnight (max. 20 h) at 37°C.
3. Disaggregate the pieces by careful pipeting, dilute into 10 mL of medium, and centrifuge in a 15-mL Falcon tube for 5 min at 170*g*.
4. Carefully remove all of the supernatant without touching the pellet; resuspend the pellet in 2 mL of medium, transfer to a 3.5-cm dish, and allow the cells to adhere and grow for 24–36 h.
5. Wash adherent cells carefully with 1X PBS to remove tissue debris; add fresh medium.
6. Grow the cells to 80% confluency. (Optimally this will take 3–5 days).

7. Split the culture into 3 dishes (3.5 cm) and keep one as stock; transfect the other two cultures with plasmid DNA encoding tTA (1.0 μg/well) in the presence or absence of Dox (1 μg/mL), respectively. Transfections can be carried out with LIPOFECTAMINE Reagent according to the manufacturer's protocol. Harvest the cells 30 h later with 100 μL of lysis buffer, and determine luciferase activity in whole cell extracts as described ***(15)***.

3.2. Administration of Dox

3.2.1. Preparation of Dox Containing Drinking Water

Dissolve doxycycline hydrochloride and sucrose in sterile distilled water to a final concentration of up to 2 mg/mL (Dox) and 2–5% (sucrose). This solution can be stored at 4°C for 1–2 weeks. Deliver the drinking water in dark bottles to protect Dox from light. Change Dox-water twice a week (*see* **Notes 3–5**).

3.2.2. Intraperitoneal Injection of Dox

Dissolve 4 mg of Dox in 1 mL 0.9% NaCl solution and filter-sterilize. Inject 0.5 mL of the solution ip.

3.3. Noninvasive Monitoring of Tet-Controlled Transcription Units

The possibility of detecting luciferase activity via bioluminescence in live animals ***(43)*** can be exploited to monitor the activity of a target gene noninvasively. By coregulating a gene of interest and the luciferase gene via a bidirectional promoter like P_{tet}bi (**Fig. 1**), luciferase bioluminescence can be used as an indicator for the activity of this bidirectional promoter ***(24)***. This approach is particularly useful in long-term experiments in which expression of the target gene is subjected to a regulatory regimen with repeated ON and OFF times or in which partial induction of a gene is intended. Furthermore, since the intensities of target gene expression can vary considerably even among litter mates ***(16)***, quantifying luciferase bioluminescence and correlating this activity with the expression of the target gene in individuals enrolled in an experiment may permit a more precise interpretation of the action of the gene under study (*see* **Note 6**).

3.3.1. Monitoring Luciferase Activity in Live Animals

1. Anesthetsize mice with avertin (*see* Chapter 2) (30 μL/g body weight of a 1.2% solution in 0.9% NaCl) and inject an aqueous solution of D-luciferin ip (100 μg/g body weight) (*see* **Note 2**).
2. After 10 min, place the mice into a light-tight chamber (*see* **Note 7**).
3. The emitted light is acquired by photon counting camera and a computer with image analysis capabilities. Body images are recorded as reference in the

daylight. The photon counting program should be initiated for periods of 1 s to 5 min. Photons are collected over time, and generated images are converted to pseudocolor, digitized with an image processor, and stored in a computer. The intensity of acquired images is calculated as relative light units per minute (rlu/min).

3.4. Controlling Cre-Recombinase via Doxycycline

Conditional gene inactivation or modification by Cre/Lox recombination has provided many insights into gene functions, as described in Chapter 9 of this book. Placing the Cre-recombinase under control of P_{tet} gives another degree of freedom: recombination events can be induced at time points that are independent of the activity spectrum of a cell type-specific promoter driving tTA/rtTA expression. Two approaches have been applied successfully. Utomo et al. ***(44)*** have described a single plasmid vector that contains the transcription units for rtTA and for Cre-recombinase under P_{tet} control, respectively. Both units are separated by a 4-kb stretch of DNA to reduce the crosstalk between P_{tet}-1 and the promoter driving rtTA expression. When individuals of the resulting mouse line were crossed with animals containing a floxed gene, ip injection of Dox induced efficient Cre/Lox recombination.

As mentioned above, our laboratory, in collaboration with K. Rajewsky and F. Schwenk, has developed a mouse line (LC-1) in which P_{tet}bi-1 coregulates expression of the genes encoding Cre-recombinase and luciferase, respectively. In any tissue examined so far (T-cells, hepatocytes, brain, kidney, lung, spleen, skeletal muscle, heart), Cre is very tightly controlled. This conclusion is based on two types of experiments. Whenever LC-1 animals are crossed with individuals containing a "floxed" β-gal gene, the double transgenic mice do not show any recombination events—as seen by *in situ* X-gal staining—in all tissues/organs examined, even after prolonged times (age of mice > 6 months). The same result is obtained when triple transgenic animals are generated that contain, in addition, the rtTA gene under control of a tissue-specific promoter. By contrast, efficient deletion of the Lox-flanked DNA segment occurs upon Cre induction: X-gal staining of all hepatocytes is observed after two ip injections of Dox (2 mg/mL in isotonic saline each) spaced 24 h apart. In conclusion, placing Cre under Tet control allows one to control the recombinase tightly and to induce highly efficiently deletion of floxed DNA even late in an animal's life without a prior buildup of background deletions over time.

3.5. Tetracyclines as Effectors of the Tet System

A major advantage of tetracyclines as effector molecules for in vivo studies is the broad knowledge we have about their pharmacologic and chemical properties, as several members of this class of compounds have been in use in

human and animal medicine for decades. The specificity of these antibiotics for prokaryotes is excellent, and accordingly they are nontoxic for eukaryotes, particularly at the concentrations required for Tet regulation. Presently, Dox is the most suitable effector molecule for Tet regulation primarily for two reasons:

1. It interacts with TetR at an about 100-fold higher binding constant ($2 \times 10^{12}\ M^{-1}$) compared with Tc.
2. It has excellent tissue penetration properties.

In tissue culture (HeLa cells), 1–5 ng/mL of Dox are sufficient to turn off the tTA system, and the new rtTA2^S-M2 is fully active at 50–80 ng/mL. By contrast, the first toxic effects in HeLa cell cultures are observed above 5 μg/mL, i.e., at 100–1000-fold higher concentrations than required for Tet regulation. How these in vitro findings relate to the in vivo situation is not entirely clear, but there are a number of indications suggesting that in vivo systems are even less susceptible to side effects of doxycycline. Here it may suffice to know that

1. Feeding mice with 200 μg/mL of Dox in the drinking water leads to saturating Dox levels in the serum within 1 h ***(27)***.
2. The steady-state levels in the serum (1 week of feeding) are around 580 ng/mL for 200 μg/mL of Dox and 130 ng/mL for 20 μg/ml of Dox in the drinking water ***(27)***;
3. Upon withdrawal of Dox from the drinking water, the serum is cleared with a half-life time of 6 h ***(27)***.

More difficult to predict is the effect of depot formation, which occurs owing to the lipophilic nature of Dox. This concern is, however, primarily of relevance for animals that were raised under Dox, in which depletion of the system from the antibiotic takes considerably longer than the cycle times in **Fig. 7** suggest (*see* **Note 5**). It is, therefore, recommended to apply the lowest Dox concentrations required, and experiments in our laboratory support the notion that 20–50 μg/mL of Dox in the drinking water is sufficient to keep tTA inactive in practically all organs of the mouse, whereas a supply of 2 mg/mL is required to activate rtTA and rtTA2^S-S2 fully in the liver. It is, however, foreseen that only 1/10 of the Dox concentration will be required for rtTA2^S-M2 animals.

As pointed out previously ***(16)***, the distribution of Dox throughout different tissues in a mouse differs; thus the Dox supply has to be calibrated accordingly, particularly when partial induction in a certain organ is intended ***(16)***. Finally, it should be emphasized again that Dox is well tolerated even at higher concentrations. Thus, we have kept and bred mice under 2 mg/mL of Dox

without noticing unusual behavior or histologic effects *(27)*, as would be expected from data where Dox was applied as antibiotic.

4. Notes

1. We have noticed that some commercially available serum products may contain some tetracycline contaminants. This can affect the regulation properties of the Tet systems, so we recommend screening new batches of sera with suitable reporter cell lines (as described in **ref.** ***14***) or purchasing only tested FCS (e.g., from Clontech, Tet System Approved Fetal Bovine Serum, cat. no. 8630-1). Even "antibiotic–free" serum that has no negative effect on cell growth may still exhibit enough tetracycline–like activity to reduce or eliminate Tet-regulated gene expression
2. D-luciferin, the substrate for the luciferase, is available either as sodium salt or as free acid (e.g., from AppliChem, D-luciferin, free acid, cat. no. A 1029). For ip injections, we routinely use 200 µL of a a 25-mM solution. The salt is readily soluble in water, but not the (cheaper) free acid. When the latter is purchased, it can be dissolved in water by titrating the acid with NaOH to a final pH of 7.3. The solution should be filter-sterilized and stored frozen in small aliquots.
3. Choice of the effector substance is critical to use of the Tet systems. Although tetracycline is efficient for deactivating tTA, it only marginally activates the rtTA-dependent system. Doxycycline is to date the most potent effector substance for both systems. Tetracyclines, which can be used used in tissue culture or mice, are purchased as hydrochlorides and thus are water soluble (Dox-HCl, Sigma, cat. no. D 9891). For cell culture experiments, it is recommended to make up 1 mg/mL stock solutions, which are filter-sterilized and stored in the dark at 4°C for 2 weeks, for use in mice (*see* **Note 4**).
4. We recommend preparation of the Dox solution in distilled sterile water. We have observed that doxycycline can form a precipitate, if normal water is used.
5. We strictly recommend use of only 20–50 µg/mL in the drinking water to inactivate the tTA system, when animals are raised under Dox. If Dox concentrations as high as 1–2 mg/mL are used, induction of gene expression by Dox depletion will take much longer.
6. Initially it is very important to examine whether the target gene is properly coregulated with the reporter gene. Sometimes unequal expression patterns occur, for a variety of reasons such as multiple insertions (head to tail, tail to tail, and so on), partial deletions at the insertion site, and outside influences of the locus. The most important parameters causing asymmetric expression are different half-life times of the respective RNAs and proteins. One example is the nZL-2 line, in which we have found strong expression of lacZ but only minor expression of the luciferase gene.
7. Mice are quite cold-sensitive; therefore we put the anesthetized animals on a warm plate before and after imaging. If internal organs like the liver or kidney are screened for luc activity, the mice should be put on their backs, when brain imaging is performed, they should lie on their bellies.

Acknowledgments

We thank Sibylle Reinig for patient support in preparing this manuscript and Yves Cully for artwork. Our work was supported by the Bioregio Program of the Federal Ministry of Education and Research and by E.C. grant BIO4-98-0203.

References

1. Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
2. Gossen, M., Freundlieb, S., Bender, G., et al. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **26,** 1766–1769.
3. Baron, U. and Bujard, H. (2000) The Tet-repressor based system for regulated gene expression in eukaryotic cells: principles and advances. *Methods Enzymol.* **327,** 401–421.
4. Gossen, M. and Bujard, H. (2001) Tetracyclines in the control of gene expression in eukaryotes. In: *Tetracyclines as Molecular Probes for Micro and Mammalian Biology* Nelson, M., Hillen, W., and Greenwald, R.A. (eds.), Birkhäuser Verlag, Basel.
5. Mansuy, I. M. and Bujard, H. (2000) Tetracycline-regulated gene expression in the brain. *Curr. Opin. Neurobiol.* **10,** 593–596.
6. Shin, M. K., Levorse, J. M., Ingram, R. S., and Tilghman, S. M. (1999) The temporal requirement for endothelin receptor-B signaling during neural crest development. *Nature* **402,** 496–501.
7. Yamamoto, A., Lucas, J., and Hen, R. (2000) Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. *Cell* **101,** 57–66.
8. Tremblay, P., Meiner, Z., Galou, M., et al. (1998) Doxycycline control of prion protein transgene expression modulates prion disease in mice. *Proc. Natl. Acad. Sci. USA* **95,** 12580–12585.
9. Felsher, D. W. and Bishop, J. M. (1999) Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol. Cell* **4,** 199–207.
10. Chin, L., Tam, A., Pomerantz, J., et al. (1999) Essential role for oncogenic Ras in tumor maintenance. *Nature* **400,** 468–472.
11. Xie, W., Chow, L. T., Paterson, A. J., Chin, E., and Kudlow, J. E. (1999) Conditional expression of the ErbB2 oncogene elicits reversible hyperplasia in stratified epithelia and up-regulation of TGFα expression in transgenic mice. *Oncogene* **18,** 3593–3607.
12. Mayford, M., Bach, M. E., Huang, Y., et al. (1996) Control of memory formation through regulated expression of a CaMKII transgene. *Science* **274,** 1678–1683.
13. Malleret, G., Haditsch, U., Genoux, D., et al. (2001) Inducible and reversible enhancement of learning, memory and longterm potentiation by genetic inhibition of calcineurin. *Cell* **104,** 675–686.

14. Freundlieb, S., Baron, U., Bonin, A.L., Gossen, M., and Bujard, H. (1997) Use of tetracycline controlled gene expression systems to study mammalian cell cycle. *Methods Enzymol.* **283,** 159–173.
15. Freundlieb, S., Baron, U., and Bujard, H. (1998) Controlling gene activities via the tetracycline regulatory systems. In: Celis, J., ed. *Cell Biology: A Laboratory Handbook*, 2nd ed., vol. 4, Academic Press, San Diego, CA, pp. 230–238.
16. Kistner, A., Gossen, M., Zimmermann, F., et al. (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc. Natl. Acad. Sci. USA* **93,** 10933–10938.
17. Hillen, W. and Berens, C. (1994) Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu. Rev. Microbiol.* **48,** 345–369.
18. Hlavka, J. J. and Bothe, J. H. (1985) *The Tetracyclines. Handbook of Experimental Pharmacology*, vol. 78, Springer-Verlag, Berlin.
19. Mansuy, I. M., Winder, D. G., Moallem, T. M., et al. (1998) Inducible and reversible gene expression with the rtTA system for the study of memory. *Neuron* **21,** 257–265.
20. Ray, P., Tang, W., Wang, P., et al. (1997) Regulated overexpression of interleukin 11 in the lung. Use to dissociate development-dependent and -independent phenotypes. *J. Clin. Invest.* **100,** 2501–2511.
21. Urlinger, S., Baron, U., Thellmann, M., et al. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc. Natl. Acad. Sci. USA* **97,** 7963–7968.
22. Baron, U., Gossen, M., and Bujard, H. (1997) Tetracycline controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res.* **25,** 2723–2729.
23. Baron, U., Freundlieb, S., Gossen, M., and Bujard, H. (1995) Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res.* **23,** 3605–3606.
24. Hasan, M. T., Schoenig, K., Berger, S., Graewe, W., and Bujard, H. (2001) Longterm, non-invasive imaging of regulated gene expression in living mice. *Genesis* **29,** 116–122.
25. Fishman, G. I., Kaplan, M. L., and Buttrick, P. M. (1994) Tetracycline regulated cardiac gene expression *in vivo*. *J. Clin. Invest.* **93,** 1864–1868.
26. Lee, P., Morley, G., Huang, Q., et al. (1998) Conditional lineage ablation to model human diseases. *Proc. Natl. Acad. Sci. USA* **95,** 11371–11376.
27. Kistner, A. (1996) Ph.D. Thesis. University of Heidelberg, Heidelberg, Germany.
28. Lamartina, S., Rinaudo, D., Roseilli, G., et al. (2001) Stringent control of gene expression *in vivo* using novel doxycycline dependent transactivators. *Hum. Gene Ther.* **13,** 199–210.
29. Corti, O., Sabaté, O., Horellou, P., et al. (1999) A single adenovirus vector mediates doxycycline controlled expression of tyrosine hydroxylase in brain gafts of human neural progenitors. *Nat. Biotechnol.* **17,** 349–354.

30. Bohl, D., Naffakh, N., and Heard, J. M. (1997) Longterm control of erythropoietin secretion by doxycycline in mice transplanted with engineered primary myoblasts. *Nat. Med.* **3,** 299–305.
31. Bond, C. T., Sprengel, R., Bissonnette, J. M., et al. (2000) Respiration and parturition affected by conditional overexpression of the Ca^{2+}-activated K^+ channel subunit, SK3. *Science* **289,** 1942–1946.
32. Gill, G. and Ptashne, M. (1988) Negative effect of the transcriptional activator GAL4. *Nature* **334,** 721–724.
33. Efrat, S., Fusco-DeMane, D., Lemberg, H., al Emran, O., and Wang, X. (1995) Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline regulated oncogene. *Proc. Natl. Acad. Sci. USA* **92,** 3576–3580.
34. Legname, G., Seddon, B., Lovatt, M., et al. (2000) Inducible expression of a p56Lck transgene reveals a central role for Lck in the differentiation of CD4 SP thymocytes. *Immunity* **12,** 537–546.
35. Scott, K., Brady, R. Jr., Cravchik, A., et al. (2001) A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell* **104,** 661–673.
36. Talbot, D., Descombes, P., and Schibler, U. (1994) The 5′ flanking region of the rat LAP C/EBP beta) gene can direct high-level, position-independent, copy number-dependent expression in multiple tissues in transgenic mice. *Nucleic Acids Res.* **22,** 756–766.
37. Perea, J., Robertson, A., Tolmachova, T., et al. (2001) Induced myelination and demyelination in a conditional mouse model of Charcot-Marie-Tooth disease type 1A. *Hum. Mol. Genet.* **10,** 1007–1018.
38. Krestel, H. E., Mayford, M., Seeburg, P. H., and Sprengel, R. (2001) A GFP-equipped bidirectional expression module well suited for monitoring tetracycline-regulated gene expression in mouse. *Nucleic Acids Res.* **29,** E39.
39. Schönig, K., Schwenk, F., Rajewsky, K., and Bujard, H. Stringent doxycycline dependent control of CRE recombinase in vivo-te LC-1 mouse line (submitted).
40. Freundlieb, S., Schirra-Müller, C., and Bujard, H. (1999) A tetracycline controlled activation/repression system for mammalian cells. *J. Gene Med.* **1,** 4–12.
41. Zhu, H., Ma, B., Homer, R. J., Zheng, T., and Elias, J.A. (2001) Use of the tetracycline controlled transcriptional silencer (tTS) to eliminate transgene leak in inducible overexpression transgenic mice. *J. Biol. Chem.* **276,** 25222–25229.
42. Clement, J. Q., Qian, L., Kaplinsky, N., and Wilkinson, M. F. (1999) The stability and fate of a spliced intron from vertebrate cells. *RNA* **5,** 206–220.
43. Contag, P. R., Olomu, I. N., Stevenson, D. K., and Contag, C. H. (1998) Bioluminescent indicators in living mammals. *Nat. Med.* **4,** 245–247.
44. Utomo, A. R., Nikitin, A.Y., and Lee, W. H. (1999) Temporal, spatial and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat. Biotechnol.* **17,** 1091–1096.
45. Passman, R. S. and Fishman, G. I. (1994) Regulated expression of foreign genes *in vivo* after germline transfer. *J. Clin. Invest.* **94,** 2421–2425.

46. Hennighausen, L., Wall, R. J., Tillmann, U., Li, M., and Furth, P. A. (1995) Conditional gene expression in secretory tissues and skin of transgenic mice using the MMTV-LTR and the tetracycline responsive system. *J. Cell. Biochem.* **59,** 463–472.
47. Manickan, E., Satoi, J., Wang, T. C., and Liang, T. J. (2001) Conditional liver-specific expression of simian virus 40 T antigen leads to regulatable development of hepatic neoplasm in transgenic mice. *J. Biol. Chem.* **276,** 13989–13994.
48. Chen, J., Kelz, M. B., Zeng, G., et al. (1998) Transgenic animals with inducible, targeted gene expression in brain. *Mol. Pharmacol.* **54,** 495–503.
49. Sarao, R. and Dumont, D. J. (1998) Conditional transgene expression in endothelial cells. *Transgenic Res.* **7,** 421–427.
50. Guo, Y., Zhao, J., Sawicki, J., Peralta Soler, A., and O'Brien, T. G. (1999) Conversion of C57Bl/6 mice from a tumor promotion-resistant to a -sensitive phenotype by enhanced ornithine decarboxylase expression *Mol. Carcinog.* **26,** 32–36.
51. Diamond, I., Owolabi, T., Marco, M., Lam, C., and Glick, A. (2000) Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators tTA and rtTA linked to the keratin 5 promoter. *J. Invest. Dermatol.* **115,** 788–794.
52. Leenders, H., Whitfield, S., Benoist, C., and Mathis, D. (2000) Role of the forkhead transcription family member, FKHR, in thymocyte differentiation. *Eur. J. Immunol.* **30,** 2980–2990.
53. Witherden, D., van Oers, N., Waltzinger, C., et al. (2000). Tetracycline-controllable selection of CD4(+) T cells. Half-life and survival signals in the absence of major histocompatibility complex class II molecules. *J. Exp. Med.* **191,** 355–364.
54. Ahmad, A., Brinson, M., Hodges, B. L., Chamberlain, J. S., and Amalfitano, A. (2000) *Mdx* mice inducibly expressing dystrophin provide insights into the potential of gene therapy for Duchenne muscular dystrophy. *Hum. Mol. Gen.* **9,** 2507–2515.
55. Nikitin, A.Y., Shan, B., Flesken-Nikitin, A., Chang, K. H., and Lee, W. H. (2001) The retinoblastoma gene regulates somatic growth during mouse development. *Cancer Res.* **61,** 3110–3118.
56. Hess, J., Nielsen, P. J., Fischer, K.-D., Bujard, H., and Wirth, T. (2001) The B lymphocyte-specific coactivator BOB.1/OBF.1 is required at multiple stages of B-cell development. *Mol. Cell. Biol.* **21,** 1531–1539.
57. Saam, J. R. and Gordon, J. I. (1999) Inducible gene knock outs in the small intestine and colonic epithelium. *J. Biol. Chem.* **274,** 38071–38082.
58. Chang, M. A., Horner, J. W., Conklin, B., et al. (2000) Tetracycline-inducible system for photoreceptor-specific gene expression. *Invest. Ophthalmol. Vis. Sci.* **41,** 4281–4287.
59. Tichelaar, J. W., Lu, W., and Whitsett, J. A. (2000) Conditional expression of fibroblast growth factor-7 in the developing and mature lung. *J. Biol. Chem.* **275,** 11858–16649.
60. Ye, L., Chan, S., Chow, Y. H., Tsui, L. C., and Hu, J. (2001) Regulated expression of the human *cftr* gene in epithelial cells. *Mol. Ther.* **3,** 723–733.

61. Ju, H., Gros, R., You, X., et al. (2001) Conditional and targeted overexpression of vascular chymase causes hypertension in transgenic mice. *Proc. Natl. Acad. Sci. USA* **98,** 7469–7474.
62. Krestel, H. E., Mayford, M., Seeburg, P. H., and Sprengel, R. (2001) An GFP-equipped bidirectional expression module well suited for monitoring tetracycline-regulated gene expression in mouse. *Nucleic Acids Res.* **29,**
63. Mansuy, I. M., Mayford, M., Jacob, B., Kandel, E. R., and Bach, M. E. (1998) Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. *Cell* **92,** 39–49.
64. Jerecic, J., Single, F., Kruth, U., et al. (1999) Studies on conditional gene expression in the brain. *Ann. NY Acad. Sci.* **868,** 27–37.
65. Huettner, C. S., Zhang, P., Van Etten, R. A., and Tenen, D. G. (2000) Reversibility of acute B-cell leukaemia induced by BCR-ABL-1. *Nat. Gen.* **24,** 57–60.
66. Bowman, J. C., Steinberg, S. F., Jiang, T., et al. (1997) Expression of protein kinase C beta in the heart causes hypertrophy in adult mice and sudden death in neonates. *J. Clin. Invest.* **100,** 2189–2195.
67. Redfern, C. H., Coward, P., Degtyarev, M. Y., et al. (1999) Conditional expression and signaling of a specifically designed G(i)-coupled receptor in transgenic mice. *Nat. Biotechnol.* **17,** 165–169.
68. Kelz, M. B., Chen, J., Carlezon, W. A., Jr., et al. (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* **16,** 272–276.
69. Clark, J. C., Tichelaar, J. W., Wert, S. E., et al. (2001) FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **280,** L705–715.
70. Lucas, J. J., Hernandez, F., Gomez-Ramos, P., et al. (2001) Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J.* **20,** 27–39.
71. Soulier, S., Stinnakre, M. G, Lepourry, L., Mercier, J.-C., and Vilotte, J.-L (1999) Use of doxycycline-controlled gene expression to reversibly alter milk-protein composition in transgenic mice. *Eur. J. Biochem.* **260,** 533–539.
72. Pan, Z. Z., Parkyn, L., Ray, A., and Ray, P. (2000) Inducible lung-specific expression of RANTES: preferential recruitment of neutrophils. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279,** 658–666.
73. Neuhold, L. A., Killar, L., Zhao, W., et al. (2001) Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. *J. Clin. Invest.* **107,** 35–44.

6

Manipulating Large Insert Clones for Transgenesis

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1. Introduction

The use of large genomic clones for transgenesis is necessary when the gene to be expressed is too large to be accommodated in a plasmid-based vector. Large insert clones can be produced with the P1 bacteriophage (P1) ***(1)***, P1 artificial chromosome (PAC) ***(2)***, bacterial artificial chromosome (BAC) ***(3)***, or yeast artificial chromosome (YAC) ***(4)*** cloning systems. Correct tissue, spatial, and developmental expression can only be achieved when all of the endogenous control elements are present in their native context, and this often requires a large genomic construct. This is particularly important for studying the expression of a gene or gene loci regulated by distant DNA elements. The use of large genomic clones to generate transgenic mice also reduces the possibility of positional effects that can be exerted by the chromosomal sequences flanking the point of transgene integration. Recently, large genomic clones have been used in mouse transgene complementation studies to discover new genes ***(5)***.

The choice of cloning vector depends on the size of the gene to be expressed and the distance of the regulatory elements from the gene. P1 clones typically contain genomic inserts of up to 100 kb, BACs up to 300 kb, and YAC vectors up to 2 Mb. All three types of clones are available from commercial sources and are generally supplied with detailed protocols for their growth and propagation. P1 and BAC clones are easiest to work with, since both are supercoiled circular plasmids, and milligram quantities of DNA can be isolated easily from conventional plasmid preparations. The preparation of YAC DNA, however, is more tedious, since one is dealing with a linear piece of DNA at low concentrations, which makes the DNA susceptible to shearing during in vitro manipulations.

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

Many applications require that the genomic insert be modified to introduce markers and/or mutations. An advantage of the YAC system is the ease with which the insert can be mutated by homologous recombination to introduce point mutations, deletions, and replacements ***(6)***. Random insertional mutations in P1 and BAC DNA can be produced with transposons ***(7,8)***. More specific mutations can be introduced with *recA*-assisted restriction endonuclease (RARE) techniques ***(9–13)***. A disadvantage of the RARE-cleavage approaches, compared with gene targeting in yeast, is that they require more extensive sequence information. The development of homologous recombination systems in *E. coli* ***(14–16)*** are very promising and will undoubtedly make it possible to manipulate P1 and BAC DNA efficiently, and without the need for extensive sequence information.

In the following subheadings, we describe how to manipulate both YAC and BAC DNA for the purpose of expressing large modified fragments of DNA in transgenic mice. We detail a protocol for the mutagenesis of YAC DNA with a "pop-in, pop-out" gene-targeting strategy that we have used to introduce point mutations and replace sequences into the apolipoprotein (apo-) B gene ***(17,18)***. We also describe a method for the deletion mutagenesis of BACs based on RARE cleavage that has been used to identify distant DNA elements controlling tissue-specific expression of the apo-B gene ***(13)***. A section detailing the preparation of YAC and BAC DNA for microinjection into murine oocytes is provided, although a number of protocols for the preparation of YAC DNA exist ***(19–21)***. For information on the preparation and manipulation of P1 DNA for generating transgenic animals, we refer the reader to Chapter 43 in *Transgenic Animals: Generation and Use* ***(22)***.

2. Materials

2.1. YAC Transgenics

2.1.1. YAC DNA Purification

1. Bio-Rad pulsed-field gel apparatus (CHEF MAPPER, CHEF DRII, or DRIII).
2. SeaPlaque GTG low-melt agarose (FMC Bioproducts, cat. no. 50101), agarose MP (Roche Molecular Biochemicals, cat. no. 1 388 983).
3. 0.5X TBE running buffer: 45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA.
4. β-Agarase and buffer (New England Biolabs, cat. no. 392L).
5. Mid-range PFG marker (New England Biolabs, cat. no. 355-1).
6. Ethidium bromide, 10 mg/mL. For staining, use 5 μL in 100 mL of 0.5X TBE running buffer.
7. Ultrafree-MC Filter Unit (Millipore, cat. no. UFC3 TTK).
8. Spermine and spermidine (hydrochloride salts, Sigma, cat. nos. S2876 and S2501): make stock solutions of 30 m*M* spermine and 70 m*M* spermidine, filter-sterilize, and store at –20°C.

9. YAC microinjection buffer: 10 m*M* Tris-HCl, pH 7.5, 250 μ*M* EDTA, pH 8.0, 100 mM NaCl, filter-sterilized. Add 30 μ*M* spermine and 70 μ*M* spermidine.

2.1.2. YAC Mutagenesis

1. pRS406 yeast integrating plasmid (Stratagene, cat. no. 217406).
2. BIO101 Yeast Spheroplast Transformation Kit (Q-Biogene, cat. no. 2210-200).
3. Synthetic dropout media. Add 6.8 g yeast nitrogen base without amino acids (Difco, cat. no. 0919-15) and the required amount of BIO101 CSM dropout mix [i.e., 0.77 g CSM-URA (Q-Biogene, cat. no. 4511-212)] to 975 mL of water and autoclave. Add 25 mL of filter-sterilized 40% glucose solution. If pouring plates, add 10 g of bacto-agar before autoclaving.
4. 5-Fluoroorotic acid plates. Add 0.68 g yeast nitrogen base without amino acids (Difco), 74 mg of BIO101 CSM-LYS dropout mix (Q-Biogene, cat. no. 4510-612), 2 g glucose, and 0.1 g 5-fluoroorotic acid (Sigma, cat. no. F5013) to 50 mL of water and filter-sterilize. Add 2 g of agar to 50 mL of water and autoclave. Bring both solutions to 52°C, mix, and pour plates.
5. Sorbitol plates. Add 182 g sorbitol, 6.8 g yeast nitrogen base without amino acids, 0.77 g CSM-URA (Q-Biogene, cat. no. 4511-212), and 20 g bacto-agar to 950 mL water and autoclave. Cool to 52°C, add 50 mL of filter-sterilized 40% glucose solution, and pour plates.
6. Sorbitol top agar. Same as sorbitol plates, but use 25 g agar instead of 20 g.

2.2. BAC Transgenics

2.2.1. BAC DNA Preparation

1. QIAGEN Plasmid Maxi Kit (Qiagen, cat. no. 12162).
2. Lysozyme (Roche Molecular Biochemicals cat. no. 107 255).
3. Luria Bertani (LB) medium, chloramphenicol, isopropyl alcohol, and phenol/chloroform (multiple suppliers).

2.2.2. Pulsed-Field Gel Purification of BAC DNA

1. Bio-Rad pulsed-field gel apparatus (CHEF MAPPER, CHEF DRII, or DRIII).
2. SeaPlaque GTG low-melt agarose (FMC Bioproducts, cat. no. 50101) and agarose MP (Roche Molecular Biochemicals, cat. no. 1 388 983).
3. 0.5X TBE running buffer: 45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA.
4. *Bss*HII, *Not*I, or *Nru*I restriction enzymes with buffers (Roche Molecular Biochemicals).
5. Mid-range PFG marker (New England Biolabs, cat. no. 355-1).
6. Gelase and buffer (Epicentre, cat. no. G09050).

2.2.3. RARE Cleavage

1. 5X RecA buffer: 125 m*M* Tris-acetate, pH 7.85, 20 m*M* $MgCl_2$, 2.5 m*M* spermidine hydrochloride (Sigma), 2 m*M* dithiothreitol (DTT).

2. Adenosine diphosphate/adenosine triphosphate (ADP/ATP)-γ-S: 22 m*M* ADP (Boehringer-Mannheim, cat. no. 1 102 164) and 6 m*M* ATP-γ-S (Boehringer-Mannheim, cat. no. 1 162 306). Keep dilutions separate. Mix immediately prior to use.
3. *Eco*RI methylase (New England Biolabs, cat. no. 211S); *Alu*I methylase (New England Biolabs, cat. no. 220L).
4. 32 m*M S*-adenosylmethionine (supplied with *Eco*RI and *Alu*I methylases).
5. *RecA* protein (United States Biochemical cat. no. E 70028Y/Z).
6. Electrocompetent *E. coli* DH10B cells (Life Technologies, cat. no. 18290-015).
7. 60-mer oligonucleotides that span *Hin*dIII or *Eco*RI sites.

3. Methods

3.1. YAC Constructs for Transgenesis

The YAC cloning system was developed by Burke et al. in 1987 ***(4)*** to allow for the cloning of Mb regions of genomic DNA. Since then a number of human and mouse YAC libraries have been generated ***(23)***, many of which are commercially available. Over the last 10 years, YACs have proved to be a valuable resource for mapping the human (and other) genomes. YACs have also been used extensively to study the structure/function relationships of large genes and gene loci, often by using YACs to generate transgenic mice ***(24)***. In most cases, the transgenic mice have been generated by pronuclear injection of purified YAC DNA.

3.1.2. Characterization of YAC Clones

YAC clones of human and mouse genes are readily available from commercial companies that provide a polymerase chain reaction (PCR)-based screening service (*see* **Subheading 3.3.**, Websites). After obtaining the required YAC clone, it is important that it be well characterized. Ideally, fluorescence *in situ* hybridization (FISH) should be performed to demonstrate that the YAC insert only maps to one chromosome (i.e., that it is not chimeric) ***(25)***. The YAC should then be subjected to restriction mapping to validate the integrity of the clone and to establish the amount of flanking sequence on either side of the gene. This should be done over several generations to check that the clone is stable since some YAC clones, particularly those containing a high level of repetitive DNA sequences, can be susceptible to rearrangement ***(26)***. The YAC should be sized by pulsed-field gel analysis. Although most YACs are easily identified as an additional chromosomal band among the *Saccharomyces cerevisiae* chromosomes, some YACs may run in the same position as a natural yeast chromosome. In this case, a Southern blot of the pulsed-field gel with a YAC arm-specific probe and a set of labeled high-molecular weight markers will establish the approximate size of the YAC. Transfer of the YAC into a

"windowed strain" in which the endogenous yeast chromosome masking the YAC has been fragmented ***(27)*** can also allow for visualization of the YAC band and subsequent manipulations. It may also be useful to "retrofit" the YAC (described in **Subheading 3.1.5.**), particularly if the YAC is to be modified by homologous recombination or if the YAC is to be transferred to mammalian cells.

3.1.3. Purification of YAC DNA for Microinjection

YAC DNA can be purified from preparative pulsed-field gels following electrophoresis of high-molecular weight yeast DNA. This presents a challenge since DNA greater than 50 kb is susceptible to mechanical shearing during in vitro manipulation. Although YACs of up to 560 kb have been successfully microinjected intact into mouse oocytes ***(28)***, it is unlikely that YACs much bigger than this could survive the microinjection procedure. Large YACs can be fragmented by Alu-mediated recombination to a smaller size for purification ***(29)***. Alternatively, transgenic mice can be produced with large YACs using embryonic stem (ES) cell techniques ***(30)***. In this case, large YAC DNA is transformed into ES cells by spheroplast fusion, and ES cells harboring the intact YAC are injected into blastocysts to obtain chimeric mice.

We have used the following method of YAC DNA purification to generate many different lines of transgenic mice harboring a 108-kb YAC containing the human apo-B gene. The key to success for this procedure is to start with agarose plugs containing a high concentration of yeast chromosomal DNA, and to protect the YAC DNA from shearing by adding polyamines and salt to all solutions after agarase digestion (*see* **Note 1**). It is also crucial that all procedures be performed under sterile conditions.

1. Prepare yeast chromosomal plugs with the protocol originally described by Southern et al. ***(31)***. A final concentration of 4×10^9 yeast cells/mL of agarose is optimal to obtain high-concentration plugs. (Approximately 1.5 mL of agarose plugs is required for each preparative gel.)
2. Equilibrate plugs with four changes of 0.5X TBE buffer.
3. Prepare 150 mL of 1% low-melt agarose solution in 0.5X TBE. Assemble the pulsed-field gel casting stand, making sure the stand is level, and put preparative comb (or taped analytical comb) in place. Pour gel at 60°C and, once solidified, place at 4°C for 30 min.
4. Remove comb and load plugs end to end along the front edge of the well. At each end of the preparative lane, load a pulsed-field gel DNA marker. Seal the well with 1% low-melt agarose.
5. Separate the yeast chromosomal DNA by pulsed-field gel electrophoresis in 0.5X TBE buffer under conditions designed to resolve yeast chromosomes in the size range of the YAC. A separation in 0.5× TBE at 6 V/cm with initial and final

switching times of 0.22 and 22 s for 16 h at 14°C is ideal to separate YACs in the 100-kb size range. Bio-Rad product information documents provide technical information regarding separation conditions for different-sized YACs.

6. Following electrophoresis, cut a strip from either side of the gel containing the marker lane and up to 1 cm of the preparative lane. Stain strips with ethidium bromide to identify the YAC band. Notch the strip to mark the location of the YAC band and realign the strips with the body of the gel. Cut across either side of the YAC band in the unstained portion of the gel with a large knife of uniform blade width. Stain the residual gel to ensure that the YAC band has been excised properly. An extra electrophoresis step can be performed at this point to concentrate the YAC DNA into 4% Nusieve ***(19)***. However, we have not found this step to be necessary.
7. Cut YAC strip into smaller lengths and equilibrate overnight in β-Agarase buffer containing 100 m*M* NaCl, 30 μ*M* spermine, and 70 μ*M* spermidine.
8. Transfer gel slices into 1.5-mL Eppendorf tubes and melt agarose at 68°C. Allow to cool to 42°C, and then add β-Agarase at 5 U/mL to melted agarose. Mix the Agarase through the solution by gently pipeting with a wide-bore 1-mL pipet tip and digest at 42°C for 2 h.
9. Centrifuge the digested agarose solution at 18,000*g* in a microfuge for 20 min to pellet any undigested agarose. Transfer the DNA solution to a Millipore Filter Unit preequilibrated with YAC microinjection buffer. Exchange the YAC DNA into 2 vol of YAC microinjection buffer by centrifugation at 17,000*g* and concentrate to 100–200 μL. Leave the DNA solution on the filter at 4°C overnight. Gently remove the DNA from the filter with a wide-bore 200-μL pipet tip and place in an Eppendorf tube.
10. Determine the YAC DNA concentration either by electrophoresis on 1% agarose gels alongside DNA markers of known concentration, DNA dipstick (Invitrogen), or fluorometry. (We routinely obtain 100–200 μL of a 5 ng/μL solution.) Assess the integrity of the purified YAC DNA on a 1% agarose pulsed-field gel (Twenty microliters should yield a YAC band that stains intensely with ethidium bromide). The YAC should migrate as a single band, with little or no evidence of shearing with ethidium bromide staining or by Southern blotting.
11. The purified YAC DNA should be used for microinjection as soon as possible; however, we have successfully microinjected YAC DNA that was stored for up to a month at 4°C. Just prior to microinjection, dilute the YAC DNA to 1 ng/μL with YAC microinjection buffer.
12. Microinject the YAC DNA according to standard procedures ***(32)***.

3.1.4. Analysis of YAC Transgenic Founders

Potential YAC founders can be identified in the same way as conventional transgenics via PCR, slot blot, or Southern blot analysis of tail DNA. Since the insertion of sheared DNA is always a possibility with YAC constructs, additional studies are required to document the structural integrity of the YAC.

PCR can be used to demonstrate the presence of the left and right YAC insert-vector junctions, although this technique alone does not *prove* that the YAC has integrated as a single, unfragmented molecule. This technique can be combined with a more extensive PCR analysis of YAC insert sequences ***(33)***. Probably the most stringent way of demonstrating an intact YAC, however, is to use long-range restriction mapping where unique restriction sites are utilized to recover the YAC insert ***(34,35)***. Methodology for a comprehensive analysis of YAC structure in transgenic mice is reviewed elsewhere ***(21)***. Only transgenic founders with an intact copy of the transgene should be studied.

3.1.5. Mutagenesis of YAC Constructs

Almost any type of mutation can be introduced into a YAC construct by homologous recombination. The two main methods used for this purpose are the "pop-in, pop-out" sequence-insertion method ***(36)*** and a sequence-replacement method ***(6,20)***. The "pop-in, pop-out" method has been used extensively to introduce mutations into YACs for functional studies ***(17,18,34,35,37–39)***. The following protocol has been used to introduce point mutations and replace sequences into a 108-kb apo-B YAC ***(17,18,40)***. This procedure requires that the YAC be "retrofitted" with appropriate selection markers. The majority of YAC libraries are made with the pYAC4 vector ***(41)***, which contains TRP1 and URA3 selection markers. Since URA3 is required for selection in both the "pop-in" and "pop-out" steps, it needs to be replaced with an alternative marker. A commonly used retrofitting vector is pRV1 ***(42)***, which replaces the URA3 marker with a LYS2 marker as well as adding a neomycin resistance gene. The latter is desirable if the YAC is to be transfected into mammalian cells. A more extensive retrofitting procedure can be performed to provide extra restriction sites for structural analysis of the YAC ***(21)***.

1. Construct a gene-targeting vector containing the desired mutated sequence in the yeast integrating vector pRS406 (Stratagene). The mutated sequence should be associated with a new restriction site to facilitate subsequent analysis of gene-targeting events. A unique restriction site preceding the mutation is also required so that the vector can be linearized to facilitate integration into the YAC. Approximately 400 bp of homology between the point of linearization and the mutation is desirable to facilitate efficient homologous recombination with the YAC in the "pop-in" step.
2. Linearize the gene-targeting vector and transform into spheroplasts prepared from yeast containing the YAC with the BIO101 Yeast Spheroplast Transformation Kit. Alternatively, use the protocol contained in *YAC Libraries: A Users Guide* ***(43)*** for generating and transforming yeast spheroplasts.
3. Following transformation, grow yeast on sorbitol plates lacking uracil to select for URA3. (It usually takes 3–4 days before transformants are visible.) Transfer

transformants to synthetic dropout plates lacking uracil, tryptophan, and lysine to maintain selection for both YAC arms and the integrated targeting vector.

4. Prepare yeast plugs containing chromosomal DNA from potential "pop-in" clones as in **Subheading 3.1.3.**, and subject them to pulsed-field gel electrophoresis. Depending on the size of the YAC and gene-targeting vector, it is sometimes possible to observe an increase in the size of the YAC after targeting. (*See* **Notes 2** and **3**.)
5. To identify correctly targeted "pop-in" clones, perform PCR reactions across the mutated region and perform a restriction digestion to test for the presence of the new site associated with the mutation. The PCR can be performed directly from yeast colonies ***(44)***. The digested PCR product will retain uncut DNA, despite successful gene targeting, because of the wild-type sequence duplicated downstream from the targeted sequence. Alternatively, a Southern blot of digested yeast genomic DNA with a flanking probe will also identify correctly targeted clones ***(17)***.
6. To complete the gene-targeting process, a "pop-out" step is performed to allow recombination between the targeted sequence and the duplicated wild-type sequence. Grow "pop-in" clones in synthetic dropout media lacking lysine and tryptophan but containing uracil (to allow for the loss of URA3 by intrachromosomal recombination). Plate yeast onto 5-fluoroorotic acid plates ***(6)*** to select for URA3-negative clones. (Colonies require 2 days to grow.) Plate potential "pop-out" clones onto dropout plates lacking lysine and tryptophan.
7. Prepare yeast plugs containing chromosomal DNA from potential "pop-out" clones and subject to pulsed-field gel electrophoresis. In some cases, the return of the YAC to its original size can be visualized. Pulsed-field gel analysis will also identify any clones that have undergone rearrangement or deletions in the "pop-out" step. (*See* **Note 4**.)
8. Perform PCR and restriction analysis or Southern blotting as in **step 5** to identify YAC clones retaining a single copy of the mutated sequence (*see* **Note 5**).

The mutant YAC clone can now be grown and the YAC DNA purified for microinjection as described in **Subheading 3.1.3** (*see* **Note 6**).

3.2. BAC Constructs for Transgenesis

In 1992, Shizuya et al. ***(3)*** described the BAC cloning system that allows maintenance of large genomic DNA fragments (up to at least 300 kb) in *E. coli*. The BAC plasmid is based on the bacterial F-factor plasmid that ensures that only one or two copies of the F plasmid are maintained in each cell. Moreover, the F factor can carry very large DNA inserts. These features of the BAC plasmid have led to a stable cloning system in which large pieces of genomic DNA can be maintained.

BAC libraries of human and mouse genomic DNA are commercially available. Several companies offer PCR or Southern blot screening services.

BACs are now widely used as a source of large genomic DNA fragments. A number of methods are available for modifying BAC inserts. Here we describe *RecA*-assisted restriction endonuclease (RARE) cleavage, which can be used efficiently for introducing specific deletions into purified BAC DNA. Recombination-based techniques have also been used for manipulating BACs in *E. coli* for transgenic mouse studies ***(14,16,45–47)***.

3.2.1. Propagation and Purification of BAC Plasmid DNA

Propagation and isolation of BAC plasmid DNA from *E. coli* can essentially be done by following standard molecular biology protocols. The major exceptions are that we always use wide-bore pipet tips and never vortex or freeze BAC DNA. We have used the QIAGEN Plasmid Maxi Kit for isolating BAC plasmid DNA from 1-L bacterial cultures. This protocol has invariably yielded high-quality BAC DNA.

1. Prepare 1 L of LB medium containing 12.5 μg/mL chloramphenicol. Inoculate the medium with an overnight BAC culture and grow for 16–18 h, shaking at 37°C.
2. Pellet the cells in a GS-3 Sorvall rotor at 13,500*g* for 15 min.
3. Decant supernatant, invert briefly to drain, and aspirate droplets. Add 13.5 mL Solution I (from the Qiagen kit) to each tube.
4. Resuspend by securing on a rotating platform, shaking at 150 rpm. This will require about 15 min and should result in a perfectly uniform suspension with no clumping.
5. Add 1.5 mL of a 10 mg/mL lysozyme solution (dissolved in Solution I). Mix by swirling for 5 min at 50 rpm on a platform shaker at room temperature.
6. Add 15 mL freshly made Solution II, and mix with a plastic pipet until solution is uniform.
7. Place on ice for 5 min while swirling at 50 rpm on the platform shaker.
8. Add 15 mL ice-cold Solution III, and mix with a pipet again until solution is completely mixed. Repeat **step 7**.
9. Transfer to a 50-mL centrifuge tube and pellet debris in a swinging bucket rotor (e.g., Sorvall HB4) at 20,000*g* for 15 min.
10. Equilibrate QIAGEN-tip 500 columns by applying 10 mL QBT buffer.
11. Pass the entire 45-mL vol through the column. It is very important that minimal debris be loaded onto the column, as this will inhibit flow. (We have used a mesh filter to filter the cell lysate before loading it onto the column.)
12. Wash the QIAGEN-tip 500 column with 2 × 30-mL washes of QC buffer.
13. Transfer column to a 50-mL centrifuge tube.
14. Elute DNA with 15 mL QF buffer.
15. Precipitate DNA with 9 mL isopropyl alcohol. Invert to mix. Pellet DNA in a Sorvall HB4 swinging bucket rotor at 20,000*g* for 20 min at room temperature.
16. Wash the pellet twice with 0.75 mL of 70% EtOH. Transfer DNA and EtOH to an Eppendorf tube.

17. Spin at 13,000*g* for 10 min.
18. Resuspend the pellet in 600 μL TE.
19. Add 70 μL 3 M NaOAc.
20. Add 700 μL of phenol/$CHCl_3$. Invert by hand for 1 min to mix well.
21. Spin at 13,000*g* for 5 min. Transfer supernatant to a new Eppendorf tube.
22. Precipitate DNA by adding 400 μL of isopropyl alcohol. Invert several times to mix well.
23. Spin at 13,000*g* for 5 min.
24. Wash with 1 mL of 70% EtOH.
25. Spin at 13,000*g* for 5 min. Aspirate. Air-dry and resuspend the BAC DNA in 150 μL TE.
26. Estimate yield from absorbance at 260/280 nm. We generally obtain 50–250 μg of BAC DNA per preparation.

3.2.2. Preparation of Linearized BAC DNA for Microinjection

BACs can frequently be linearized within the vector sequences with *Bss*HII, *Nru*I, or *Not*I (*see* **Note 7**). The linearized DNA can then be purified from pulsed-field gels. Prior to choosing one of these enzymes, the absence of these restriction sites in the BAC insert should be established by restriction enzyme cleavage and pulsed-field gel analysis of BAC DNA. *Not*I cleaves the BAC at the cloning site, removing all of the BAC vector sequences. Both *Bss*HII and *Nru*I cleave twice within the BAC vector and leave some BAC vector sequences with the insert. Linearization with *Bss*HII or *Nru*I makes it possible to use Southern blots, with BAC vector probes, to establish that the entire BAC transgene has integrated into the mouse genome. *Bss*HII-cleaved BAC transgenes can be recovered with *Nru*I or *Not*I. *Nru*I-prepared fragments can be recovered with *Not*I. If the BAC insert is cleaved by all three enzymes, an alternative is to utilize the unique λ terminase recognition site in the BAC vector to linearize the BAC ***(48)***. Although it is frequently recommended that all plasmid DNA sequences be removed from conventional transgenes prior to microinjection, we have never observed any interference of the retained BAC vector sequences on expression patterns of apo-B transgenes.

We have used the protocol below to prepare 70–205-kb linearized BAC DNA for microinjections ***(13,49)***.

1. Cut 10–30 μg of BAC DNA with *Bss*HII, *Nru*I, or *Not*I in a 200-μL reaction volume by incubating at 37°C for 3 h.
2. Melt 2 g low-melt agarose with 200 mL 0.5X TBE. Cast gel in a 15 × 15-cm gel casting tray. Prepare one wide lane corresponding to six normal lanes (approx 4 cm wide) with a taped gel comb. After the agarose has solidified, load the digest in the wide lane with pulsed-field gel markers (e.g., Midrange PFG marker

from New England Biolabs) on either side of the gel so that the size of the DNA fragment for microinjection can be accurately assessed. Run the pulsed-field gel in 0.5X TBE at 6 V/cm with initial and final switching times of 1 and 10 s, respectively, for 14 h at 14°C.

3. After electrophoresis, slice a gel strip containing a small part of the preparative lane and stain with ethidium bromide. After staining for 30 min, mark the position of the linearized BAC band under UV light. Realign the gel strip with the body of the gel, and cut out the BAC band from the unstained part of the gel. The band should be cut out to maximize the concentration of DNA, even if this occurs at the expense of DNA recovery.
4. Equilibrate gel slice with gelase buffer containing 100 m*M* NaCl.
5. Transfer the gel blocks to a microcentrifuge tube. Place the tube at 65°C for 10 min to melt the gel. Bring the tube and its contents to 42°C in a water bath. Add 5 U of gelase (5 μL) per 200 μL of gel. Incubate at 42°C for at least 1 h. Place tube on ice for 2 min in order to solidify any undigested agarose. Centrifuge tube at 20,000*g* for 2 min to pellet any undigested agarose. If there is a significant fraction of undigested (i.e., visible) agarose remaining, the digestion should be repeated.
6. Estimate the concentration of the linearized fragment, preferably with a fluorometric method (alternatively, by agarose gel electrophoresis). Adjust the concentration of DNA to 3 ng/μL with gelase buffer containing 100 m*M* NaCl and store at 4°C. We have generated transgenic mice from BACs with final DNA concentrations of 3, 2, and 0.5 ng/μL with no discernible effect on transgenesis efficiency or transgene copy number. The linearized BACs can be stored in the gelase buffer containing 100 m*M* NaCl for at least 5 days before microinjection.
7. Prior to microinjection, analyze the isolated DNA fragment on a 1% pulsed-field agarose gel to ensure that the fragment is not sheared and that it is the correct size.
8. Microinject as per standard procedures ***(32***; *see* **Note 8)**.

3.2.3. Assessing the Intactness of BAC Transgenes

Shearing of the linearized BAC DNA used for microinjection may result in the generation of transgenic mice that lack portions of the transgene. Therefore, it is advisable to evaluate the intactness of the BAC transgene in transgenic mouse lines. This can be done by Southern blot analysis of high-molecular genomic DNA from the transgenic mice ***(34,35)***. Alternatively, we have used PCR analysis of transgenic mouse genomic DNA ***(13,49)***. Amplification of BAC vector sequences at the 5′ and 3′ ends of the BAC transgene was used to ensure that both ends of the BAC had integrated into the transgenic mouse genome. However, the latter approach does not *prove* that the two ends have integrated into the same chromosomal location.

3.2.4. Making Specific Deletions in a BAC with RARE Cleavage

For some transgenic studies with BACs, it may be an advantage to delete a portion of the BAC prior to microinjection. For instance, genes that coreside with the gene of interest in a BAC can be removed to avoid putative unwanted effects of such genes in a transgenic experiment. Another application of transgenic studies is to define distant DNA elements that control gene expression. We have used deleted BACs to define distant DNA regulatory sites that control the intestinal expression of the apo-B gene ***(13,49)***. For these studies, we used RARE cleavage, a methodology that can be used efficiently to delete a portion of a BAC between two *Eco*RI or two *HindIII sites* ***(9,11–13,49–51)***. In the presence of *recA*, specific oligonucleotides can interact with the double-stranded BAC DNA and protect a given restriction site from methylation by a restriction-site specific methylase. This method has been used extensively to delete 16–65-kb fragments in an approx 145-kb BAC containing the approx 45-kb human apo-B gene.

1. Add these reagents to a sterile microcentrifuge tube at room temperature in the following order: the required volume of H_2O to make a final volume of 160 µL, 32 µL 5X *recA* buffer, 18.2 µL *recA*, 16 µL ADP/ATP-γ-S, 2.25 µL of each oligonucleotide (160 ng/mL; or 4.5 µL if only one oligonucleotide is used; *see* **Note 9**), 4 µg BAC DNA, 8 µL acetylated bovine serum albumin (BSA; 2 mg/mL in H_2O).
2. Mix gently by flicking the tube.
3. Incubate for 10 min at 37°C.
4. Add 8 µL *Eco*RI methylase diluted 1:10 with H_2O (if digesting with *Eco*RI) or 6.4 µL *Alu*I methylase undiluted (if digesting with *Hin*dIII).
5. Add 8 µL S-adenosylmethionine, diluted 7.5:100 with H_2O.
6. Mix gently.
7. Incubate for 30 min at 37°C.
8. Incubate for 15 min at 65°C (to inactivate the methylase and *recA* protein).
9. Centrifuge for 2 min at 20,000*g*.
10. Filter-dialyze the sample for 30 min against 0.5X TE on Millipore type VS filter-0.025 µm.
11. Add 1:9 vol of restriction endonuclease buffer and 80 U of *Eco*RI or *Hin*dIII.
12. Incubate for 1 h at 37°C.
13. Examine 1:10 of the RARE cleavage product by pulsed-field gel electrophoresis.
14. To purify the RARE cleavage reaction product, use pulsed-field gel electrophoresis in low-melt agarose as described in **steps 2–5** in **Subheading 3.2.2.** The only difference is that the lane should be approx 2 cm wide rather than the 4-cm wide lanes that are used for preparing BAC DNA for microinjection.
15. Mix 90 µL of the digested agarose containing BAC DNA, 10 µL of 10X ligation buffer, and 1.5 µL of T4-ligase (New England Biolabs). Incubate at 16°C overnight to ligate the deleted BAC DNA.

16. Filter-dialyze the ligation reaction against 0.5X TE for at least 30 min at room temperature before transformation.
17. Electrotransform 30 μL of competent DH10B *E. coli* cells with 2 μL of the ligation reaction. Follow the instructions that come with the cells, except use 1.3 V/cm instead of 1.8 V/cm in the electroporation step.
18. Inoculate cells in SOC medium for 45–60 min. Plate 80–90% of the ligation product on an LB plate containing chloramphenicol (12.5 μg/mL). Incubate overnight at 37°C.
19. Pick 10 colonies and prepare minipreps as described below to analyze the deleted BACs.

3.2.5. Minipreps of BAC DNA for Restriction Enzyme Mapping

This method yields approx 4 μg of BAC DNA. The DNA should be kept at 4°C and used for analyses within 24 h. We have used standard solutions from the QIAGEN Plasmid Maxi Kit for the BAC minipreps.

1. Pick a colony and inoculate into 4.5 mL of LB medium containing chloramphenicol (12.5 μg/mL). Grow overnight.
2. Pellet cells (5500*g* for 10 min). Remove supernatant.
3. Prepare lysozyme solution (10 mg/mL, in Solution I from the Qiagen kit).
4. Add 100 μL Solution I. Vortex to resuspend.
5. Add 10 μL lysozyme solution (10 mg/mL). Leave at room temperature for 3 min.
6. Add 200 μL Solution II. Vortex very briefly.
7. Add 150 μL Solution III.
8. Transfer to an Eppendorf tube and centrifuge for 5 min at 20,000*g*.
9. Transfer supernatant to a new tube and add 2X vol of 100% EtOH. Flick the tube to mix and precipitate DNA.
10. Centrifuge for 5 min at 20,000*g*. Decant EtOH.
11. Wash the pellet in 1 mL 70% EtOH and centrifuge for 5 min at 20,000*g*. Decant supernatant.
12. Let the DNA pellet dry for 5 min at room temperature and redissolve in 20 μL of TE.
13. Analyze BAC DNA with 5 μL in each restriction-digest reaction.

3.2.6. Sequencing of BAC DNA

To map the direction of an insert in a BAC clone, we have used DNA sequencing with T7 and SP6 primers. We have also sequenced fragments of the insert in BACs to ensure the fidelity of RARE-cleavage deletions. For BAC sequencing, we have used the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin-Elmer according to the manufacturer's protocol, except that we use 2 or 4 μg of BAC DNA in each sequencing reaction. We have successfully sequenced BAC DNA isolated both by the mini-

prep (*see* **Subheading 3.2.5.**) and the MAXI-PREP protocol (*see* **Subheading 3.2.1.**).

3.2.7. Storage of BAC Clones

BAC plasmid DNA isolated as described in **Subheading 3.2.1.** can be kept at 4°C for months in TE. We have kept glycerol stocks of BAC clones for years at –80°C.

3.3. Websites

For relevant websites, *see* **Notes 10** and **11**.

4. Notes

1. Polyamines and salt are included in the isolated YAC DNA solutions to compact the DNA and therefore protect against breakage. There is some controversy as to whether both are necessary. A recent study suggests that only the polyamines are necessary ***(52)***, whereas Petersen et al. ***(21)*** have successfully produced many lines of β-globin mice in microinjection buffer containing only the salt component.
2. We have occasionally observed yeast clones containing two copies of the YAC. This was only apparent after pulsed-field gel analysis revealed the presence of a mutated and a wild-type YAC in the same "pop-in" colony ***(20)***. A KAR cross ***(53)*** will efficiently allow for segregation of the mutant and the wild-type YAC.
3. Occasionally, multiple copies of the gene-targeting vector can cointegrate into the YAC in the "pop-in" step. This is usually apparent after pulsed-field gel analysis reveals a greater-than-expected increase in YAC size. Clones containing multiple integrations should be avoided.
4. We have identified deleted YACs in the "pop-out" step after pulsed-field gel electrophoresis revealed YACs of reduced size. This was presumably a result of recombination between the integrated URA3 gene and remnants of the URA3 gene flanking the LYS2 on the retrofitted YAC arm. An improved retrofitting procedure ***(21)*** should prevent this problem.
5. In general, we find that about 40% of the URA3/TRP1/LYS2-positive clones in the "pop-in" step contain a single correctly targeted YAC. A lower efficiency is expected in the "pop-out" step, since a certain portion of the clones will revert back to wild-type due to the location of the intrachromosomal recombination event. We have experienced efficiencies of up to 25% in the "pop-out" step, i.e., 25% of the URA3 negative clones contain correctly sized YACs retaining the mutant sequence.
6. In our experience, the efficiency of transgenesis with YAC DNA has ranged between 10 and 20%. All of the mice produced from our experiments expressed the full-length gene product, except for one founder mouse that expressed a truncated form of the protein, presumably from the incorporation of sheared YAC DNA.

7. One group has reported generation of transgenic mice by directly injecting nonlinearized BAC plasmid DNA ***(54)***. This approach, however, could result in the breaking of the BAC insert within a region important for the function of the gene of interest.
8. In generating more than 40 lines of transgenic mice from BACs, the transgenesis efficiencies have been approx 15%, and the transgene copy number has ranged from 1 to 4. In our experiments, the integrated BACs have been intact, except in one case.
9. For RARE cleavage reactions, in some cases we have used a *single* 60-mer oligonucleotide to protect two *Hin*dIII sites: one arm of the oligonucleotide covered 30 nucleotides adjacent to one *Hin*dIII site; the other arm of the oligonucleotide covered 30 nucleotides adjacent to another *Hin*dIII site.
10. A number of YAC screening services are available on the internet including screening from the CEPH library (http://www.cephb.fr/services/), the ICI library (http://w3dibit.hsr.it/YAC/first-yac.html), and the Washington library (http://www.resgen.com/products/WASHUYAC.php3). Yeast vectors for modifying YACs can be found at the American Type Tissue Collection website (http://www.atcc.org/). A range of useful YAC protocols can be found at the protocols online web site (http://www.protocol-online.net/molbio/Yeast/yac.htm).
11. Several protocols for BAC plasmid isolation and sequencing are available on the web: http://gsu.med.ohio-state.edu/bac_prep_Plass.html; http://www.tree.caltech.edu/protocols/BAC-DNA-Isolation.html; and http://www.nig.ac.jp/labs/EarlyEmb/midline/protocol/BAC.html. An excellent compilation of protocols and technical hints for recombination-based modification of BACs in *E. coli* is available on http://www.embl-heidelberg.de/ExternalInfo/stewart/index.html.

References

1. Sternberg, N. (1990) Bacteriophage P1 cloning system for the isolation, amplification, and recovery of DNA fragments as large as 100 kilobase pairs. *Proc. Natl. Acad. Sci. USA* **87,** 103–107.
2. Ioannou, P. A., Amemiya, C. T., Garnes, J., et al. (1994) A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat. Genet.* **6,** 84–89.
3. Shizuya, H., Birren, B., Kim, U. J., et al. (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. USA* **89,** 8794–8797.
4. Burke, D. T., Carle, G. F., and Olson, M. V. (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* **236,** 806–812.
5. Zhu, Y., Jong, M. C., Frazer, K. A., et al. (2000) Genomic interval engineering of mice identifies a novel modulator of triglyceride production. *Proc. Natl. Acad. Sci. USA* **97,** 1137–1142.

6. Rothstein, R. (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* **194,** 281–301.
7. Sternberg, N. (1994) The P1 cloning system: past and future. *Mamm. Genome* **5,** 397–404.
8. McCormick, S. P. A., Linton, M. F., and Young, S. G. (1994) Expression of P1 DNA in mammalian cells and transgenic mice. *Genet. Anal. Tech. Appl.* **11,** 158–164.
9. Ferrin, L. J. and Camerini-Otero, R. D. (1991) Selective cleavage of human DNA: RecA-assisted restriction endonuclease (RARE) cleavage. *Science* **254,** 1494–1497.
10. Callow, M. J., Ferrin, L. J., and Rubin, E. M. (1994) Single base, site-directed mutagenesis of a 90 kilobase-pair P1 clone. *Nucleic Acids Res.* **22,** 4348–4349.
11. Boren, J., Lee, I., Callow, M. J., Rubin, E. M., and Innerarity, T. L. (1996) A simple and efficient method for making site-directed mutants, deletions, and fusions of large DNA such as P1 and BAC clones. *Genome Res.* **6,** 1123–1130.
12. Allan, C. M., Taylor, S., and Taylor, J. M. (1997) Two hepatic enhancers, HCR.1 and HCR.2, coordinate the liver expression of the entire human apolipoprotein E/C-I/C-IV/C-II gene cluster. *J. Biol. Chem.* **272,** 29113–29119.
13. Nielsen, L. B., Kahn, D., Duell, T., et al. (1998) Apolipoprotein B gene expression in a series of human apolipoprotein B transgenic mice generated with recA-assisted restriction endonuclease cleavage-modified bacterial artificial chromosomes. An intestine-specific enhancer element is located between 54 and 62 kilobases 5′ to the structural gene. *J. Biol. Chem.* **273,** 21800–21807.
14. Yang, X. W., Model, P., and Heintz, N. (1997) Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat. Biotechnol.* **15,** 859–865.
15. Jessen, J. R., Meng, A., McFarlane, R. J., et al. (1998) Modification of bacterial artificial chromosomes through chi-stimulated homologous recombination and its application in zebrafish transgenesis. *Proc. Natl. Acad. Sci. USA* **95,** 5121–5126.
16. Muyrers, J. P., Zhang, Y., Testa, G., and Stewart, A. F. (1999) Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res.* **27,** 1555–1557.
17. McCormick, S. P. A., Ng, J. K., Taylor, S., et al. (1995) Mutagenesis of the human apolipoprotein B gene in a yeast artificial chromosome reveals the site of attachment for apolipoprotein(a). *Proc. Natl. Acad. Sci. USA* **92,** 10147–10151.
18. McCormick, S. P. A., Ng, J. K., Cham, C. M., et al. (1997) Transgenic mice expressing human ApoB95 and ApoB97. Evidence that sequences within the carboxyl-terminal portion of human apoB100 are important for the assembly of lipoprotein. *J. Biol. Chem.* **272,** 23616–23622.
19. Schedl, A., Larin, Z., Montoliu, L., et al. (1993) A method for the generation of YAC transgenic mice by pronuclear microinjection. *Nucleic Acids Res.* **21,** 4783–4787.
20. McCormick, S. P. A., Peterson, K. R., Hammer, R. E., Clegg, C. H., and Young, S. G. (1996) Generation of transgenic mice from yeast artificial chromosome DNA that has been modified by gene targeting. *Trends Cardiovasc. Med.* **6,** 16–24.

21. Peterson, K.R. (1999) Use of yeast artificial chromosomes to express genes in transgenic mice. *Methods Enzymol.* **306,** 186–203.
22. McCormick, S. P. A., Allan, C. M., Taylor, J. M., and Young, S. G. (1997) The use of P1 bacteriophage clones to generate transgenic animals, in *Transgenic Animals: Generation and Use* (Houdebine, L. M., ed.), Harwood Academic Publishers, Amsterdam, The Netherlands, pp. 273–281.
23. Larin, Z., Monaco, A. P., and Lehrach, H. (1994) Large insert yeast artificial chromosome libraries, in *YAC Libraries: A User's Guide* (Nelson, D. L. and Brownstein, B. H., eds.), W. H. Freeman, New York, pp. 43–56.
24. Peterson, K. R., Clegg, C. H., Li, Q., and Stamatoyannopoulos, G. (1997) Production of transgenic mice with yeast artificial chromosomes. *Trends Genet.* **13,** 61–66.
25. Green, E. D., Riethman, H. C., Dutchik, J. E., and Olson, M. V. (1991) Detection and characterization of chimeric yeast artificial-chromosome clones. *Genomics* **11,** 658–669.
26. Kouprina, N., Eldarov, M., Moyzis, R., Resnick, M., and Larionov, V. (1994) A model system to assess the integrity of mammalian YACs during transformation and propagation in yeast. *Genomics* **21,** 7–17.
27. Hamer, L., Johnston, M., and Green, E. D. (1995) Isolation of yeast artificial chromosomes free of endogenous yeast chromosomes: construction of alternate hosts with defined karyotypic alterations. *Proc. Natl. Acad. Sci. USA* **92,** 11706–11710.
28. Huxley, C., Passage, E., Manson, A., et al. (1996) Construction of a mouse model of Charcot-Marie-Tooth disease type 1A by pronuclear injection of human YAC DNA. *Hum. Mol. Genet.* **5,** 563–569.
29. Pavan, W. J., Hieter, P., Sears, D., Burkhoff, A., and Reeves, R. H. (1991) High-efficiency yeast artificial chromosome fragmentation vectors. *Gene* **106,** 125–127.
30. Jakobovits, A., Moore, A. L., Green, L. L., et al. (1993) Germ-line transmission and expression of a human-derived yeast artificial chromosome. *Nature* **362,** 255–258.
31. Southern, E. M., Anand, R., Brown, W. R., and Fletcher, D. S. (1987) A model for the separation of large DNA molecules by crossed field gel electrophoresis. *Nucleic Acids Res.* **15,** 5925–5943.
32. Hogan, B., Beddington, R., and Constantini, F. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
33. Frazer, K. A., Narla, G., Zhang, J. L., and Rubin, E. M. (1995) The apolipoprotein(a) gene is regulated by sex hormones and acute-phase inducers in YAC transgenic mice. *Nat. Genet.* **9,** 424–431.
34. Peterson, K. R., Li, Q. L., Clegg, C. H., et al. (1995) Use of yeast artificial chromosomes (YACs) in studies of mammalian development: production of beta-globin locus YAC mice carrying human globin developmental mutants. *Proc. Natl. Acad. Sci. USA* **92,** 5655–5659.
35. Peterson, K. R., Clegg, C. H., Navas, P. A., et al. (1996) Effect of deletion of 5′HS3 or 5′HS2 of the human beta-globin locus control region on the developmental

regulation of globin gene expression in beta-globin locus yeast artificial chromosome transgenic mice. *Proc. Natl. Acad. Sci. USA* **93,** 6605–6609.
36. Scherer, S. and Davis, R. W. (1979) Replacement of chromosome segments with altered DNA sequences constructed in vitro. *Proc. Natl. Acad. Sci. USA* **76,** 4951–4955.
37. Duff, K., McGuigan, A., Huxley, C., Schulz, F., and Hardy, J. (1994) Insertion of a pathogenic mutation into a yeast artificial chromosome containing the human amyloid precursor protein gene. *Gene Ther.* **1,** 70–75.
38. Bungert, J., Dave, U., Lim, K. C., et al. (1995) Synergistic regulation of human beta-globin gene switching by locus control region elements HS3 and HS4. *Genes Dev.* **9,** 3083–3096.
39. Liu, Q., Bungert, J., and Engel, J. D. (1997) Mutation of gene-proximal regulatory elements disrupts human epsilon-, gamma-, and beta-globin expression in yeast artificial chromosome transgenic mice. *Proc. Natl. Acad. Sci. USA* **94,** 169–174.
40. Cheesman, E. J., Sharp, R. J., Zlot, C. H., et al. (2000) An analysis of the interaction between mouse apolipoprotein B100 and apolipoprotein(a). *J. Biol. Chem.* **275,** 28195–28200.
41. Smith, D. R. (1994) Vectors and host strains for cloning and modification of yeast artificial chromosomes, in *YAC Libraries: A User's Guide* (Nelson, D. L. and Brownstein, B. H., eds.), W. H. Freeman, New York, pp. 1–31.
42. Srivastava, A. K. and Schlessinger, D. (1991) Vectors for inserting selectable markers in vector arms and human DNA inserts of yeast artificial chromosomes (YACs). *Gene* **103,** 53–59.
43. McCormick, M. K. (1994) Yeast spheroplast transformation with large DNA, in *YAC Libraries: A User's Guide* (Nelson, D. L. and Brownstein, B. H., eds.), W. H. Freeman, New York.
44. Huxley, C., Green, E. D., and Dunham, I. (1990) Rapid assessment of *S. cerevisiae* mating type by PCR. *Trends Genet.* **6,** 236.
45. Zhang, Y., Buchholz, F., Muyrers, J. P., and Stewart, A. F. (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.* **20,** 123–128.
46. Yu, W., Misulovin, Z., Suh, H., et al. (1999) Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5′ of RAG2. *Science* **285,** 1080–1084.
47. Nefedov, M., Williamson, R., and Ioannou, P. A. (2000) Insertion of disease-causing mutations in BACs by homologous recombination in *Escherichia coli*. *Nucleic Acids Res.* **28,** E79.
48. Chrast, R., Scott, H. S., and Antonarakis, S. E. (1999) Linearization and purification of BAC DNA for the development of transgenic mice. *Transgenic Res.* **8,** 147–150.
49. Nielsen, L. B., McCormick, S. P., Pierotti, V., et al. (1997) Human apolipoprotein B transgenic mice generated with 207- and 145-kilobase pair bacterial artificial chromosomes. Evidence that a distant 5′-element confers appropriate transgene expression in the intestine. *J. Biol. Chem.* **272,** 29752–29758.

50. Ferrin, L. J., and Camerini-Otero, R. D. (1994) Long-range mapping of gaps and telomeres with RecA-assisted restriction endonuclease (RARE) cleavage. *Nat. Genet.* **6,** 379–383.
51. Ferrin, L. J. (1995) Manipulating and mapping DNA with RecA-assisted restriction endonuclease (RARE) cleavage. *Genet. Eng.* **17,** 21–30.
52. Bauchwitz, R. and Costantini, F. (1998) YAC transgenesis: a study of conditions to protect YAC DNA from breakage and a protocol for transfection. *Biochim. Biophys. Acta.* **1401,** 21–37.
53. Spencer, F., Hugerat, Y., Simchen, G., et al. (1994) Yeast kar1 mutants provide an effective method for YAC transfer to new hosts. *Genomics* **22,** 118–126.
54. Antoch, M. P., Song, E. J., Chang, A. M., et al. (1997) Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell* **89,** 655–667.

7

Reporter Molecules in Genetically Engineered Mice

Thomas L. Saunders

1. Introduction

Reporter molecules are commonly used in transgenic mice to follow in vivo gene expression patterns through all developmental stages of the life cycle in all tissues. Numerous reporters are available for use in transgenic mice. The selection of a particular marker for gene expression will affect the type of information that can be obtained from a genetically engineered mouse model. For example, some reporters are suitable for observations of living cells and others for tissue sections or protein extracts. Depending on the reporter molecule, gene expression can be detected by the addition of an enzyme substrate, visualized with an ultraviolet (UV) light, or analyzed with sophisticated instrumentation for flow cytometry, magnetic resonance imaging, and positron emission tomography ***(1–6)***.

Reporter genes can be used in transgenic mice for a number of different purposes. These include the identification of genetic elements that control tissue-specific and temporally correct gene expression, the marking of cells so that the developmental fate of the cells can be described, or the isolation of purified cell populations. A reporter such as the *E. coli* enzyme β-galactosidase is coupled with a heterologous promoter to determine the expression pattern conferred by the promoter sequence. Once the expression pattern of a promoter is known, it can be developed for therapeutic use ***(7)***. In cell lineage studies, a promoter can be used to express a toxic gene such as the diphtheria A chain ***(8)***. Relationships between progenitor cells and descendants are made after the cell types that develop or fail to develop are identified. Cells from transgenic mice expressing reporter genes can be processed for vital staining, and flow cytometry can be used to isolate specific, highly purified differentiated cell

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen © Humana Press Inc., Totowa, NJ

types for further analysis ***(4)***. Transgenic mice with reporter genes are used to determine Cre expression patterns in transgenic models for conditional gene knockouts and tetracycline-inducible gene expression ***(9–12)***.

There are a number of factors that influence the choice of a reporter gene in a particular experiment. Ideal characteristics of a reporter molecule include sensitive detection methods, ease of detection, and absence of toxicity. In principal, any gene can be a reporter gene, as long as an assay exists to detect the gene product. For instance, antibodies to the myc tag, synthetic peptide sequences, and human growth hormone have all been used to detect transgene expression ***(13–15)***. Antibodies to these proteins can be used in immunocytochemistry and Western blot analysis. The prokaryotic chloramphenicol O-acetyl transferase (CAT) enzyme was an early favorite of transgenic researchers because it is sensitive, stable, and specific for transgene expression. Typically, assays for CAT activity are measured in tissue extracts, which makes it difficult to identify individual cells that express CAT. The biochemical assay for CAT is laborious and uses a radioactive substrate. Thin-layer chromatography and X-ray film exposure are required for detection. CAT has been supplanted by other enzymatic reporters such as β-galactosidase, human alkaline phosphatase, and luciferase (**Fig. 1**). Luciferase is a reporter that resembles CAT in that it is sensitive, there is no endogenous enzyme with the same activity, and it is measured in tissue extracts. The luciferase assay is simpler to conduct and is nonradioactive. Commercial kits with all of the necessary reagents for luciferase detection are available. Both CAT and luciferase continue to be used to measure the expression levels of gene promoters in cultured cells. However, with respect to transgenic mice, they are less versatile than other reporters, because it is difficult to identify specific cells that express CAT or luciferase.

At this time, the most widely used reporter molecule in transgenic mice is the β-galactosidase enzyme of *E. coli* (β-gal) encoded by the *lacZ* gene (**Fig. 1**). Assays for its enzymatic activity are highly sensitive. It is a stable protein, and there is low background activity in mouse tissue ***(16)***. One of the reasons for the increased application of β-gal is its adaptability to different uses. For example, as a reporter in transgenic and gene-targeted mice, β-gal has been used to 1) identify expression patterns in whole-mount embryos and organs ***(17)***; 2) identify individual cells within tissues that express the transgene ***(18)***; 3) stain tissue sections both for β-gal and antibody binding ***(18)***; 4) quantitate expression levels in tissue extracts ***(19)***; and 5) purify viable cell populations with vital staining and flow cytometry analysis ***(4)***. β-gal is more versatile than the CAT or luciferase genes because single cells that express this reporter gene can be identified.

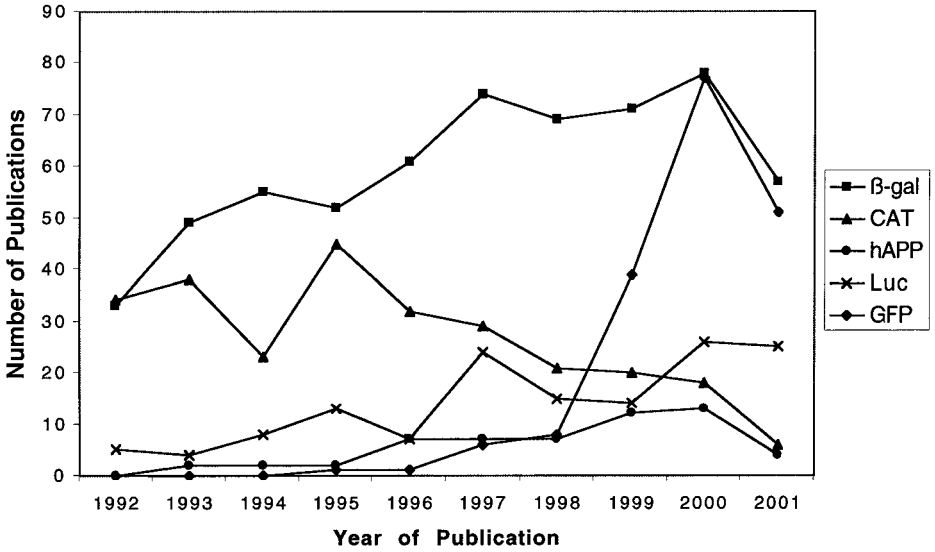

Fig. 1. Trends in reporter gene use. The data in this figure were generated by searching the Medline Database. The numbers of publications shown were found by crossing the Mesh heading "Mice, Transgenic" with the following Mesh headings: galactosidase (β-gal), chloramphenicol *O*-acetyltransferase (CAT), alkaline phosphatase (hAPP), luciferase (Luc), or the key word "green fluorescent protein" (GFP). The results indicate an increasing use of GFP reporter genes in transgenic mice.

Another reporter molecule used with transgenic mice is human alkaline placental phosphatase (hAPP). This enzyme is heat-stable and can be used to mark cells in whole-mount samples or tissue sections. It resembles β-gal in its versatility and may have some advantages over β-gal. For example, it has been suggested that β-gal, a prokaryotic gene, is susceptible to silencing when incorporated into the mouse genome ***(20)***. Comparison of in vivo expression patterns produced by β-gal and hAPP ***(21)*** suggest that expression patterns observed with hAPP and green fluorescent protein (GFP; see below) more closely resemble endogenous gene expression patterns than promoters driving β-gal expression.

GFP is an interesting reporter that has received a lot of attention recently. It is useful for the study of living cells since expression can be assayed simply by illumination with a UV light source provided by a fluorescence microscope or even a hand-held long-wave UV source ***(2)***. The use of confocal microscopy can reveal highly detailed images and allow the identification of GFP-expressing cells ***(22,23)***. One issue to consider is that GFP may not be as sensitive as the enzyme reporters (β-gal, hAPP, luciferase, and CAT). The sensitivity of an enzyme can

be increased by prolonged incubation in enzyme substrates. GFP signals cannot be amplified in the same way. Expression patterns produced by GFP transgenes appear to resemble endogenous patterns more closely than those produced by β-gal transgenes ***(24)***. Unlike the other reporters, which do not affect mouse physiology, evidence of GFP toxicity has been presented ***(25,26)***. GFP is a small molecule and readily diffuses out of permeabilized tissue samples. This makes it difficult to localize to individual cells within an organ. On the other hand, GFP is a powerful reporter for analyzing living cells. For example, Yang et al. ***(27)*** used GFP to image the growth and metastasis of tumor cells in living mice.

A number of molecules can be used to detect transgene expression in genetically modified mice. These animals may be generated by pronuclear microinjection of transgene constructs or they may be produced from mouse embryonic stem cells that have undergone homologous recombination with a targeting vector. In gene knockin mice, the replacement of a gene's coding sequence with a reporter such as β-gal can be used to mark the cells that normally express the gene. All regulatory DNA sequences on the chromosome will be present, and the cells should precisely recapitulate the expression pattern of the endogenous gene ***(28)***. At this time, the most widely used reporter in genetically engineered mice is β-gal. In the past, it was CAT. In the future other reporters will find widespread application; perhaps GFP will be the next widely used reporter. The essential properties of any reporter molecule will continue to be sensitivity of detection, ease of detection, and lack of secondary effects on animal physiology.

2. Materials

2.1. β-Galactosidase Detection

2.1.1. Whole-Mount Histochemical β-gal Detection

1. Fixative for whole-mount samples: 4% paraformaldehyde (Sigma) in 0.1 *M* sodium phosphate buffer, pH 7.3, 5 m*M* EGTA, 2 m*M* $MgCl_2$. Fix can be stored at –20°C for 3 months or stored at 4°C for 2 weeks.
2. X-gal stock (4-chloro-5-bromo-3-indoyl-β-D-galactopyranoside; Sigma): Dissolve in dimethylformamide at 25 mg/mL and store in 1-mL aliquots at –20°C.
3. Wash buffer: 0.1 *M* Sodium phosphate buffer, pH 7.3, 2 m*M* $MgCl_2$, and 0.01% sodium deoxycholate.
4. X-gal stain: make up 5 m*M* potassium ferrocyanide (Sigma), 5 m*M* potassium ferricyanide (Sigma), and 1 mg/mL X-gal in wash buffer.

2.1.2. Histochemical β-Gal Detection in Cryostat Sections

1. Fixative for tissue sections: 0.2% glutaraldehyde in 0.1 *M* sodium phosphate buffer, pH 7.3, 5 m*M* EGTA, and 2 m*M* $MgCl_2$. Add fresh glutaraldehyde when preparing fix.

2. Wash buffer: 0.1 *M* sodium phosphate buffer, pH 7.3, 2 m*M* $MgCl_2$, and 0.02% NP-40.
3. OCT (Miles Scientific).
4. Poly-lysine-coated microscope slides (Fisher Scientific).
5. X-gal stain: *see* **Subheading 2.1.1.**
6. Counterstain: 0.5% neutral red in 50 m*M* sodium acetate, pH 3.3.

2.1.3. β-Gal Detection in Tissue Extracts by Fluorescence

1. Tissue homogenization buffer: 250 m*M* Tris-HCl, pH. 8.0. (*See* **Note 1**.)
2. MUG stock (4-methylumbelliferyl-β-D-galactoside; Sigma). Make up 18 m*M* MUG in distilled water and store in 1-mL aliquots at –20°C.
3. MUG reaction buffer: 25 m*M* Tris-HCl, pH 7.5, 125 m*M* NaCl, 2 m*M* $MgCl_2$, 12 m*M* 2-mercaptoethanol, and 1.8 m*M* MUG to make 10 mL.
4. β-gal standards: prepare 5×10^3 to 5×10^5 units β-gal (Sigma) in reaction buffer when samples are tested.
5. MUG stop buffer: 0.25% Trichloroacetic acid (Sigma).
6. Fluorescence buffer: 0.133 *M* Glycine, 0.083 *M* sodium carbonate, pH 10.7.

2.1.4. β-Gal Detection in Tissue Extracts by Spectrophotometry

1. Phosphate buffer: 3 m*M* $MgCl_2$, 300 m*M* sodium phosphate buffer, pH 7.5.
2. 1.0 *M* 2-mercaptoethanol.
3. ONPG: 14 m*M* O-nitrophenyl-β-D-galactopyranoside (Sigma) in 10 m*M* $MgCl_2$, 10 m*M* Tris-acetate, pH 7.5. Prepare fresh.
4. β-gal standard curve: Dissolve 20–100 μg β–gal in 10 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 10 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.5.

2.1.5. β-Gal detection by Fluorescence in Single Cell Suspensions

1. Single cell suspensions are prepared with appropriate methods for the tissue.
2. 2 m*M* FDG (fluorescein di-β-D-galactopyranoside; Molecular Probes) in sterile water.
3. FDG buffer: Phosphate-buffered saline (PBS), pH 7.3, 10 m*M* HEPES, pH 7.3, 5% fetal bovine serum (FBS).
4. Propidium iodide buffer: FDG staining buffer that contains 1 μg/mL propidium iodide (Sigma).

2.2. Human Alkaline Phosphatase Detection

2.2.1. hAPP in Whole-Mount Samples and Tissue Sections

1. Fixative for whole-mount samples: 4% Paraformaldehyde, 2 m*M* $MgCl_2$ in PBS, pH 7.4.
2. NBT stock (nitro blue tetrazolium; Roche Molecular Biochemicals). Dissolve in dimethylformamide at 50 mg/mL and store in 1-mL aliquots at –20°C.
3. BCIP stock (5-bromo-4-chloro-3-indoyl-phosphate; Roche Molecular Biochemicals). Dissolve in distilled water at 5 mg/mL and store in 1-mL aliquots at –20°C.

4. hAPP stain: 0.1 *M* Tris-HCl, pH 9.5, 0.1 *M* NaCl, and 10 m*M* $MgCl_2$; 1 mg/mL NBT and 0.1 mg/mL BCIP.
5. AP rinse buffer: PBS, pH 7.4 containing 0.2% Tween 20 (Sigma) and 2 m*M* $MgCl_2$.

2.2.2. hAPP Detection in Tissue Extracts by Fluorescence

1. Tissue homogenization buffer: 250 m*M* Tris-HCl, pH. 8.0.
2. MUP stock (4-methylumbelliferyl phosphate; Sigma). Make up 3.6 m*M* MUP fresh daily in 50 m*M* Tris-HCl, pH 8.0, 0.1% bovine serum albumin (BSA).
3. MUP reaction buffer: 36 μ*M* MUP in 50 m*M* Tris-HCl, pH 8.0, 0.1% BSA. Prepare fresh daily.
4. Calf alkaline phosphatase (Roche Molecular Biochemicals) standards: Prepare 1–1500 U/μL in 50 m*M* Tris-HCl, pH 8.0, 0.1% BSA. Prepare fresh daily.
5. MUP stop buffer: 200 m*M* sodium carbonate in distilled water.

2.2.3. hAPP Detection in Tissue Extracts by Spectrophotometry

1. Tissue homogenization buffer: 250 m*M* Tris-HCl, pH. 8.0.
2. PNP stock: 670 m*M* *p*-Nitrophenyl phosphate Na salt (Sigma) is prepared in distilled water.
3. Diethanolamine buffer: 1000 m*M* Diethanolamine, pH 9.8, at 37°C, 0.5 m*M* $MgCl_2$.
4. PNP reaction diluent: 100 m*M* triethylamine-HCl, pH 7.6, 10 m*M* $MgCl_2$, 1 m*M* $ZnCl_2$.
5. Calf alkaline phosphatase (Roche Molecular Biochemicals) standards: Prepare 1–1500 U/μL in 50 m*M* Tris-HCl, pH 8.0, 0.1% BSA. Prepare fresh daily.

2.3. Reagents for Chloramphenicol O-Acetyl Transferase Detection

2.3.1. CAT Detection in Tissue Extracts

1. Tissue homogenization buffer: 250 m*M* Tris-HCl, pH 7.8.
2. CAT reaction buffer: 250 m*M* Tris-HCl, pH 7.8.
3. 4 m*M* acetyl coenzyme A, prepare fresh.
4. [^{14}C]chloramphenicol (50 μCi/mmol; New England Nuclear).
5. Ethyl acetate.
6. Thin-layer chromatography solvent for silica gel plates: 95:5 chloroform/methanol.

2.3.2. CAT Detection in Tissue Sections

1. Fixative for tissue sections: 2% paraformaldehyde in PBS.
2. Cryoprotectant buffers: 10% Sucrose in PBS and 30% sucrose in PBS.
3. Gelatin-coated microscope slides.
4. Stain stock A: 8 m*M* sodium citrate, 5 m*M* copper sulfate, 100 m*M* Na maleate, pH 6.0. Store at 4°C.
5. Stain stock B: 50 m*M* Potassium ferricyanide. Store at 4°C.

6. Stain stock C: 16 m*M* Chloramphenicol. Prepare fresh.
7. Stain stock D: 15 m*M* Acetyl coenzyme A (ICN Biochemicals). Prepare fresh.
8. Stain: Mix together stocks A/B/C/D in a ratio of 630 : 100 : 250 : 20.

2.4. Green Fluorescent Protein Detection

1. GFP buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0, 0.02% sodium azide.
2. GFP standard: Purified GFP (Clontech) is diluted to 5 μg/mL in GFP buffer, with a final volume of 4 mL or sufficient to cover the excitation/emission ports of the fluorometer.

2.5. Luciferase Detection in Tissue Extracts

1. Homogenization buffer: 15 m*M* Potassium phosphate buffer, pH 7.5, 1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 10% glycerol.
2. 5X lysis buffer: 125 m*M* Tris-PO_4, pH 7.8, 10 m*M* DTT, 10 m*M* 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 50% glycerol, 5% Triton X-100 detergent.
3. Luciferase reaction buffer: 167 m*M* Potassium phosphate, pH 7.8, 17 m*M* $MgCl_2$, 8.3 m*M* adenosine triphosphate (ATP). Final concentrations in the reaction: 100 m*M* potassium phosphate, pH 7.8, 10 m*M* $MgCl_2$, 5 m*M* ATP.
4. Luciferin stock: 1 m*M* Luciferin (Sigma).

2.6. Information on the Internet

Reagents and equipment can be found at the sources listed below. Please note that this list is not intended to be exhaustive and there will be inevitable omissions.

1. Biological Laboratory Equipment Maintenance and Services: http://www.bls-ltd.com/. GFP visualization tools.
2. Clontech: http://www.clontech.com. Example: GFP color variants.
3. Fisher Scientific: https://www1.fishersci.com.
4. ICN Biochemicals: http://www.icnbiomed.com/.
5. Invitrogen: http://www.invitrogen.com. Example: antibody to β-galactosidase.
6. Molecular Probes: http://www.molecularprobes.com/. Fluorescent reagents for chemiluminescent and bioluminescent assays.
7. New England Nuclear: http://www.nen.com/. [^{14}C]chloramphenicol.
8. Perkin-Elmer Instruments. http://instruments.perkinelmer.com/. Example: instrumentation such as luminometers to detect reporter molecules.
9. Promega: http://www.promega.com/. Example: reagents for enzymatic assays.
10. Roche Molecular Biochemicals: http://biochem.roche.com/. Example: reagents for histochemical detection of CAT.
11. Sigma Chemical Company: http://www.sigma-aldrich.com. Example: reagents for enzymatic assays.
12. Stratagene: http://www.stratagene.com. Example: humanized GFP (hrGFP).
13. Tropix: http://www.appliedbiosystems.com/tropix/. Reagents for chemiluminescence.

Table 1
Assays to Detect Reporter Expression

Reporter molecule	Direct observation	Chromogenic substrate	Antibody for immunohistochemistry	Fluorescent/ luminescent substrate	Radioactive substrate
β-galactosidase (β-gal)	–	+	+	+	–
Human alkaline phosphatase (hAPP)	–	+	+	+	–
Chloramphenicol-o-acetyltransferase (CAT)	–	+	–	–	+
Luciferase	–	–	+	+	–
Green fluorescent protein (GFP)	+	–	–	–	–

14. Turner Designs: http://www.turnerdesigns.com/. Instruments to detect luminescence.
15. Vector Laboratories: http://www.vectorlabs.com/. Reagents for immunohistochemistry.

3. Methods

Several methods can be used to detect reporter expression (**Tables 1** and **2**). The most direct is to visualize the reporter protein (GFP) directly. Indirect methods to detect expression in cells include 1) addition of a chromogenic substrate (β-gal, hAPP); 2) immunohistochemistry with a commercial antibody directed to the reporter molecule (β-gal, hAPP, luciferase); 3) addition of a luminescent or fluorescent substrate to tissue homogenates (β-gal, hAPP, luciferase); or 4) addition of a radioactive substrate (CAT).

3.1. β-Gal Detection

β-gal is the most widely used reporter in transgenic mice (**Fig. 1**; *see* **Note 2**). The many assays for β-gal allow for staining of whole-mount embryos or tissues or single cells expressing the transgene; quantitation of activity in tissue extracts; expression at the single cell level in combination with immunohistochemistry; and quantitation of expression in tissue extracts. In addition, it is possible to use viable cell suspensions in fluorescence-activated cell sorting (FACS). Endogenous galactosidase activity can be limited by incubating reactions at a pH above 7.5 ***(16)***. β-gal hydrolyzes a number of β-galactosidase linkages. The hydrolysis of the chromogenic substrate X-gal (5-bromo-4-chloro-3-indoyl β-D-galactoside) is used for whole-mount staining

Table 2
Methods of Detecting Reporter Expression

Reporter molecule	Whole-mount observation	Tissue section observation	Tissue homogenates	Flow cytometry
β-galactosidase (β-gal)	+	+	+	+
Human alkaline phosphatase (hAPP)	+	+	+	+
Chloramphenicol-o-acetyltransferase (CAT)	–	–	+	–
Luciferase	–	–	+	–
Green fluorescent protein (GFP)	+	+	+	–

and histochemistry ***(29)***. The hydrolysis of MUG is used for quantitative measurements of enzyme activity ***(16)***. The hydrolysis of FDG (fluorescein di-β-D-galactopyranoside) in viable cells is used in FACS ***(3,4)***. In addition, commercially available antibodies to β-gal can be used to detect the enzyme in a number of ways [enzyme-linked immunosorbent assay (ELISA), immunofluorescence, immunohistochemistry, and so forth].

3.1.1. β-*Gal Detection by Histochemistry:* Whole Mount

1. Rinse mouse embryos or tissues in 0.1 *M* sodium phosphate buffer, pH 7.3, prior to fixation. Place tissues in fix for 30 min on ice with gentle shaking. Larger tissues and older embryos can be bisected after 30 minutes and returned to fix for 30 min to ensure penetration. (*See* **Note 3**.)
2. Rinse specimens in wash buffer 3 times and stain overnight at 37°C with gentle shaking in X-gal stain.
3. After the stain has developed, rinse the samples 3 times in 0.1 *M* PBS, pH 7.3 (10 min each rinse) and store at 4°C.
4. If desired, embryos or tissues can be postfixed for 2–4 h, embedded in paraffin, and sectioned for immunohistochemistry. (*See* **Note 4**.)

3.1.2. β-*Gal Detection by Histochemistry:* Tissue Sections

1. Remove fresh tissue and freeze in OCT (Miles Scientific) on dry ice. Freeze brains whole in 2-methyl butane at –25° to –35°C. Frozen tissues can be stored at –70° to –80°C until sections are cut. (*See* **Note 2**.)
2. Section frozen tissues with a cryostat. Mount 10–20-μm sections on poly-lysine slides. Sections can be stored at –70° to –80°C until they are fixed.
3. Add fix to sections for 5–10 min.

4. Rinse slides 3 times in wash buffer and incubate at 37°C from 0.5 to 24 h in X-gal stain.
5. Rinse slides in PBS and dehydrate through sequential washes in increasing concentrations of ethanol. Mount cover slips on the sections with Permount.
6. To enhance contrast, sections can be counterstained with 0.5% neutral red prior to mounting cover slips. (*See* **Note 5**.)

3.1.3. β-Gal Detection in Tissue Extracts by Fluorescence

1. Prepare tissue extracts in Dounce homogenizers. Add 100 mg of tissue or cells to 0.9 mL buffer and homogenize for 10–20 strokes.
2. Freeze-thaw extracts for three cycles with dry ice and a 37°C bath. Sediment debris by centrifugation at top speed in a refrigerated microfuge for 10 min. (*See* **Note 6**.)
3. Mix 1 part of the supernatant with 4 parts of MUG reaction buffer and incubate at 37°C. At the same time, prepare a standard curve with β-galactosidase.
4. After 4 h, stop the reaction by adding 0.05 mL stop buffer and pellet precipitated proteins by spinning at top speed in a microfuge for 10 min.
5. Measure fluorescence with a fluorometer (excitation wavelength 360, detection wavelength 450). Dilute part of the supernatant (0.02–0.4 mL) in 2.0 mL of fluorescence buffer.
6. After the protein concentration of the homogenates is measured ***(30)***, the amount of β-galactosidase activity is expressed as units of enzyme per mg protein extract.

3.1.4. β-Gal Detection in Tissue Extracts by Spectrophotometry

1. Prepare tissue extracts as in **Subheading 3.1.3.**
2. Prepare reaction buffer: mix together phosphate buffer, mercaptoethanol, ONPG, and distilled water in a ratio of 50 : 15 : 25 : 55.
3. Prepare a standard curve with known amounts of β-gal enzyme.
4. Add 50 μL of extract or enzyme standard to 1 mL of reaction buffer and incubate at room temperature for 5–10 min.
5. Measure absorbance at 420 nm in a spectrophotometer and calculate β-gal concentration in extracts.
6. After the protein concentration of the homogenates is measured ***(30)***, the amount of β-galactosidase activity is expressed as units of enzyme per mg protein extract. (*See* **Note 7**.)

3.1.5 β-Gal detection by Fluorescence in Single Cell Suspensions

1. Load cells with FDG (Molecular Probes) by osmotic shock. Adjust cell concentration to 1×10^7/mL in FDG buffer and incubate 0.1-mL aliquots of cells at 37°C for 15 min. Add 0.1 mL of 2 m*M* FDG and incubate at 37°C for 60 s. Stop the staining with 2 mL of propidium iodide buffer.

2. Incubate cells on ice for 2–4 h to allow conversion of the FDG substrate.
3. Cells can be processed by FACS or visualized with a confocal microscope or with a fluorescence microscope set for fluorescein.
4. Lysed, nonviable cells will have blue nuclei stained with propidium iodine.

3.2. hAPP Detection

The characteristics of hAPP that make it a good reporter include its stability at high temperatures and activity at high pH. Heat treatment prior to addition of substrate at alkaline pH effectively eliminates interference from endogenous phosphatases. There are a number of ways that hAPP activity can be detected. Whole-mount specimens or cells in sections can be stained with BCIP. Commercially available antibodies to hAPP can be used to detect the enzyme by immunohistochemistry or in Western blots. Enzymatic activity in tissue extracts can be analyzed with fluorescent substrates. Cells expressing hAPP can be analyzed by FACS. If desired, sequential staining for β-gal and hAPP can be performed in the same tissue sections.

3.2.1. hAPP Detection by Histochemistry in Whole-Mount Samples

1. Rinse mouse embryos or tissues in PBS, pH 7.4 prior to fixation. Place tissues in fix for 30 min on ice with gentle shaking. Larger tissues and older embryos can be bisected after 30 min and returned to fix for 30 min to ensure penetration.
2. Rinse specimens in PBS 3 times and then heat at 65°C in PBS for 30 min to inactivate endogenous, heat-labile phosphatases.
3. Place samples in AP stain and incubate at 4°C for 1–24 h. Incubating at 37°C can accelerate staining.
4. After the stain has developed, rinse the samples for 10 min 3 times in rinse buffer and store at 4°C.
5. If desired, embryos or tissues can be dehydrated through sequential washes in increasing concentrations of ethanol, embedded in paraffin, and sectioned for immunohistochemistry.

3.2.2. hAPP Detection in Tissue Sections by Histochemistry

1. Prepare tissues for sections as in **Subheading 3.2.1.**
2. Rinse slides in PBS, pH 7.4 after fixation and incubate at 65°C for 30 min to inactivate endogenous phosphatases. (*See* **Note 8**.)
3. Incubate in AP stain at 37°C for 10 min up to 1 h. Staining can be done at lower temperatures, but color development will take longer.
4. Rinse slides in PBS and dehydrate through sequential washes in increasing concentrations of ethanol; mount cover slips on the sections with Permount. (*See* **Note 9**.)
5. To enhance contrast, sections can be counterstained with 0.5% neutral red in 50 m*M* sodium acetate, pH 3.3, prior to mounting cover slips.

3.2.3. hAPP Detection in Tissue Extracts by Fluorescence

1. Prepare tissue extracts as in **Subheading 3.1.3.** (*See* **Note 8**.)
2. Add 500 µL MUP reaction buffer to each cuvet.
3. Add 100 µL of sample to a cuvet, mix, and incubate at room temperature for 2 min.
4. Add 1900 µL MUP stop buffer to the cuvette and immediately measure fluorescence. Fluorescence is measured in a fluorometer (excitation wavelength 360, detection wavelength 450).
5. Set up an enzyme standard curve from 1×10^{-5} to 1×10^{-3} U/mL and use it to determine the number of units in each experimental sample.
6. After the protein concentration of the homogenates is measured ***(30)***, the amount of hAPP activity is expressed as units of enzyme per mg protein extract.

3.2.4. hAPP Detection in Tissue Extracts by Spectrophotometry

1. Prepare tissue extracts as in **Subheading 3.1.3.** (*See* **Note 8**.)
2. Mix together in cuvets: 2.9 mL diethanolamine buffer and 50 µL PNP. Equilibrate at 37°C for 5–10 min.
3. Prepare alkaline phosphatase standard in diluent to 0.05 U/mL and add 100 µL to cuvet for use as standard. Prepare unknowns in diluent. Add 100 µL of standard or unknown to cuvet and measure change in the absorbance at 405 nm at 37°C over 5 min.
4. Calculate the number of units in the sample in the following way:
   units/mL = [(change in Abs_{405}/min)(reaction volume)(sample dilution)]/[(E_{405})(sample volume)]. E_{405} is the millimolar extinction coefficient of 18.3 of *p*-nitrophenol measured at 405 nm.
5. After the protein concentration of the homogenates is measured ***(30)***, the amount of hAPP activity is expressed as units of enzyme per mg protein extract.

3.3. Chloramphenicol O-acetyl Transferase Detection

Assays for CAT expression were one of the earliest methods devised to test DNA sequences for gene expression with in vitro systems. Subsequently the CAT reporter was used for analysis of gene expression in transgenic mice ***(31)***. CAT was used because there is no endogenous mouse enzyme with the same activity, a sensitive assay is available, and CAT expression does not adversely affect mouse physiology. Assays for the enzyme in tissue extracts and in tissue sections are presented. CAT acetylates chloramphenicol at the 3-hydroxyl position. In tissue extracts, the native and acetylated forms of [C^{14}]chloramphenicol are separated by thin-layer chromatography (TLC) (***32***; *see* **Note 10**). In tissue sections CAT activity produces a brown precipitate ***(33)***.

3.3.1. CAT Detection in Tissue Extracts by Radioactive Assay

1. Prepare protein extracts in Dounce homogenizers. Add 100 mg of tissue or cells to 0.9 mL buffer and homogenize for 10–20 strokes.

2. Debris is sedimented by centrifugation at top speed in a refrigerated microfuge for 10 min. The extracted proteins in the supernatant are used in the assay. (*See* **Note 11**.)
3. Preincubate 100 μL of reaction buffer with 20 μL of protein extract and 1 μCi [^{14}C]chloramphenicol at 37°C for 5 min.
4. Initiate the reaction by adding 20 μL of acetyl CoA .
5. After 0.5–1 h, stop the reaction by adding 2 mL cold ethyl acetate. The chloramphenicol and acetylated chloramphenicol will dissolve in the ethyl acetate.
6. Separate the ethyl acetate from the aqueous phase. Concentrate the reaction products by evaporation of the organic phase. Redissolve in 30 μL ethyl acetate and separate the chloramphenicol from acetylated chloramphenicol by silica gel TLC.
7. Use the TLC plates to expose X-ray film. If desired, the spots can be scraped from the TLC plate and quantitative measurements can be made with a scintillation counter.

3.3.2. CAT Detection in Tissue Sections by Histochemistry

1. Fix tissues in 2% paraformaldehyde at 4°C for 1 h.
2. Rinse in PBS and immerse in 10% sucrose for 1 h and then 30% sucrose overnight.
3. Freeze and prepare 5–10 μm sections on a cryostat.
4. Cover sections with stain and incubate at room temperature for 2–24 h until stain develops. Wash in PBS and mount slides in Permount for microscopy.

3.4. Green Fluorescent Protein Detection

There are four variants of GFP available from Clontech based on *Auqueoria* and one from Stratagene based on *Renilla* (HrGFP) ***(34)***. Each has different excitation and emission maxima (**Table 3**). EGFP is the most widely used variant in transgenic mice ***(2,11,35,36)***. (*See* **Note 12**.) The availability of additional GFP variants makes it possible to use multiple reporters in a single animal. There are as many ways to visualize GFP expression and capture images as there are microscope manufacturers. Nikon, Leica, Leitz, and Olympus all produce suitable equipment. A stereomicroscope equipped for fluorescence can be used to image large specimens at low magnification. EGFP fluorescence can be visualized with a conventional inverted fluorescent microscope with a fluorescein isothiocyanate (FITC) filter set ***(37)***. Confocal microscopy can also be used to acquire high-resolution images ***(23)***. Since EGFP is a small molecule, it will easily diffuse out of tissue permeabilized with ethanol or methanol. To enhance detection in sections, it is important that the EGFP molecules be fixed and imaged before they can diffuse out ***(2,22)***. Optimizing fixation may be necessary to detect EGFP expression in sections. The highest sensitivity is obtained with confocal microscopes adjusted to match the excitation/emission spectra of EGFP.

Table 3
GFP Variants

GFP variant	Color	Excitation maxima	Emission maxima
EBFP	Blue	380	440
EGFP	Green	488	509
ECFP	Cyan	433	453
		475	501
EYFP	Yellow	513	527
HrGFP	Green	500	506

3.4.1. GFP Detection in Whole-Mount Samples

1. Fix mouse embryos or tissues as above in **Subheading 3.1.1.** Fix early embryos (up to E9.5) for 30–60 min on ice. Larger samples are fixed for up to 4 h.
2. The sample can be flattened between a glass slide and a cover slip for observation. GFP fluorescence can be detected with instruments that range in complexity from a hand-held long-wave UV lamp to a fluorescence microscope set for fluorescein to a confocal microscope.

3.4.2. GFP Detection in Tissue Sections

1. Thick sections: cut 50-μm sections in ice-cold PBS with a vibratome. Mount in 50% glycerol in PBS and image immediately.
2. Thin sections: embed fixed tissue in OCT (Miles), freeze in liquid nitrogen, cut 12-μm sections with a cryostat, and image immediately.
3. Observe GFP fluorescence with a confocal or fluorescence microscope. (*See* **Notes 13** and **14**.)

3.4.3. GFP Detection in Tissue Extracts

1. Prepare the tissue extract as described in **Subheading 3.1.3**.
2. Use a GFP standard to set the reading of the fluorometer to the middle of its range.
3. Dilute the tissue extract in GFP buffer to obtain a signal in the linear range of the fluorometer that can be compared with the standard. Adjust the excitation/emission wavelength of the fluorometer to match the form of GFP in use (**Table 3**).
4. After the protein concentration of the homogenates is measured **(30)**, the amount of GFP activity is expressed as μg GFP per mg protein extract.

3.4.4. GFP Detection in Single Cell Suspensions

1. Single cell suspensions are prepared with methods appropriate to the tissue in question.
2. Cells can be processed by FACS or visualized in a confocal or fluorescence microscope.

3.5. Luciferase Detection

As a reporter molecule, luciferase has several advantages. It is easy to detect, extremely low levels of the enzyme can be detected, and there is no background activity in mouse tissue, unlike β-gal or hAPP. Luciferase produces a flash of light when it oxidizes its substrate, luciferin, in the presence of ATP. Luminometers are typically used to detect the light (*see* **Note 15**). The emission of light from single cells transfected with luciferase plasmids and incubated in luciferin has been measured with sensitive cameras ***(38)***. Luciferase plasmids and assay kits are commercially available. The limitation of luciferase is that it is principally used with tissue extracts; consequently direct visualization of luciferase expression in sections is not possible. Commercially available antibodies for firefly luciferase can be used in immunohistochemistry to detect luciferase expression at the cellular levels. The luminescence of the enzyme has been used to image gene expression in vivo ***(39)***, similar to the use of GFP ***(27)***. In this case, the mouse is treated with the substrate for luciferase, and the luminescence of the cells is imaged with a camera system.

3.5.1. Luc Detection in Tissue Extracts by Luminescence

1. Homogenize 15 mg tissue for 30 s in 1 mL buffer with a polytron (Brinkmann Instruments).
2. Add 250 μL of 5X lysis buffer and mix at room temperature for 15 min.
3. Pellet debris by centrifugation at top speed in a microfuge for 15 min.
4. Add 20 μL of the cleared protein extract to the 180 μL luciferase reaction buffer. The enzymatic reaction is initiated by injection of 100 μL of 1 m*M* luciferin. The amount of light generated is integrated by the luminometer for 20 s.
4. After the protein concentration of the homogenate is measured ***(30)***, results can be expressed as arbitrary light units per microgram extract per 20 s. Alternatively, a standard curve can be constructed with purified luciferase (Sigma) diluted in 1X lysis buffer. Then results can be expressed as units of enzyme per mg extract or as the amount of luciferase per mg extract.

4. Notes

1. To increase the stability of β-galactosidase, add DTT 0.005 *M* and phenylmethylsulfonyl fluoride (PMSF) to 0.002 *M* in the extract buffer. DTT may increase background of luminescent assays.
2. Nuclear localized β-gal produces a punctate staining pattern in tissues and allows antibody staining of cytosolic proteins. This form of β-gal can simplify the identification of specific cell types that express the reporter ***(40)***.
3. To reduce background activity of endogenous β-gal in whole-mount specimens, increase the pH of the phosphate buffer to 8.0. Alternatively, 0.1 *M* HEPES, pH 8.0, can be used in the wash buffers. Bacterial β-galactosidase retains more activity at pH 8.0 than endogenous enzymes.

4. Clearing tissue or embryos can be done to increase the visibility of structures stained for β-gal ***(41)***.
5. When examining slides of tissue sections stained for β-gal, remember to stain the cells first and then embed them in paraffin and section them. This enzyme will not survive the embedding process. It may be necessary to slice tissues into thinner pieces with a scalpel or razor blade to allow even penetration of solutions.
6. To reduce background activity of endogenous β-gal in tissue extracts, in addition to raising the pH of the assay buffer to pH 8.0 (see above), extracts can be heated to 50°C for 30 min prior to the assay. This will selectively inactivate the tissue β-galactosidases without inactivating the bacterial enzyme. Care should be used not to exceed 50°C since the bacterial enzyme is labile at 55°C. Clear tissue extracts by centrifugation prior to assay.
7. The fluorometric assay of β-gal will detect fewer molecules than the spectrophotometric assay. You may be unable to detect activity in tissue extracts with the spectrophotometric assay although you can see stained cells in sections. You can increase the sensitivity of the β-galactosidase assay by changing to the fluorometric assay for the test.
8. If background activity is too high after 65°C heat treatment, increase the heat to 70° or 75°C. Clear heated tissue extracts by centrifugation prior to assay.
9. When processing slides of tissue sections, cut thin sections of paraffin-embedded tissues prior to staining. Addition of substrate after sectioning will ensure complete and even penetration of the cells on the section. Background is reduced by heating the slides to 70° or 75°C.
10. An alternative to TLC to measure CAT activity is to substitute butyryl-CoA in the reaction substrate. Phase extraction can then be used to separate the butyrylated [^{14}C]chloramphenicol from unreacted [^{14}C]chloramphenicol ***(42)***.
11. To reduce background activity of endogenous acetylases, heat tissue extracts or sections to 65°C for 5 min prior to the assay. Clear tissue extracts by centrifugation prior to assay.
12. To increase the sensitivity of GFP reporter experiments, make sure that you are using the enhanced GFP molecule (Clontech: pEGFP-N1), which has been engineered to fluoresce more intensely than wild-type GFP when excited at 488 nm.
13. Imaging EGFP in sections may be tricky. After brief fixation, thick cryosections (12 μm) should be imaged immediately to prevent loss of cellular localization of EGFP. If necessary, a vibratome can be used to sections of fresh tissue from 50 to 150 μm thick for imaging.
14. Confocal microscopy provides better images of GFP-expressing cells than fluorescence microscopy.
15. A dedicated luminometer will give the best results with the chemiluminescent assays. If a luminometer is not available, then a scintillation counter can be used in its place ***(43)***. The sensitivity of the assay will probably be lower. The linear range of detection in a luminometer or scintillation counter will vary according

to expression levels. If necessary, vary the amount of tissue extract to obtain values in the linear range.

References

1. Allen, N. D., Cran, D.G., Barton, S. C., et al. (1988) Transgenes as probes for active chromosomal domains in mouse development. *Nature* **333,** 852–855.
2. Hadjantonakis, A. K., Gertsenstein, M., Ikawa, M., Okabe, M., and Nagy, A. (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech. Dev.* **76,** 79–90.
3. Nolan, G. P., Fiering, S., Nicolas, J. F., and Herzenberg, L. A. (1988) Fluorescence-activated cell analysis and sorting of viable mammalian cells based on beta-D-galactosidase activity after transduction of *Escherichia coli lacZ. Proc. Natl. Acad. Sci. USA* **85,** 2603–2607.
4. Abe K., Kashiyama, M., Macgregor, G., and Yamamura, K. I. (1996) Purification of primordial germ cells from TNAP beta-geo mouse embryos using FACS-gal. *Dev. Biol.* **180,** 468–472.
5. Omerovic, E., Basetti, M., Bollano, E., et al. (2000) In vivo metabolic imaging of cardiac bioenergetics in transgenic mice. *Biochem. Biophys. Res. Commun.* **271,** 222–228.
6. Herschman, H. R., MacLaren, D. C., Iyer, M., et al. (2000) Seeing is believing: non-invasive, quantitative and repetitive imaging of reporter gene expression in living animals, using positron emission tomography. *J. Neurosci. Res.* **59,** 699–705.
7. Hartigan-O'Connor, D. and Chamberlain, J.S. (2000) Developments in gene therapy for muscular dystrophy. *Microsc. Res. Tech.* **48,** 223–238.
8. Burrows, H. L., Birkmeier, T. S., Seasholtz, A. F., and Camper, S. A. (1996) Targeted ablation of cells in the pituitary primordia of transgenic mice. *Mol. Endocrinol.* **10,** 1467–77.
9. Furth, P. A., St. Onge, L., Boger, H., et al. (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306.
10. Lobe, C. G., Koop, K. E., Kerppner, W., et al. (1999) Development. Z/AP, a double reporter for Cre-mediated recombination. *Dev. Biol.* **208,** 281–292.
11. Kawamoto, S., Niwa, H., Tashiro, F., et al. (2000) A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett.* **470,** 263–268.
12. Lomeli, H., Ramos-Mejia, V., Gertsenstein, M., Lobe, C. G., and Nagy, A. (2000) Targeted insertion of Cre recombinase into the TNAP gene: excision in primordial germ cells. *Genesis* **26,** 116–117.
13. Feltri, M. L., D'Antonio, M., Quattrini, A., et al. (1999) A novel P0 glycoprotein transgene activates expression of lacZ in myelin-forming Schwann cells. *Eur. J. Neurosci.* **11,** 1577–1586.
14. Kamat, A., Graves, K. H., Smith, M. E., Richardson, J. A., and Mendelson, C. R. (1999) A 500-bp region, approximately 40 kb upstream of the human CYP19

(aromatase) gene, mediates placenta-specific expression in transgenic mice. *Proc. Natl. Acad. Sci. USA* **96,** 4575–4580.
15. Telling, G. C., Tremblay, P., Torchia, M., et al. (1997) N-terminally tagged prion protein supports prion propagation in transgenic mice. *Protein Sci.* **6,** 825–833.
16. Young, D. C., Kingsley, S. D., Ryan, K. A., and Dutko, F. J. (1993) Selective inactivation of eukaryotic beta-galactosidase in assays for inhibitors of HIV-1 TAT using bacterial beta-galactosidase as a reporter enzyme. *Anal. Biochem.* **215,** 24–30.
17. Bonnerot, C., Grimber, G., Briand, P., and Nicolas, J. F. (1990) Patterns of expression of position-dependent integrated transgenes in mouse embryo. *Proc. Natl. Acad. Sci. USA* **87,** 6331–6335.
18. Brinkmeier, M. L., Gordon, D. F., Dowding, J. M., et al. (1998) Cell-specific expression of the mouse glycoprotein hormone alpha-subunit gene requires multiple interacting DNA elements in transgenic mice and cultured cells. *Mol. Endocrinol.* **12,** 622–33.
19. Kendall, S. K., Gordon, D. F., Birkmeier, T. S., et al. (1994) Enhancer-mediated high level expression of mouse pituitary glycoprotein hormone alpha-subunit transgene in thyrotropes, gonadotropes, and developing pituitary gland. *Mol. Endocrinol.* **8,** 1420–1433.
20. Lau, S., Jardine, K., and McBurney, M. W. (1999) DNA methylation pattern of a tandemly repeated LacZ transgene indicates that most copies are silent. *Dev. Dyn.* **215,** 126–138.
21. DePrimo, S. E., Stambrook, P. J., and Stringer, J. R. (1996) Human placental alkaline phosphatase as a histochemical marker of gene expression in transgenic mice. *Transgenic Res.* **5,** 459–466.
22. Kisseberth, W. C., Brettingen, N. T., Lohse, J. K., and Sandgren, E. P. (1999) Ubiquitous expression of marker transgenes in mice and rats. *Dev. Biol.* **214,** 128–138.
23. Paddock, S. W. (ed.) (1998) *Confocal Microscopy Methods and Protocols*. Humana, Totowa, NJ.
24. Chiocchetti, A., Tolosano, E., Hirsch, E., Silengo, L., and Altruda, F. (1997) Green fluorescent protein as a reporter of gene expression in transgenic mice. *Biochem. Biophys. Acta.* **1352,** 193–202.
25. Huang, W.-Y., Aramburu, J., Douglas, P. S., and Izumo, S. (2000) Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat. Med.* **6,** 482–483.
26. Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H., and Ke, N. J. (1999) Is green fluorescent protein toxic to the living cells? *Biochem. Biophys. Res. Commun.* **260,** 712–717.
27. Yang, M., Baranov, E., Jiang, P., et al. (2000) Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. *Proc. Natl. Acad. Sci. USA* **97,** 1206–1211.
28. Mansour, S. L., Thomas, K. R., Deng, C. X., and Capecchi, M. R. (1990) Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination. *Proc. Natl. Acad. Sci. USA* **87,** 7688–7692.

29. Sanes, J. R., Rubenstein, J. L., and Nicolas, J. F. (1986) Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5,** 3133–3142.
30. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of nanogram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.
31. Overbeek, P. A., Lai, S. P., Van Quill, K. R., and Westphal, H. (1986) Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. *Science* **231,** 1574–1577.
32. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2,** 1044–1051.
33. Donoghue, M. J., Alvarez, J. D., Merlie, J. P., and Sanes, J. R. (1991) Fiber type- and position-dependent expression of alpha myosin light chain-CAT transgene detected with a novel histochemical stain for CAT. *J. Cell Biol.* **115,** 423–434.
34. Tsien, R. Y. (1998) The green fluorescent protein, in *Annual Review of Biochemistry*, vol. 67 (Richardson, C. C., Abelson, J. N., Raetz, C. R., and Thorner, J. W., eds.), Annual Reviews, Palo Alto, CA, pp. 509–544.
35. Ikawa, M., Kominami, K., Yoshimura, Y., et al. (1995) Green fluorescent protein as a marker in transgenic mice. *Dev. Growth Differ.* **37,** 455–459.
36. Okabe, M., Ikawa.M., Kominami. K., Nakanishi. T., and Nishimune. Y. (1997) 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407,** 313–319.
37. Allan, V. (ed.) (1999) *Protein Localization by Fluorescence Microscopy: A Practical Approach*. Oxford University Press, London.
38. Maire, E., Lelievre, E., Brau, D., et al. (2000) Development of an ultralow-light-level luminescence image analysis system for dynamic measurements of transcriptional activity in living and migrating cells. *Anal. Biochem.* **280,** 118–127.
39. Sweeney, T. J., Mailander, V., Tucker, A. A., et al. (1999) Visualizing the kinetics of tumor-cell clearance in living animals. *Proc. Natl. Acad. Sci. USA* **96,** 12044–12049.
40. Bonnerot, C., Grimber, G., Briand, P., and Nicolas, J. F. (1990) Patterns of expression of position-dependent integrated transgenes in mouse embryo. *Proc. Natl. Acad. Sci. USA* **87,** 6331–6335.
41. Turkay, A., Saunders, T. L., and Kurachi, K. (1999) A method for histochemical analysis of cleared mouse embryos and adult tissues expressing β-galactosidase. *J. Histotech.* **22,** 323–324.
42. Seed, B. and Sheen, J/Y. (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene* **67,** 271–7.
43. Nguyen, V. T., Morange, M., and Bensaude, O. (1988) Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. *Anal. Biochem.* **171,** 404–8.

8

Gene Targeting in Mouse Embryonic Stem Cells

Jan van Deursen

1. Introduction

Gene targeting in mouse embryonic stem cells is now a well-established technique that is widely used to create animal models for human disease or to study gene function at the level of the whole animal. The purpose of this chapter is to provide a detailed protocol to investigators without previous experience in gene targeting and embryonic stem (ES) cell technology. This protocol is an adaptation of previously published protocols ***(1,2)*** and has been successfully applied by investigators at the Mayo Clinic in Rochester and in several other institutions.

2. Materials

2.1. Equipment

ES cell culture requires a tissue culture area that contains the following pieces of equipment:

1. A tissue culture hood with ultraviolet (UV) light.
2. A water-jacketed 37°C incubator with 5% CO_2 and 20% O_2 gas and saturated humidity.
3. A high-capacitance electroporator. A Bio-Rad Gene Pulser II is commonly used for this purpose.
4. An inverted microscope equipped with 4× (or 5×) and 10× phase-contrast objectives.
5. A tabletop centrifuge with a swing-out rotor for 15-mL tubes.
6. A 20-µL pipet.
7. Multichannel pipet with a 20–100-µL volume range.

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen © Humana Press Inc., Totowa, NJ

2.2. Disposables

1. The following tissue culture-treated flasks, dishes, and plates are used: 25-cm^2 and 75-cm^2 culture flasks (use 5 and 10 mL medium per flask, respectively); 10-cm dishes; 24- and 96-well flat-bottomed plates and 96-well round-bottomed plates.
2. Electroporation cuvets with an electrode gap of 0.4 cm (Bio-Rad, cat. no. 65-2088).
3. 1-mL cryogenic vials.

2.3. Tissue Culture Media and Solutions

2.3.1. MEF-DMEM

Prepare this medium by adding the following sterile components to 500 mL Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4500 mg/L) and low bicarbonate (17 g $NaHCO_3$ mg/mL) (e.g., Gibco-BRL, cat. no. 11960-044; stored at 4°C):

1. 50 mL ES-tested fetal calf serum (FCS; stored in 50-mL aliquots at –20°C).
2. 5 mL 200 m*M* L-Glutamine (Gibco-BRL, cat. no. 25030-081; stored at –20°C).
3. 0.5 mL 10 mg/mL Gentamycin (Gibco-BRL, cat. no. 15710-064; stored at 4°C).
4. 0.5 mL 5.5×10^{-2} *M* β-Mercaptoethanol (Gibco-BRL, cat. no. 21985-023; stored at 4°C).

2.3.2. ES-DMEM

Prepare this medium by adding the following sterile components to 500 mL DMEM containing high glucose (4500 mg/L) and low bicarbonate (17 g $NaHCO_3$ mg/mL) (e.g., Gibco-BRL, cat. no. 11960-044; stored at 4°C):

1. 100 mL ES-tested FCS (stored in 50-mL aliquots at –20°C; *see* **Note 1**).
2. 30 μL 10.000.000 U/mL Leukemia inhibitory factor (LIF; (e.g., Gibco-BRL, cat. no. 13275-029; stored at 4°C; *see* **Note 2**).
3. 5 mL 200 m*M* L-Glutamine (Gibco-BRL, cat. no. 25030-081; stored at –20°C).
4. 5 mL 100 m*M* Sodium pyruvate (Gibco-BRL, cat. no. 113600-070; stored at 4°C).
5. 5 mL 10 m*M* Nonessential amino acids (Gibco-BRL, cat. no. 11140-050; stored at 4°C).
6. 0.5 mL 10 mg/mL Gentamycin (Gibco-BRL, cat. no. 15710-064; stored at 4°C).
7. 0.5 mL 5.5×10^{-2} *M* β-Mercaptoethanol (Gibco-BRL, cat. no. 21985-023; stored at 4°C).

Both MEF- and ES-DMEM should be stored at 4°C protected from light and can be used for up to 1 month if L-glutamine is replaced weekly. You can complete the entire gene-targeting protocol within 4 weeks.

2.3.3. 2× Freezing Medium

Supplement 40 mL DMEM with 40 mL ES-FCS and 20 mL dimethylsulfoxide (DMSO; Fisher, cat. no. D128-500). When stored at 4°C protected from light, this medium stays fresh for 2 months.

2.3.4. PBS

Calcium- and magnesium-free phosphate-buffered saline (PBS; Gibco-BRL, cat. no. 14190-144), stored at room temperature.

2.3.5. Trypsin-EDTA

Dissolve 5 g trypsin-2.0 g EDTA-4Na (Gibco-BRL, cat. no. 15405-012) in 250 mL calcium- and magnesium-free PBS. PBS-trypsin-EDTA is stable at room temperature for at least 6 months.

2.3.6. PBS-0.1% Gelatin

Add 0.5 g porcine skin gelatin (Sigma, cat. no. G-1890) to 500 mL calcium- and magnesium-free PBS. Dissolve and sterilize by autoclaving (stored at room temperature).

3. Methods

3.1. Preparation of Targeting Vector DNA for Electroporation into ES Cells

1. Isolate targeting vector DNA from bacteria by alkaline lysis and anion-exchange chromatography (e.g., use columns supplied by Qiagen).
2. Linearize about 25–40 μg of targeting vector DNA with an appropriate restriction enzyme according to the manufacturer's instructions.
3. Precipitate linearized DNA with ethanol. Wash once with 80% ethanol. Aspirate the 80% ethanol in a tissue culture hood and resuspend the DNA pellet at 40 μg/mL in sterile PBS. Store the targeting vector at –20°C.

3.2. Preparation of Mouse Embryonic Fibroblasts

Targeting vectors almost always contain a neomycin phosphotransferase gene, a hygromycin phosphotransferase gene, or a puromycin-*N*-acetyltransferase gene for positive selection of recombinant ES cells. Transgenic mouse strains that carry these particular drug-resistance genes are commercially available for the production of drug-resistant MEF cells. MEF cells are prepared from 13.5-day-old mouse embryos.

1. Set up timed pregnancies with drug-resistant mice.
2. Sacrifice pregnant female mice at 13.5-days post coitum. Spray the abdomen with 70% ethanol and dissect the abdomen. Collect the uterine horns and dissect the mouse embryos in a tissue culture hood.
3. Wash the embryos in a 10-cm dish with sterile PBS. Then decapitate the embryos and remove the internal organs. Wash the carcasses 2 times in PBS.
4. Place each embryo carcass in a separate 10-cm dish. Thoroughly mince each carcass with a sterile razor blade.
5. Suspend each minced embryo in 5 mL of trypsin-EDTA and transfer it to a 15-mL tube.
6. Incubate the tubes in a 37°C water bath for 10 min. Vigorously shake the tubes at 2-min intervals.
7. Add 5 mL of MEF-DMEM and spin in a tabletop centrifuge for 5 min at 300*g*.
8. Resuspend each cell pellet into 10 mL MEF-DMEM and transfer the suspension to a 75-cm^2 flask. Incubate at 37°C and leave the dishes undisturbed overnight.
9. The next day, cultures should be 30–50% confluent. Change the medium to remove loose debris.
10. When the flasks become highly confluent (2–4 days after initial seeding), expand the cultures. Treat the flasks individually as follows. Wash twice with PBS and add 2 mL trypsin-EDTA. After 5 min at 37°C, resuspend the cells in 40 mL MEF-DMEM. Seed in three 75-cm^2 flasks.
11. When the cultures again are highly confluent, frozen stocks should be prepared. Wash twice with PBS and add 4 mL trypsin-EDTA. After 5 min at 37°C, resuspend cells in each 75-cm^2 flask in 4 mL MEF-DMEM and transfer the trypsinized cells to 15-mL tubes.
12. Spin the cells for 5 min at 300*g* and resuspend the pellet in 4 mL freezing medium. Divide equally over four cryogenic vials (1 mL). Wrap the vials in tissue towels and place them in a Styrofoam box at –80°C.
13. The next day, store vials in liquid nitrogen.

3.3. Preparation of Feeder Monolayers of Mouse Embryonic Fibroblasts

MEF cells are primary cells and have a limited life span. They divide rapidly for about 4–5 passages (approx. 20 cell divisions) and then become senescent. The frozen MEF stocks are of passage 2 and can be grown for an additional 2–3 passages after reseeding (to amplify the number of cells for feeder layer production). Although feeder monolayers can be prepared from a vial of MEF cells within 24 h after seeding, the use of MEF feeder cells will be more efficient if thawing and culture of MEF cells is scheduled 4–5 days before their use as feeder monolayers. The procedure below details how to produce the appropriate amounts of feeder monolayers for a single gene-targeting experiment.

Table 1
Feeder Monolayer Preparation on Various Tissue Culture Surfaces

Culture plate/dish/flask	No. of feeder cells	mL of 1.5×10^5 cell suspension
96-well plate	1.5×10^4/well	0.1 mL/well
48-well plate	3.0×10^4/well	0.2 mL/well
24-well plate	6.0×10^4/well	0.4 mL/well
12-well plate	1.2×10^5/well	0.8 mL/well
6-well plate	2.4×10^5/well	1.6 mL/well
10-cm dish	1.5×10^6	10 mL
25 cm^2 flask	0.75×10^6	5 mL
75 cm^2 flask	2.0×10^6	13.3 mL

1. Thaw a vial of frozen MEFs and transfer the cell suspension to a 15-mL tube with 5 mL MEF-DMEM. Spin for 5 min at 300*g*.
2. Resuspend the pellet in 10 mL of MEF-DMEM and transfer the suspension to a 75-cm^2 flask. Incubate at 37°C.
3. The next day the flask should be confluent, but it generally takes 1 more day before it reaches high confluency ($6–7 \times 10^6$ cells/75-cm^2 flask).
4. When highly confluent, wash 2 times with PBS and add 1.5 mL trypsin-EDTA. After 5 min at 37°C, resuspend cells in 30 mL MEF-DMEM. Divide the cells over three 75-cm^2 flasks.
5. When the flasks are highly confluent again, trypsinize the cells and resuspend them at a density of 2×10^5 cells/mL. Transfer the suspension to a γ-irradiator (e.g., a ^{137}Cesium source) and expose the MEF cells to 3000 rads (*see* **Note 3**).
6. Seed the irradiated cells as indicated in **Table 1**. The cells will attach within 10–20 min and form a homogenous monolayer within 8–12 h. If MEF cells are plated at too high a density, the feeder layer has a tendency to curl up when the ES cells on top reach (semi)confluency. The mitotically inactivated cells can be used as ES cell feeder monolayers up to 5–6 days after irradiation. Replace the MEF-DMEM with ES-DMEM before use (*see* **Note 4**).

3.4. Preparation of ES Cells for Electroporation

It is important to be sure that your stocks of frozen ES cells are truly pluripotent. They should have a demonstrated ability to produce germline-transmitting male chimeras after their injection into host C57BL/6 blastocysts. Therefore, if cells are newly obtained from another laboratory or company, it is of critical importance to prepare a large number of frozen stocks (for example, 15–30 vials). Thaw one of these stocks for microinjection of the ES cells into C57BL/6 blastocysts. Suitable ES cell stocks should produce a high frequency of germline-transmitting chimeric males in your hands.

There is no uniform guideline for the culture of ES cells because each independently generated cell line seems to have slightly different growth properties. Usually ES cells need to be diluted when the cultures reach approximately 70–80% confluency. A 70–80% confluent culture is generally diluted three- to sixfold. If diluted 3 times, the culture will be 70–80% confluent again within 24–36 h. If diluted 6 times, this will take about 48 h. If one inadvertently seeds ES cells that are too diluted, the culture will not reach 70–80% confluency within 3–4 days. Because the formation of large ES cell clusters may trigger differentiation, it is advisable to trypsinize the ES cells on the fourth day after seeding. Dilute them only twofold to increase the cell density of the culture.

1. Four to 5 days prior to the scheduled seeding of ES cells, thaw one vial of MEFs in a 37°C water bath, and seed into a 75-cm^2 flask. Expand the MEFs as indicated in **Subheading 3.3**. Then use these MEFS to make one 25-cm^2 flask, one 75-cm^2 flask, and five 10-cm dishes with irradiated feeder monolayers.
2. Then, thaw a vial of ES cells in the 37°C water bath. Transfer the ES cell suspension to a 15-mL tube with 5–10 mL ES-DMEM. Pellet the ES cells at 300*g* for 5 min.
3. Aspirate the supernatant, gently resuspend the cells in 6 mL ES-DMEM, and transfer to the 25-cm^2 flask with irradiated feeders. Incubate at 37°C.
4. When the ES cells cover 70–80% of the surface area, transfer them to a 75-cm^2 flask with irradiated feeders. To do so, aspirate the medium, wash the cell culture 3 times with 5 mL PBS (swirl dishes to remove dead cells and traces of ES-DMEM), and add 750 μL trypsin-EDTA (evenly distribute liquid over the cell layer). Incubate at 37°C for 5 min.
5. Remove the 25-cm^2 flask from the incubator and vigorously shake it to disaggregate the cells. Verify under low-power magnification that ES cells are single or in small clumps of two to six cells.
6. Add 12 mL of ES-DMEM and transfer the cell suspension to a 75-cm^2 flask with irradiated feeders. Incubate at 37°C.
7. The next day, ES cells will cover 70–80% of the surface area and are ready for electroporation. (Medium should be orange.) The flask now contains about 20–30 × 10^6 ES cells. Aspirate the medium, wash the cell culture 3 times with 5 mL PBS (swirl dishes to remove dead cells and traces of ES-DMEM), and add 2 mL of trypsin-EDTA. Distribute the trypsin-EDTA evenly and incubate at 37°C for 5 min.
8. Remove the 75-cm^2 flask from the incubator and vigorously shake it to generate a single cell suspension. Add 8 mL of ES-DMEM, transfer the suspension to a 15-mL tube, and pellet the ES cells at 300*g* for 5 min.
9. Aspirate the supernatant, gently resuspend the cells in 5 mL PBS, and pellet the cells again. Repeat this wash once.
10. After the last wash, resuspend the cells in 750 μL of the stored DNA solution (40 μg/mL linearized targeting vector in PBS). Transfer the suspension to an

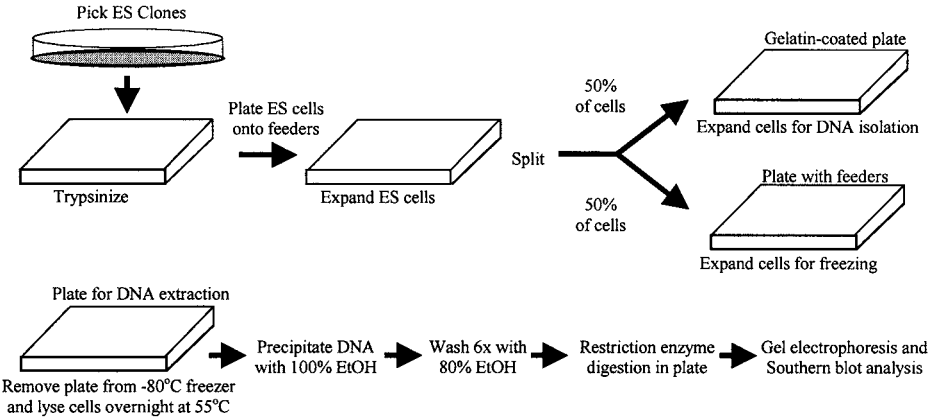

Fig. 1. Schematic overview of the procedure for picking drug-resistant ES clones and preparation of replica plates.

eletroporation cuvet with a 0.4-cm gap size. Remove potential air bubbles (*see* **Note 5**).

11. Place the cuvet in the Bio-Rad Gene Pulser and electroporate at 230 V/500 μF. The time constant should be between 6 and 8 msec. Place the cuvet in the tissue culture hood and let it sit for 5 min (*see* **Note 6**).
12. In the mean time, fill a 50-mL tube with 50 mL ES DMEM. Five minutes after electroporation, gently transfer the ES cells to the 50-mL tube.
13. Aspirate the media from the five 10-cm dishes with MEF feeders and plate 10-mL aliquots from the ES cell suspension in the 50-mL tube.
14. Exactly 24 h after the electroporation, replace the ES-DMEM on the plates with drug-containing ES-DMEM. The following final drug concentrations are used: 350 μg/mL G418; 140 μg/mL hygromycin B; 2.75 μg/mL puromycin; 0.2 μ*M* 1-[2-Deoxy]2-fluoro-β-D-arabinofuranosyl (FIAU); 2 μ*M* ganciclovir.
15. Refresh the selective medium daily. Generally the ES colonies will be ready for picking 8–9 days after electroporation.

3.5. Picking of Drug-Resistant ES Clones and Preparation of Replica Plates

It takes some experience to identify truly drug-resistant ES colonies rapidly. The shape of the colonies can be dependent on the drug combinations used for ES cell selection. Suitable ES colonies have a uniform appearance with relatively sharp edges, owing to three-dimensional growth. The center of an ES colony has usually a higher cell density and is somewhat darker in color. ES colonies with a flat pancake-like appearance should not be picked because they consist of differentiated ES cells. See **Fig. 1** for a schematic overview of the picking procedure.

1. Five days prior to the scheduled picking of the ES cell colonies, thaw one vial of MEFs in a 37°C water bath, and seed into a 75-cm^2 flask. Expand the MEFs as indicated in **Subheading 3.3.** Use these MEFS to prepare eight 96-well flat-bottomed plates with irradiated feeder monolayers (four plates with 2×10^4 feeder cells/well and four with 1×10^4 cells/well).
2. Fill a sterile 50-mL reagent reservoir with trypsin-EDTA solution and use a multichannel pipet to put 25 μL trypsin-EDTA in each of the wells of a round-bottomed 96-well plate. Prevent evaporation of the trypsin-EDTA solution by placing the plates in the 37°C incubator.
3. Next, load a sterile 50-mL reagent reservoir with ES-DMEM selection medium and remove the MEF-DMEM from the four 96-well flat-bottomed plates with 2×10^4 feeders/well. Add 200 μL ES-DMEM selection medium to each of the wells and place the plates back in the 37°C incubator.
4. Take a 10-cm dish from the incubator and divide it into four quadrants with a permanent marker (to allow systematic screening of the dish for drug-resistant colonies). Leave the ES-DMEM selection medium in the dish to preserve the normal morphology of the ES clones during the picking (*see* **Note 7**).
5. Set an adjustable 20-μL pipet at 2.5–3 μL and apply a standard 200-μL tip. Start scanning the first quadrant for suitable ES colonies. When a suitable colony appears, use the tip of the pipet to interrupt the feeder monolayer around the colony (by "drawing" circles around the colony). Then detach the colony and draw it into the pipet tip with 2.5 μL ES-DMEM selection medium (*see* **Note 8**). Transfer the colony to the first well of the round-bottomed 96-well plate with trypsin-EDTA solution.
6. Pick up a total of 48 colonies (assuming that the picking time for 48 colonies is less than 30 min). Change pipet tips between colonies to avoid cross-contamination. After the picking, incubate the plate for 5 min at 37°C.
7. Take the trypsinized ES cells and one of the 96-well plates with feeder monolayers out of the incubator. Disaggregate the ES cells in row 1 of the round-bottomed 96-well plate using a multichannel pipet (pipet up and down at least 10–20 times). Then, transfer the suspended ES cells to row 1 of the 96-well plate with feeders. Repeat this procedure for the remaining rows, and incubate the plate in a 37°C incubator.
8. Add 48 additional colonies to this plate by repeating **steps 4–6**. In a standard targeting experiment, we pick a total of 4×96 ES colonies.
9. On the second day, small colonies of ES cells start to appear in the wells. The ES cell growth rate of individual ES cell colonies will vary. At day 3 after picking, the medium in some of the wells will start to turn orange to yellowish. Refresh these wells individually (200 μL ES-DMEM). At day 4 or 5 after picking, the medium on 70–80% of the colonies will generally turn orange or yellowish within 24 h. When this happens, the ES cell clones are ready to be split into two new flat-bottomed 96-well dishes: one plate has 2×10^4 feeders/well and will be used to prepare frozen ES cell stocks, and the other plate is gelatin-coated and will be used for isolation of genomic DNA (for identification of homologous recombinants via Southern blot analysis; *see* **Note 9**).

10. Before splitting ES cells, add 100 μL 0.1% gelatin-PBS to four 96-well plates with flat bottoms. Incubate for 30 min at 37°C and subsequently replace the gelatin with 200 μL ES-DMEM (without drugs). Then replace the MEF-DMEM of the four 96-well plates with 1×10^4 feeder cells for ES-DMEM (250 μL/well). We will refer to the latter plates as the master plates.
11. Remove one of the 96-well dishes with subconfluent ES cells from the incubator. Take away most of the ES-DMEM media from the wells by using vacuum suction. Remove the remainder of the medium with a multichannel pipet. (Use clean tips for each row.) Wash ES cells by pipeting 100 μL of PBS down and up with a multichannel pipet.
12. After all washes are completed, add 50 μL trypsin-EDTA to each well and incubate the dish for 5 min at 37°C.
13. Check the plate for proper trypsinization of the ES cells. Then take 50 μL ES-DMEM from row 1 of the fresh 96-well feeder plate and disaggregate the trypsinized ES cells using the multichannel pipet. (Pipet vigorously up and down at least 10–20 times.) Then, transfer 50 μL ES cell suspension to row 1 of a fresh feeder plate and the remaining 50 μL to the gelatin-coated plate. When ES cells of all 8 rows have been split, incubate both plates at 37°C. Split the ES clones in the remaining three plates by repeating **steps 10–12** for each of the plates.
14. Within 2 days after the splitting, the medium on the cells will turn orange to yellow. Then it is time to prepare frozen stocks.
15. Remove the first master plate from the incubator. Aspirate most of the ES-DMEM media from the wells using vacuum suction. Remove the remainder of the medium with a multichannel pipet. (Use clean tips for each row.) Wash ES cells by pipeting 100 μL PBS down and up in the wells using a multichannel pipet.
16. After washing all wells, add 30 μL trypsin-EDTA to each of the wells and incubate the plate for 5 min at 37°C.
17. Take 75 μL ES-DMEM from a reagent reservoir and disaggregate the trypsinized ES cells using a multichannel pipet. Pipet vigorously up and down at least 10–20 times. Then, mix the contents of each well with 100 μL ice-cold 2X freezing medium. (Pipet vigorously up and down at least 5 times to dilute the 2X freezing medium.) Quickly seal the plate with masking tape, wrap it in several layers of tissue towel, and transfer it to a Styrofoam box with a lid. Place the box for at least 24 h in a –80°C freezer.
18. Within 2–3 days after splitting, the medium on the gelatin-coated plates will turn orange to yellow. At this point, refresh the ES-DMEM medium daily until most ES clones have become superconfluent (usually 4–6 days after splitting).
19. To harvest the superconfluent cells, simply remove the 96-well plate lid, invert the plate, and start vigorously clapping it into a stack of tissue towels until all residual medium has been removed. (Do not be afraid to lose ES cells, as they are firmly attached to the plates.) No washing with PBS is necessary. Tape the lid to the plate and freeze it for at least 2 h at –80°C (*see* **Note 10**).

3.6. Identification of Targeted ES clones

3.6.1. Extraction and Restriction Enzyme Digestion of Genomic DNA from ES Cells

Allen Bradley and coworkers *(3)* have developed the following procedure for extraction and restriction enzyme digestion of genomic DNA *(3)*.

1. Incubate 96-well plates stored in a –80°C freezer for 5 min at room temperature.
2. Then, add 50 µL lysis buffer [10 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA, 10 m*M* NaCl, 0.5% sarcosyl, and freshly added 1 mg/mL proteinase K (e.g., Sigma, cat. no. P-2308)] to each of the wells, using a multichannel pipet (*see* **Note 11**).
3. Seal the edges of the 96-well plates with masking tape and incubate them overnight at 55°C in a box with water-soaked tissue towels.
4. The next day, precool the plates on ice. Slowly add 100 µL of ice-cold absolute ethanol per 96-well plate using a multichannel pipet. Allow plates to stand at room temperature for at least 3 h. Then, screen wells for the presence of precipitated genomic DNA using a microscope with low-power magnification. Precipitated genomic DNA typically has a web-like appearance. If DNA is not observable in all wells, continue the incubation for at least another hour (*see* **Note 12**).
5. Discard the supernatant by inversion of the plates. The DNA will adhere to the 96-well plate. Gently add 100 µL 80% ethanol to each well with a multichannel pipet to wash the precipitated DNA. Invert the plates onto paper towels to discard the wash solution thoroughly. Repeat the ethanol wash 6 times.
6. After the final wash, invert the plates to remove as much wash solution as possible. Leave the plates tilted to air-dry for about 1 h. Screen wells for complete evaporation of the ethanol wash solution. If traces of ethanol remain, restriction enzymes will cut the genomic ES cell DNA incompletely.
7. While plates are drying, prepare a restriction digest cocktail. A typical mixture contains the optimal 1X restriction buffer for the enzyme used, 1 m*M* spermidine, 100 µg/mL acetylated BSA, 50 µg/mL RNase, and 10–15 U enzyme per sample.
8. Add 35 µL restriction digest cocktail per well with a multichannel pipet. Tap the plates to ensure that the cocktail completely covers the surface of each well.
9. Seal plates with masking tape and incubate them overnight in a humidified closed plastic container at the temperature specified by the manufacturer.

3.6.2. Gel Electrophoresis and Southern Blot Analysis

1. Take the plates out of the incubator and add 7 µL DNA loading buffer to each digest using a multichannel pipet. Place the plates in the refrigerator until electrophoresis (or freeze if longer storage is required).
2. Prepare 0.8–1.0% agarose gels in 1X tris-acetate EDTA (TAE) for electrophoresis of the digests. Use an electrophoresis system that has capacity for many samples.

Preferably, gel combs should yield slots with a loading capacity 30–40 μL and allow sample loading with a multichannel pipet.

3. Perform electrophoresis at 20–40 V until the diagnostic wild-type and mutant fragments are adequately segregated (usually requires overnight electrophoresis).
4. Photograph the gels and mark the positions of DNA marker band with a Pasteur pipet. (Punch holes in the gel.) Using a scalpel, cut the area of the gel to be blotted.
5. To hydrolyze the DNA, soak the gel pieces in 0.25 *M* HCl for 45 min. Mix constantly using a shaking platform (e.g., a Belly Dancer).
6. While gels are incubating, cut a Hybond N^+ nylon membrane for each gel piece. Engrave a blot ID in the upper left corner of each membrane (date, gene, investigator).
7. Carefully pour off the 0.25 *M* HCl (avoid breaking of gels) and incubate in 0.4 *M* NaOH for 20 min on a shaking platform. Refresh the 0.4 *M* NaOH solution and incubate for another 20 min.
8. Fast, efficient, and reproducible transfer of DNA fragments from gel to nylon membrane is achieved by vacuum blotting (e.g., Stratagene, cat. no. 400330). Transfer for 2–3 h.
9. Stop the blotting and rinse the membrane in 3X standard saline citrate (SSC) for about 30 s. Repeat the rinse one more time.
10. Carry out hybridization in roller bottles in a hybridization oven (e.g., Stovall Life Science) at 65°C. Transfer the membranes to hybridization tubes with the DNA side facing the inner tube. Prehybridize for 15–60 min in a rapid hybridization solution (Amersham, cat. no. RNP1635). Use a 12 mL rapid hybridization solution for a 12-inch hybridization tube, and 6 mL for a 6-inch tube.
11. While prehybridizing the membranes, radioactively label the DNA probe.
12. Add the denatured probe and hybridize for 2–16 h at 65°C.
13. Wash the membrane with 1X SSC/0.1%, sodium dodecyl sulphate (SDS) for 20–30 min at 65°C with agitation. Then wash with 0.3X SSC/0.1% SDS for 20–30 min at 65°C. A final wash in 0.1X SSC/0.1% SDS may be necessary depending on the probe. Wrap the membranes in plastic, carefully remove excess liquid, and expose the films at –80°C.

3.7. Expansion and Verification of Targeted ES clones

Targeted ES clones must be thawed and expanded for 1) Southern blot confirmation of the targeted mutagenesis; 2) karyotyping; and 3) preparation of multiple frozen stocks.

1. Five days prior to the scheduled expansion of the targeted ES cell clones, thaw one vial of MEFs in a 37°C water bath, and seed into a 75-cm^2 flask. Expand the MEFs as indicated in **Subheading 3.3.** Use these MEFS to prepare 96-, 24-, and 6-well plates with irradiated feeder monolayers (**Table 1**).
2. Remove one 96-well plate with targeted ES clones from the –80°C freezer and place it in a 37°C water bath. Prevent water from leaking into the plate.

3. When the freezing medium in the plate has melted, verify that the cells are settled at the bottom of the plate. If so, remove all but 20 μL freezing medium from wells containing targeted ES cell clones.
4. Resuspend each independently targeted ES clone in 200 μL ES-DMEM and transfer the cell suspension to a well in the 96-well plate with feeders. Incubate at 37°C.
5. The next day, change the medium on the 96-well plate to remove any traces of DMSO.
6. Monitor the growth of the ES cells closely and split clones that have reached 70–80% confluency. Aspirate the medium, wash the cell culture 3 times with 200 μL PBS, add 35 μL of trypsin-EDTA, and incubate at 37°C for 5 min.
7. Remove the plate from the incubator and thoroughly suspend the trypsinized cells to generate a single cell suspension. Add 1 mL of ES-DMEM and transfer the suspension to a 24-well plate with irradiated feeders.
8. Once ES clones reach 70–80% confluency, transfer each clone to a 6-well plate for further expansion. When ES clones are at the appropriate cell density, rinse the wells 2 times with PBS, add 1 mL of trypsin-EDTA, and incubate 5 min at 37°C.
9. Resuspend cells in 5 mL ES-DMEM, transfer the cell suspension to a 15-mL tube, and spin for 5 min at 300*g*.
10. Aspirate the medium from the tube and suspend the cell pellet in 2 mL ES-DMEM. Transfer 3 × 0.5 mL to cryogenic vials and add 0.5 mL 2× freezing medium to each cell suspension. Wrap the vials in tissue towels and transfer them to a –80°C freezer in a Styrofoam box. Divide the remaining 0.5 mL cells over two wells of a 24-well plate with MEF cells. Grow one well to superconfluency for DNA extraction and confirmation of targeted mutagenesis. Use the ES cells in the other well to measure whether individual ES clones contain 40 chromosomes (*see* **Note 13**).

3.8. Preparation of ES Clones for Blastocyst Injection

1. Prepare a 24-well plate with the irradiated feeder cells as described in **Subheading 3.4.** Approximately 5 days before the scheduled microinjection, thaw a vial of ES cells in a 37°C water bath. Transfer the ES cell suspension to a 15-mL tube with 4 mL ES-DMEM and pellet the ES cells at 300*g* for 5 min.
2. Aspirate the supernatant, gently resuspend the cells in 1 mL ES-DMEM, and transfer to a well of the 24-well plate with MEF feeders. Incubate at 37°C.
3. Once the targeted clones reach 75–90% confluence (usually within 2–3 days after seeding), trypsinize and reseed them at different dilutions (1:4 and 1:6).
4. On the day of blastocyst injection, select a well that is 40–80% confluent. Rinse the well 3 times with PBS, add 0.5 mL of trypsin-EDTA, and incubate 5 min at 37°C.
5. Resuspend the cells in 2 mL ES-DMEM. Transfer 2 mL of the cell suspension to a 15-mL tube and spin it at 300*g* for 5 min. (Reseed the remaining 0.5 mL

of the cell suspension in a well of a 24-well plate with feeder cells to allow for reinjection of the ES clone, if necessary.)

6. Aspirate the supernatant and resuspend the cells in 250 µL of microinjection medium [HEPES-buffered (20 m*M*) ES-DMEM without LIF]. Place the cells on ice. Use them for microinjection within 1–2 h following trypsinization.
7. Typically, 16–24 embryos are injected per targeted clone. Optimally, three to four independently targeted ES clones will be injected.

4. Notes

1. It is important to test serum lots for high ES cell plating efficiency. Companies will send out free aliquots of ES-qualified serum that you can use for selecting the serum with the highest plating efficiency. Test the plating efficiency by seeding 5000 ES cells onto 10-cm dishes with MEF feeder layers. After 5–7 days, high-quality sera will show a colony-forming efficiency of about 30% or more of the plated cells.
2. Although MEF cells should produce sufficient LIF to inhibit differentiation of ES cells, many investigators using feeder layers add an extra 1000 U/mL LIF to the culture medium to ensure that LIF levels remain consistently high.
3. Instead of preparing feeder monolayers at this step, you can also choose to split the MEFs once again 1 in 3 (to amplify them further).
4. If feeder cells are plated at too high a density, cells will start detaching and "roll up" when the number of ES cells on top of the feeder layer increases.
5. The ES cell/targeting vector mixture can immediately be shocked. Preincubations of the ES cell/targeting vector mixture have no beneficial effect on the ultimate targeting frequency.
6. Substantial cell lysis will occur during electroporation, which give rise to a somewhat viscous cell suspension. This is a normal phenomenon.
7. Replacing the medium with PBS will round up ES cells and make it more difficult to select pluripotent ES colonies.
8. The volume of 2.5 µL ES-DMEM does not interfere with the proper trypsinization of the ES colony.
9. Normally about 90% of the wells will contain expanded ES cells at this stage. If not, try to improve your picking efficiency, reducing your picking speed.
10. Storage at –80°C will crack the cells. This facilitates cell lysis and proteinase K digestion and increases the DNA yield.
11. Lysis buffer lacking proteinase K (incomplete lysis buffer) can be stored at room temperature. Prepare a 10 mg/mL stock of proteinase K in milliQ and freeze it in 500-µL aliquots. Add 500 µL proteinase K solution to 5 mL incomplete lysis buffer. The resulting solution is sufficient for one 96-well plate.
12. If the proteinase K digestion is incomplete, it will take longer for the DNA to precipitate.

13. The genetic stability varies per parental ES cell line. If a highly stable ES cell line is used, most of the clones will be euploid, and karyotype analysis can be omitted.

References

1. Abbondanzo, S. J., Gadi, I., and Stewart, C. L. (1993) Derivation of embryonic stem cell lines. *Methods Enzymol.* **225,** 803–823.
2. Ramirez-Solis, R., Davis, A. C., and Bradley, A. (1993) Gene targeting in embryonic stem cells. *Methods Enzymol.* **225,** 855–878.
3. Ramirez-Solis, R., Rivera-Perez, J., Wallace, J. D., et al. (1992) Genomic DNA microextraction: a method to screen numerous samples. *Anal. Biochem.* **201,** 331–335.
4. Kontgen, F. and Stewart, C. L. (1993) Simple screening procedure to detect gene targeting events in embryonic stem cells. *Methods Enzymol.* **225,** 878–890.

9

Conditional Knockout Mice

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1. Introduction

1.1. Principle of Conditional Gene Targeting

In the last decade, gene targeting in embryonic stem (ES) cells has been extensively used as a powerful tool to study gene function in the mouse, as a mammalian model organism. As initially developed, the technique allows the disruption of a target gene in the murine germline by the insertion of a selectable marker ***(1)***. The vast majority of the more than 1000 knockout mice in existence have been created following this design. Many of these strains have given valuable information on the biologic function of the genes studied ***(2)***. Since these "conventional" knockout mice are usually homozygous for a null allele in the germline, they provide an appropriate model for inherited diseases, leading to embryonic or early postnatal lethality in about 30% of cases. Apart from this application, germline knockout mice do not necessarily represent the best technical approach for studying other aspects of gene function in vivo, in particular in adult mice. A refined knockout strategy termed conditional gene targeting has been developed ***(3)*** that permits the inactivation of the target gene to be restricted to a certain organ and/or developmental stage ***(4)***. **Figure 1** depicts the principal difference between the two strategies, comparing a germline knockout mouse with two types of conditional mutants in which the target becomes inactivated either early on in a particular organ without temporal control or upon induction at a chosen time point. The inactivation of the target gene in a conditional mutant is achieved by the expression of a site-specific DNA recombinase (Cre or FLP) in mice in conjunction with the introduction of two recombinase recognition sequences

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

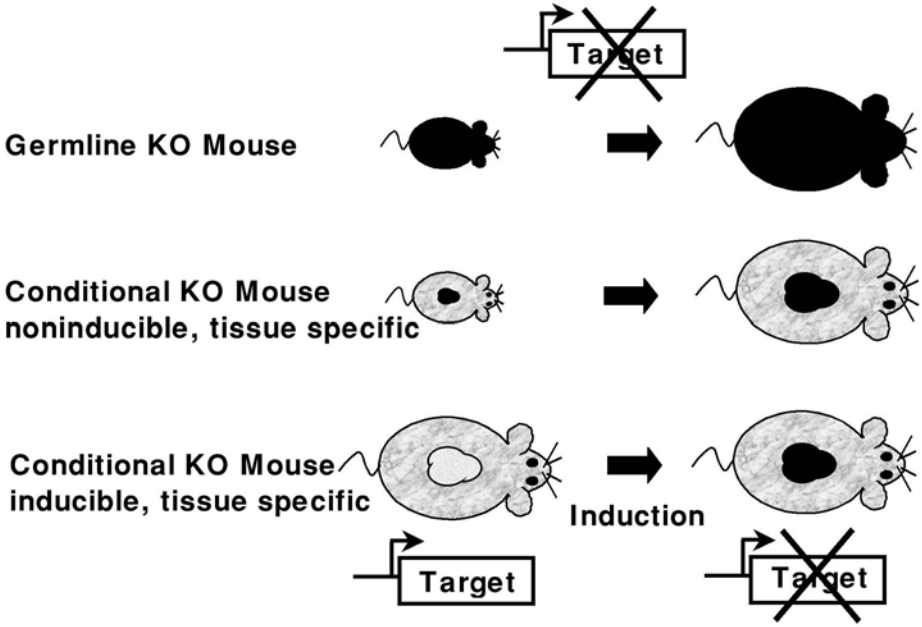

Fig. 1. Gene-targeting strategies. Upper row: the knockout (KO) is transmitted through the germline, resulting in a null allele mutant strain (conventional KO); middle row: the KO is introduced in somatic cells and restricted to a specific tissue (conditional, cell type-specific KO); bottom row: the KO is introduced upon induction (conditional, inducible KO). Tissues in which the target gene is inactivated are shown in black.

[loxP or FLP recognition target (FRT)] into noncoding regions of the target gene (**Fig. 2**). These sites are usually placed in the same orientation into introns such that recombination results in gene inactivation through the deletion of the loxP- or FRT-flanked exon(s). Although the generation of completely ES cell-derived mice has been recently significantly improved ***(5)***, the current standard method to derive a conditional mutant requires the generation of two mouse strains: one mouse strain harboring a loxP- or FRT-flanked gene segment by gene targeting in ES cells and a second transgenic strain expressing Cre or FLP either constitutively, or upon induction, in one or several organs. The conditional mutant is generated by crossing these two strains such that the inactivation of the target gene will be restricted in a spatial and temporal manner, following the pattern of Cre or FLP expression in the transgenic strain (**Fig. 2**, right side). As the homozygous loxP- or FRT-flanked allele must be combined with a heterozygous recombinase transgene, it often requires additional generation time (3 months) to obtain reasonable numbers

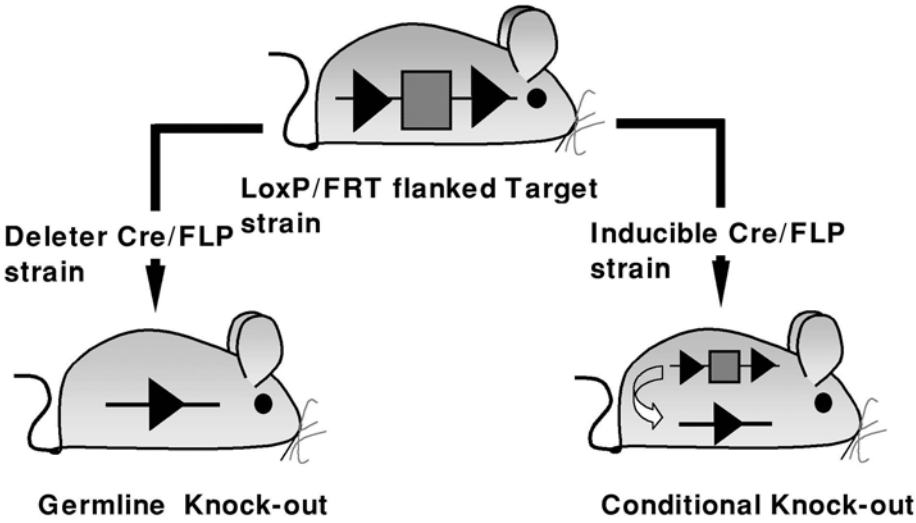

Fig. 2. Generation of a conditional mutant. Upper mouse: a strain harboring a loxP-flanked (triangles) gene segment (square). To derive a conditional mutant, the strain is crossed to mice expressing Cre recombinase constitutively or upon induction in specific cell types or organs (right side). Gene modification occurs by Cre-mediated recombination according to the expression pattern of the recombinase transgene. The loxP-containing strain can be converted into a null allele mutant by a single cross to a deleter strain expressing Cre in germ cells or the early embryo (left side).

of conditional mutants, compared with germline KO mice. The loxP-containing strain can be also converted into a null allele mutant by a single cross to a deleter strain expressing Cre in germ cells or the early embryo (**Fig. 2**, left side). There are no general rules to decide whether the germline or the conditional mutation is more appropriate for a particular experiment since this depends on the biologic question. In fact, both types of mutants are usually generated at the same time to investigate gene function both during embryonic development and in the adult animal. However, following the conditional mutagenesis scheme described in **Subheading 1.4.**, germline and conditional mutants can be generated for a particular gene using a single targeted ES cell clone. Thus, we would recommend the conditional gene targeting scheme for all knockout projects, as this offers a greater flexibility compared with the conventional approach without additional efforts once the necessary reagents are assembled.

1.2. Use of Conditional Mutants

Conditional mutants have been used to address various biologic questions that could not be resolved with germline mutants, often because a null allele

results in either an embryonic or a neonatal lethal phenotype. If the main question to answer concerns the role of different cell types in a physiologic process, the use of noninducible, cell type-specific Cre strains is presently the most pragmatic choice, as a collection of tissue-specific strains is already available. A good example of this approach is comparison of the cell type-specific inactivation of the insulin receptor in skeletal muscle, pancreas, and liver. Cell type-specific gene targeting has revealed a prime role for the liver, and not skeletal muscle, in glucose homeostasis and also the role of insulin signaling in pancreatic insulin secretion (**Table 1**, examples 1–3). In the selection of a Cre transgenic strain for a particular experiment, it is important to be aware of the developmental stage at which the chosen line starts to delete the target, as many of the promoter regions used for Cre expression are active before birth. To illustrate this point, two examples of genes that would most likely lead to embryonic lethality in a prenatal neuron-specific knockout are shown in **Table 1** (examples 4 and 5). The use of transgenic strains with postnatal neuronal Cre expression allowed dissection of the role of these two receptors in long-term memory in adult mice.

Inducible gene targeting is especially helpful to analyze gene function in adults, as it facilitates performance of gene inactivation studies in animals that have undergone normal embryonic development. Furthermore, it permits investigation of the effect of gene inactivation after the onset of a chronic or acute disease. This aspect is of particular interest in the validation of genes for pharmaceutical drug development, as genes can be inactivated in the context of a fully developed disease. The use of inducible gene targeting in mouse models of human disease can provide an ideal surrogate for the treatment of patients with antagonistic drugs. **Table 1** (examples 6–8) gives three recent examples in which the induced inactivation of genes has provided information on the biologic function of the respective proteins in adult tissues. Germline mutations in these genes have been shown to be lethal for the embryo or cell type studied. Note that in example 8, Cre-mediated deletion and inversion has been used to switch between the expression of two target genes.

1.3. Site-Specific DNA Recombinases

Cre recombinase is a P1 phage-derived site-specific DNA recombinase that recognizes and mediates recombination between 34-bp sequences referred to as loxP sites. As Cre does not require any cofactors or accessory proteins to mediate loxP-specific recombination and exhibits optimal activity at 37°C ***(14)***, the Cre/loxP system is a well-suited and widely used recombination system for genetic engineering in ES cells and mice ***(15)***. A loxP site is composed of two 13-bp inverted repeats separated by an 8-bp asymmetric spacer that determines the orientation of the loxP site (***16***,***17***; **Fig. 3A**). Cre-mediated recombination

Table 1
Conditional Gene-Targeting Experiments

Target gene	Cell type expressing Cre	Modification	Expression mode / onset of deletion	Germline Knockout	Physiologic Question	Reference
1 Insulin receptor	Muscle	Deletion	Constitutive/prenatal	Neonatal lethal	Glucose homeostasis	***(6)***
2. Insulin receptor	β-cells	Deletion	Constitutive/prenatal	Neonatal lethal	Glucose homeostasis	***(7)***
3. Insulin receptor	Hepatocytes	Deletion	Constitutive/prenatal	Neonatal lethal	Glucose homeostasis	***(8)***
4. NMDA receptor I	Forebrain neurons	Deletion	Constitutive/2 weeks on	Embryonic lethal	Spatial memory	***(9)***
5. TrkB receptor	Forebrain neurons	Deletion	Constitutive/2 weeks on	Embryonic lethal	Spatial memory	***(10)***
6. Notch 1	T-lymphocytes	Deletion	Inducible/neonatal	Embryonic lethal	T-cell development	***(11)***
7. RXRα	Keratinocytes	Deletion	Inducible/juvenile	Embryonic lethal	Skin development	***(12)***
8. Ig-H V-gene	B-lymphocytes	Inversion	Inducible/adults	Lethal for cell type	B-cell memory	***(13)***

NMDA, *N*-methyl-D-aspartate; RXRα, retinoid-x-receptor alpha.

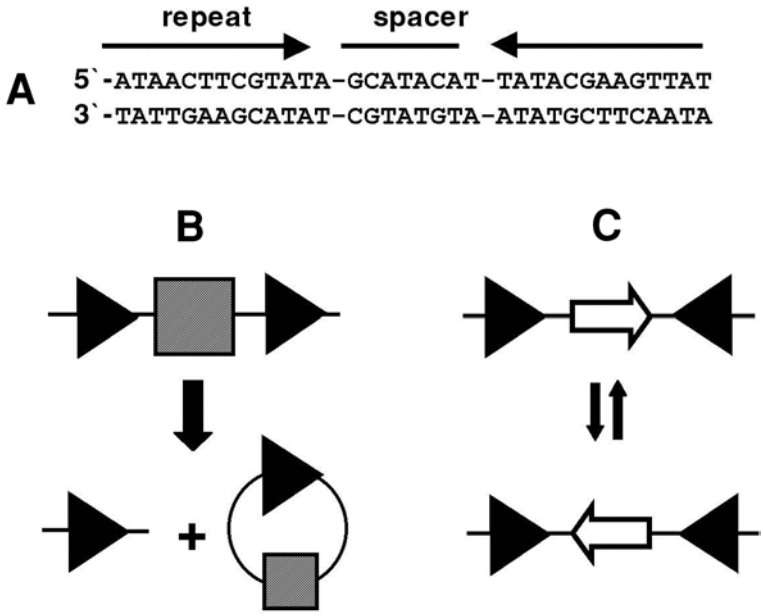

Fig. 3. The Cre/loxP recombination system. **(A)** Sequence of a loxP site. **(B)** Cre-mediated deletion of a DNA segment (square) flanked by two loxP sites (triangles) of the same orientation. **(C)** Cre-mediated inversion of a DNA segment flanked by two loxP sites in opposite orientation.

between two loxP sites results in the reciprocal exchange of DNA strands between these sites. Depending on the orientation and location of the two sequences, different products are produced. When two loxP sites are located in the same orientation on a linear DNA molecule, Cre-mediated recombination results in the excision of the loxP-flanked DNA segment as a circular molecule leaving a single loxP site on each reaction product (**Fig. 3B**). Although this reaction is reversible, the equilibrium is strongly biased toward excision as the circular molecule can diffuse away. When two loxP sites are located on the same DNA molecule in opposite orientation to each other, Cre mediates the inversion of the loxP-flanked sequence (***18***; **Fig. 3C**). As this reaction is also reversible, equal amounts of each inversion product are generated. Finally, if the loxP sites are located on two linear DNA molecules, Cre can mediate intermolecular translocations. Mutant loxP sites that recombine with each other, but not with wild-type loxP sites ***(19)***, can be used to recombine two pairs of lox sites independently at different loci in the genome without crossreaction ***(13)***. The equilibrium of Cre-mediated inversion and integration can be shifted toward one side by the recombination between two different single mutants,

which generates a double mutant and a loxP site ***(20)***. This reaction has been applied for the targeted integration of plasmids into a genomic lox site ***(21)***. The efficiency of recombination between two loxP sites on the same DNA molecule is a function of the distance between them, according to the current view that recombination occurs after the random collision of the two substrate sequences. Whereas the minimum distance required between two loxP sites to allow recombination was found to be 82 bp ***(22)***, the exact relationship between recombination efficiency and loxP distance has not yet been determined for the Cre/loxP system. In a study using the related FLP/FRT recombinase system, optimal recombination between a pair of FRT sites in a genomic region was observed at a distance of 200 bp, whereas a progressively reduced efficiency was observed with increasing distances between FRT sites ***(23)***. Furthermore, the recombination efficiency is also influenced by the genomic integration site ***(24)***. The recombination mechanism and the structure of a Cre/loxP intermediate are described in **ref. *25***.

A useful set of Cre expression and loxP substrate vectors is available from Life Technologies. Other Cre expression vectors along with a comparison of their expression levels in ES cells, a Cre-GFP fusion protein, a vector for coexpression of Cre, and a drug resistance gene to enrich for transiently transfected cells can be found in **refs. *26–29***. Since the Cre protein carries endogenous nuclear targeting determinants, the addition of a nuclear localization signal does not increase its activity in mammalian cells ***(30)***. The coding sequence of Cre is available under the EMBL database accession number X03453. A codon-optimized Cre gene that increases its expression in mammalian cells has recently been described ***(31)***.

In contrast to the diversity of genome manipulations that have been developed for the Cre/loxP system, limited efforts have been made to utilize other site-specific recombination systems in mammalian cells. Only a few other recombinases besides Cre have been shown to exhibit activity in mammalian cells ***(32–34)***. Their practical value is presently unclear, as their efficiency has not been thoroughly compared with the Cre/loxP system. The best characterized example is the yeast-derived FLP recombinase, which exhibits a temperature optimum at 30°C but which is unstable at 37°C ***(35)***. The affinity of FLP to the FRT target site is much lower compared with the affinity of Cre to loxP sites ***(108)***. A mutational analysis of FLP resulted in the mutant FLPe, which shows improved thermostability and activity at 37°C, but this mutant is still considerably more heat-labile compared with Cre ***(35)***. However, FLP has been used for tissue-specific genomic deletions in mice ***(36,37)***, and a more recent report on the first efficient FLPe transgenic "deleter" mouse ***(38)*** may encourage the further use of the FLPe/FRT system in mice.

1.4. Generation of LoxP-flanked Alleles; Vector Design

A requirement for Cre/loxP-based conditional mutants is the generation of an ES cell-derived mouse strain that harbors a loxP-flanked DNA region of interest. Although at least two strategies have been used for the conditional inactivation of endogenous genes *(4,39)*, we recommend the experimental design outlined in **Fig. 4** and described in **ref.** *39*. This strategy employs Cre/loxP recombination for the conditional inactivation of the target gene and FLP/FRT recombination to remove the selection marker gene from the ES cell genome. Both recombinase-mediated steps can be performed either in ES cells by transient recombinase expression or, which is more convenient, in vivo using Cre and FLP-deleter mouse strains.

The main objectives in designing a conditional construct are that the expression of the target gene should not be disturbed by the presence of the loxP sites, but should become inactivated (or otherwise modified) upon Cre-mediated recombination. A conditional construct contains a selectable marker gene (i.e., a neomycin resistance gene) flanked by two approx. 1–5 kb regions of homology to the target gene and optionally a thymidine kinase gene for negative selection (*see* **Note 1**). A short homology arm of 1 kb or less is required only if the transfected ES clones will be tested by polymerase chain reaction (PCR). In contrast to a conventional gene targeting construct, the resistance marker does not disrupt or replace one of the exons but is placed into an intron and flanked by two FRT sites, next to a single loxP site (*see* **Note 2**). A second loxP site is placed into one of the homology arms such that both loxP sites are flanking one or more exons of the target gene (**Fig. 4**; *see* **Note 3**). The conditional gene-targeting construct should be fully sequenced before the transfection of ES cells to avoid the transfer of undesired mutations into the target gene (*see* **Note 4**). This double-lox/double-FRT arrangement is introduced into ES cells through homologous recombination and transmitted through the germline of chimeric mice. Upon crossing to an FLP deleter strain *(38,39)*, the neo gene is deleted during germline transmission, resulting in the conditional, loxP-flanked allele; crossing to a Cre deleter strain (**Table 2**) results in the conventional null allele. Crossing to both, Cre and FLP deleter strains would also remove the selection marker from the germline mutant if the potential interference with other genes located near the target is regarded as an issue *(40)*.

Alternatively, both alleles can be obtained by transient transfection of the homologous recombined ES cells with a Cre or FLP expression vector, as described in **Subheading 1.3.** However, this additional manipulation during tissue culture requires more effort and increases the chance that the targeted clones may lose their germline potency. With regard to the position of the loxP sites within the target gene, we recommend placing them approx. 200 bp or

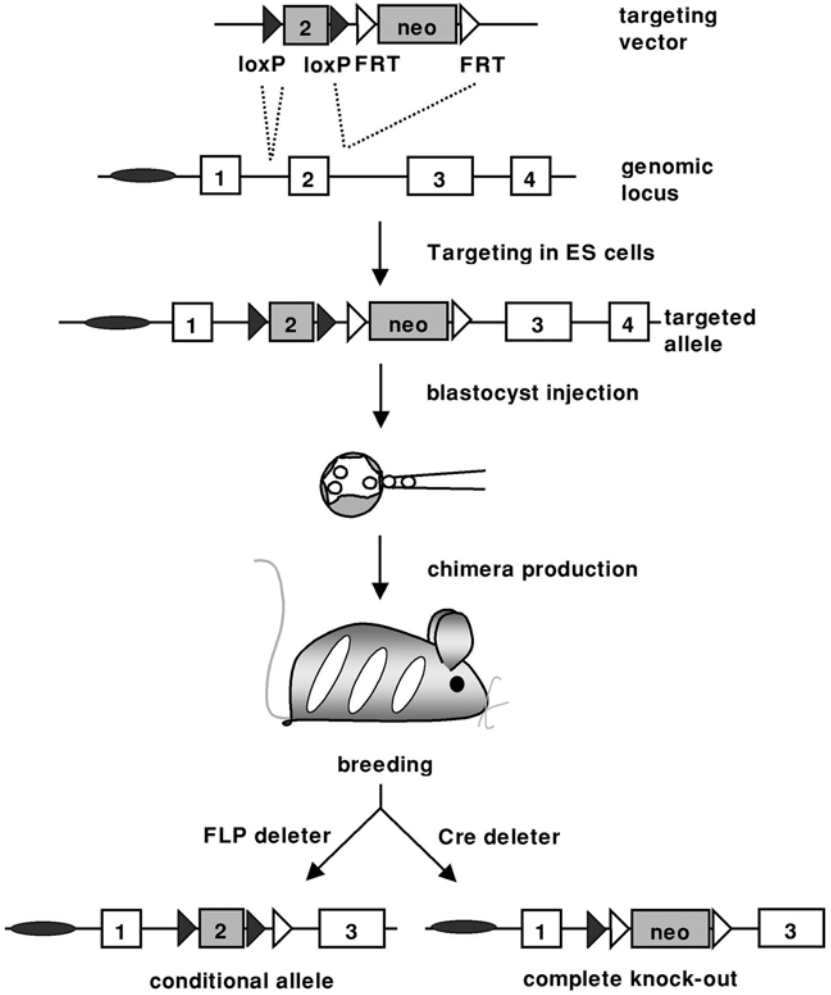

Fig. 4. Strategy to generate a loxP-flanked and a deleted allele of a target gene using a single construct. The gene-targeting vector contains an FRT-flanked neomycin resistance gene (neo), and the second exon (numbered rectangle) of the target gene is flanked by two loxP sites. The thymidine kinase expression cassette (tk) allows negative selection of ES cell clones carrying randomly integrated copies of the targeting vector. Upon homologous recombination, the second exon of the target gene and the neo gene are flanked by the two loxP sites. Chimeric mice are generated through injection of the targeted ES cell clones into blastocysts. By crossing of the chimeras to a FLP deleter strain, the loxP-flanked allele for conditional gene targeting is generated. Mating of the chimeras with Cre deleter mice results in a germline knockout mouse strain. Thin dashed lines connecting the targeting vector and genomic locus show the sites of insertion of the loxP sites and the FRT-flanked selection marker.

Table 2
Recombinase-Mediated Genomic Manipulations

Genomic manipulation	Purpose	Reference
Removal of selection marker	Introduction of small mutation	***(42)***
	Generation of homozygous knockout ES cells	***(43)***
Chromosomal deletion/inversion	Genomic studies	***(44)***
Chromosomal translocation	Modeling oncogenic translocations	***(45)***
Targeted integration	Transgene expression	***(21)***
Modification of gene trap vector	Exchange of reporter gene	***(46,47)***
Gene replacement	Expression of modified genes	***(47–49)***

more apart from the exon boundaries to reduce the risk of interference with splicing signals. In most cases a region of 1–3 kb comprising one or a few exons of the target gene has been chosen for deletion. Although it is generally easier to inactivate a gene functionally by the excision of many exons, the deletion of a large loxP-flanked gene segment may be less efficient, as discussed in **Subheading 1.3.** Sources for useful Cre plasmids are described in **Subheading 3.**, and a set of FLP/FRT constructs can be found in **ref.** ***41***.

The production of a conditional allele of an endogenous gene was first described in 1994 ***(4)*** for the DNA polymerase β gene, in which a 1.5-kb region containing the promoter and first exon was flanked by loxP sites. The authors describe a strategy that employs three loxP sites and requires the selection marker removal in ES cells. Although a number of genes have been successfully modified in this way, we believe that most experiments in the future will follow the outline in **Fig. 4**, promoted by the recent description of efficient FLPe deleter mice ***(38)***. Presently, conditional gene targeting has been applied to a large number of genes, some of which are listed on the home page of A. Nagy (http://www.mshri.on.ca/nagy/cre.htm).

1.5. Other Applications of Cre/loxP in ES Cells

Besides conditional gene inactivation, the Cre/loxP system can be applied to derive a variety of other genetic manipulations in ES cells, which can be introduced into the murine germline without further modification. By appropriately positioning the two loxP sites, almost any type of mutation can be produced, including point mutations, gene replacements, insertions, and chromosomal translocations. Since the main focus of this chapter is the generation of conditional mutants, other Cre/loxP applications are summarized as an overview in **Table 2**. For a detailed description of these technologies, we refer the reader to **refs.** ***50*** and ***51***.

1.6. Cre Transgenic Mice

1.6.1. Constitutive Recombinase Expression

Cre transgenic mice can be produced through either the pronuclear microinjection of randomly integrating transgene constructs or the targeted introduction of the Cre gene in frame with the start codon of an endogenous gene. The first approach is more straightforward, considering the effort involved in vector construction and its introduction into the germline. However, an appropriate promoter region tested for transgenic expression is required (*see* **Note 5**). Since the level and pattern of transgene expression often varies greatly depending on its copy number and integration site, a number of different founder lines needs to be tested in order to identify a useful strain. To reduce position effects on transgene expression, bacterial artificial chromosomes (BACs) carrying genes with an appropriate expression pattern can be used as vectors, providing that all regulatory elements are present on the genomic fragment ***(52,53)***. In any case, the expression pattern of the promoter region used will determine the onset and cell type specificity of Cre-mediated gene modification, whereas the expression level will determine the efficiency of gene modification in a given cell type. However, the exact relationship between the number of Cre molecules in a cell and the efficiency of deletion of a loxP-flanked gene segment has not been established. Furthermore, the extent of Cre-mediated recombination can be expected to decrease as the distance between two loxP sites is increased, and it seems also to depend on the chromosomal position of the target, as discussed in **Subheading 1.3.**

For identification of a Cre transgenic strain suitable for conditional gene targeting, it is presently necessary to produce a series of transgenic mice and compare the deletion efficiency among these lines by crossing them to an indicator strain possessing a loxP-flanked gene segment. The extent of deletion in different organs can be directly assessed at the DNA level by Southern blot analysis. To determine the pattern of recombination at the level of single cells in histologic sections, mice transgenic for a β-galactosidase or green fluorescent protein (GFP) recombination vector can be used as an indicator strain ***(54–59)***. For ubiquitous expression of the recombined reporter construct, the Rosa26-knockin approach seems to be most appropriate ***(56,57,59)***. Transgene constructs for the expression of Cre recombinase can be designed similarly to cDNA expression vectors for transgenic mice, i.e., a promoter region coupled to the Cre coding sequence, preceded by a hybrid intron providing 5′ splice donor/acceptor sites to increase cDNA expression ***(60)***, followed by an efficient polyadenylation signal sequence (***61***; **Fig. 2**). We do not recommend placing splice donor/acceptor sites 3′ of the Cre gene, as we found that codon 145 can be used as a cryptic splice donor site in combination

with an SV40 intron located downstream of the coding region (R. Kühn, unpublished data).

As an alternative to the transgenic approach, the Cre gene has been inserted downstream of the promoter of an endogenous gene by homologous recombination in ES cells (**Table 3**). By this strategy, Cre expression becomes optimally regulated since all control elements of the targeted gene are present at their natural chromosomal environment. Furthermore, the targeting approach can be applied to any gene for which genomic clones are available without the need for characterized promoter regions. Gene targeting is, however, more laborious compared with conventional transgenesis, and the generation of a nonfunctional allele of the gene used for targeted Cre expression may be a disadvantage in certain cases. The latter potential complication can be addressed through the expression of Cre via an internal ribosomal entry site without interfering with the endogenous gene, as described in **ref. *62***.

A replacement-type vector for targeted Cre insertion should be constructed such that the initiation codon of the targeted gene is replaced by the coding region of Cre (**Fig. 2**). We suggest adding a polyadenylation signal at the end of the Cre coding region to prevent transcription of downstream exons of the targeted gene. It has been reported that premature termination codons can destabilize the mRNA owing to a mechanism termed nonsense-mediated decay ***(63)***. A selection marker gene must be included within the targeting vector for the identification of targeted ES clones, which should later be removed from the genome to minimize disturbance of the targeted locus ***(40)***. We recommend not using a loxP-flanked selection gene for this purpose to avoid potential chromosomal rearrangements, which may occur when the Cre-expressing mouse strain is crossed to another strain possessing the loxP-flanked target gene. Thus, for targeting vectors that insert Cre, the selection marker should be flanked either with FRT sites to remove the selection cassette in recombinant ES clones using FLP recombinase or by mutant lox sites, which do not recombine with wild-type loxP sites ***(19)***. An FRT-flanked neo cassette has been described in **ref. *64***.

A number of constitutive, cell type-specific promoters have been successfully used for Cre expression in transgenic mice, which allows conditional gene targeting in a variety of tissues (**Table 3**; http://www.mshri.on.ca/nagy/cre.htm). Other types of recombinase-expressing strains have been generated to delete a loxP- or FRT-flanked gene segment from the mouse genome in the germline, e.g., for removal of a selection marker or to convert a conditional into a conventional, complete mutant (*see* **Subheading 1.4.** and **Fig. 5**). These so called deleter strains express the recombinase in germ cells or during early

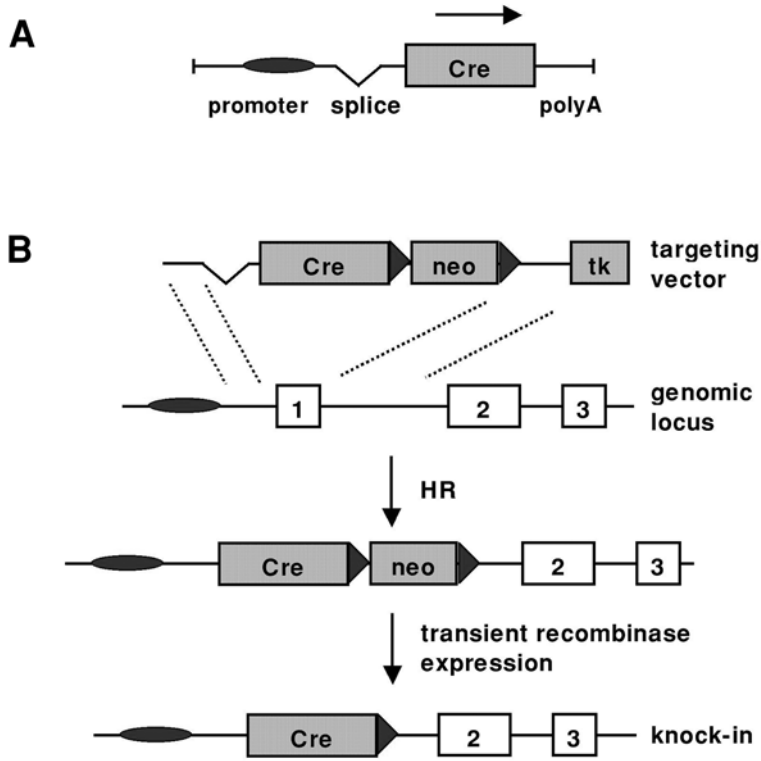

Fig. 5. Generation of recombinase-expressing mouse strains. **(A)** Diagram of a Cre expression cassette as used for the production of transgenic mice by pronucleus injection. A promoter region (filled ellipse), which has been previously characterized for gene expression in transgenic mice, directs transcription of the 1-kb coding region of Cre recombinase (stippled rectangle). The Cre coding region is followed by a polyadenylation signal sequence (polyA). Splice donor and acceptor sites (splice) are placed in the 5′ region of the Cre gene for efficient transgene expression. **(B)** Diagram of a vector for the targeted insertion of Cre. Upon homologous recombination, the first exon of the target gene (numbered rectangle) is replaced in the targeting vector by the coding region of Cre such that the position of the translation initiation codon is unchanged. A neomycin resistance gene (neo) flanked by FRT (or mutant lox) sites (black triangles) is inserted downstream of Cre. The FRT (lox)-flanked neo gene must be deleted from the targeted locus by transient expression of FLP (Cre) recombinase. Thin dashed lines connecting the targeting vector and genomic locus show the sites of insertion of the Cre gene and the selection marker. HR, homologous recombination.

Table 3
A Collection of Tissue-Specific Cre-Expressing Mouse Strains

Specificity	Control element	Reference
Thymocytes	Proximal lck promoter	***(65)***[a]
Eye lense	A-crystallin promoter	***(66)***
B-lymphocytes	CD19 gene (knockin)	***(67)***
Forebrain neurons	CamKIIa promoter	***(68)***
Midbrain/hindbrain neurons	En2	***(69)***
Schwann cells	P0 promoter	***(70)***
Pituitary gland	POMC promoter	***(70)***
Retina	IRBP promoter	***(70)***
Adipocytes	aP2 promoter	***(71)***
Hepatocytes	Albumin promoter/enhancer	***(72***[a]***, 73)***
Pancreatic β-cells	Rat insulin 2 promoter	***(74)***[a]
Cardiac muscle	αMHC promoter	***(75)***
Cardiac muscle	MLC2v gene (knockin)	***(76)***
Skeletal muscle	MCK promoter	***(6)***
Chondrocytes	Col2a1	***(77)***[a]
Epidermis	Keratin-5 promoter	***(78)***
Macrophages	Lysosyme M gene (knockin)	***(64)***
Mammary gland	WAP promoter	***(79)***[a]
Deleter strains		
Oocyte	ZP3 promoter	***(80***[a]***, 81***[a]***)***
Embryo, mosaic	Nestin promoter/enhancer	***(82)***[a]
Early embryo	Human CMV promoter	***(83)***[a]
Zygote	Adenovirus EIIa promoter	***(84)***[a]
Male germline	Pritamin 1 promoter	***(85)***[a]

[α]MHC, α-myosin heavy chain; CMV, cytomegalovirus; IRBP, interphotoreceptor retinoid-binding protein; MLC2v, myosin light chain 2v; POMC, propiomelanocortin; WAP, whey acidic protein; ZP3, mouse zona pellucida 3 gene.

[a]The strain is available from The Jackson Laboratories (http://lena.jax.org).

embryogenesis so that all or a part of the offspring contain the deleted target gene in germ cells (***38,39***; **Table 3**).

1.6.2. Inducible Recombinase Expression

In the first example of inducible gene targeting, Cre was placed under the control of the interferon-α/β-inducible promoter of the mouse Mx1 gene ***(86)***. Complete deletion of a loxP-flanked allele is achieved in the liver and lymphocytes 2 days after treatment with interferon, indicating that induced Cre-mediated deletion can proceed both rapidly and efficiently in an organ

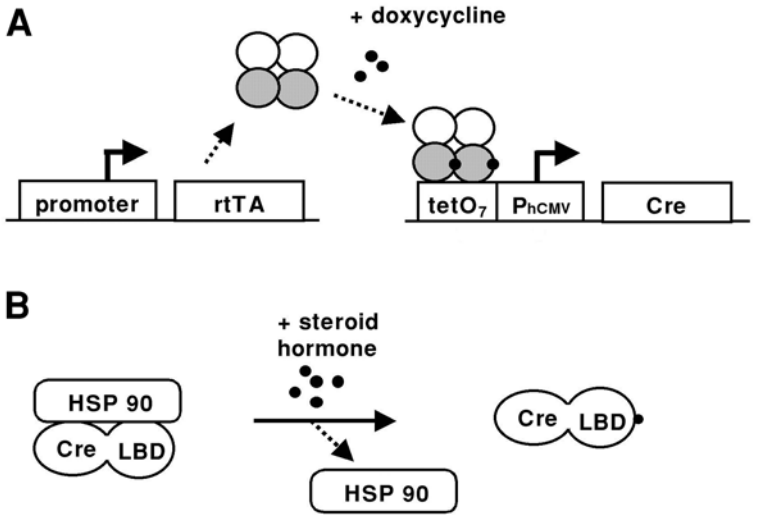

Fig. 6. Inducible systems. **(A)** Regulation of Cre expression by doxycycline. The reverse transactivator (rtTA) is expressed through a constitutive ubiquitous or cell type-specific promoter. Without doxycycline, the rtTA is inert and unable to activate Cre transcription. In the presence of doxycycline, rtTA binds to the $tetO_7$/human cytomegalovirus (hCMV) promoter, leading to Cre expression. **(B)** Principle of the hormone-regulated Cre-steroid receptor ligand binding domain (LBD) fusion protein. Cre is fused to a mutant LBD, which is unresponsive to its natural ligand but can be activated by a synthetic ligand. The fusion protein is inactive because the LBD is bound by the ubiquitous heat shock protein 90 (HSP 90) complex. Upon binding of the ligand (synthetic hormone) to the LBD, the fusion protein is released from the inactive state, allowing recombination between two loxP sites in the genome.

composed mainly of resting cells. Since many tissues respond to interferon, induced deletion in Mx cre mice is not restricted to a single cell type.

Cell type-specific transcriptional control can be achieved by the tet system developed by the group of Hermann Bujard. In this system, the activity of an engineered minimal promoter region can be activated by a specific transactivating protein, which is in turn regulated by the inducer doxycycline (***87***; **Fig. 6A**). By placing the Cre gene behind the minimal promoter and directing ubiquitous or tissue-specific expression of the transactivator, site-specific recombination in mice can be controlled by the administration of doxycycline. A novel transactivator with an increased sensitivity for the inducer has recently been described ***(88)***. To achieve sufficient expression of Cre in all cells of a given tissue during the time of induction, the promoter driving transactivator expression must be carefully selected. The activity of most promoter fragments used for transgenic gene expression in mice is not tested at the single cell

level on tissue sections. The tet regulatory system has been successfully used to control the activity of Cre in several transgenic mice, which are listed in **Table 4**.

Inducible activation of Cre protein can also be achieved by the cell type-specific expression of a fusion protein consisting of Cre and the ligand binding domain (LBD) of steroid hormone receptors. In the absence of hormone, the steroid receptor LBDs are bound by heat shock proteins, which inactivate the recombinase, presumably by sterical hindrance. The recombinase can be activated by the addition of hormone, which releases the heat shock proteins from the fusion protein (**Fig. 6B**). To derive a system in mice that is unresponsive to natural steroids, Cre was fused to mutant LBDs of the estrogen ***(105)*** and progesterone receptors ***(106)***, which are unresponsive to their natural ligands but can be activated by synthetic hormone antagonists. A number of transgenic mouse strains expressing Cre-LBD fusion proteins have been published, demonstrating that this method works in different tissues (**Table 4**). To reduce the amount of hormone required to induce quantitative recombination, a new mutant estrogen receptor LBD with an increased sensitivity to the inducer has been developed ***(93,107)***.

Alternatively to the use of inducible transgenic expression vectors, Cre can be delivered to somatic tissues through infection of mice with viral expression vectors (**Table 4**). These Cre-expressing viruses can be applied either locally, infecting the cells around the injection site, or by intravenous injection reaching many cells, mainly in the liver and spleen. Viral vectors can be applied at a given time point into adult mice, but they do not act in a cell type-specific manner and elicit a strong immune response against infected cells.

2. Materials

2.1. Electroporation of ES Cells

1. Exponentially growing ES cells ($2–10 \times 10^6$), plated out the day before electroporation (*see* Chapter 8).
2. Two 9-cm Petri dishes with mitomycin C (MMC)-treated embryonic fibroblasts (4×10^6/plate).
3. Electroporation device (Bio-Rad Gene Pulser II with Capacitance Extender II).
4. Electroporation cuvets (Bio-Rad Gene Pulser Cuvette, 0.4-cm electrode distance, cat. no. 165-2088).
5. Cre expression vector pIC-Cre, pMC-Cre, pgk-Cre, or CAG-Cre, or FLP expression vector pCAGGS-FLPeIRESpuro. An amount of 5–40 µg supercoiled DNA is required per electroporation, purified, e.g., by Quiagen columns. The DNA for electroporation is sterilized on the day of electroporation by ethanol precipitation,

Table 4
Inducible Cre Mice

System/specificity	Control element	Reference
Mx promoter		
Ubiquitous	Mx1 promoter	***(86)***[a]
CreER		
Ubiquitous	CMV promoter	***(89)***
Embryonic neural tube	Wnt promoter/enhancer	***(90)***
B-lymphocytes	SV40 promoter/Ig enhancer	***(91)***
Keratinocytes	K14 promoter	***(92,93)***
Hepatocytes	α_1-antitrypsin promoter	***(94)***
Smooth muscle	SM22 promoter (knockin)	***(109)***
CrePR		
Brain	CamKII promoter; CrePR	***(95)***
Cerebellum	NMDR GluRε3 subunit promoter; CrePR	***(96)***
Keratinocytes	K14 promoter	***(97)***
Tet system		
Ubiquitous	hCMV minimal promoter	***(98)***
Intestinal epithelium	rat Fabpl promoter	***(99)***
Neurons	Rb	***(100)***
Mammary gland	WAP	***(100)***
Viral vectors		
Brain	HSV-IE promoter/herpesvirus	***(101)***
Liver	CMV-IE/adenovirus	***(102)***
Liver/brain	HSV-tk promoter/adenovirus	***(103)***
Colorectal epithelium	SRα promoter/adenovirus	***(104)***
Many tissues	hCMV-IE promoter/adenovirus	***(70)***

CMV, cytomegalovirus; HSV, herpes simplex virus; IE, immediate early; LAP, liver-enriched activator protein; NMDR, NMDA-type glutamate receptor GLuR-3 subunit gene; SM22, smooth muscle protein 22; SV40, simian virus 40; WAP, whey acidic protein; Wnt, wingless-type MMTV integration site family, member 1.

[a]The strain is available from The Jackson Laboratories (http://lena.jax.org).

washed with 70% ethanol, dried under sterile air and resolved in phosphate-buffered saline (PBS) at 1 μg/μL.

6. Electroporation buffer: 20 m*M* HEPES, pH 7.0 (1 *M* stock, Gibco, cat. no. 15630-056), 137 m*M* NaCl (1 *M* stock), 5 m*M* KCl (1 *M* stock), 0,7 m*M* Na_2HPO_4 (0.5 *M* stock), 6 m*M* glucose (0.5 *M* stock), 0.1 m*M* 2-β-mercaptoethanol (50 m*M* stock, Gibco, cat. no. 31350-010).
7. Puromycin: 1 mg/mL in PBS (Calbiochem, cat. no. 540222).

2.2. Isolation and Phenotyping of Transfected Colonies

1. Stereomicroscope.
2. Sterilized pipet tips.
3. PBS (Gibco, cat. no. 20012-019).
4. U-bottomed 96-well plate (Falcon, cat. no. 3070).
5. Flat-bottomed 96-well plates (Falcon, cat. no. 3072) with MMC-treated fibroblasts.
6. Flat-bottomed 96-well plates incubated with gelatin solution.
7. Gelatin solution: add 1 g gelatin (porcine skin, type A; Sigma, cat. no. G1890) to 1 L water and sterilize by autoclaving.
8. Trypsin (0.25%)/EDTA solution (Gibco, cat. no. 25200-056).
9. G418 (50 mg/mL solution; Gibco, cat. no. 10131-019).

3. Methods

3.1. Transient Expression of CRE/FLP in ES Cells

To delete or invert a loxP- or FRT-flanked DNA segment in vitro, Cre or FLP is transiently expressed in ES cell clones by electroporation with a supercoiled recombinase expression vector. These cells are plated out, and a number of clones can be analyzed for the desired recombination event by PCR, Southern blotting, or selecting replica clones for the loss of a drug resistance marker. Since the frequency of clones carrying a Cre/FLP-mediated deletion is usually in the range of 5–50%, it is not necessary to enrich for such clones by inclusion of a negative selection marker, such as thymidine kinase, within the loxP/FRT-flanked sequence. To generate conditional or null alleles following the gene targeting strategy shown in **Fig. 4**, transient Cre or FLPe expression in ES cells is straightforward, as only two loxP and FRT sites are included in the vector. The recombination between these sites results in only one deletion event per recombinase, both of which remove the neomycin resistance gene from the targeted locus. Thus, for this application, it is most efficient to transfect the ES cells with a relatively large amount of a strong Cre or FLPe expression vector to maximize recombinase expression and to analyze only 96 colonies. If a construct with three loxP or FRT sites is used and the goal is to achieve a partial deletion between two of these sites, the amount of recombinase expression vector must be limited (*see* **Subheading 3.2.**), to avoid all transfected clones having a complete deletion within the outer sites.

3.2. Transfection Protocol

1. Transfect 2–10 × 10^6 homologous recombinant ES cells mixed with 5–40 μg of supercoiled Cre or FLPe recombinase vector in 800 μL electroporation buffer using a Bio-Rad electroporation cuvet. This number of transfected cells is in vast excess of what will actually be screened for deletion (see below), but the

remaining cells can be frozen down for a later analysis if the first is unsuccessful. Useful Cre expression vectors are pIC-Cre or pMC-Cre ***(26)*** but constructs with the pgk or CAG promoter give even higher expression ***(27)***. To obtain complete deletion, use 40 μg of either plasmid; to obtain partial deletion, use 5–10 μg of pIC-Cre. A further enrichment of recombinant clones can be obtained through the use of a expression vector producing both Cre and puromycin resistance ***(29)***. A useful FLPe expression vector is pCAGGS-FLPeIRESpuro (www.embl-heidelberg.de/ExternalInfo/stewart/plasmids.html), a bicistronic expression vector for FLPe and puromycin resistance. To obtain complete deletion, use 40 μg of vector combined with puromycin selection. For transfection, we use a Bio-Rad electroporator set to 240 V and 475 μF.

2. Plate out transfected cells (on mitomycin C-treated feeder cells) at approx. 5×10^6 cells/9-cm dish and allow to grow for 2 days with normal ES medium or medium containing 1 μg/mL puromycin ***(29)***. Even wild-type feeder cells will tolerate the low amount of puromycin used for ES cell selection. This initial plating is to allow the recombinase to be expressed and to avoid mixed colonies if the deletion occurs only after a transfected cell has divided.
3. After 2 days, trypsinize the plate and replate (on mitomycin C-treated feeder cells) on two plates at 1000 cells/9-cm plate. Freeze remaining transfectants for subsequent plating and picking, if required. In general, one plate should suffice to pick 200 colonies, which should include 10–100 deleted clones.
4. Six to 9 days after plating, when the center of the colonies turns dark, replace medium with PBS and pick the colonies under a stereomicroscope with sterile pipet tips into 50 μL trypsin solution in a 96-well U-bottomed plate. Incubate the plate at 37°C for 10 min, and then add 50 μL medium/well. Pipet up and down 5 times, and transfer half of the solution into a 96-well plate with embryonic feeder cells and ES cell medium for further culture. The other half is transferred into a gelatin-treated 96-well plate containing ES cell medium with G418 at a concentration that is 25% higher than that used for the initial selection of homologous recombinants. For further details on ES cell culture, see Chapter 8 or **ref. *51***.
5. G418-sensitive dying colonies that have undergone recombinase mediated deletion are clearly identifiable after 3 days of selection and will not have any viable cells remaining in the well. At this time a number of G418-sensitive clones should be expanded (from the duplicate master plate) for freezing and genomic DNA isolation in order to verify the deletion event by Southern blot analysis. Alternatively to the G418 selection step, deleted colonies could be identified with a specific PCR reaction ***(51)***.

4. Notes

1. Whenever possible, we recommend using the same mouse strain for vector construction and gene targeting in ES cells, as the frequency of cointegration of a distant loxP site may be strongly reduced in the case of strain differences. A small distance between loxP sites (approx. 500 bp) also ensures a better deletion rate in vivo.

2. Before starting a conditional gene-targeting experiment, think carefully about the Southern blot strategy for detection of the various alleles, and pretest genomic probes for function. It is also advisable to plan a construct in such a way that all alleles can be detected upon digestion with a single, cheap restriction enzyme (e.g., *Bam*HI, *Eco*RI).
3. LoxP or FRT sites required within conditional gene-targeting vectors can also be conveniently introduced by cloning annealed, phosphorylated oligonucleotides that include a unique restriction site.
4. Constructs for conditional gene-targeting vectors should be tested for the functionality of the loxP or FRT sites by transformation into Cre- or FLP-expressing bacteria befor starting ES cell work (for strains, see F. Stewart's web page: www.embl-heidelberg.de/ExternalInfo/stewart/plasmids.html).
5. Not every promoter region described for the use in transgenic mice is also successfully employed to derive a new strain of Cre mice, as some genes that are well known to be expressed in differentiated cells are also active in early embryonic development. In this case, all strains will show a "deleter" phenotype. If you intend to use a strain described in the literature, its best to recheck the strain properties in time, using one of the Rosa Cre reporter strains.

References

1. Capecchi, M. R. (1989) The new mouse genetics: altering the genome by gene targeting. *Trends Genet.* **5,** 70–76.
2. Mak, T. W. (ed.) (1998) *The Gene Knockout Facts Book*. Academic, San Diego, CA.
3. Rajewsky, K., Gu, H., Kühn, R., et al. (1996) Conditional gene targeting. *J. Clin. Invest.* **98,** 600–603.
4. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type specific gene targeting. *Science* **265,** 103–106.
5. Eggan, K., Akutsu, H., Loring, J., et al. (2001) Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc. Natl. Acad. Sci. USA* **98,** 6209–6214.
6. Brüning, J. C., Michael, M. D., Winnay, J. N., et al. (1998) A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol. Cell* **2,** 559–569.
7. Kulkarni, R. N., Brüning, J. C., Winnay, J. N., et al. (1999) Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* **96,** 329–339.
8. Michael, M. D., Kulkarni, R. N., Postic, C., et al. (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol. Cell* **6,** 87–97.
9. Tsien, J. Z., Huerta, P. T., and Tonegawa, S. (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* **87,** 1327–1338.

10. Minichiello, L., Korte, M., Wolfer, D., et al. (1999) Essential role for TrkB receptors in hippocampus-mediated learning. *Neuron* **24,** 401–414.
11. Radtke, F., Wilson, A., Stark, G., et al. (1999) Deficient T cell fate specification in mice with an induced inactivation of notch 1. *Immunity* **10,** 547–558.
12. Li, M., Indra, A. K., Warot, X., et al. (2000) Skin abnormalities generated by temporally controlled RXRα mutations in mouse epidermis. *Nature* **407,** 633–636.
13. Maruyama, M., Lam, K. P., and Rajewsky, R. (2000) Memory B-cell persistence is independent of immunizing antigen. *Nature* **407,** 636–642.
14. Buchholz, F., Ringrose, L., Angrand, P.-O., Rossi, F., and Stewart, A. F. (1996) Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. *Nucleic Acids Res.* **24,** 4256–4262.
15. Kilby, N. J., Snaith, M. R., and Murray, J. A. H. (1993) Site specific recombinases: tools for genome engineering. *Trends Genet.* **9,** 413–421.
16. Hoess, R. H., Ziese, M., and Sternberg, N. (1982) P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc. Natl. Acad. Sci. USA* **79,** 3398–3402.
17. Hoess, R. H. and Abremski, K. (1984) Interaction of the bacteriophage P1 recombinase Cre with the recombining site loxP. *Proc. Natl. Acad. Sci. USA* **81,** 1026–1029.
18. Abremski, K., Hoess, R., and Sternberg, N. (1983) Studies on the propoerties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell* **32,** 1301–1311.
19. Hoess, R. H., Wierzbicki, A., and Abremski, K. (1986) The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14,** 2287–2300.
20. Albert, H., Dale, E. C., Lee, E., and Ow, D. W. (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *Plant J.* **7,** 649–659.
21. Araki, K., Araki, M., and Yamamura, K. (1997) Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res.* **25,** 868–872.
22. Hoess, R., Wierzbicki, A., and Ambremski, K. (1985) Formation of small circular DNA molecules via an in vitro site-specific recombination system. *Gene* **40,** 325–329.
23. Ringrose, L., Chabanis, S., Angrand, P.-O., Woodroofe, C., and Stewart, A. F. (1999) Quantitative comparison of DNA looping in vitro and in vivo: chromatin increases effective DNA flexibility at short distances. *EMBO J.* **23,** 6630–6641.
24. Baubonis, W. and Sauer, B. (1993) Genomic targeting with purified Cre recombinase. *Nucleic Acids Res.* **21,** 2025–2029.
25. Guo, F., Gopaul, D. N., and van Duyne, G. D. (1997) Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. *Nature* **389,** 40–46.
26. Gu, H., Zou, Y.-R., and Rajewsky, K. (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* **73,** 1155–1164.

27. Araki, K., Imaizumi, T., Okuyama, K., Oike, Y., and Yamamura, K. (1997) Efficiency of recombination by Cre transient expression in embryonic stem cells: comparison of various promoters. *J. Biochem.* **122,** 977–982.
28. Gagneten S., Le, Y., Miller, J., and Sauer, B. (1997) Brief expression of a GFPcre fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions. *Nucleic Acids Res.* **25,** 3326–3331.
29. Taniguchi, M., Sanbo, M., Watanabe, S., et al. (1998) Efficient production of Cre-mediated site-directed recombinants through the utilization of the puromycin resistance gene, pac: a transient gene-integration marker for ES cells. *Nucleic Acids Res.* **26,** 679–680.
30. Le, Y., Gagneten, S., Tombaccini, D., Bethke, B., and Sauer, B. (1999) Nuclear targeting determinants of the phage P1 Cre DNA recombinase. *Nucleic Acids Res.* **27,** 4703–4709.
31. Koresawa, Y., Miyagawa, S., Ikawa, M., et al. (2000) Synthesis of a new Cre recombinase gene based on optimal codon usage for mammalian cells. *J. Biochem.* **127,** 367–372.
32. Groth, A. C., Olivares, E. C., Thyagarajan, B., and Calos, M. P. (2000) A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* **97,** 5995–6000.
33. Kolot, M., Silberstein, N., and Yagil, E. (1999) Site-specific recombination in mammalian cells expressing the Int recombinase of bacteriophage HK022. *Mol. Biol. Rep.* **26,** 207–213.
34. Schwikardi, M. and Dröge, P. (2000) Site-specific recombination in mammalian cells catalyzed by gammadelta resolvase mutants: implications for the topology of episomal DNA. *FEBS Lett.* **471,** 147–150.
35. Buchholz, F., Angrand, P.-O., and Stewart, A. F. (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nature Biotech.* **16,** 657–662.
36. Dymecki, S. M. and Tomasiewicz, H. (1998) Using FLP recombinase to characterize expansion of Wnt-1-expressing neural progenitors in the mouse. *Dev. Biol.* **201,** 57–65.
37. Vooijs, M., van der Valk, M., te Riele, H., and Berns, A. (1998) FLP-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. *Oncogene* **17,** 1–12.
38. Rodriguez, C. I., Buchholz, F., Galloway, J., et al. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nature Genet.* **25,** 139–140.
39. Meyers, E. N., Lewandoski, M., and Martin, G. R. (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat Genet.* **18,** 136–141.
40. Olson, E. N., Arnold, H. H., Rigby, P. W., and Wold, B. J. (1996) Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* **85,** 1–4.
41. Dymecki, S. M. (1996) A modular set of Flp, FRT and lacZ fusion vectors for manipulating genes by site-specific recombination. *Gene* **171,** 197–201.

42. Vazquez, J. C., Nogues, C., Rucker, E. B., and Piedrahita, J. A. (1998) Factors affecting the efficiency of introducing precise genetic changes in ES cells by homologous recombination: tag-and-exchange versus the Cre-loxP system. *Transgenic Res.* **7,** 181–193.
42. Farley, F. W., Soriano, P., and Steffen, L. S. (2000) Dymecki SM. Widespread recombinase expression using FLPeR (Flipper) mice. *Genesis* **3–4,** 106–110.
43. Abuin, A. and Bradley, A. (1996) Recycling selectable markers in mouse embryonic stem cells. *Mol. Cell. Biol.* **16,** 1851–1856.
44. Ramirez-Solis R., Liu P., and Bradley, A. (1995) Chromosome engineering in mice. *Nature* **378,** 720–724.
45. Van Deursen, J., Fornerod, M., Van Rees, B., and Grosveld, G. (1995) Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. *Proc. Natl. Acad. Sci. USA* **92,** 7376–7380.
46. Hardouin, N. and Nagy, A. (2000) Gene-trap-based target site for Cre-mediated transgenic insertion. *Genesis* **26,** 245–252.
47. Araki, K., Imaizumi, T., Sekimoto, T., et al. (1999) Exchangeable gene trap using the Cre/mutated lox system. *Cell. Mol. Biol.* **45,** 737–750.
48. Zou, Y. R., Muller, W., Gu, H., and Rajewsky, K. (1994) Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies. *Curr. Biol.* **4,** 1099–1103.
49. Soukharev, S., Miller, J. L., and Sauer, B. (1999) Segmental genomic replacement in embryonic stem cells by double lox targeting. *Nucleic Acids Res.* **27,** e21.
50. Nagy, A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26,** 9–109.
51. Torres, R. M. and Kühn, R. (1997) *Laboratory Protocols for Conditional Gene Targeting*. Oxford University Press, Oxford, UK.
52. Yang, X. W., Model, P., and Heintz, N. (1997) Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat. Biotechnol.* **15,** 859–865.
53. Muyrers, J. P., Zhang, Y., Testa, G., and Stewart, A. F. (1999) Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res.* **27,** 1555–1557.
54. Thorey, I. S., Muth, K., Russ, A. P., Otte, J., Reffelmann, A., and von Melchner, H. (1998) Selective disruption of genes transiently induced in differentiating mouse embryonic stem cells by using gene trap mutagenesis and site-specific recombination. *Mol. Cell. Biol.* **18,** 3081–3088.
55. Lobe, C. G., Koop, K. E., Kreppner, W., et al. (1999) Z/AP, a double reporter for cre-mediated recombination. *Dev. Biol.* **208,** 281–292.
56. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21,** 70–71.
57. Mao, X., Fujiwara, Y., and Orkin, S. H. (1999) Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc. Natl. Acad. Sci. USA* **96,** 5037–5042

58. Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C. G. (2000) Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon cre-mediated excision. *Genesis* **3–4,** 147–155.
59. Mao, X., Fujiwara, Y., Chapdelaine, A., Yang, H., and Orkin, S. H. (2001) Activation of EGFP expression by cre-mediated excision in a new ROSA26 reporter mouse strain. *Blood* **97,** 324–326.
60. Choi, T., Huang, M., Gorman, C., and Jaenisch, R. (1991) A generic intron increases gene expression in transgenic mice. *Mol. Cell. Biol.* **11,** 3070–3074.
61. Pfarr, D. S., Rieser, L. A., Woychik, R. P., et al. (1986) Differential effects of polyadenylation regions on gene expression in mammalian cells. *Gene* **5,** 115–122.
62. Lindeberg, J. and Ebendal, T. (1999) Use of an internal ribosome entry site for bicistronic expression of Cre recombinase or rtTA transctivator. *Nucleic Acids Res.* **27,** 1552–1554.
63. Hentze, M. W. and Kulozik, A. E. (1999) A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* **96,** 307–310.
64. Clausen, B. E., Burkhardt, C., Reith, W., Renkawitz, R., and Forster, I. (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* **8,** 265–277.
65. Hennet, T., Hagen, F. K., Tabak, L. A., and Marth, J. D. (1995) T-cell-specific deletion of a polypeptide N-acetylgalactosaminyl-transferase gene by site-directed recombination. *Proc. Natl. Acad. Sci. USA* **92,** 12070–12074.
66. Lakso, M, Sauer, B, Mosinger, B Jr, et al. (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89,** 6232–6236.
67. Rickert, R. C., Roes, J., and Rajewsky, K. (1997) B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res.* **25,** 1317–1318.
68. Tsien, J. Z., Chen, D. F., Gerber, D., et al. (1996). Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* **87,** 1317–1326.
69. Zinyk, D. L., Mercer, E. H., Harris, E., Anderson, D. J., and Joyner, A. L. (1998) Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. *Curr. Biol.* **8,** 665–668.
70. Akagi, K., Sandig, V., Vooijs, M., et al. (1997) Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res.* **25,** 1766–1773.
71. Barlow, C., Schroeder, M., Lekstrom-Himes, J., et al. (1997) Targeted expression of Cre recombinase to adipose tissue of transgenic mice directs adipose-specific excision of loxP-flanked gene segments. *Nucleic Acids Res.* **25,** 2543–2545.
72. Postic, C. and Magnuson, M. A. (2000) DNA excision in liver by an albumin-Cre transgene occurs progressively with age. *Genesis* **26,** 149–150.
73. Kellendonk, C., Opherk, C., Anlag, K., Schutz, G., and Tronche, F. (2000) Hepatocyte-specific expression of Cre recombinase. *Genesis* **26,** 151–153.
74. Gannon, M., Shiota, C., Postic, C., Wright, C. V., and Magnuson, M. (2000) Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. *Genesis* **26,** 139–142.

75. Agah, R., Frenkel, P. A., French, B. A., et al. (1997) Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J. Clin. Invest.* **100,** 169–179.
76. Chen, J., Kubalak, S. W., and Chien, K. R. (1998) Ventricular muscle-restricted targeting of the RXRalpha gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis. *Development* **125,** 1943–1949.
77. Ovchinnikov, D. A., Deng, J. M., Ogunrinu, G., and Behringer, R. R. (2000) Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. *Genesis* **26,** 145–146.
78. Tarutani, M., Itami, S., Okabe, M., et al. (1997) Tissue-specific knockout of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin development. *Proc. Natl. Acad. Sci. USA* **94,** 7400–7405
79. Wagner, K. U., Wall, R. J., St-Onge, L., et al. (1997) Cre-mediated gene deletion in the mammary gland. *Nucleic Acids Res.* **25,** 4323–4330.
80. Lewandoski, M., Wassarman, K., M., and Martin, G. R. (1997) Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. *Curr. Biol.* **7,** 148–151.
81. de Vries, W. N., Binns, L. T., Fancher, K. S., et al. (2000) Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. *Genesis* **26,** 110–112.
82. Betz, U. A. K., Voβhenrich, C. A. J., Rajewsky, K., and Müller, W. (1996) Bypass of lethality with mosaic mice generated by Cre-loxP-mediated recombination. *Curr. Biol.* **6,** 1307–1316.
83. Schwenk, F., Baron, U., and Rajewsky, K. (1996) A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.* **23,** 5080–5081.
84. Lakso, M., Alt, F., and Westphal, H. (1996) Efficient in vivo manipulation of mouse genomic sequences at the zygotic stage. *Proc. Natl. Acad. Sci. USA* **93,** 5860–5865.
85. O'Gorman, S., Dagenais, N. A., Qian, M., and Marchuk, Y. (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **94,** 14602–14607.
86. Kühn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995) Inducible gene targeting in mice. *Science* **269,** 1427–1429.
87. Gossen, M., Freundlieb, S., Bender, G., et al. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268,** 1766–1769.
88. Urlinger, S., Baron, U., Thellmann, M., et al. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc. Natl. Acad. Sci. USA* **97,** 7963–7968.
89. Feil, R., Brocard, J., Mascrez, B., et al. (1996) Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci. USA* **93,** 10887–10890.
90. Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K., and McMahon, A. P. (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8,** 1323–1326.

91. Schwenk, F., Kuhn, R., Angrand, P. O., Rajewsky, K., and Stewart, A. F. (1998) Temporally and spatially regulated somatic mutagenesis in mice. *Nucleic Acids Res.* **26,** 1427–1432.
92. Vasioukhin, V., Degenstein, L., Wise, B., and Fuchs, E. (1999) The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc. Natl. Acad. Sci. USA* **96,** 8551–8556.
93. Indra, A. K., Warot, X., Brocard, J., et al. (1999) Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res.* **27,** 4324–4327.
94. Imai, T., Chambon, P., and Metzger, D. (2000) Inducible site-specific somatic mutagenesis in mouse hepatocytes. *Genesis* **26,** 147–148.
95. Kellendonk, C., Tronche, F., Casanova, E., et al. (1999) Inducible site-specific recombination in the brain. *J. Mol. Biol.* **285,** 175–1782.
96. Tsujita, M., Mori, H., Watanabe, M., et al. (1999) Cerebellar granule cell-specific and inducible expression of Cre recombinase in the mouse. *J. Neurosci.* **19,** 10318–18323.
97. Berton, T. R., Wang, X. J., Zhou, Z., et al. (2000) Characterization of an inducible, epidermal-specific knockout system: differential expression of lacZ in different Cre reporter mouse strains. *Genesis* **26,** 160–161.
98. Holzenberger, M., Zaoui, R., Leneuve, P., Hamard, G., Le Bouc, Y. (2000) Ubiquitous postnatal LoxP recombination using a doxycycline auto-inducible Cre transgene (DAI-Cre). *Genesis* **26,** 157–159.
99. Saam, J. R. and Gordon, J. I. (1999) Inducible gene knockouts in the small intestinal and colonic epithelium. *J. Biol. Chem.* **274,** 38071–38082.
100. Utomo, A. R., Nikitin, A. Y., and Lee, W. H. (1999) Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat. Biotechnol.* **17,** 1091–1096
101. Brooks, A. I., Muhkerjee, B., Panahian, N., Cory-Slechta, D., and Fedoroff, H. J. (1997) Nerve growth factor somatic mosaicism produced by herpes virus-directed expression of cre recombinase. *Nat. Biotechnol.* **15,** 57–62.
102. Anton, M. and Graham, F. L. (1995) Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J. Virol.* **69,** 4600–4606.
103. Wang, Y., Krushel, L. A., and Edelman, G. M. (1996) Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene. *Proc. Natl. Acad. Sci. USA* **93,** 3932–3936.
104. Shibata, H., Toyama, K., Shioya, H., et al. (1997) Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science* **278,** 120–123.
105. Zhang, Y., Riesterer, C., Ayrall, A. M., et al. (1996) Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res.* **24,** 543–548.
106. Kellendonk, C., Tronche, F., Monaghan, A. P., et al. (1996) Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res.* **24,** 1404–1411.

107. Feil, R., Wagner, J., Metzger, D., and Chambon, P. (1997) Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem. Biophys. Res. Commun.* **237,** 752–757.
108. Ringrose, L., Lounnas, V., Ehrlich, L., et al. (1998) Comparative kinetic analysis of FLP and Cre recombinases: mathematical models for DNA binding and recombination. *J. Mol. Biol.* **284,** 363–384.
109. Kuhbandner, S., Brummer, S., Metzger, D., et al. (2000) Temporally controlled somatic mutagenesis in smooth muscle. *Genesis* **28,** 15–22.

10

Knockin Approaches

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1. Introduction

Molecular genetic approaches toward the study of gene function in mice have traditionally relied on either overexpression or gene ablation using transgenic or knockout mouse strategies, respectively. More recently, the knockin approach, which often combines features of both techniques, has allowed a more subtle analysis of genes. The protein coding region of a gene can be replaced by, e.g., a reporter gene, or a homologous gene of a different species, or related genes from the same organism. Even (point) mutations can be introduced into a gene, affecting the gene product itself.

This chapter provides a brief overview of examples and applications of this novel and powerful technique of answering scientific questions. Further successful application of the knockin approach might lead in the future to a greater necessity to target the same gene with different modifications. A knockin strategy using recombination-mediated cassette exchange (RMCE) might be a good, time-saving alternative for repeated application of a conventional knockin strategy in order to generate different modifications of the same gene. This RMCE strategy is discussed in detail.

2. Knockin Technologies: *Potential Uses*

2.1. Reporter Knockins

The first knockin experiments involved insertion of an exogenous reporter gene into the genome such that it acquired the transcriptional regulation of the targeted gene locus. The reporter knockin is actually a simple variation of standard knockout protocols, utilizing the activation of the inserted gene as an immediate readout for proper insertion downstream of an active promotor. The

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen © Humana Press Inc., Totowa, NJ

reporter construct usually lacks promotor and enhancer control elements and thus relies on controls provided by the targeted gene for appropriate expression. Reporter knockins allow easy determination of the expression of the targeted gene in the reconstituted mouse. Heterozygotes can be used to determine the timing and patterns of expression of the targeted gene, since in most cases, such heterozygotes develop and function normally even when the gene is required for normal mouse development. Only in case of haplotype insufficiency will normal development and expression be compromised. In principle, the homozygotes no longer express a functional wild-type allele and can be studied as a classical germline knockout. For this kind of analysis, many investigators have used the bacterial *lacZ* gene, because excellent reagents exist to determine expression of β-galactosidase using antibodies or biochemical reactions. In addition, individual isolated cells can be analyzed for β-galactosidase using a fluorescence-based flow cytometry analysis. For example, Elefanty et al. ***(1)*** used a lacZ reporter knocked in to the *SCL* locus to fractionate bone marrow cells into low, medium, and high SCL-expressing subsets.

2.2. Functional Knockins

The second type of knockin experiment involves insertion of an altered or exogenous gene directly into a specific locus in order to test its effects on mouse development or function. Usually, but not necessarily, this process ablates expression of the original gene at that location, often by completely replacing all or an important part of the coding sequence. Three aspects of this procedure tend to make it superior to the more traditional transgenic approach to analysis of gene function: 1) the simultaneous removal of the endogenous normal gene can greatly simplify the analysis; 2) as in the case of a reporter knockin, the spatial and temporal patterns of expression should be close to or identical to that of the replaced endogenous gene; and 3) there is much less risk of mutagenizing an unrelated gene at the time of plasmid insertion, a potential problem with the random DNA integration typical of transgenic mice.

A frequent use of functional knockin technology is to determine whether a gene can be replaced by a homolog, either the same gene from a different species, or a related gene from the same organism. As an example of the former, Rozmahel et al. ***(2)*** studied the ability of the human cystic fibrosis transmembrane conductance (CFTR) channel to work in mice. Previous attempts to perform "gene therapy" to cure mice lacking a functional CFTR protein by transgenic or viral mediated insertion of a human *CFTR* gene were unsuccessful, for unknown reasons. By generating a knockin of the human *CFTR* gene into the homologous mouse locus, these researchers found that even when the gene was in the correct chromosomal location and expressed in

the correct spatial and temporal manner, mice did not recover normal function. This provided a model system to study the putatively important differences between the mouse and human CFTR protein.

Studies to replace a gene with related family members include the demonstrations that myogenin can replace Myf5 in rib cage development ***(3)*** and that En-2 can replace the requirement for En-1 in hind brain development ***(4)***. Similarly, GATA-3 (GATA binding protein 3) can partially replace the highly homologous transcription factor GATA-1 in erythroid cell development. In a related but different analysis, Geng et al. ***(5)*** tested the specific role of cyclin D by replacing its coding region with that of cyclin E, which is normally induced by the former. Although a cyclin D knockout causes embryonic lethality, supplying cyclin E in place of cyclin D completely reversed the phenotypic effects of the D knockout. Thus, the authors concluded that the essential role of cyclin D1 is to induce synthesis of cyclin E (and other cyclin D-specific effects are not necessary.)

A more refined use of this strategy was performed by Reichardt et al. ***(6)*** to specifically test defined regions of the glucorticoid receptor in vivo. These investigators directed the creation of a specific point mutation within the endogenous glucocorticoid receptor, which ablates its ability to activate transcription but does not interfere with its transcriptional repression activity. This has facilitated the understanding of the mechanism of steroid action in multiple different pathways, including lung development, hypothalamic action, and lymphocyte apoptosis ***(6)***.

Functional knockin technology is also an excellent method for creating mouse models of human diseases. Any well-characterized human mutation can be best studied by generating the equivalent in a mouse homolog. Recent examples of such experiments include the creation of a knockin of multiple CAG repeats within the huntingtin gene, which recapitulates juvenile Huntington's disease ***(7)***. Similarly, Guo et al. ***(8)*** generated a model of Alzheimer's disease by knocking in a specific mutation into the presenilin-1 gene. Lastly, Lorenzetti et al. ***(9)*** knocked in an expanded tract of 78 CAG repeats into the spinocerebellar ataxia type 1 (*Sca*I) locus to create a mouse model of the human disease by the same name. In all these examples, it was essential to ensure that expression of the mutant gene would parallel (in both spatial and temporal patterns) that which occurs in the human condition. These sorts of models have been used to understand better the molecular mechanisms that cause pathophysiologic states and also to test different strategies (diet, for example) that might ameliorate the severity of disease.

Knockin technology appears to be tailor-made for studying a naturally occurring knockin, the development of translocation breaks in oncogenesis. The

earliest translocation breakpoint discovered in leukemia was the Philadelphia chromosome, which results from a 9:22 translocation fusing the *BCR* gene to the *ABL* protooncogene. For years, transgenic mice expressing this aberrant protein failed to recapitulate the human disease. It was not until a knockin was created ***(10)*** that a reasonably accurate mouse model of human leukemia induced by this fusion protein could be generated.

Okuda et al. ***(11)*** created an acute myelogenous leukemia *AML1-ETO* knockin mouse and found a severe effect on fetal definitive hematopoiesis. Because these mice were embryonic lethal, even in the context of a hemizygous mutant state, the situation in which a translocation develops in a fully formed mouse was not completely recapitulated. The power of this protocol was further refined in work by Rhoades et al. ***(12)***, who repeated this study using a tetracycline-repressible promotor. This study in fact confirmed the finding that AML1-ETO interferes with normal hematopoiesis but also showed that the transforming ability of the fusion protein on its own is very weak. Okuda et al. ***(13)*** used a different knockin strategy to show that the AML1b isoform (when expressed under the control of the *AML1* regulatory sequences) can rescue the embryonic lethal phenotype and can contribute to lymphohematopoiesis in a chimera.

Dobson et al. ***(14)*** similarly created a model for AML by generating an appropriate *Mll-AF*9 fusion knockin, which recapitulates fusions found in many types of human leukemia. Likewise, Castilla at al. ***(15)*** created a knockin *CBFB-MYH11* mouse to determine the function of the INV16 chromosomal rearrangement, which is common in AML.

3. Strategy and Design

3.1. Design of Commonly Used Knockin Targeting Vectors

The experimental design of a gene knockin project uses the same basic principles as for a knockout. Thus all the general guidelines and considerations for a standard knockout also apply in this situation. Detailed gene targeting protocols are discussed elsewhere in this volume.

The key strategy in a knockin experiment is to place the expression of an exogenous gene or a modification of the endogenous gene under the transcriptional control of cis-acting elements belonging to the endogenous gene. Therefore, knockin targeting vectors should be constructed such that the knockin genes faithfully reproduce the normal transcription patterns, yet encode a mutated of otherwise "different" coding region. As is the case in standard knockout protocols, a drug-resistance selection cassette needs to be present, usually downstream of the knockin gene. Use of a strong ubiquitously

expressed promoter, such as phosphoglycerate kinase (PGK) ensures that the recombined cells will grow appropriately under drug selection.

In order to restore the state of the targeted gene to as close to undisturbed as possible, it is best to enable subsequent removal of the drug-selection marker by loxP flanking of the selection cassette. **Figure 1** outlines the general layouts of commonly used knockin targeting vectors. As for knockout strategies, the cloning sites used for replacement by knockin and selection marker sequences can be either exonic or intronic, or a combination of both. If intronic cloning is used, the coding sequence of the knocked-in gene should be flanked on its 5′ end with a splice acceptor and at its 3′ end with a spice donor sequence to ensure proper stable expression of its transcript within cells.

When a knocked-in coding sequence is designed to direct synthesis of a fusion with an endogenous gene, it is essential to make certain that the fusion will be in-frame and will include all necessary transcription and translation signals (including polyadenylation sequence, initiation codon, internal ribosome entry site (IRES), and so on). Furthermore, cDNA, or genomic sequences, or a combination of both can be incorporated into the targeting construct as part of the knockin. This depends largely on the size, the organization, and the complexity of the genes involved (of both the endogenous gene to be targeted and the knockin gene), as well as the objectives of the knockin project. If the knockin is designed to introduce a subtle mutation within a small, easily manipulated region, one selects an appropriate genomic fragment containing the mutation of interest so that the knockin gene is a close mimic of the wild-type gene (**Fig. 1G**). On the other hand, if the goal is to replace an entire gene (with a homolog from another species or with a different gene), often a cDNA sequence is used to replace the targeted coding sequence. **Figure 1A–F** shows the major strategies when using cDNAs as knockin coding sequences. Both partial as well as complete cDNA replacements have been shown to work in many studies. Thus, the choice of which to use can be made freely depending on the purpose and specifics of a particular project.

3.2. Recombination-Mediated Cassette Exchange

The successful application of knockin approaches for studying gene function might invite researchers to generate different modifications of the same gene for further subtle analysis of its function. The laborious, time-consuming, and costly procedure of targeting a construct into embryonic stem (ES) cells and of isolation and characterization of ES cell clones resulting from proper homologous recombination needs to be done repeatedly in case different modifications (mutations) have to be introduced. Application of RMCE might

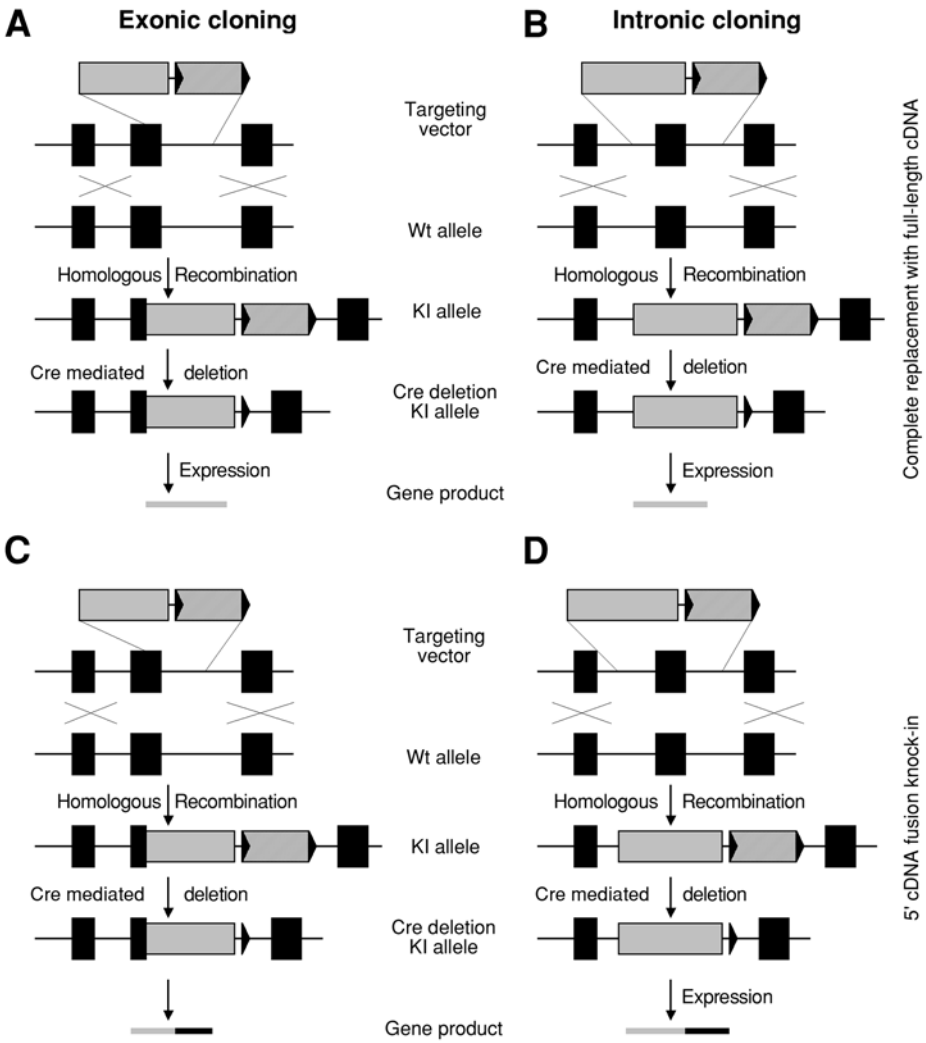


Fig. 1. Schematic representation of commonly used knockin constructs. A complete knockin gene cassette along with a downstream PGK promotor-driven neo cassette is cloned into an exonic **(A)** or intronic **(B)** restriction site. Besides the complete coding sequences, they also contain IRES, polyadenylation signal, and in-frame start and stop codons for proper translation. For fusion knockins, the knockin gene sequence is introduced to a site either in an exon **(C, E)** or in an intron **(D, F)** to generate in-frame fusion with the 3′ **(C, D)** or 5′ **(E, F)** portion of the endogenous gene. If an intronic site is used, an appropriate splicing acceptor (SA) and/or splicing donor (SD) must be included. **(G)** Subtle mutation (*) can be knocked in using a mutation containing a genomic fragment instead of cDNA. In all cases, the Neo cassette is flanked by two LoxP DNA sequences that are in the same orientation (filled triangles), so that it can

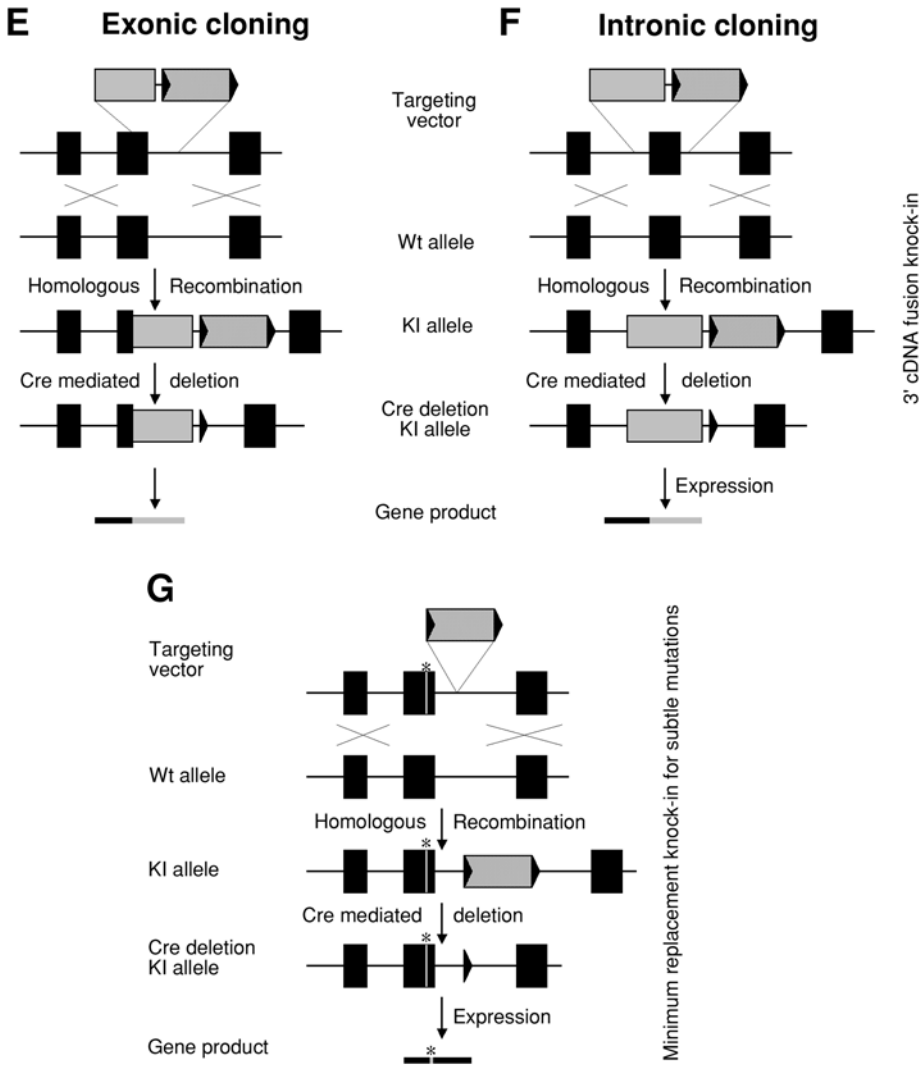


Fig. 1. *(continued)* be removed from targeted ES cell clones by the expression of cre recombinase. The cloning can be either insertion or replacement depending on the restriction sites.

be a good time- and money-saving alternative in case multiple different modifications need to be knocked in. RMCE refers to a highly efficient recombinase-mediated exchange of an initial cassette introduced into a gene by secondary cassettes encoding different modifications of this gene. For application of RMCE to knockins, the first cassette is initially introduced into

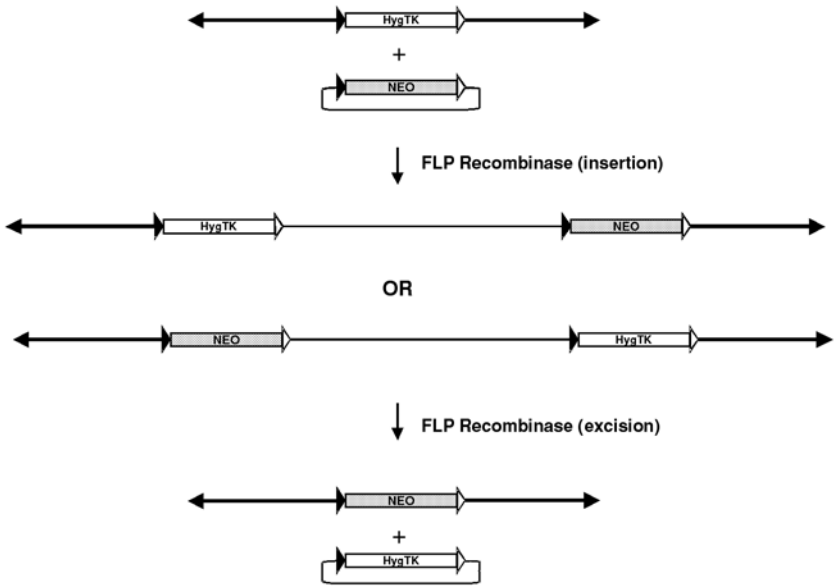


Fig. 2. Recombination-mediated cassette exchange (RMCE). By the activity of a transiently expressed FLP recombinase, a circular plasmid encoding a neomycin resistance gene flanked by two heterospecific FRT sites is inserted by recombination between either one of the FRT sites and the respective identical FRT site flanking the HygTK cassette integrated in the host genome. By a second recombination event, the FLP recombinase again excises a circular plasmid. By the appropriate negative (gancyclovir) and/or positive selection (G418), only cells resulting from the intended exchange will survive.

the gene of interest by homologous recombination. Subsequently, this parental ES cell line can be used repeatedly to introduce different modifications in the gene of interest, as will be explained.

3.2.1. Principle of RMCE

The RMCE method referred to is based on the work of Jürgen Bode and colleagues ***(16–18)***. Mutant FLP recognition target (FRT) sites were shown to recombine efficiently with identical mutant FRT sites but no longer with wild-type FRT sites. This finding resulted in the design of an FRT/FLP recombinase RMCE method: an integrated expression cassette consisting of a HygTK (hygromycin B-positive/ ganciclovir-negative) selection marker flanked by two such heterospecific FRT sites could be efficiently exchanged by a Neo expression cassette flanked by the same two heterologous FRT sites. As shown in **Fig. 2**, the exchange method exists of two FLP recombination events: an

initial insertion event followed by an excision event. Positive and/or negative selection is used to select for the intended exchange. Application of only negative selection allows even the replacement by constructs without a selection marker. This method was also shown to work in ES cells with a single, randomly integrated HygTK cassette. Mutant LoxP sites are also described that recombine with each other, but not with wild-type LoxP sites ***(19)***. Similar RMCE methods are described for the LoxP/Cre system ***(20–24)***. These FRT/FLP- and LoxP/Cre-based RMCE methods were shown to work efficiently in ES cells and were recognized as potentially powerful tools for the generation of modified and knockin mice. Nevertheless, to date there are no reports of the application of RMCE to the generation of genetically modified mice from the manipulated ES cells. However, RMCE has potentially a wide applicability, as reviewed in **refs.** ***25*** and ***26***.

3.2.2. Potential Application of RMCE for the Generation of Knockin Mice

The starting point for the application of RMCE for the generation of knockin mice is the introduction by homologous recombination of an exchangeable cassette in ES cells replacing parts of the target gene. After targeting of the HygTK cassette flanked by heterospecific FRT sites, such a parental ES cell line could subsequently be used repeatedly to generate different knockins in the same gene by an exchange of the HygTK cassette for different sequences. The HygTK cassette flanked with the hetereospecific FRT sites is preferably introduced intronic at the 5′-end and intronic or downstream of the last exon at the 3′-end. The FRT sites remain present in the locus after the exchange, so they should at least not be present in the protein coding region. As for the commonly used knockin targeting vectors, the RMCE exchange plasmid should contain the appropriate knockin sequences and preferably an excisable selection marker gene flanked by LoxP sites. However, it should be noted that in case only negative selection with ganciclovir against the HygTK cassette is applied, the selection marker gene could be omitted from the exchange plasmid.

A theoretical example of RMCE application is depicted in **Fig. 3**, which shows a gene with 5 exons. Exon 2 (encoding the initation codon) to the last exon (encoding the stop codon and polyadenylation site) are replaced by homologous recombination by the HygTK cassette flanked by the heterospecific FRT sites. By subsequent RMCE, this cassette can be replaced by knockin sequences encoding a point mutation, sequences encoding a specific splice variant ablating a particular exon, sequences encoding a green fluorescent protein (GFP) fusion protein, or sequences encoding a complete different protein coding sequence (e.g., reporter gene), and so on. The knockin sequences will mostly consist of both cDNA and genomic sequences. In principle, the

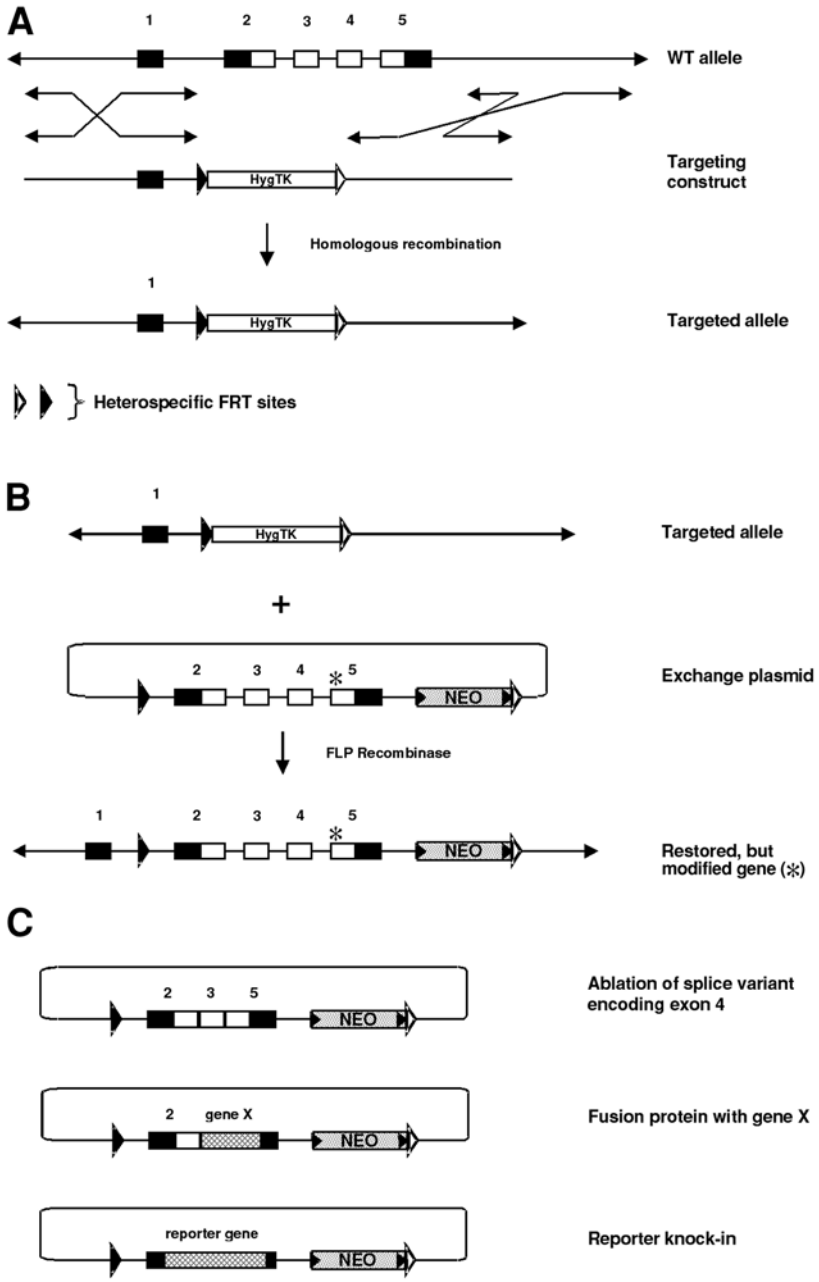
targeted gene could also be restored wild-type with extra LoxP sites, allowing conditional inactivation later on.

Of course it is obvious that the size and the complexity of the gene of interest may restrict the number of possible modifications for a particular parental ES cell. For smaller genes, the complete gene (including transcription regulatory sequences) could be removed and eventually restored with a modified promoter region. For larger genes, it might be necessary to replace only a part of the gene and to focus on modification of that particular part of the gene (e.g., transcription regulatory sequences, particular protein domain, and so on). However, the application of this RMCE method should only be considered if multiple modifications are envisaged, since it always requires two steps of ES cell targeting. On the other hand, a parental ES cell line will very often be suitable for the generation of a classical germline knockout, leaving the possibility for future modifications open.

3.2.3. RMCE in the LRP Gene

So far, the above-mentioned potential applications of RMCE up to the actual generation of genetically modified mice lack documented experimental proof. The idea of using the RMCE method of Jürgen Bode and colleagues for the generation of knockins in a particular locus arose within the scope of a research project to study the significance of many potential protein interaction domains found in the large cytoplasmic tail of the lipoprotein receptor LRP. Their significance will be studied by in vivo analysis of different LRP tail mutants. The LRP gene is very large, consisting of of 89 very small exons encoding a transcript of about 15 kb and a protein of 4545 amino acids. Using homologous recombination in ES cells, the 3′-end part of intron 75, the exons 76–89, and some downstream genomic sequences of the LRP gene were successfully replaced in ES cells by the HygTk cassette flanked by the heterospecific FRT sites. In the parental ES cell line, subsequent RMCE by the FLP recombinase was used to

Fig. 3. *(See facing page)* Application of RMCE for the generation of knockins in a theoretical gene consisting of five exons (exons 2–5 protein coding, open boxes). **(A)** Generation of a parental ES cell line suitable for RMCE. A large part of the gene is replaced, owing to homologous recombination by the HygTK cassette flanked by heterospecific FRT sites. **(B)** RMCE is used to restore the gene by reintroduction of the replaced sequences now encoding a mutation in exon 5. The 3′-end FRT site in the exchange plasmid is preceded by a Neo selection marker gene (flanked by LoxP sites for removal later on) to allow positive selection for cells with the intended exchange. **(C)** a Limited number of alternative exchange plasmids are shown to illustrate the usefulness of the parental ES cell line for the generation of different modifications.


A
1
2
3
4
5
WT allele
HygTK
Targeting construct
Homologous recombination
1
HygTK
Targeted allele
Heterospecific FRT sites
B
1
HygTK
Targeted allele
+
2
3
4
5
NEO
Exchange plasmid
FLP Recombinase
1
2
3
4
5
NEO
Restored, but modified gene (*)
C
2
3
5
NEO
Ablation of splice variant encoding exon 4
2
gene X
NEO
Fusion protein with gene X
reporter gene
NEO
Reporter knock-in

replace the HygTK cassette by the 3′-end part of intron 75, wild-type or modified exons 76–89 cDNA sequences, downstream genomic sequences, and a neomycin selection marker gene flanked by LoxP sites. This should restore the LRP gene, either wild-type or mutant. Instead of genomic sequences, predominantly cDNA sequences were used in the replacement vector to reduce the complexity of the construct and to facilitate the introduction of many different or multiple mutations in sequences corresponding to different exons of the endogenous gene. To date the RMCE method has been applied successfully in seven different replacement constructs (Roebroek et al., unpublished data).

The efficiency of the method seems to be high and reproducible: 45–70% of the G418-resistent clones resulted from intended recombination in the different experiments. Thus only a limited number of G418-resistant ES cell clones need to be picked and analyzed in order to isolate a right one. The parental ES cell line and two derivative ES cell lines, one with a wild-type restored LRP gene (positive control) and one with a modified restored LRP gene, have already been used to generate mice. Heterozygote mice have been obtained for these three ES cell lines. The parental ES cell line is expected to result in a homozygously embryonic lethal phenotype like the classical LRP knockout ***(27,28)***, since a large and essential carboxyl-terminal part of the LRP protein is missing. The preliminary results from a first intercross breeding experiment indicate that homozygous mice from the ES cell line with the restored wild-type LRP allele are viable and express a normal LRP protein. Thus the RMCE procedure seems to work as expected (*see* **Note 1**).

4. Notes

1. Some further remarks considering the RMCE experimental procedure might be helpful for potential users. The procedures for ES cell propagation and targeting are in principle according the general guidelines and protocols discussed elsewhere in this volume. This is especially true for the initial targeting of the HygTK cassette (kindly provided by Jürgen Bode) by homologous recombination. For transient expression of the FLP recombinase, the pCAGGS-FLPeIRESpuro FLPe expression vector was used (kindly provided by Francis Stewart). In the RMCE procedure itself, a mixture of 100 μg FLPe expression plasmid and 30 μg replacement construct plasmid DNA (both supercoiled circular DNA) in a volume of 30 μL TE was electroporated into 5×10^6 ES cells. Cells were plated on feeders in a 250-mL culture flask for about 30–48 h (depending on the density and growth of the ES cells) without selection pressure, to allow the recombination events happen. Subsequently, ES cells were trypsinized and replated in a density of 2.5×10^5 ES cells/75-mL culture flask. The selection for G418-resistant clones was started immediately or after 12 h. G418-resistant clones (about 5–25 surviving clones per flask) were picked for further growth and analysis after 8–10 days. As mentioned, the efficiency was about 40–70%

of the surviving clones. In a pilot experiment, a combination of G418-positive and ganciclovir-negative selection was used, resulting in 100% efficiency, but ES cells grew poorly under the conditions tested. To avoid a potentially negative impact on the capacity of the manipulated ES cell lines to go germline, this double selection is no longer applied, especially because the high efficiency of G418 selection alone proved to be sufficient.

References

1. Elefanty, A. G., Begley, C. G., Metcalf, D., Barnett, L., Köntgen, F., and Robb, L. (1998) Characterization of hematopoietic progenitor cells that express the transcription factor SCL, using a lacZ "knock-in" strategy. *Proc. Natl. Acad. Sci. USA* **95,** 11897–11902.
2. Rozmahel, R., Gyomorey, K., Plyte, S., et al. (1997) Incomplete rescue of cystic fibrosis transmembrane conductance regulator deficient mice by the human CFTR cDNA. *Hum. Mol. Genet.* **6,** 1153–1162.
3. Wangm Y., Schnegelsbergm P. N., Dausmanm J., and Jaenisch, R. (1996) Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. *Nature* **379,** 823–825.
4. Hanks, M. C., Loomis, C. A., Harris, E., et al. (1998) Drosophila engrailed can substitute for mouse Engrailed1 function in mid-hindbrain, but not limb development. *Development* **125,** 4521–4530.
5. Geng, Y., Whoriskey, W., Park, M. Y., et al. (1999) Rescue of cyclin D1 deficiency by knockin cyclin E. *Cell* **97,** 767–777.
6. Reichardt, H. M., Kaestner, K. H., Tuckermann, J., et al. (1998) DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* **93,** 531–541.
7. Wheeler, V. C., White, J. K., Gutekunst, C. A., et al. (2000) Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. *Hum. Mol. Genet.* **9,** 503–513.
8. Guo, Q., Fu, W., Sopher, B. L., et al. (1999) Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. *Nat. Med.* **5,** 101–106.
9. Lorenzetti, D., Watase, K., Xu, B., et al. (2000) Repeat instability and motor incoordination in mice with a targeted expanded CAG repeat in the Sca1 locus. *Hum. Mol. Genet.* **9,** 779–785.
10. Castellanos, A., Pintado, B., Weruaga, E., et al. (1997) A BCR-ABL(p190) fusion gene made by homologous recombination causes B-cell acute lymphoblastic leukemias in chimeric mice with independence of the endogenous bcr product. *Blood* **90,** 2168–2174.
11. Okuda, T., Cai, Z., Yang, S., et al. (1998) Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. *Blood* **91,** 3134–3143.
12. Rhoades, K. L., Hetherington, C. J., Harakawa, N., et al. (2000) Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. *Blood* **96,** 2108–2115.

13. Okuda, T., Takeda, K., Fujita, Y., et al. (2000) Biological characteristics of the leukemia-associated transcriptional factor AML1 disclosed by hematopoietic rescue of AML1-deficient embryonic stem cells by using a knock-in strategy. *Mol. Cell. Biol.* **20,** 319–328.
14. Dobson, C. L., Warren, A. J., Pannell, R., et al. (1999) The mll-AF9 gene fusion in mice controls myeloproliferation and specifies acute myeloid leukaemogenesis. *EMBO J.* **18,** 3564–3574.
15. Castilla, L. H., Wijmenga, C., Wang, Q., et al. (1996) Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. *Cell* **87,** 687–696.
16. Schlake, T. and Bode, J. (1994) Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. *Biochemistry* **33,** 12746–12751.
17. Seibler, J. and Bode, J. (1997) Double-reciprocal crossover mediated by FLP-recombinase: a concept and an assay. *Biochemistry* **36,** 1740–1747.
18. Seibler, J., Schübeler, D., Fiering, S., Groudine, M., and Bode, J. (1998) DNA cassette exchange in ES cells mediated by FLP recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs. *Biochemistry* **37,** 6229–6234.
19. Hoess, R. H., Wierzbicki, A., and Abremski, K. (1986) The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14,** 2287–2300.
20. Araki, K., Araki, M. and Yamamura, K. (1997) Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res.* **25,** 868–872.
21. Bethke, B. and Sauer, B. (1997) Segmental genomic replacement by Cre-mediated recombination: genotoxic stress activation of the p53 promoter in single-copy transformants. *Nucleic Acids Res.* **25,** 2828–2834.
22. Bouhassira, E. E., Westerman, K., and Leboulch, P. (1997) Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange. *Blood* **90,** 3332–3344.
23. Feng, Y. Q., Seibler, J., Alami, R., et al. (1999) Site-specific chromosomal integration in mammalian cells: highly efficient FRE recombinase-mediated cassette exchange. *J. Mol. Biol.* **292,** 779–785.
24. Kolb, A. F. (2001) Selection-marker-free modification of the murine beta-casein gene using a lox2722 site. *Anal. Biochem.* **290,** 260–271.
25. Fiering, S., Bender, M. A., and Groudine, M. (1999) Analysis of mammalian cis-regulatory DNA elements by homologous recombination. *Methods Enzymol.* **306,** 42–66.
26. Bode, J., Schlake, T., Iber, M., et al. (2000) The transgeneticist's toolbox: novel methods for the targeted modification of eukaryotic genomes. *Biol. Chem.* **381,** 801–813.
27. Herz, J., Couthier, D. E., and Hammer, R. E. (1992) LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell* **71,** 411–421.
28. Herz, J., Couthier, D. E., and Hammer, R. E. (1993) Correction: LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell* **73,** 428.

11

Aggregation of Embryos and Embryonic Stem Cells

Ronald Maatman, Marina Gertsenstein, Emile de Meijer, Andras Nagy, and Kristina Vintersten

1. Introduction

The human genome has been almost completely sequenced, and at least 30,000 genes have been found ***(1)***. Systematic studies of gene expression patterns by using cDNA microarrays have provided a powerful approach to molecular dissection of cells and tissues by comparing expression levels of tens of thousands of these genes at a time. Even insight into signaling pathways has been gained ***(2,3)***. However, information about the in vivo function of the various genes, especially disease genes, still requires the development of animal models carrying particular mutations. Several mouse mutagenesis projects (*see* Chapter 13) have been started during the last decade, and the number of mutant mice generated by targeted mutagenesis in mouse embryonic stem (ES) cells has increased exponentially ***(4–7)***.

Generation of mutant mice from genetically modified ES cells has become a standardized technique over the years, and basically two methods have routinely been used to derive animals (chimeras) to transmit the ES cell genome to the offspring: injection of ES cells into blastocysts or aggregation with blastomere stage embryos. Blastocyst injection (*see* Chapter 2), in which ES cells are introduced into the blastocoel cavity by the use of microinjection pipets and micromanipulators, although most commonly used, requires high skills and is expensive to establish in a laboratory. Using the aggregation technique, ES cells are aggregated with cleavage stage embryos, followed by culture of the aggregates under optimal conditions to the blastocyst stage. This procedure does not require expensive instrumentation, is technically less demanding, and can be performed manually under a dissecting microscope.

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

In the ideal situation, in which the ES cells have excellent developmental potential, both techniques will produce germline transmitting chimeras equally well. If the quality of the ES cells is very low, neither technique will work. However, there is a certain difference between the two techniques when suboptimal ES cell clones have to be used. During transfection, passaging, cloning, and selection, some ES cells lose their ability to contribute at a high level to multiple cell lineages. Consequently, they will no longer have the capacity to colonize the germline of the resulting animals. Such a deficiency often alters the physical parameters of ES cells, e.g., the size. In this case, the injection technique, by which individual cells are selected under high magnification, could have a certain advantage over the aggregation technique, in which random clumps of cells are used.

Optimally, the two techniques should be used to complement each other. It is, however, important to stress that neither technique can compensate for poor culture conditions. It is crucial to attempt to keep the ES cells under as optimal conditions as possible, in order to retain their full developmental potential (*see* **Note 1**). We also found that a higher number of embryos needs to be aggregated than injected to achieve the same number of chimeras. However, the aggregation procedure is far less expensive and faster to perform ***(8)***.

There is no reliable way to predict the developmental capacity of an ES cell line without actually introducing it into an embryonic environment. However, certain criteria may give a strong indication of the quality of the cells, like the morphology of the ES cells in culture. The cells should grow in dense, three-dimensional colonies, with sharp edges. Flattening colonies, fibroblast-like outgrowths, and unusually high or low growth rates are all bad signs. Another criterion is the correct euploid karyotype. ES cells kept in culture for extended passages very often gain chromosomal abnormalities. The most common alteration is the loss or gain of one entire chromosome. Unfortunately, aneuploid cells often gain in growth speed and can quickly overgrow the normal cells in the culture. To find out the ratio of euploid cells within a cell line, chromosome counting can be performed on a large number of metaphase spreads, and the ratio of normal cells can be calculated (*see* **Subheading 3.1.**). Obviously, this method does not give any information about other chromosomal alterations (such as translocations), but it is simple and fast and gives a good general measure of the quality of an ES cell line. If a targeting experiment has resulted in multiple positive clones, one can use the information from karyotyping to choose the clones best suitable for further experiments, and by these means increase the efficiency of producing good chimeras.

Experimental chimeras were first generated between two diploid blastomere stage embryos, mostly in order to assay the developmental potential of the components. The degree of chimerism from the offspring was determined by

the use of genetic markers ***(9,10)***. Later it was shown that intact embryos are not the only components from which chimeras can be made. Isolated inner cell mass (ICM) and teratocarcinoma (EC) cell lines were able to contribute to different tissues, if they were introduced back into the embryonic environment. EC cell contribution, however, often resulted in abnormal embryonic development, and ES cells were practically not compatible with germ cells ***(11)***. Two decades ago, Evans, Kaufman, and Martin were able to culture ES cells isolated from the primitive ectoderm component of blastocysts ***(12,13)***. Shortly thereafter, it was shown that ES cells were capable of contributing to all cell lineages derived from the primitive ectoderm, such as the entire embryo proper and the resulting chimeric adult, including the germline. Interestingly, however, they fail to differentiate into the trophoblast and primitive endoderm lineage. These experiments were done by injecting ES cells into blastocysts, which were returned to pseudopregnant recipients ***(14,15)***. A few years later, the observed capabilities and restrictions of ES cells were confirmed by aggregating ES cells with 1 day earlier (8-cell) stage embryos (aggregation ES cell chimeras) ***(8)***.

Recently the establishment of permanent cell lines from trophectoderm cells of the blastocyst has been made possible ***(16)***. These cells [trophoblast stem (TS) cells] can contribute to chimeras in a similar way as ES cells. However, the contribution is directed to different compartments. They contribute only to the placenta, showing the developmental potential/restriction of their origin, the trophectoderm cells.

The variation in possible chimera components has been expanded on the embryo side as well. Simple techniques for duplicating the number of chromosomes in the developing early stage embryos have been developed, for example, electrofusion of the blastomeres of 2-cell stage embryos ***(17)***. These embryos have limited developmental potential and rarely develop beyond the early somite stage. In chimeras with diploid embryos, however, they contribute to the trophoblast and primitive endoderm lineages. As we mentioned earlier, ES cells have a complementary developmental potential. The combination of these two components in chimeras results in entirely ES cell-derived fetuses developing on mostly tetraploid extraembryonic membranes. (**Fig. 1**) ***(18)***.

Given that chimeras need two components to be placed together and that these two could have different developmental restrictions and/or genotypes, several types of chimeras can be produced with various component allocation biases. These variations provide a powerful tool for dissecting multiple functions of mutations ***(19)***. The ES cell embryo chimeras still lead the list in importance, and in this chapter we describe in detail the standard methods of generating aggregation chimeras between ES cells and diploid or tetraploid embryos.

The successful production of any chimera is highly dependent on the care with which both ES cells and embryos are handled. Absolute sterile technique

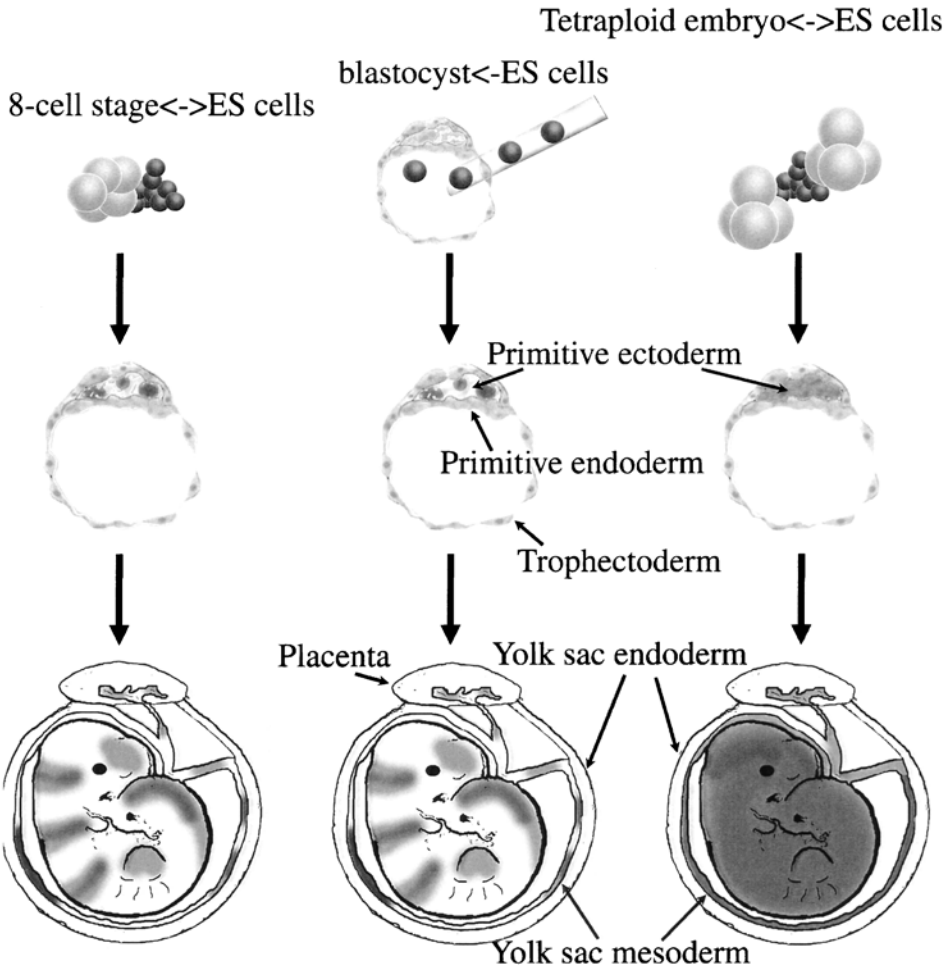


Fig. 1. Diagram of lineage contributions in different kinds of aggregation chimeras. Solid colors indicate nonchimeric tissues; light/dark mixed area indicates chimeric tissues. Light gray/white areas indicate embryonically derived tissue. Dark gray areas indicate ES-derived tissue.

has to be adopted in the tissue culture and every possible risk of contamination avoided. Newly produced batches of mouse embryonic fibroblasts (MEFs) for feeder cells and all cells arriving from outside the lab should be tested for the presence of *Mycoplasma*. One should keep in mind that several subtypes of *Mycoplasma* can contaminate cultured cells. Some of these subtypes grow very slowly and will not be detected unless the screening is performed on enriched supernatant, cultured for up to 3 weeks under optimal conditions. For this

reason, commercially available test kits may in some cases not detect a subtle contamination. An example of this situation is the widely used E14.1 wild-type ES cell line, which is known to be contaminated with *Mycoplasma homini* ***(20,21)***.

Each batch of fetal bovine serum used for ES cell culture medium has to be carefully tested both for possible toxicity and for its ability to support proliferation of ES cells in an undifferentiated state (*see* **Subheading 3.3.**).

Embryos should be handled separately from ES cells until the point of aggregation. They should be cultured in an incubator designated only for embryos, and care should be taken to minimize the time they spend in normal room environment.

2. Materials

2.1. Karyotyping of ES Cell Clones (see Note 2)

1. ES cells in a 35-mm dish, subconfluent culture.
2. Phosphate-buffered saline (PBS).
3. 0.25% trypsin/1 m*M* EDTA (1X solution; Life Technologies, cat. no. 25200).
4. ES cell medium (*see* **Subheading 2.2.**).
5. Colcemid (Life Technologies, cat. no. 15210-012).
6. 0.56 % KCl.
7. Methanol/glacial acetic acid in a 3 : 1 ratio, freshly prepared and kept at 4°C.
8. Clean glass slides.
9. Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Vector, cat. no. H-1200).

2.2. Preparation of ES Cells for Aggregation with Diploid or Tetraploid Embryos

1. EF medium:
   a. Dulbecco's modified Eagle's medium (DMEM) high glucose, (Life Technologies, cat. no. 10566-016).
   b. 10% fetal bovine serum (FBS; Life Technologies, cat. no. 10120-160; Sigma, HyClone).
   c. 1 m*M* sodium pyruvate (optional; 100X stock; Life Technologies, cat. no. 11360) .
   d. 2 m*M* L-glutamine (optional; 100X stock; Life Technologies, cat. no. 25030).
   e. Penicillin and streptomycin, final concentration 50 μg/mL each, [Life Technologies cat. no. 15140 (100X stock) or 15070 (200X stock)].
2. ES medium:
   a. DMEM high glucose (Life Technologies, cat. no. 10566-016).
   b. 15% FBS (ES cell-tested; *see* **Subheading 3.3.**).
   c. 1 m*M* sodium pyruvate (100X stock; Life Technologies, cat. no. 11360).
   d. 2 m*M* L-glutamine (100X stock; Life Technologies, cat. no. 25030).

e. Penicillin and streptomycin, final concentration 50 μg/mL each [Life Technologies, cat. no. 15140 (100X stock) or 15070 (200X stock)].
f. 0.1 m*M* nonessential amino acids (100X stock; Life Technologies, cat. no. 11140).
g. 100 μM β-mercaptoethanol (Sigma, M cat. no. 7522).
h. 1000–2000 U leukemia inhibiting factor (LIF)/mL (Chemicon, cat. no. ESG 1107).

3. 0.25% trypsin/1 m*M* EDTA (1X solution; Life Technologies, cat. no. 25200).
4. PBS without Ca and Mg.
5. 0.1% gelatin (Sigma, cat. no. G-2500).
6. Sterile tissue culture plates.
7. Organ culture dishes (Falcon, cat. no. 3037).
8. Humidified incubator, 5% CO_2 and 37°C.

2.3. Testing of Fetal Bovine Serum for ES Cell Use

1. ES medium.
2. Control and test batches of FBS.
3. Methylene blue (3.3 g methylene blue, 1.1 g basic fuchsin per liter methanol).
4. PBS without Ca and Mg.

2.4. Production and Isolation of 2- and 8-Cell Stage Embryos for Aggregation

2.4.1. Superovulation

1. Pregnant mare serum gonadotropin (PMSG; Folligonan® 1000 IU, InterVet Intergonan® 1000 IU).
2. Human chorionic gonadotropin (hCG; Chorulon® 1500 IU, Sigma, cat. no. CG-10).
3. Sterile water or physiologic saline solution.
4. 1-mL syringes with 26- or 30-gage needle.

2.4.2. Isolation of 2- and 8-Cell Stage Embryos

1. Dissecting microscope with transmitted light source (light box with frosted glass).
2. Flushing needle (the sharp tip of a 30-gage needle is removed and/or rounded with a sharpening stone or sand paper).
3. 1-mL syringe.
4. Surgical instruments: sharp and fine pointed straight scissors, and Dumont No. 5 watchmaker's forceps.
5. Alcohol or Bunsen burner.
6. 70% EtOH.
8. Mouth pipet assembly for embryo manipulation. A Pasteur pipet is pulled by hand over a flame and broken. The tip is flame-polished to avoid any sharpness, which

could damage the embryos. The pipet is connected to an aspirator mouthpiece (HPI Hospital Products, cat. no. 11501) through a latex or silicon tubing.
8. Sterile plastic tissue culture dishes (35-mm Easy Grip Falcon, cat. no. 3001).
9. M2 medium (Specialty Media, cat. no. MR-015P-5F), M16 medium (Specialty Media, cat. no. MR-010P-5F), or K Simplex Optimization Medium (KSOM; Specialty Media, cat. no. MR-020P-5F).
10. Organ culture dishes (Falcon, cat. no. 3037), alternatively, microdrops of M16 or KSOM media covered with embryo-tested light mineral oil (Sigma, cat. no. M8410).
11. Humidified incubator with 5% CO_2 and 37°C.

For the production of viable chimeras, the quality of the media for isolation of the embryos and culture of the aggregates is crucially important (**Table 1**; *see* **Note 3**. If media are prepared from basic components or from concentrated stocks, the following factors should be considered: Water should be of the highest quality grade and should be embryo tested; the chemicals should also be of the highest grade and preferably used only for media preparation. All media should be made up and stored in plastic, since glass often contains detergents or other organic solvents. The concentrated stock solutions can be stored at –80°C for a few months. After preparation, the medium is filtered through a 0.2-μm Millipore filter, aliquoted, and stored at 4°C for no longer than 2 weeks. Optimally, KSOM, developed by Lawitts and Biggers ***(22,23)***, and M16 medium should be gassed with 5% CO_2 in air to adjust the pH to 7.4 before storage. Both KSOM and M16 media must be equilibrated by incubation at 37ºC/5% CO_2 prior to use. A variety of embryo culture media are available commercially from several suppliers. These ready-made alternatives are usually of high quality; however, newly purchased supplies as well as media prepared from basic components should always be tested prior to use. To test the media and culture conditions, collect 1-cell stage embryos (0.5 days post coitum) and culture them to the blastocyst stage. (Most of the embryos should reach this stage.)

2.4.3. Preparation of Tetraploid Embryos for Aggregation; Preparation of the Fusion Chamber and the Nonelectrolyte Solution

1. Dissecting microscope(s).
2. Cell fusion instrument (BLS Ltd., Hungary, cat. no. CF-150B, http://www.bls-ltd.com) with electrodes GSS-250 or GSS-1000.
3. M2 medium (*see* **Subheading 2.4.2.**).
4. High vacuum grease (Dow Corning).
5. Microdrops of KSOM or M16 medium (*see* **Subheading 2.4.2.**) covered by embryo-tested mineral oil (Sigma, cat. no. M8410) in 35-mm tissue culture dishes (Easy Grip Falcon, cat. no. 3001).

Table 1
Composition of KSOM, M16, and M2 Media

Component	Concentration (g/L)		
	KSOM	M16	M2
NaCl	5.55	5.53	5.53
KCl	0.186	0.356	0.356
KH_2PO_4	0.0476	0.162	0.162
$MgSO_4$	0.0493	0.293	0.293
Nalactate	1.12 or 1.87 g of 60% syrup	4.349 g of 60% syrup	4.349g of 60% syrup
D(+)glucose	0.036	1.00	1.00
Sodium pyruvate	0.022	0.036	0.036
$NaHCO_3$	2.10	2.10	0.349
$CaCl_2$ (dihydrous)	0.251	0.252	0.252
L-Glutamine (Life Technologies, cat. no. 25030)	0.146	—	—
EDTA (tetrasodium salt)	0.0038	—	—
Bovine serum albumin (Sigma, cat. no. A3311)	1.0	4.00	4.00
Penicillin-G	0.060	0.060	0.060
Streptomycinsulfate	0.050	0.050	0.050
Phenol red	—	0.01	0.01
HEPES buffer	—	—	4.969

6. 0.3 *M* mannitol (Sigma, cat. no. M4125), dissolved in ultrapure water containing 0.3% BSA (Sigma, cat. no. A3311). The solution should be filter-sterilized through a 0.22-μm Millipore filter and aliquots stored at –20°C. Freshly thawed solution should be used for each experiment; do not refreeze thawed aliquots.
7. Humidified incubator, 5% CO_2 and 37°C.

2.4.4. Preparation of Aggregation Plate

1. Sterile 35- or 60-mm tissue culture dishes (Easy Grip Falcon, cat. nos. 3001 or 3004)
2. KSOM or M16 medium (*see* **Subheading 2.4.2.**)
3. 1-mL syringe with 26-gage needle or micropipeter.
4. Embryo-tested mineral oil (Sigma, cat. no. M8410).
5. 70% EtOH.
6. Aggregation needle DN-09 (BLS Ltd., cat. no. DN-09).
7. Humidified incubator, 5% CO_2 and 37°C.

2.4.5. Removal of Zona Pellucida by Acid Tyrode's Solution

1. Dissecting microscope.
2. Sterile 100-mm tissue culture dish.
3. KSOM or M16 medium.
4. M2 medium.
5. 1-mL syringe with 26-gage needle or micropipeter.
6. Sterile 1% albumin solution. [Dissolve embryo-tested albumin (Sigma, cat. no. A-3311) PBS, filter-sterilize, and store at 4°C].
7. Tyrode's acid solution (Sigma, cat. no. T-1788) or own preparation (see below) The solution should not be warmer then room temperature when used.
8. Mouth pipet assembly (*see* **Subheading 2.2.2.**)
9. Humidified incubator, 5% CO_2 and 37°C.

Tyrode's acid solution:

NaCl	8.00g/L
KCl	0.20g/L
$CaCl_2 \cdot 2H_2O$	0.24g/L
$MgCl_2 \cdot 6H_2O$	0.10g/L
Glucose	1.00g/L
Polyvinylpyrrolidone (PVP)	4.00g/L

Prepare at room temperature, and adjust the pH to 2.5 with Analar HCl. Filter-sterilize and store in aliquots at –20°C.

2.4.6. Assembly of ES Cell ↔ Diploid or Tetraploid Embryo Aggregation Chimeras

1. Dissecting microscope.
2. Aggregation plate (*see* **Subheading 2.4.4.**).
3. Sterile 1% albumin solution. (Use embryo-tested albumin, filter-sterilize, and store at 4°C.)
4. Mouth pipet assembly (*see* **Subheading 2.4.2.**).
5. Humidified incubator with 5% CO_2 and 37°C.

2.4.7. Embryo Transfer

1. Dissecting microscope.
2. Surgical instruments: sharp and fine pointed straight scissors, and Dumont no. 5 watchmakers forceps, fine pointed forceps with serrated tip, serrefine clip (FST, cat. no. 18050-28 bulldog-type serrated serrafine vascular clamp).
3. Wound clips and Autoclip applier (Clay Adams, cat. nos. B-D 7631 and B-D 763007).
4. Mouth pipet assembly (*see* **Subheading 2.4.2.**).
5. M2 medium and 1% BSA solution.

6. Ketamine/xylazine or avertin anesthetics.
7. 70% EtOH.

2.4.8. Cesarean Section

1. Heating pad or desk lamp.
2. Surgical instruments: sharp and fine pointed straight scissors, fine pointed forceps.
3. Fine and soft tissue paper.
4. Replacement foster mother: female mouse with young pups (ideally newborn or 1 day old). If possible, the female should be chosen from a strain that is known to produce good fosters, such as CD1 or ICR.

3. Methods

3.1. Karyotyping of ES Cell Clones

We regard clones that show a euploidy of at least 85% as a "good" choice for ES cell injection/aggregation to produce chimeric mice. Clones with a euploidy lower than 60% are not worth using.

1. Passage ES cells on to a gelatinized 35-mm tissue culture dish 1 day prior to the preparation. Make sure to preplate away all feeders carefully.
2. Next day, add 1 μg/ml colcemid to the culture medium, and incubate for 60 min at 37°C.
3. Trypsinize the cells, and resuspend to a single cell suspension in ES media.
4. Centrifuge for 5 min at 270*g*.
5. Remove the medium and tap the tube to disrupt the pellet. Carefully resuspend the cells in 5 mL of hypotonic solution (0.56% KCl).
6. Incubate at room temperature for 5 min.
7. Add 1 mL of ice-cold fixative (methanol/glacial acetic acid). Mix by inverting the tube.
8. Centrifuge for 5 min at 270*g*.
9. Discard the supernatant, leaving 0.5 mL in the tube. Tap the tube to disrupt the pellet.
10. Add 5 mL of cold fixative. Mix by inverting the tube. Incubate for 10 min on ice.
11. Centrifuge as before. Remove all fixative except 0.5 mL. Disrupt the pellet and add 5 mL of fixative. Invert to mix, and centrifuge as before. Repeat this step twice.
12. Resuspend pellet in 0.5–1 ml of cold fixative.
13. Drop the cell suspension onto a glass slide from 10-cm distance.
14. Air-dry at room temperature.
15. Add a drop of mounting medium with 4,6-diamidino-2-phenylindole (DAPI) to each slide, and seal with a cover slip. Chromosomes can be visualized under ultraviolet (UV) light.
16. Count 30–40 chromosome spreads. Euploid spreads should contain 40 chromosomes.

3.2. Preparation of ES Cells for Aggregation with Diploid or Tetraploid Embryos

Both diploid and tetraploid aggregation experiments require ES cells to be cultured under optimal conditions, in order to maintain their pluripotency. The cells should be passaged at least once after thawing, prior to the experiment. However, the time in culture and number of passages should be kept as low as possible. For detailed protocols on culturing ES cells *see* also Chapter 8. The protocols below were developed for R1 cells ***(18)***, but they can also be used for other ES cell lines (e.g., E14-1). When preparing the ES cells for aggregation, the goal is to produce clumps of 8–15 loosely connected cells. The timing of the passage has to be determined from case to case. ES cells do not like to grow sparsely. However, they will quickly start to differentiate if left to grow at high densities. Optimally, one should attempt to split them every other day at a semiconfluent state (**Fig. 2**).

1. Day –3: thaw ES cells on an appropriately sized tissue culture dish with an inactivated MEF feeder layer.
2. Day –2: change the medium.
3. Day –1:
   a. Trypsinize the cells and resuspend into a single cell suspension.
   b. Centrifuge at 270*g* for 5 min.
   c. Discard the supernatant.
   d. Loosen the pellet by flicking the tube.
   e. Resuspend the cells in 5 mL medium, let any large clumps settle down for a few minutes, and plate the top portion of the cell suspension onto gelatinized tissue culture dishes. Prepare several plates with varying densities, to ensure the formation of small colonies until the next day. For example, plate 0.1, 0.3, and 0.6 mL of suspension on three 60-mm gelatinized dishes.
4. Day ± 0: day when embryos are ready for aggregation:
   a. Wash the growing ES cell plates once with PBS and once quickly with trypsin.
   b. Add a minimal amount of trypsin to the plates and let them trypsinize for 3 min at room temperature. Watch the cells under a microscope and inactivate the trypsin by adding ES media as soon as gentle swirling lifts up clumps of loosely connected cells.
   c. Do not pipet the cell suspension unless the clumps are too large for aggregation. If this would be the case, transfer aliquots to new dishes, and treat them with one, two, or three gentle pipetings up and down to decrease the clump sizes.
   d. Keep the cells in the dishes at room temperature until aggregates have been assembled (up to 2 hours).

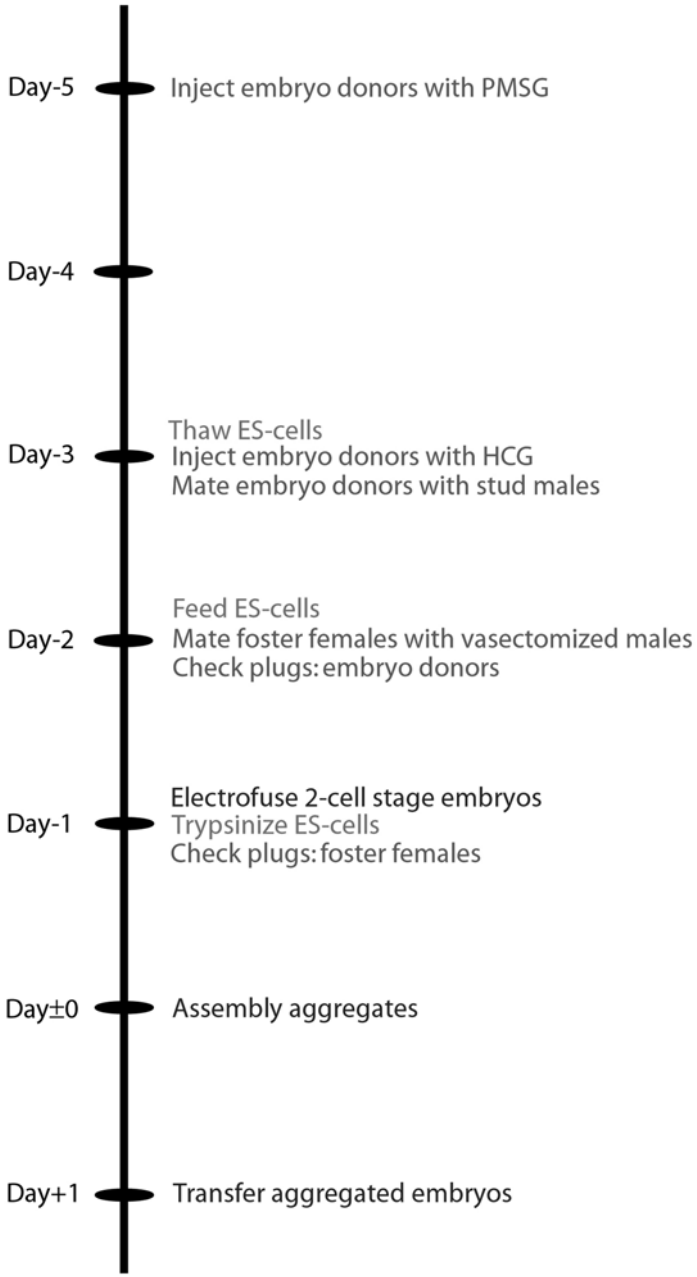


Fig. 2. Diagram describing the time schedule for aggregation in terms of embryonic stem (ES) cell culture, embryo production, aggregation, and transfer. HCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin.

3.3. Testing of Fetal Bovine Serum for ES Cell Use

The quality of the FBS used for ES cell culture is an issue of major importance. The variation between batches can be high, which makes the proper testing of a suitable batch necessary. A batch of known high-quality serum capable of optimally supporting ES cell growth should be used as a control. Once a "good" batch has been found, it is advisable to purchase a larger quantity for future use. Serum can be stored at –20°C for a long time.

1. Trypsinize exponentially growing ES cells as described in **Subheading 3.2.**
2. Perform an extensive preplating in order to discard the MEFs.
3. Prepare three 35-mm tissue culture dishes for each batch of serum to be tested (include three dishes for the control).
4. Prepare 3 aliquots of ES medium without LIF for each test and control batch. Add 10, 15, and 30% serum, respectively.
5. Seed out approximately 10^3 ES cells per 35-mm tissue culture dish without MEFs.
6. Let the colonies grow, and change the media every day, each dish with its own designated batch/serum concentration.
7. When the colonies have grown to a size at which they can be readily judged, check the colony morphology and note which culture looks healthiest by visual judgment, compared with the control culture.
8. Rinse with PBS and stain with methylene blue for 5 min at room temperature.
9. Examine the colonies again with respect to density and morphology.
10. Choose the serum batch that shows no toxicity at 30% concentration and that shows as good support for colony growth/morphology maintenance as the control batch in 10%. (The 15% serum test will help verifying the optimal serum concentration).

3.4. Production and Isolation of 2- and 8-Cell Stage Embryos for Aggregation

The mouse strain used to generate chimeras by aggregation seems not to be as critical as it is for ES cell injection into blastocysts, in which the most common choice of strain is C57BL/6 inbred. This may be explained by the different developmental stage at which the ES cells are introduced into the host embryo. Mouse strains that give good results for aggregation are the albino outbred strains CD-1 and ICR, but F1 hybrids between C57 × CBA, C57 × DBA, and even C57 × CD1 outbred also seem to do well. Use of pure inbred strains for aggregation is less recommended since chimeras will be largely ES cell-derived, which might decrease their viability. The advantages of outbred or F1 hosts are the low costs and excellent yields of embryos after superovulation. The superovulation of young female mice increases the yield of embryos significantly. However, the process can be greatly influenced by several factors

such as the age, strain, and health status of the females and the stud males, the general conditions in the animal room, and the proper handling of the hormones.

The highest number of embryos is usually obtained from very young (prepuberty aged) females. However, very small females may suffer significantly during the stressful experience of being mated by a large and often less careful male. Hence we recommend using 5–6 week-old females, even if the number of embryos recovered may be slightly reduced.

The timing of the hormone injections and the light cycle in the animal room will influence the ovulation and fertilization time point, which in turn will have a great effect on the developmental stage of the embryos (*see* **Note 4**). It is recommended to determine empirically which combination suits one's needs the best. However, one important factor to pay attention to is to keep the time between PMSG and hCG between 45 and 48 h. As a baseline, the following parameters can be used and modified: light cycle 05.00–19.00, PMSG 11.00, hCG 10.00.

1. Day 0: assemble aggregates.
2. Day 5: begin superovulation of young females by injecting 5 IU PMSG ip.
3. Day 3: inject the females with 5 IU hCG ip and mate them with stud males.
4. Day 2: check plugs in the early morning (0.5 days post coitum).

The procedure for oviduct flushing of 2- or 8-cell stage embryos is essentially the same. Avoid long exposure to room temperature. The time between sacrificing the embryo donors and placing the embryos in culture should be kept to a minimum (*see* **Note 5**).

To generate tetraploid embryos by blastomere fusion, late 2-cell stage embryos are isolated at 1.5 days post coitum (day –1). The presence of 10–15% 3/4-cell stage embryos among the 2-cell stages at the time of flushing indicates appropriate timing. This can usually be achieved by collecting the embryos 46–48 h after the hCG injection.

To obtain 8-cell stage embryos suitable for aggregation, 2.5 days post coitum females are sacrificed at day 0. For both stages, the embryos are recovered from the oviduct.

1. Sacrifice plug-positive females by cervical dislocation.
2. Wash the abdomen with 70% EtOH.
3. Make an incision in the skin across the midline at the most caudal part of the abdomen. Grab the skin on both sides of the incision, and tear it away from the abdomen.
4. Open the abdominal wall, and push away the intestines so that the uterus becomes visible.

5. Grab the upper part of the uterus, near to the oviduct with fine forceps.
6. Tear away the connective tissues attached to the oviduct with fine scissors.
7. Cut through the upper part of the uterus and between the ovary/oviduct. Take good care not to damage the oviduct coils!
8. Move the oviduct to a tissue culture dish with M2 medium.
9. Fill a 1-mL syringe with M2 medium.
10. Round the tip of a 30-gage needle with fine sandpaper, and attach to the syringe. Alternatively, the tip of the 30-gage needle can be completely removed before the end is smoothed with fine sandpaper or a sharpening stone.
11. Insert the needle in the infundibulum, and flush with approx. 0.1 mL medium. The oviduct coils should visibly expand, and embryos should swim out of the uterus part.
12. Collect the embryos with a mouth pipet assembly.
13. Wash the embryos through several drops of M2 media, sort, and count them.
14. Transfer the embryos to M16 or KSOM media (wash again through several drops), and place the dish in the incubator.

3.5. Preparation of Tetraploid Embryos for Aggregation

As described above in ES cell ↔ tetraploid embryo aggregation, tetraploid cells do contribute to the primitive endoderm and trophectoderm derivatives, and they are selected against in other lineages in the presence of diploid cells. On the other hand, ES cells only contribute to the primitive ectoderm-derived lineages. The combination of these two components makes "polarized" chimeras, with an exclusively tetraploid contribution to the trophoblast of the placenta and the parietal and visceral endoderm. The contribution from the ES cells is directed exclusively to the embryonic mesoderm component of the placenta, allantois, yolk sac mesoderm, amnion, and the entire embryo proper. This type of chimeras has been shown to be extremely helpful in addressing tasks like the following:

1. Distinguishing extraembryonic from embryonic phenotypes of a mutation.
2. Rapid testing of the developmental potential of new ES cell lines.
3. Analysis of phenotypes of mutations directly from deficient ES cells, without germline transmission ***(24)***.

3.5.1. Electrofusion

Several different methods have been described to produce tetraploid mouse embryos. Electrofusion, as devised by Kubiak and Tarkowski ***(17)***, is very simple and efficient, making it superior to other techniques. Here we describe this method in detail. The fusion of the two blastomeres of a 2-cell stage embryo is achieved by applying an electric pulse at a right angle to the cell membrane between the two blastomeres. It is crucial that the embryo is perfectly oriented,

to achieve a "melt-down" of the connecting plane and at the same time prevent the embryo from lysing. This orientation can easily be achieved by placing the embryos in a nonelectrolyte solution and applying a very low, constant high-frequency AC electric field. It is, however, possible to orient the embryos by hand, and in this case the fusion can take place in M2 medium. Since this method is much more time-consuming (each embryo has to be oriented individually), we recommend the use of nonelectrolyte solution. As soon as the embryos are orient, a single or double pulse is applied to the fusion chamber. After returning the embryo to normal culture conditions, the fusion will take place within the next 15–60 min.

We recommend the use of the CF-150/B electrofusion apparatus (BLS Ltd., 31 Zselyi Aladar utca, Budapest, H-1165, Hungary) and an electrode chamber with a fixed distance between the electrodes of 250 or 1000 μm.

The exact setting of the electrofusion parameters does slightly vary between individual machines. It is influenced by the strain of the donor embryos, and, most importantly, it depends on the distance between the electrodes. For each combination, a pilot experiment should be performed in which the optimal parameters are determined. The aim should be to prevent any embryos from lysing and still achieve a fusion in at least 90% of the cases. If the lysing rate is high, either the pulse is too strong or too long, or the AC field is too high. If the fusion rate is too low, the problem is that the pulse is too weak or too short.

As a guideline, the following settings can be used for electrodes with 250 μm distance:

1. 1 or 2 pulses.
2. 30-V and 40-μs pulse.
3. 1–2-V AC field.

The following settings can be used for electrodes with 1000 μm distance:

1. 2 pulses.
2. 137-V and 26-μs pulse.
3. 2-V AC field.

It is also important to keep in mind that the embryos should be kept in the nonelectrolyte solution for as short time as possible.

1. If available, it is convenient to use one microscope for the fusion and a second for embryo washes. Otherwise, one microscope is enough, as described below.
2. Switch on the electrofusion apparatus about 30 min in advance. Set the appropriate parameters for the number of pulses, AC or DC field, and the pulse duration.

3. Place the electrode chamber in a 100-mm tissue culture dish. It is advisable to prevent the chamber from moving in the dish by applying a small amount of high-vacuum grease under the plate.
4. Place 2 large drops of M2 medium in the dish. Place 1 large drop of mannitol solution in the dish and another large drop on the electrodes.
5. Place all the embryos in one of the drops of M2.
6. From this step onward, work with no more than 20–30 embryos at a time.
7. Move the first group of embryos to the mannitol drop in the dish. Pick them up again, and move them to the mannitol solution between the electrodes.
8. The embryos will orient correctly within seconds in most cases. Embryos that have not managed to do so can be maneuvered by blowing mannitol solution on them with the mouth pipet.
9. Push the trigger as soon as all embryos have taken the right position.
10. Move the embryos to the second M2 drop in the dish.
11. Repeat the process with all remaining embryos.
12. Wash the embryos through several drops of M16 or KSOM media, and return them to the incubator.

The mannitol solution in drop C should be replaced with fresh solution every 15 min (*see* **Note 6**).

3.5.2. Fusion Process, Selection, and Culture of Fused Embryos

Very soon after the electrofusion treatment, the cell membrane between the two blastomeres will dissolve, and the embryos will slowly change shape in a way reminiscent of a "reverse cell division" (**Fig. 3**). Some embryos will start this process very soon, whereas others may take as long as 60 min to complete the fusion.

Since the embryos are already near to their second mitotic division when the fusion is being performed, they will start dividing again soon after. Hence it is important to monitor the embryos carefully every 10–15 min and to move the successfully fused embryos away from the nonfused ones. If this is not done, tetraploid divided embryos may get confused with diploid nondivided ones. One should also carefully monitor the morphology of each individual embryo in order to screen away those in which one blastomere has lysed during fusion. Under optimal conditions, the rate of unfused and lysed embryos should not exceed 5–10%.

3.6. Aggregation of Cleavage Stage Embryos with ES Cells

3.6.1. Preparation of the Aggregation Plate

The aggregation plate is used for assembly and overnight culture of aggregates consisting of ES cells and diploid morula stage embryos or tetraploid

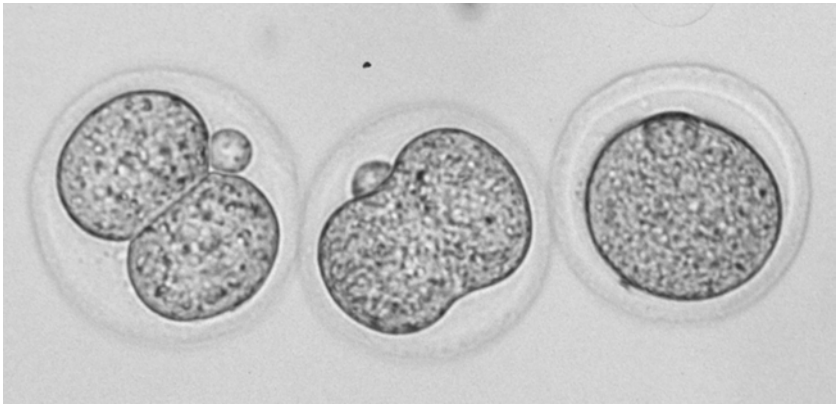


Fig. 3. Embryos undergoing the electrofusion process.

embryos. Using an aggregation needle, small depressions are indented into the plastic. The plate is set up as in **Fig. 4A**, for example. The top and bottom contain 3 drops of KSOM or M16, for the selection of ES cell clumps of the correct size. The middle contains 10 drops, with indentations for setting up the aggregates. One of these latter drops is shown in **Fig. 4B** and **C**.

The size and shape of the wells are very important parameters. Wells that are too shallow will not keep the embryos in place, whereas it is difficult to collect aggregated blastocysts from wells that are too deep. The wells should also have a very smooth surface to prevent embryos from attaching to the plastic (which causes damage during collection; *see* **Note 7**). Good aggregation needles can be purchased from BLS (*see* **Subheading 2.4.4.**).

1. Place microdrops (3–10 mm in diameter) of KSOM media into a 35- or 60-mm plastic tissue culture dish. The size of the dish and the organization of the drops is a matter of personal preference. However, it is important to reserve only 1–3 drops for the final selection of ES cell clumps (**Fig. 4A**).
2. Cover the drops with embryo-tested light paraffin oil.
3. Number the majority of the drops by using a thin-tipped marker pen and writing in a mirrored fashion from below on the bottom of the dish (optional).
4. Clean the darning needle with 70% EtOH.
5. Make six to eight compression wells in each of the numbered drops by pressing the darning needle into the plastic and making a circular movement. Do not twist the needle. Check that the wells are smooth and have the right depth to hold an embryo without getting too deep (**Fig. 4B**). Leave the remaining drops without wells.
6. Place the dish in the incubator for at least 30 min before use. (It is better to prepare the dishes 1–2 h in advance, or even let them incubate overnight.)

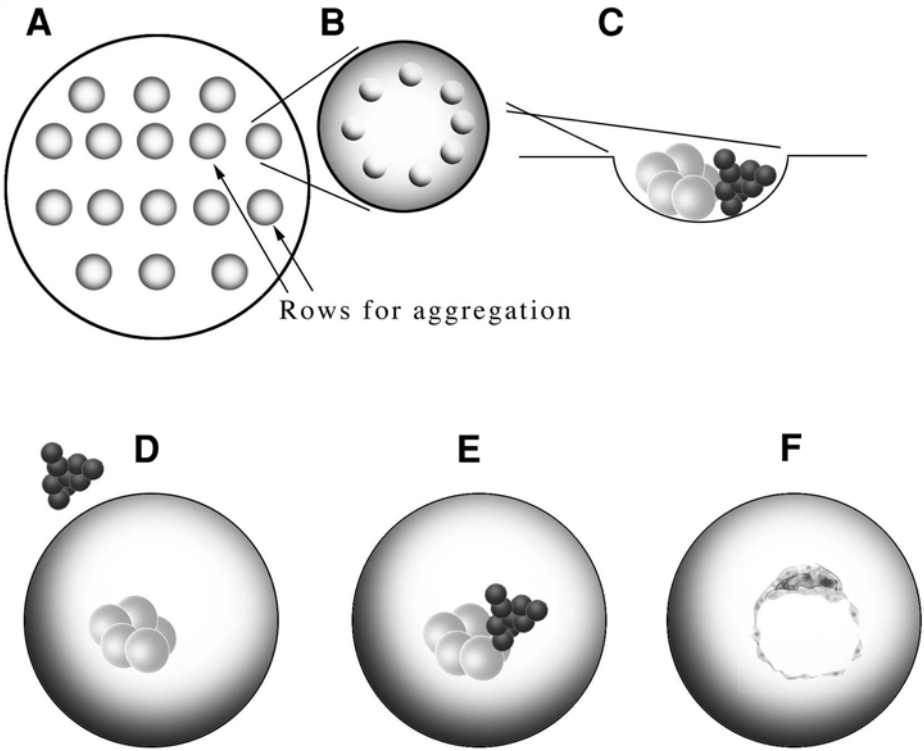


Fig. 4. **(A–C)** Aggregation plate and arrangement of depression wells and aggregates **(A)** Aggregation plate with microdrops. **(B)** Microdrop with depression wells **(C)** Sideview of one microdrop containing a diploid embryo and an ES cell clump. **(D–F)** Assembly of aggregates. **(D)** Embryo in depression well after removal of the zona pellucida. A clump of ES cells is placed near the well for final selection. **(E)** The ES cell clump is moved into the well in tight connection to the embryo. **(F)** Blastocyst stage embryo after overnight culture.

3.6.2. Preparation of Embryos for Aggregation

3.6.2.1. Judgment of Embryo Quality

Only embryos of the highest quality should be used in aggregation experiments. Diploid embryos should be at the 8-cell to morula stage, and tetraploid embryos should have three or four blastomeres. The embryos should be spherical, without any dead blastomeres. A portion of the tetraploid embryos will fall into delayed development after fusion. These embryos will not have reached the 3- or 4-cell stage at the time of aggregation. Delayed embryos should be discarded, since they are less efficient in supporting embryonic development.

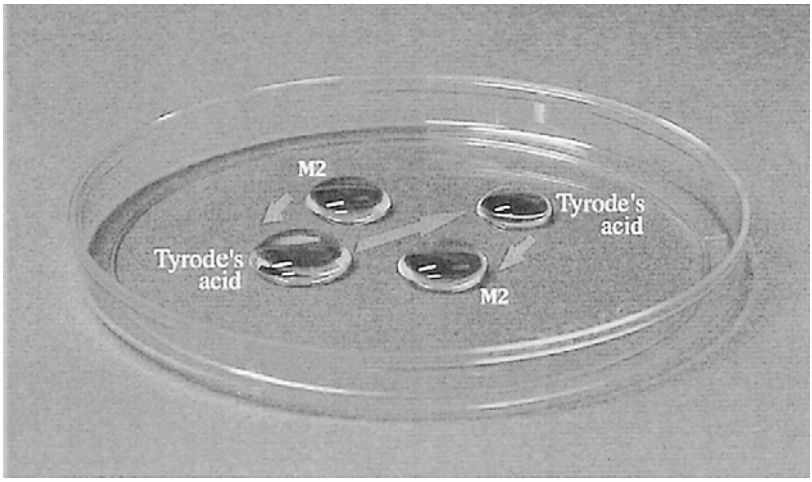


Fig. 5. Tissue culture dish with drops of M2 media and Tyrode's acid solution for removal of the zona pellucida.

3.6.2.2. Removal of The Zona Pellucida

The zona pellucida can be removed chemically with acidic Tyrode's solution (pH = 2.5) (**Fig. 5**). Since this method works fast, it requires exact timing to avoid damaging the embryos. Essential for the procedure is to avoid transfer of too much media to the Tyrode's solution. Critical for the viability of the embryos are multiple washes with KSOM or M16 media at the end of the procedure to remove the Tyrode's solution (*see* **Note 8**).

1. Move the embryos to M2 medium.
2. For each batch of 15–20 embryos: place 2 drops of M2 and 2 drops of Tyrode's acid in a sterile 100-mm tissue culture dish.
3. Place a group of embryos in the first M2 drop.
4. Move the embryos with as little media as possible to the first Tyrode's acid drop, and then immediately to the second.
5. Agitate the embryos carefully by pipeting them around with the mouthpipet, while carefully observing the zona pellucida. Move the embryos as soon as the zona disappears to the second drop of M2 media.
6. Repeat the procedure with the remaining groups of embryos.

3.6.3. Assembly of Embryo/ES Cell Aggregates

The same protocol can be used for setting up aggregations with both diploid and tetraploid embryos. For R1 ES cells to be most efficiently internalized, the clump should consist of 10–15 loosely connected cells. For other ES cell lines,

the number of cells might be different and has to be tested. In our experiments using E14 ES cells, we found that clumps of 5–10 cells gave the best results. An alternative to the aggregation method is to coculture the embryos on a lawn of ES cells for 3–4 h and subsequently divide the embryos to separate wells. Both methods work equally well; however, the amount of ES cells attached to each embryo in the coculture method is harder to control than in the aggregation technique ***(25)***. Aggregation of two embryos with each other, without any ES cells, can be done the same way as described below, by placing two embryos and no ES cells in each well. It is important to continue with the assembly of the aggregates as soon as possible after the zona removal.

1. Wash the zona-free embryos through a few drops of equilibrated culture media, and then move them to the aggregation plate.
2. Pick up each embryo individually, and place them carefully into the depresssion wells.
3. Trypsinize the ES cells as described in **Subheading 3.1.1.**
4. Select a number of clumped ES cells of the approximately right size, take them up in a transfer capillary, and divide them into the drops, which do not contain wells/embryos. Do not place more than 80–100 clumps per drop.
5. Carefully select clumps with 10–15 loosely connected cells, and move them into the drops with embryos, at a distance from the wells (*see* **Note 9**; **Fig. 4D**). Move one clump at a time into each well, and let it fall onto the embryo (**Fig. 4E**).
6. Make sure that the embryo is in close contact with the ES cell clump (**Fig. 6**).
7. Continue in the same manner with the remaining embryos, and place the aggregation dish in the incubator overnight.

ES cell ↔ tetraploid aggregates are generally set up by "sandwiching" the ES cells between two tetraploid embryos (although one embryo is sufficient) ***(26)***. The rational for this is that tetraploid embryos have only half the number of blastomeres of diploid embryos.

Aggregations between clumps of ES cells and tetraploid embryos are performed as described above, with the exception that two 4-cell stage tetraploid embryos should be transferred into each depression.

In this case, the ES cell clumps can be placed in the wells first and the two embryos carefully dropped in afterward. The final picture should resemble a triangle, in which the two embryos and the cell clumps all have direct contact with each other. This assembly can also be used for aggregating three embryos together without any ES cells, for example, two tetraploid and one diploid embryo.

3.6.4. Embryo Culture and Transfer

Most of the aggregated embryos should develop into blastocysts after overnight culture (**Fig. 4F**; *see* **Note 10**). Some aggregates may be delayed

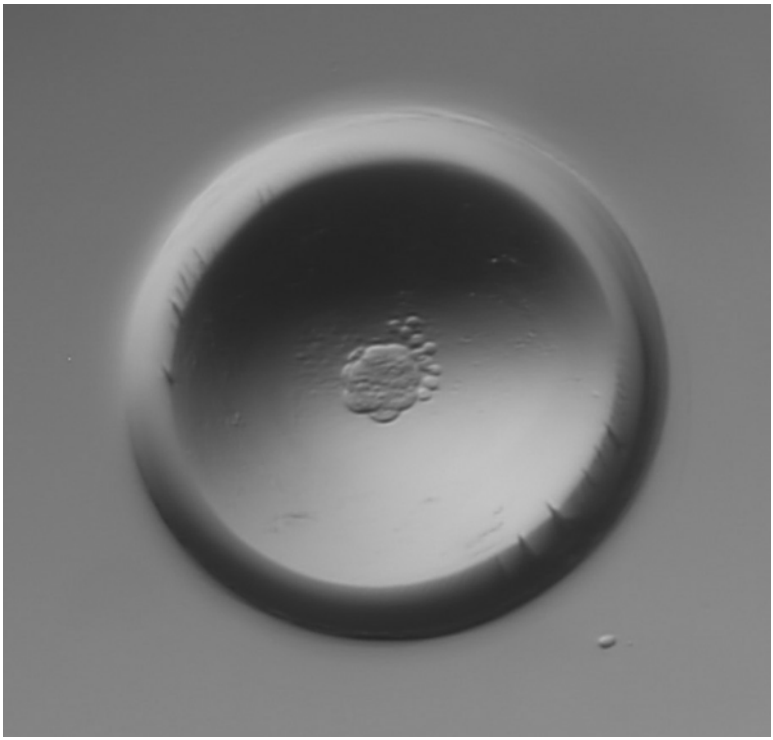


Fig. 6. Diploid embryo and ES cell clump in depression well.

and may more resemble a compacted morula. These embryos will catch up with time and can either be transferred together with the blastocysts or given another day in culture before they are transferred.

Blastocysts are generally transferred into the uterus of E2.5 pseudopregnant foster females. However, it is also possible to transfer them to the oviduct of E0.5 pseudopregnant females. The concern that embryos without a zona would stick together during their way through the oviduct to the uterus is ungrounded. The efficiency of such transfers in terms of number of pups born is comparable to the results from the classical uterus transfer regimen. There is in fact a wide range of possibilities for matching different embryo stages into the reproductive tract of pseudopregnant females (**Table 2**). Common to them all is the fact that the mother should be synchronized with or "behind" the embryos. The pregnancy will always follow the foster mother's timing, and embryos with a more advanced developmental stage will simply be slightly delayed at the stage of implantation. Hence, these pups will be born later than if they had been left in the donor female (*see* **Note 11**).

Table 2
Possible Combinations of Embryo Age, Transfer Method, and Pregnancy Stage of Foster Females[a]

	Embryo				
Recipient	E0.5	E1.5	E2.5	E3.5	E4.5
E0.5	Oviduct	Oviduct	Oviduct	Oviduct	Oviduct
E1.5	—	—	—	—	—
E2.5	—	—	Uterus	Uterus	Uterus

[a]Transfer of embryos into the reproductive tract of E1.5 pseudopregnant females is possible but not recommended due to low and unpredictable efficiency.

The number of embryos to transfer per female is also rather flexible. It is advisable to aim for at least 4–5 pups born, and preferably not many more than 10–12. If the litter size is too small, the few pups will grow very large *in utero*, causing difficulties at delivery. If, on the other hand, the litter is very large, some pups may fail to survive, owing to competition over milk with their littermates. Optimally, we recommend transferring 8 embryos per uterine horn, which gives a total of 16 embryos per female. Assuming that on average only 50% of the transferred embryos will go to term, the litter size will thus be optimal. However, it is possible to transfer as few as 6 embryos into a single uterine horn, or as many as 12 embryos in each horn in case there is a shortage of foster females or embryos available.

3.6.4.1. Preparation of Avertin (Anesthetic)

Avertin is a widely used anesthetic for mice, which provides surgical anesthesia with a duration of approximately 30 min. A 100% stock solution is prepared by mixing 10 g 2,2,2-tribromoethyl alcohol (Sigma Aldrich) with 10 mL tert-amyl alcohol (Sigma Aldrich). A 2.5% working solution is prepared by diluting the concentrated stock in sterile water. Concentrated stock solutions should be stored at–20°C and the working solution at 4°C. Avoid repeated freeze/thawing, and protect the solutions from light. Each batch should be tested both for possible toxicity and determination of required dosage. The dose varies slightly for each preparation, with a normal range of 14–18 μL/g bodyweight.

3.6.4.2. Preparation of Ketomine /Xylazine (Anesthetic)

A mixture of ketamine and xylazine can be used as an alternative to avertin. The duration of the anesthesia is approximately the same, or slightly shorter.

A working solution is prepared by mixing 100 mg ketamine hydrochloride (Bayer) with 800 µL xylazine 2% solution (Bayer), and adding 7 mL sterile water. The dose for surgical anesthesia is 10 µL/g body weight.

1. Wash the embryos through 2 drops of M2 medium, using a transfer capillary soaked in 1% albumin solution.
2. Take up M2 medium in a transfer capillary, and load two big and one small air bubble in it. Take up the embryos after the small air bubble. Take up another small air bubble and a very small amount of medium. This way, good control is achieved in the transfer capillary, and one does not risk losing the embryos.
3. Anesthetize the mouse by injecting the appropriate dose of ketamine/xylazine or avertin ip. Moisten its eyes with sterile PBS. (This is important, since the eyes will remain open during the anesthesia, and it is extremely painful for the mouse if the cornea dries out!)
4. Place the mouse on its side. Shave (optional) and wash the incision area with 70% EtOH.
5. Make an incision just behind the last rib, right under the spine. Clean away all loose hair.
6. Locate a whitish area through the abdominal wall. This is the fatpad connected to the ovary. Make an incision, and pull out the fatpad.
7. Cut through the connective tissue to the kidney, and place the upper part of the uterus outside the incision.
8. Make a hole in the uterus wall with a 27- or 30-gage needle, and immediately insert the transfer capillary containing the embryos.
9. Blow in the embryos with the two small air bubbles–not more!
10. Put the organs back under the abdominal wall, and seal the wound in the skin with a clip.
11. Place the mouse in warmth and in peace and quiet until it has woken up.

Oviduct transfers are performed similarly, with the exception that the embryos are placed in the oviduct through the infundibulum. The bursa surrounding the ovary is carefully ruptured, and the transfer capillary (with the loaded embryos) is inserted in the infundibulum (*see* **Note 12**).

3.7. Cesarean Section

The percentage of embryos that develop to term might depend on the degree of chimerism and is usually far lower than the number of embryos transferred to the recipient. When only one or two embryos develop to term, the embryos tend to be larger than normal and the delivery might therefore be difficult. To save such litters, the recipient has to be sacrificed and the fetuses delivered by cesarean section and cross-fostered to a new mother. It is essential to have replacement foster mothers available by setting up normal matings that precede the pseudopregnant recipient's day of delivery by 1 or 2 days.

A cesarean section is not easy to perform, but with some practice, it is a very reliable procedure, and viable pups are rarely lost. It is essential to work fast, accurately, and with great care. The pups are surprisingly resistant to hypoxia, but they should be cleaned and dried as soon as possible after recovery from the uterine environment to avoid hypothermia.

If ES cells have been aggregated with tetraploid embryos, the chance of getting live-born and surviving pups is very low. In these cases, planned cesarean sections are recommended to avoid the possibility of the mother eating any stillborn pups (*see* **Note 13**).

1. Kill the foster mother on her day E18.5 if a cesarean section is planned. If the section is performed due to difficulties in giving birth, it should be performed at the latest 12 h after expected delivery.
2. Place the mother on her back, and make an incision in the skin and abdominal wall as described in **Subheading 3.2.**
3. Locate the pups in the uterus, and determine their orientation. (The dark placenta can be seen through the uterus wall and indicates the pup's ventral part.)
4. Grasp the uterus wall with sharp scissors at the middle dorsal part of the first pup.
5. Cut a small hole in the uterus wall right at the forceps. Very carefully, without hurting the pup, insert the tip of the scissors in the hole, and cut the uterus open so that the pup can be squeezed out.
6. Push the pup out of the uterus with the forceps, and cut the umbilical cord.
7. When all pups have been removed from the uterus, collect them into soft tissue paper, and dry them carefully.
8. The yolk sac can by used for genotyping by PCR or Southern analysis if required.
9. Place the pups onto a heat pad or under a desk lamp.
10. Keep moving, wiping, rubbing, and turning them around until they breath regularly and turn pink.
11. Place the pups with a replacement foster mother, and mix them with her own pups. It is advisable to use a foster mother who has pups with a different coat color marker than the fostered pups, for distinguishing them later.

3.8. Analysis of Offspring

The detection of components in chimeras made by diploid embryo aggregation was initially achieved by using coat color markers. Since then, several markers have been developed to be able to follow *in situ* the spatial distribution of the two components in a chimera. The markers most commonly used are glucose phosphate isomerase (GPI), human placental alkaline phosphatase (hPLAP) ***(27)***, *lacZ* ***(28,29)***, and green fluorescent protein (GFP) ***(30)***. GPI is a traditional marker for chimera studies. It is based on polymorphisms in

the GPI gene. Three electrophoretically distinct alleles of the gene have been found in laboratory strains. Using different strains for embryo aggregations yields chimeras with varying amounts of the different isozymes, allowing one to follow the contribution of mutant cells to the various tissues. Although GPI is a highly sensitive marker, it is not suitable for analyzing chimeras at the single cell level unless the tissue was brought into single cell suspension and cell sorting is applied. The other markers listed above do not have this disadvantage.

The most popular marker is the bacterial β-galactosidase enzyme encoded by the *lacZ* gene. It is used in many targeting constructs to tag the targeted gene expression in ES cell derivatives in the chimera and to follow the fate of deficient cells in the mutant offspring. Embryos up to day 11.5 days post coitum can easily be stained in whole mount. Later stages of development require cryostat sectioning of the embryos and subsequent staining. The ubiquitous *lacZ* expressor ROSA 26 gene trap line ***(31)*** is frequently used when tagging one of the chimera components is required. hPLAP, in contrast to mouse alkaline phosphatase, is heat-resistant and therefore an excellent marker in the mouse system. A short incubation at 70°C destroys all endogenous mouse phosphatases, which are widely expressed during embryogenesis and adult life, while the reporter enzymatic activity remains. In comparison with lacZ, the hPLAP stain does not penetrate tissues that well, but its detection is at least as sensitive as that of lacZ.

GFP is a protein derived from the jellyfish *Aequorea victoria*, which emits green light upon illumination with blue light. Use of GFP (and other color variants) in genetic marker systems allows one to follow temporal and spatial expression in vivo in developing embryos and adult mice and to sort GFP-positive cells by fluorescence-activated cell sorting (FACS). These are unique features in comparison with the other markers. However, GFP activity is lost by most fixation procedures or sectioning and therefore its use is mostly limited to studying whole embryos, thick sections, or single cell suspensions. GFP has already been successfully used as a vital transgenic reporter system in the mouse to identify and purify chimeric compartments in specific cell types ***(32)***, to determine the sex of the embryo as early as preimplantation stages ***(33)***, and to report on specific gene expression ***(34)***. More detailed protocols on reporter systems are provided in Chapter 7.

Apart from the use of ES cell aggregation chimeras as a laboratory tool to analyze mutant phenotypes represented by either the ES cell or the embryo compartment, these chimeras are efficiently used to obtain germline transmission from the ES cell component (*see* **Note 14**). We expect that more and more cell lines, which are compatible with this means of introducing mutations in vivo, will be identified. The ease and efficiency of generating chimeras by

aggregation will then be recognized in more and more applications and fields of experimental biology and medical research using the mouse as a model system. We hope that this collection of protocols will encourage the reader to establish this method as a standard laboratory technique available for phenotype dissection or the introduction of ES cell-mediated genome alterations in the mouse.

4. Notes

1. ES cell culture. All possible care should be taken when culturing and handling ES cells. This includes strict sterile techniques, the use of freshly and accurately prepared media (kept for a maximum of 14 days at 4°C), very careful daily monitoring and media change, and passaging at the appropriate density.
2. Karyotyping. Care should be taken not to damage the cells during fixation. Avoid extensive pipeting, and add the fixative in steps according to the protocol.
3. Embryo culture conditions. The embryos are exposed to in vitro conditions for a few hours if they are injected, but for more than 24 h if they are aggregated (more than 48 h for tetraploids). Therefore, having optimal culture conditions is much more critical in the latter case. Frequently, suboptimal culture conditions are the reasons behind failure to establish the aggregation technique.
4. Superovulation. It is essential to keep prepared hormones ready at a constant –20°C or –80°C. Avoid repeated freeze-thawing. It is also very important to keep the light-dark cycle constant in the animal room. The timing of hormone injection and embryo collections is also critical.
5. Embryo handling. The time during which the embryos are exposed to the bench environment should be kept to a minimum. Care should be taken to wash the embryos through at least 3 drops of media when moving them between M2 and M16/KSOM. M16/KSOM media used for washes should be equilibrated in the incubator.
6. Electrofusion. It is important not to leave the embryos in the mannitol solution for longer than absolutely necessary, and they should be placed back at 37°C as soon as possible after the electric pulse has been applied. Embryos in which one blastomere has been damaged should be sorted out immediately after fusion. Successfully fused embryos should be moved to a new drop or dish as soon as the fusion is completed. The whole process takes a considerable amount of time, and it should be followed and handled with great care to ensure that no embryos are wasted and that no diploid embryos are used due to confusion.
7. Microdrops and depression wells. The size of the microdrops should be kept small, and the depression wells should be deep and smooth, to keep embryos from "jumping out" when moving the dish to and from the incubator. For beginners, we recommend no more than 40–50 aggregates in the dish at a time, to keep the time of manipulation at room temperature minimal.
8. Removal of the zona pellucida. The embryos will become very sticky immediately after removal of the zona pellucida. It is advisable to keep them floating by blowing them around in the acid and for 20–30 s in the M2 after the zona has

disappeared. Do not use warmed-up Tyrode's acid; it should be kept at room temperature maximum. Use bacteriologic grade plastic or the lid of a tissue culture grade dish, since the surface treatment of TC dishes will makes the embryos stick to the plate.

9. Aggregation. The embryos should not be overloaded with ES cells. Clumps containing a maximum of 10–15 cells should be chosen. Too high numbers of ES cells will kill the embryo.
10. Harvesting aggregates. When moving the aggregates out of the wells (after overnight culture), care should be taken not to damage them. Instead of aspirating them directly from the wells with the mouthpipet, it is better to "blow" media into the wells. This way, the embryos will smoothly "jump out" of the wells and can then be easily collected for transfer.
11. Choice of recipient females. It is important to use pseudopregnant foster females of a stain/stock known to produce good mothers. They should optimally be 8–12 weeks old and weigh 28–35 g if CD1 or ICR outbred animals are used. Only females in perfect health should be chosen.
12. Postsurgery care. Females that have undergone surgery should be kept warm until they regain consciousness. This can be achieved by placing the cage on a heating pad or under a warming lamp and/or by loosely wrapping the mice in tissue paper.
13. Cesarean section. If the recipient female has not littered down in the afternoon of her expected date of delivery, a cesarean section has to be performed immediately. The most likely reason for the difficulty is small litter size, which lets a few pups grow very large. These pups can be rescued if they are sectioned out in time.
14. Germline transmission. It is not unusual for chimras to produce only wild-type pups in their first litters and show germline transmission only later. It is therefore advisable not to mate chimeric mice too early (minimum 6 weeks of age) and not to expect germline transmission immediately. In case of aggregation chimeras, mixed germline transmission (when both host and ES cell compartments are transmitted) is rare. Most of the time the chimeras are either overall ES cell or nontransmitters. The nontransmitters mature earlier; therefore the first litters of the germline test could be disappointing. Do not panic, and wait for the performance of the slowpokes.

References

1. Venter, J. C., Adams, M. D., Myers, E. W., et al. (2001) The sequence of the human genome. *Science* **291,** 1304–1351.
2. Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P., and Trent, J. M. (1999) Expression profiling using cDNA microarrays. *Nat. Genet.* **21,** 10–14.
3. Ferea, T. L. and Brown, P. O. (1999) Observing the living genome. *Curr. Opin. Genet. Dev.* **9,** 715–722.
4. Justice, M. J., Zheng, B., Woychik, R. P., and Bradley, A. (1997) Using targeted large deletions and high-efficiency N-ethyl-N-nitrosourea mutagenesis for functional analyses of the mammalian genome. *Methods* **13,** 423–436.

5. You, Y., Browning, V. L., and Schimenti, J. C. (1997) Generation of radiation-induced deletion complexes in the mouse genome using embryonic stem cells. *Methods* **13,** 409–421.
6. Nolan, P. M., Peters, J., Strivens, M., et al. (2000) A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nat. Genet.* **25,** 440–443.
7. Hrabe de Angelis, M. H., Flaswinkel, H., Fuchs, H., et al. (2000) Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat. Genet.* **25,** 444–447.
8. Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A., and Nagy, A. (1993) Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365,** 87–89.
9. McLaren, A. *Mammalian Chimeras*, book section. Cambridge University Press, Cambridge, UK, 1976.
10. Tarkowski, A. K. (1961) *Nature* **190,** 857.
11. Moustafa, L. A. and Brinster, R. L. (1972) Induced chimaerism by transplanting embryonic cells into mouse blastocysts. *J. Exp. Zool.* **181,** 193–201.
12. Evans, M. J. and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154–156.
13. Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78,** 7634–7638.
14. Beddington, R. S. and Robertson, E. J. (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* **105,** 733–737.
15. Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309,** 255–256.
16. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A., and Rossant, J. (1998) Promotion of trophoblast stem cell proliferation by FGF4. *Science* **282,** 2072–2075.
17. Kubiak, J. Z. and Tarkowski, A. K. (1985) Electrofusion of mouse blastomeres. *Exp. Cell Res.* **157,** 561–566.
18. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90,** 8424–8428.
19. Nagy, A. and Rossant, J. (2001) Chimeras and mosaics for dissecting complex mutant phenotypes. *Int. J. Dev. Biol.* **45,** 577–582.
20. Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* **326,** 292–295.
21. Torres, R. M. and Kühn, R. (1997) *Laboratory Protocols for Conditional Gene Targeting*. Oxford University Press, Oxford.
22. Lawitts, J. A. and Biggers, J. D. (1993) Culture of preimplantation embryos. *Methods Enzymol.* **225,** 153–164.

23. Erbach, G. T., Lawitts, J. A., Papaioannou, V. E., and Biggers, J. D. (1994) Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol. Reprod.* **50,** 1027–1033.
24. Carmeliet, P., Ferreira, V., Breier, G., et al. (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435–439.
25. Wood, S. A., Pascoe, W. S., Schmidt, C., Kemler, R., Evans, M. J., and Allen, N. D. (1993) Simple and efficient production of embryonic stem cell-embryo chimeras by coculture. *Proc. Natl. Acad. Sci. USA* **90,** 4582–4585.
26. Shimada, H., Kaname, T., Suzuki, M., et al. (1999) Comparison of ES cell fate in sandwiched aggregates and co-cultured aggregates during blastocyst formation by monitored GFP expression. *Mol. Reprod. Dev.* **52,** 376–382.
27. DePrimo, S. E., Stambrook, P. J., and Stringer, J. R. (1996) Human placental alkaline phosphatase as a histochemical marker of gene expression in transgenic mice. *Transgenic Res.* **5,** 459–466.
28. Goring, D. R., Rossant, J., Clapoff, S., Breitman, M. L., and Tsui, L. C., (1987) In situ detection of beta-galactosidase in lenses of transgenic mice with a gamma-crystallin/lacZ gene. *Science* **235,** 456–458.
29. Le Mouellic, H., Lallemand, Y., and Brulet, P. (1990) Targeted replacement of the homeobox gene Hox-3.1 by the Escherichia coli lacZ in mouse chimeric embryos. *Proc. Natl. Acad. Sci. USA* **87,** 4712–4716.
30. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997) 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407,** 313–319.
31. Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G., and Soriano, P. (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **94,** 3789–3794.
32. Hadjantonakis, A. K., Gertsenstein, M., Ikawa, M., Okabe, M., and Nagy, A. (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech. Dev.* **76,** 79–90.
33. Hadjantonakis, A. K., Gertsenstein, M., Ikawa, M., Okabe, M., and Nagy, A. (1998) Non-invasive sexing of preimplantation stage mammalian embryos. *Nat. Genet.* **19,** 220–222.
34. Godwin, A. R., Stadler, H. S., Nakamura, K., and Capecchi, M. R. (1998) Detection of targeted GFP-Hox gene fusions during mouse embryogenesis. *Proc. Natl. Acad. Sci. USA* **95,** 13042–13047.

12

Adenovirus-Mediated Gene Transfer

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1. Introduction

The injection of a recombinant adenoviral vector into the tail vein of a mouse results in highly preferential infection of the liver and subsequent liver-specific expression of the genes that are inserted into the adenoviral backbone. These characteristics of systemic adenovirus injection, and the fact that adenoviral vectors are relatively easy to generate and amplify to high titers, provide an exquisite and extremely powerful means to investigate the effects of liver-specific expression of a given gene. Moreover, since any transgenic mouse model can be injected with adenoviral vectors, this technology allows rapid analysis of (trans)gene-gene interaction. This chapter focuses on the generation and application of first-generation adenoviral vectors to express genes specifically in the livers of mice. First-generation vectors reach peak transgene expression typically 4–5 days after tail vein injection, and expression can be detected for up to 2 weeks after injection. Thus, the biologic effects of the transgene product should be detectable within this relatively short period of transgene expression. However, it has been demonstrated for many physiologic processes, including, for example, lipoprotein metabolism and blood coagulation, that this period of gene expression is sufficient to determine the effect of overexpression of the protein under investigation.

Several approaches can be taken to modulate gene expression in the liver. Overexpression of the unmodified gene of interest is the most straightforward. This gene may or may not be normally expressed in the liver and can encode either an intracellular protein, a membrane protein, or a secreted protein. Alternatively, expression of a dominant variant of a gene allows analysis in the presence of a functional endogenous gene. In this setting the dominant

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

variant of the protein overrides and modulates normal function. This could, for example, be achieved by mutating the gene in regions that encode protein domains or sites that are essential for proper function such as dimerization, DNA binding, phosphorylation, or internalization. Naturally occurring dominant mutants are of course the most obvious candidates for such an approach.

In the following sections, the different steps necessary to go from a candidate gene to injection of the adenoviral vector into a mouse will be discussed. These steps include construct design, generation of the adenoviral vector, expansion and purification of the adenoviral vector, in vitro analysis of gene expression, and finally injection into the mouse. If an adenoviral vector has been obtained by collaboration, the later sections on expansion, purification, and application may be helpful. Since the development of adenoviral vectors is progressing rapidly, two short sections are added discussing novel generation of adenoviral vectors and the retargeting of adenoviral vectors to infect tissues other than the liver.

2. Materials

2.1. Generation of Adenoviral Vectors by Homologous Recombination in Bacteria (Ad-Easy System)

2.1.1. Generation of the Adenoviral Plasmid Construct

1. Adenoviral backbone vector: pAdEasy-1 or pAdEasy-2.
2. Shuttle vectors: pShuttle, pAdTrack, pShuttle-CMV or pAdTrack-CMV.
3. Restriction endonucleases of specific interest: *Pme*I, *Pac*I, *BstX*I.
4. High-efficiency electrocompetent *E. coli* BJ5183 cells.
5. 2.0-mm electroporation cuvets.
6. Gene Pulser electroporator (Bio-Rad).
7. L-broth (LB) medium.
8. LB-agar plates containing 50 μg/mL kanamycin.

2.1.2. Generation of Infectious Adenovirus

1. 293 or 911 cell line.
2. Tissue culture medium: Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, cat. no. 41966), supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 m*M* biotin, 20 m*M* glutamine.
3. 6-well tissue culture dishes.

2.2. Expansion of Adenoviral Vectors

1. 293 or 911 cells and tissue culture medium (*see* **Subheading 2.1.2.**)
2. Various sizes of tissue culture flasks.
3. Phosphate-buffered saline (PBS) with calcium and magnesium [Gibco-BRL, cat. no. 14040 (PBS++)].

4. Horse serum (HS).
5. PBS++ with 2% HS (PBS/HS).
6. Tissue culture medium with 2% HS.

2.3. Purification of Adenoviral Vectors

1. CsCl solutions for ultracentrifugation:
   d = 1.45 g/cm^3: 610 g/L in TE, pH 8.0.
   d = 1.2 g/cm^3: 277 g/L in TE, pH 8.0.
   d = 1.33 g/cm^3: 450 g/L in TE, pH 8.0.
2. Ultracentrifugation tubes (Beckmann, cat. nos. 344059 and 342413).
3. Beckman SW41 rotor and Ti70 rotor or equivalent.
4. 10X TD buffer: 1.37 m*M* NaCl, 50 m*M* KCl, 7.3 m*M* Na_2HPO_4, 250 m*M* Tris, pH 7.8, autoclaved.
5. 200X Ca^{2+}/Mg^{2+}: 0.18 *M* $CaCl_2$, 0.1 *M* $MgCl_2$, autoclaved.
6. Dialysis buffer: 1X TD with 1X Ca^{2+}/Mg^{2+} in ddH_2O.
7. 10X sucrose solution in H_2O (50%), filter-sterilized.
8. 10X salt buffer: 1.4 *M* NaCl, 49 m*M* $Na_2HPO_4 \bullet 2H_2O$, 15 m*M* KH_2PO_4, pH 7.8, filter-sterilized.
9. Sucrose dialysis buffer: 1X sucrose solution with 1X salt buffer in ddH_2O.
10. Slide-a-lyzer cassettes (Pierce, MWCO: 10,000, volume: 0.5–3 mL, cat. no. 66425).

2.4. Analysis of Adenoviral Vector Preparations

2.4.1. Titration

1. PBS/HS (*see* **Subheading 2.3.**).
2. 2X agar solution: 1.7% Agar Noble (DIFCO, cat. no. 0142-01) in 40 m*M* HEPES, pH 7.2 (Gibco-BRL , cat. no. 15630), autoclaved.
3. 2X F-15 medium: double concentration medium made from powder (Gibco-BRL, cat. no. 52100); add pyruvate and sodium bicarbonate, filter-sterilize.

2.4.2. Detection of Replication-Competent Adenovirus

1. Forward primer: 5′-GGGTGGAGTTTGTGACGTG -3′ (=RCAf1).
2. Reverse primer: 5′- TCGTGAAGGGTAGGTGGTTC -3′ (=RCAr2).
3. 10X polymerase chain reaction (PCR) buffer: Perkin-Elmer buffer without $MgCl_2$.
4. Taq polymerase (AmpliTaq, Perkin-Elmer).
5. 25 m*M* $MgCl_2$.
6. 20 mg/mL Proteinase K.
7. 10 m*M* (2.5 m*M* per nucleotide) dNTPs.
8. 10 mg/mL tRNA.
9. H5dl7001, or wild-type Ad5, proteinase K-treated.
10. 10 ng/μL pJM17 DNA.
11. $T_{10}E_1$: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA.

3. Methods

Adenoviruses were first isolated as causal agents for adenoid inflammation in children; respiratory infection by adenovirus generally results in common cold symptoms. Close to 50 different serotypes of adenovirus have been described, of which serotype 5 is the most commonly used for vector construction. Adenoviruses are nonenveloped, regular icosahedrons that are 65–80 nm in diameter. Twelve fibers extend from the core of the virus, and these fibers consist of a shaft and a terminal knob. The terminal knob contains the domain that is recognized by the coxackie adenovirus receptor (CAR). Adenoviruses contain some 36 kb of linear DNA flanked by two inverted terminal repeats. The left inverted terminal repeat partially overlaps with the sequence that is necessary to package the DNA into the virion. Upon infection of a host cell by adenovirus, the DNA is unpackaged, and early transcription is initiated. The first and essential transcripts derive from the E1A region. The E1A proteins control transcription of the additional early region genes, E1B, E2, E3, and E4. Expression of the late genes, many of which encode structural components of the virion, is under control of these early genes. The early region genes also control viral DNA replication. Interestingly, the E1A proteins also have transforming capacity, and several immortalized human cell lines have been generated by E1A transformation. Of these, the HEK293 cell line is the most widely used. For reviews of adenovirus biology, *see* **refs. *1*** and ***2***.

First-generation adenoviral vectors are based on the premise that adenoviral vectors devoid of the E1A region are replication deficient and that by growing these E1A-deleted (ΔE1A) adenoviral vectors on a cell line that expresses the E1A proteins, such as 293, the ΔE1A vectors will be complemented and will replicate. The most commonly used systems to generate adenoviral vectors are based on a two-component system. Given the large size of the adenoviral vector DNA (36 kb), it is impractical to clone constructs directly into the full-length backbone using plasmid-derived vectors. Therefore, the gene of interest is cloned into a relatively small shuttle vector containing a terminal region of the adenovirus DNA. The shuttle vector plus insert is cotransfected with a large plasmid containing the remainder of the adenovirus DNA into either the 293 cells ***(3)*** or bacteria ***(4)***. Upon homologous recombination between the overlapping regions of both plasmids, full-length adenovirus DNA is regenerated (**Fig. 1**). The method, as developed by He et al. ***(4)*** in the Vogelstein laboratory and termed the Ad-easy system, was proved to be very reliable and practical in our hands (described below in **Subheading 3.7.**). Alternative methods for the generation of adenoviral vectors have been noted to be successful ***(5–10)*** but will not be discussed in this paper.

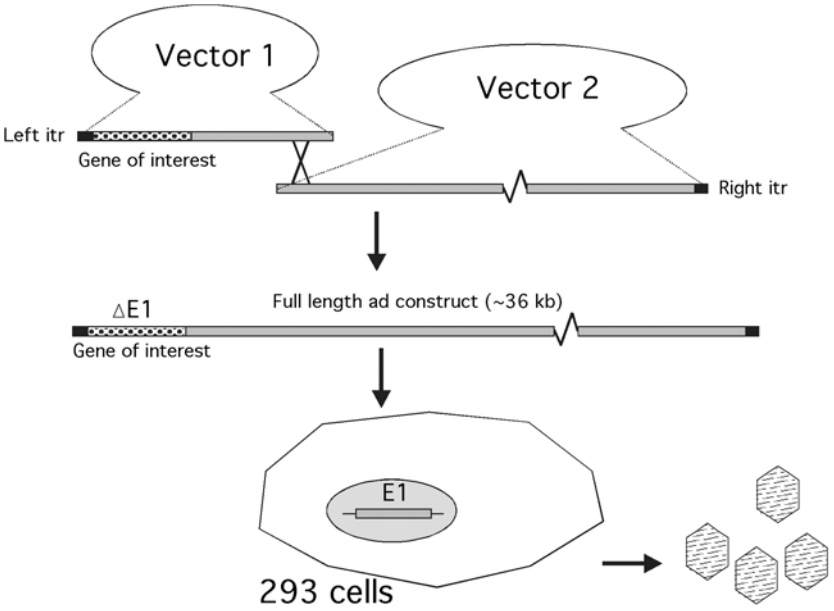


Fig. 1. Schematic representation of the generation of recombinant adenoviral vectors by homologous recombination. The gene of interest is cloned into shuttle vector 1, which contains the adenovirus left inverted terminal repeat (itr) and a region of several kbs immediately downstream from the E1 region. The deleted E1 region provides space necessary for insertion of the gene of interest. Upon homologous recombination with vector 2, which contains the remainder of the adenovirus genome up to the right inverted terminal repeat, full-length adenoviral vectors are generated. These E1-deleted vectors can be amplified on an E1-expressing cell line such as 293.

3.1. Generation of Adenoviral Vectors by Homologous Recombination in Bacteria: The Ad-Easy System

The general outline of the Ad-easy system is as follows: The gene of interest is first cloned into a shuttle vector. Four types of shuttle vectors are available, with and without a cytomegalovirus (CMV) promoter/polyadenylation site, and with and without separate CMV-driven green fluorescent protein (GFP) genes. The resultant (kanamycin-resistant) plasmid is linearized by digesting with the restriction endonuclease *Pme*I. Subsequently, this linearized vector and an (ampicillin-resistant) adenoviral backbone plasmid are cotransformed into *E. coli* BJ5183 cells. Two backbone plasmids are available differing in

the maximum size of the insert they will accept.* Recombinants are selected for kanamycin resistance, and recombination is confirmed by restriction endonuclease analyses. The use of *E. coli* BJ5183 cells for recombination is critical for efficient recombination to occur. The final step consists of transfecting linearized recombinant plasmid into adenovirus-packaging cell lines. Upon transfection into E1A-expressing cells, recombinant adenoviruses are typically generated within 7–12 days.

The Ad-easy system can be obtained from the Vogelstein laboratory (vogelbe@welchlink.welch.jhu.edu). They also maintain an excellent website for additional information, help, and protocols: www.coloncancer.org/adeasy/protocol.htm

The E1A-expressing 293 cell line is widely available or can be purchased through the ATCC (www.atcc.com). This cell line is appropriate for the generation and amplification of adenoviral vectors. An alternative cell line that should be considered is the 911 cell line ***(11)***. In contrast to the 293 cell line, the Ad5 genome region present in the 911 cell line is completely known. Moreover, 911 cells frequently give better virus yields and vastly outperform 293 cells in the plaque assay (*see* **Subheading 3.4.1.**). The 911 cell line is available from CruCell B.V., Leiden, The Netherlands (www.crucell.com).

3.1.1. Generation of the Adenovirus Plasmid Construct

1. Subclone your gene of interest into the multiple cloning site of the appropriate shuttle vector.
2. Linearize the newly constructed plasmid with *Pme*I (make sure digestion is complete and/or gel-purify fragment, *see* **Note 1**).
3. Ethanol-precipitate and resuspend the DNA in ddH_2O.
4. Prepare adenoviral backbone plasmid DNA (pAdEasy-1) resuspended in ddH_2O (preferably, use *E. coli* Stbl2™ cells from Gibco-BRL to propagate large plasmids at 30°C).
5. Mix 1.0 μg linearized shuttle plasmid DNA with 100 ng supercoiled adenoviral plasmid DNA in a total volume of 5–10 μL.
6. Add 100 μL of electrocompetent BJ5183 cells and electroporate in 2.0-mm cuvets at 2.5 kV, 200 W, and 25 mF in a Bio-Rad Gene Pulser. (These settings should give a time constant between 4 and 5 ms.)
7. Immediately add 1 mL of LB medium and incubate at 30°C for 30 min.
8. Plate different amounts of cell suspension on LB kanamycin plates and incubate at 30°C for 24 h.
9. Pick colonies and prepare miniprep DNA according to standard procedures. Note that the smaller colonies often represent the recombinants.

*Please note: the adenoviral backbone that is suitable for larger inserts requires a special cell line for vector generation and expansion; see web site for details.

10. Screen clones by analyzing their supercoiled sizes on agarose gels, comparing them with pAdEasy-1 and shuttle vector controls.
11. Subsequently, digest DNA from positive clones with *Bst*XI. If the insert does not harbor *Bst*XI sites, the 8.2-kb DNA fragment found in pAdEasy-1 digested with *Bst*XI should be larger in the recombined clones (*Note:* sometimes the generated larger fragment runs as a doublet along with the 11-9 kb DNA fragment from pAdEasy-1).
12. Finally, confirm recombinant adenoviral clone(s) with additional restriction enzyme digests.

3.1.2. Generation of Infectious Adenovirus

1. Grow 911 or 293 cells to approximately 80% confluency on a 6-well plate (approx. 1×10^6 cells/well).
2. Transfect 10 μg of recombinant adenoviral vector DNA, digested with *Pac*I, ethanol-precipitated, and resuspended in ddH_2O/well using the calcium phosphate coprecipitation technique or your favorite transfection agent.
3. After 24 h, transfected cells can be monitored for GFP expression (if "Track" vectors have been used). (Twenty to 30% of the cells should be fluorescent.)
4. Harvest the well on day 12 post transfection by vigorous pipeting. (Five to seven adenovirus-producing comet-like foci should be present.)
5. Subject the cell suspension to three cycles of freezing at –20°C and rapid thawing at 37°C. Use 100 μL of this viral lysate (approx. 2 mL) to infect 1 well of a 24-well plate with 293 or 911 cells (approx. 4×10^5 cells).
6. Transfected cells are monitored for GFP expression and collected on day 7 post infection.
7. The obtained viral lysate (approx. 1 mL) is used for titration in a plaque assay (*see* **Subheading 3.5.1.**) and for subsequent expansion.

3.2. Expansion of Adenoviral Vectors

The expansion of adenoviral vectors consists of repeated rounds of infection and harvesting of increasingly larger batches of cells. General rules of thumb are (1) that infection should be done at a multiplicity of infection (MOI) of 5–10 infectious particles per cell and (2) that a successful infection will result in approximately 1000 infectious viral particles per cell. Adenovirus infection is lytic, and two options are available to harvest the amplified virus:

Option 1

1. If infection is performed at a very low MOI, the infection is left to proceed until all cells are floating. This will take 4–7 days, and during this period the virus that is produced from the first rounds of infection will infect the previously noninfected cells. In this protocol, most of the virus will be in the medium. The cellular debris is removed from the virus-containing medium by centrifugation, and virus can be recovered from the supernatant by ammonium sulphate precipitation ***(12)***.

2. If infection is performed at an MOI of 5–10, most cells will be infected simultaneously. Approximately 48 h after infection, the vast majority of amplified virus is still present intracellularly. By gently tapping the flask, the cells are dislodged from the plastic (as a result of the infection they will be rounded and loosely attached to the plastic) and centrifuged at low speed. By removing most of the supernatant and subjecting the pellet to three rounds of freeze/thawing, the adenovirus is released from the cell pellet. Cellular debris is removed from this virus-containing supernatant by vigorous centrifugation.

Option 2

1. The lysate obtained from a single well of a 24-well plate (*see* **Subheading 3.1.2., step 7**) is used to infect 50–75-cm^2 flasks with 293 or 911 cells at an MOI of 5–10. For infection, always use just enough virus suspension to cover all cells. Dilute virus if necessary in PBS/HS or medium with HS. (*See* **Note 2**).
2. Infection is left to proceed for 1 h at 37°C in the CO_2 incubator.
3. Replace viral suspension with tissue culture medium with 2% HS.
4. 40–48 h after infection, cells should be rounded and loosely attached to the plastic. Gently tap flasks to dislodge cells.
5. Harvest cell pellet by gentle centrifugation (10 min at 800 rpm in a tabletop centrifuge).
6. Aspirate nearly all of the supernatant and resuspend pellet in attached medium.
7. Subject cell suspension to three rounds of freeze/thawing.
8. Centrifuge vigorously to pellet cell debris (10 min at 2500 rpm in a table-top centrifuge).
9. Remove virus-containing supernatant. This crude lysate can be stored at –20°C until further processing.

After titration of the prep, repeat **steps 1–7** on 3–5 175-cm^2 plates and subsequently on 10–30 175-cm^2 plates. If tissue culture incubator space is a problem, Nalge Nunc (Naperville, IL) produces a 500-cm^2 triple flask with a volume comparable to that of a 175-cm^2 flask.

3.3. Purification of Adenoviral Vectors

The crude lysate obtained after freeze-thawing is subject to two rounds of centrifugation over CsCl gradients. After the second centrifugation, the virus-containing band is isolated, and CsCl is removed by dialysis. In the final dialysis step, the buffer contains 5% sucrose, which is necessary for virus stability at –80°C.

1. Add 2 mL of CsCl (d = 1.45 g/cm^3) to a clear ultracentrifugation tube (Beckmann, cat. no. 344059) and overlay with 4 mL of CsCl (d = 1.2 g/cm^3).
2. Carefully overlay the CsCl block gradient with the virus-containing supernatant (up to 4 mL of supernatant).

3. In a Beckmann ultracentrifuge, spin for 2 h at a speed of 30,000 rpm. Use the SW41 swinging bucket rotor (k-factor 230.9).
4. Aspirate the white band(s) that is located roughly at the interface between the two densities using an 18-gage needle attached to a 2-mL syringe.
5. Transfer the solution to a quick-seal ultracentrifugation tube (Beckmann, cat. no. 342413) and completely fill the tube with CsCl (d = 1.33 g/cm^3). Seal the tube.
6. Spin overnight in a Beckmann Ti70 rotor, at 48,000 rpm (k-factor 92.8).
7. Aspirate the lowest white band using an 18-gage needle attached to a 2-mL syringe, and transfer to a slide-a-lyzer cassette. The second, somewhat higher, white band consists of defective and empty viral particles.
8. Dialyze against 500 mL 1X TD buffer, at 4°C. Change the buffer after 2 and 4 h. After the last buffer, change dialyze overnight.
9. The next day, remove the old 1X TD buffer, add fresh, cold, 1X sucrose buffer, and dialyze for an additional 3 h, at 4°C.
10. Remove virus solution from the dialysis cassette using an 18-gage needle attached to a 2-mL syringe. Aliquot 200–400 µL samples in cryovials and flash-freeze in liquid nitrogen. Store cryovials at –80°C (*see* **Note 3**). Make 2–3 30-µL aliquots for titration of the virus (*see* **Subheading 3.4.1.**).

3.4. Analysis of Adenoviral Vector Preparations

It is recommended that at all stages of virus amplification the infectious titer of the virus be determined. The cell line 911 is superior to the 293 cell line for this purpose. Plaques appear much faster on 911 cells, and the success rate is much higher. It is also recommended that at least after the final stage of amplification the preparations be tested for the presence of replication-competent virus (RCA). RCA can be generated by recombination between the adenoviral DNA present in the genomes of 293 or 911 cell lines and the ΔE1A recombinant adenoviral vector DNA. Such a recombination will yield an adenovirus that has captured the E1A region from the cellular genome. The overlap between the adenoviral DNA in the genome of the cell lines and the Ad-easy vectors enables this event to occur. Once RCA is present in a preparation, it will quickly overgrow the recombinant virus.

3.4.1. Titration by Plaque Assay

1. Grow 911 cells in a 6-well plate to 95–100% confluency.
2. Make a serial dilution of virus in PBS/HS. Add 1 mL of PBS/HS to each of six 10-mL tubes. Use the following pipeting scheme, mix thoroughly at each step:
   a. Add 20 µL of virus directly to the first tube, labeled 2.
   b. Transfer 10 µL from the 2 tube to a new tube, labeled 4.
   c. Transfer 10 µL from the 4 tube to a new tube, labeled 6.
   d. Transfer 10 µL from the 6 tube to a new tube, labeled 8.
   e. Transfer 100 µL from the 8 tube to a new tube, labeled 6.
   f. Transfer 100 µL from the 9 tube to a new tube, labeled 10.

3. Aspirate medium from the 6-well plate and add 500 µL of diluted virus from the -8, -9, and -10 tubes to three of the wells in duplicate. (Use whatever dilution is estimated appropriate.) Incubate for 1 h in a 37°C CO_2 incubator.
4. Warm 2X F-15 medium to 37°C. Melt the agar solution in the microwave and cool melted agar to 42°C. (Use water baths).
5. After 1 h of infection, mix 2X F-15 medium and agar solution in a 1 : 1 ratio.
6. Aspirate the virus solution from each well, and immediately add 3 mL of F-15/agar mix.
7. Leave the plate in the TC hood until the F-15/agar mix solidifies. Then transfer to a 37°C CO_2 incubator. Wait 10–14 days (or until plaques appear), and count the plaques. Titer [in plaque-forming units (pfu)/mL] = (number of plaques in highest dilution) × $10^{(-\text{dilution factor})}$.

3.4.2. Particle Count

A second important indicator of the quality of an adenovirus batch is the particle over pfu ratio. This gives an indication of the number of noninfectious particles per infectious particle. This ratio should be around 100, but ratios of up to 10,000 have been used successfully.

1. Add 25 µL of virus stock to 475 µL of sucrose (dialysis) buffer and measure the OD_{260}. Total virus particles = $OD_{260} \times 20 \times 5 \times 10^{11}$ (20 is dilution factor).

An OD_{260} of 1 equals 5×10^{11} of total virus particles in the cuvet.

3.4.3. Detection of Replication-Competent Adenovirus

It is extremely important to screen any virus batch for the presence of RCA. RCAs are defined as adenoviruses that can replicate independently in normal human cells. In most cases, RCAs arise as a result of recombination between homologous sequences in the recombinant vector and the 293- or 911-helper cells. As a result, the transgene is replaced by the E1 region, thus alleviating the need for E1 *in trans* (for a review of RCAs, *see* **ref. *13***). We have developed a sensitive method for detection of RCA, using PCR ***(14***; also *see* **Notes 4** and **5*)***.

1. Preparation of the virus:
   a. Use 45–90 µL of a freeze-thaw isolate (see **Subheading 3.2., step 9**). Note that crude lysate from adenoviral vectors grown on 293 cells may *not* be used, since the (abundant) 293 genomic DNA will give a false-positive signal. In this case, use 1–2 µL of CsCL-purified adenovirus (*see* **Subheading 3.3., step 10**).
   b. Add 10X PCR buffer to 1x final concentration.
   c. Add 1 µL proteinase K and incubate for 16 h at 42°C.
   d. Boil for 5 min to inactivate proteinase K.
   e. Centrifuge for 5 min at max speed in a microfuge and retain supernatant.
2. Preparation of control samples: make a serial dilution of H5dl7001 or wild-type Ad5 virus in $T_{10}E_1$, resulting in 700, 70, 7, and 0.7 pfu/µL.

Table 1
Composition of the 1X PCR Reaction Mixture (Amounts for Analysis of One Sample)

Component	1X PCR mix (μL)
10X PE buffer	5
25 mM $MgCl_2$	3.5
dNTPs	2
RCAf1 (10 pmol/μL)	2
RCAr2 (10 pmol/μL)	2
tRNA (10 mg/mL)	0.5
Taq polymerase (PE)	0.3
Water	29.7
Total amount	45

Table 2
Setup of the PCR reactions for detection of RCA. The positive control (pos ctrl) can be any virus containing the E1 region.

	PCR reactions				
	0.7 pfu Pos ctrl	7 pfu Pos ctrl	70 pfu Pos ctrl	700 pfu Pos ctrl	Virus sample
Amount of virus prep	1	1	1	1	5
1X PCR-mix	45	45	45	45	45
Water	4	4	4	4	0

3. Prepare 1x PCR mix and perform the various PCR reactions according to the pipeting schemes shown in **Tables 1** and **2**.
4. PCR settings:
   5 min at 94°C
   40 s at 94°C
   45 s at 63°C 32 cycles
   50 s at 72°C
   8 min at 72°C
   Hold at 4°C
5. Load 20 μL of each PCR reaction on an agarose gel. RCA-band: 615 bp (Ad5 nucleotides 52–693).

Note that only if the diagnostic band (615 bp) in the reaction with 0.7 pfu is visible can the assay can be considered optimal.

3.5. In Vitro Application of Adenoviral Vectors

The expression and function of the gene cloned into the adenoviral vector is generally first analyzed in vitro. Most cell lines that are susceptible to adenovirus infection can be used for this purpose. However, for a direct comparison of in vivo and in vitro data, a (preferably mouse) hepatic cell line should be used. Alternatively, the adenoviral vectors may be used as a general and highly efficient means to transfer the gene of interest to a cell line of interest. Most cell lines can be infected with adenovirus, but the necessary MOI to obtain a certain level of infection will vary from cell line to cell line. In this regard, the "Track" series of vectors that are part of the Ad-easy system enable a quick analysis of the optimum infection efficiency of a given cell line by virtue of the endogenous GFP marker. Note that adenovirus infection at high MOIs will result in cellular toxicity and that the sensitivity to this phenomenon varies from cell line to cell line. Usually, gene expression peaks at 24–48 h after in vitro infection. Both crude lysates and purified viral preps can be used for in vitro infections, and the decision on what to use mostly depends on the sensitivity of the cell line to adenovirus-induced toxicity (the more sensitive the cell, the purer the preparation should be).

3.6. In Vivo Administration of Adenoviral Vectors

For in vivo administration, the adenovirus preparations should be purified according to **Subheading 3.3.** Generally, a dose of 2×10^9 pfu per mouse injected into the tail vein will result in high-level liver-specific gene expression. Doses of 5×10^{10} pfu or more per mouse can be expected to give significant liver toxicity. However, depending on the preparation, the insert, and the recipient mouse (strain, age, sex, and so on), the virus dose that will result in liver toxicity will vary. It is useful to monitor liver enzymes (such as ALAT) for the presence of damage to the liver at the dose that is used in the mouse of interest.

At a dose of 2×10^9 pfu, the mouse will show transient discomfort, starting at 10–20 min after injection and lasting for approximately 30–60 min. At day 3 after injection, more than 99% of the injected virus will be cleared from the circulation. Gene expression peaks at days 4 and 5, is significantly decreased at day 8, and is generally detectable up to days 12–14. The rapid decline in gene expression seems mostly related to downregulation of the CMV promoter that drives gene expression. However, immune responses to infected cells that have leaky expression of adenovirus backbone genes also contribute to downregulation of gene expression.

1. If necessary, dilute the CsCl-purified and dialyzed virus prep with sterile PBS to 2×10^9 pfu/200 μL. This volume may be increased to 400 μL.

2. Immobilize the mouse in a restraining tube in such a manner that the tail protrudes from the tube. Anesthesia is not necessary.
3. Inject the diluted virus into the left or right tail vein using an insulin syringe with a preattached needle. (This avoids excessive dead volume in the syringe.) Do not use the artery that runs along the top of the tail. If the tail veins are hard to see, the mouse may be warmed under a lamp.
4. Observe the mouse regularly over a period of 2 h for excessive side effects.

3.7. Developments in Adenovirus-Mediated Gene Transfer

3.7.1. Novel Generation of Adenoviral Vectors

The first-generation adenoviral vectors discussed so far are not suitable to obtain long-term gene expression. This is generally attributed to downregulation of the CMV promoter and to an immune response to virus-infected cells. These infected cells are recognized by the immune system since some of the adenoviral backbone genes, despite the absence of the early regulator switch E1, are clearly expressed. If the expressed transgene is also highly immunogenic (such as the bacterial *lacZ* gene), this will further contribute to the immune response.

To prolong gene expression after adenovirus-mediated gene transfer, systemic immune suppression has been applied ***(15,16)***. In addition, adenoviral vectors have been modified to prevent late adenoviral gene expression. These viral vector modifications consist of progressive deletions of the adenovirus backbone and thus more effective crippling of the virus.

Second-generation adenoviral vectors are based on the observation that the adenovirus mutant TS125 only grows at 32°C owing to a temperature-sensitive mutation in the E2 protein. The vector system designed from this E2 mutant thus consists of an adenovirus backbone plasmid containing the TS-E2 variant. The adenoviral vectors generated from this plasmid can be amplified at 32°C but not at 37°C. Thus, in the liver at the mouse body temperature of 37°C, these vectors will be double deficient (ΔE1A and TS-E2). Using these vectors, prolonged expression times have been reported ***(17,18)***.

Helper-dependent or gutted adenoviral vectors lack all endogenous adenoviral genes ***(19)***. The most successful system is based on a helper adenoviral vector that is E1A-deficient and in which the packaging signal is surrounded by LoxP sites (floxed). This helper vector can be amplified in regular 293 or 911 cells. The gene of interest is cloned in a vector flanked on both sides by inverted terminal repeats and on one side by a functional packaging signal. Inserts of up to 36 kb can be cloned in this "gutted" vector.

The helper-dependent vector is amplified on a 293 cell line that expresses the Cre-recombinase protein. Upon infection of this cell line with the floxed helper vector, the packaging signal is deleted and the helper DNA will not be

packaged into the virion. Transfection of the linearized vector with a functional packaging signal will result in preferential packaging of this vector into the virion. Long-term expression has been demonstrated with helper-dependent vectors ***(20)***.

3.7.2. Altering Tissue Specificity of Adenoviral Vectors

Recombinant adenovirus vectors can be generated in a relatively short period, can be amplified to high titers, and can infect susceptible cells very efficiently, resulting in high levels of gene expression. Since systemic injection of recombinant adenoviral vectors results in effective hepatic gene delivery, they have been applied to address a whole variety of scientific questions. To expand the utility of adenovirus vectors, several different approaches have been taken to redirect the tropism of adenoviral vectors from the liver to alternative tissues.

The cellular entry route used by type 2 and type 5 adenoviruses has been described in some detail ***(21,22)***. Initially, the knob of the fiber protein binds a cellular receptor called CAR ***(23)***. Subsequently, the pentonbase binds αVβ1 or αVβ3 integrins via an RGD motif, which leads to internalization of the virus particle by endocytosis. Finally, the virus disrupts the endosome and migrates to the nucleus. In the process, capsid components are released.

To modify the tropism of adenoviral vectors, both the natural tropism for CAR needs to be ablated and a novel specificity needs to be added. The most successful approaches to date rely on the recruitment of an alternative cellular receptor with or without ablation of specificity for CAR. Peptide ligands have been inserted into the HI-surface loop of the fiber knob ***(24)***, added to the C-terminus of the fiber knob ***(25,26)***, and inserted into the hexon protein ***(27)***. These approaches have been shown to be effective, but there is a rather strict requirement for the maximum size of the peptide that can be added or inserted. As an alternative approach, strategies employing antibodies have been used ***(28–30)***. Also, the fibers of the CAR binding adenoviral vectors have been replaced by fibers from adenoviruses belonging to different serotypes and recognizing other receptors ***(31–34)***. The proof of principle for many of these approaches has been delivered; however, much work remains to be done specifically regarding the development of short cell type-specific peptides. For a recent review on adenovirus retargeting, *see* **ref. *35***.

4. Notes

1. Probably the most critical step in the Ad-easy system is the recombination of the shuttle vector with the adenoviral backbone plasmid in the BJ5183 bacteria. Digestion of the shuttle vector with *Pme*I should be complete, since undigested vector will transform the bacteria with very high efficiency and result in failure

to detect recombinants. Prolonged restriction endonuclease incubation, repeated addition of enzyme, and/or agarose gel purification of the linear plasmid will decrease the background of undigested shuttle vector.

2. In the presence of bivalent cations and/or excess proteins, adenovirus preparations are very stable (i.e., in crude lysates). Once the adenovirus is CsCl-purified and dialyzed, it is much less stable, and repeated freezing and thawing should be avoided since this will affect titer.
3. It is recommended that at all stages of virus amplification the infectious titer of the virus be determined. Infection at an MOI of 5–10 generally results in optimal virus amplification. If the MOI is too low or too high, no or little amplification will occur. Very high titers in particular will result in massive cell death caused by viral toxicity.
4. Once RCA is detected in a virus preparation, it is pertinent to stop amplification and perform plaque purification. This consists of three rounds of titration (*see* **Subheading 3.4.1.**) whereby the titrations are done using isolated plaques that have been tested for the absence of RCA and the presence of the gene of interest. Use a glass pipet to stamp out the plaque and elute the agarose cylinder in 100 µL of PBS/HS. Use the eluate for PCR and subsequent titration.
5. To avoid formation of RCA, novel E1A-expressing cell lines have been developed that lack overlap between the E1A region present in the cellular genome and the recombinant adenovirus and thus prohibit recombination between these two sequences ***(36,37)***.

References

1. Horwitz, M. S. (1990) Adenoviridae and their replication, in *Virology*, 2nd ed. (Fields, B. N., Knipe, D. M., et al., eds.), Raven, New York, pp. 1679–1721.
2. Horwitz, M. S. (1990) Adenoviruses, in *Virology*, 2nd ed. (Fields, B. N., Knipe, D. M., et al., eds.), Raven, New York, pp. 1723–1740.
3. McGrory, W. J., Bautista, D. S., and Graham, F. L. (1988) A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* **163,** 614–617.
4. He, T. C., Zhou, S., da Costa, L. T., et al. (1998) A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* **95,** 2509–2514.
5. Fu, S. and Deisseroth, A. B. (1997) Use of the cosmid adenoviral vector cloning system for the in vitro construction of recombinant adenoviral vectors. *Hum. Gene Ther.* **8,** 1321–1330.
6. Ng, P., Parks, R. J., Cummings, D. T., et al. (1999) A high-efficiency Cre/loxP-based system for construction of adenoviral vectors. *Hum. Gene Ther.* **10,** 2667–2672.
7. Ng, P., Cummings, D. T., Evelegh, C. M., and Graham, F. L. (2000). Yeast recombinase FLP functions effectively in human cells for construction of adenovirus vectors [In Process Citation]. *Biotechniques* **29,** 524–526, 528.
8. Tashiro, F., Niwa, H., and Miyazaki, J. (1999) Constructing adenoviral vectors by using the circular form of the adenoviral genome cloned in a cosmid and the Cre-loxP recombination system. *Hum. Gene Ther.* **10,** 1845–1852.

9. Ng, P., Parks, R. J., Cummings, D. T., Evelegh, C. M., and Graham, F. L. (2000) An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. *Hum. Gene Ther.* **11,** 693–699.
10. Aoki, K., Barker, C., Danthinne, X., Imperiale, M. J., and Nabel, G. J. (1999) Efficient generation of recombinant adenoviral vectors by Cre-lox recombination in vitro. *Mol. Med.* **5,** 224–231.
11. Fallaux, F. J., Kranenburg, O., Cramer, S. J., et al. (1996) Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* **7,** 215–222.
12. Schagen, F. H., Rademaker, H. J., Rabelink, M. J., et al. (2000) Ammonium sulphate precipitation of recombinant adenovirus from culture medium: an easy method to increase the total virus yield [In Process Citation]. *Gene Ther.* **7,** 1570–1574.
13. Fallaux, F. J., van der Eb, A. J., and Hoeben, R. C. (1999) Who's afraid of replication-competent adenoviruses? [editorial]. *Gene Ther.* **6,** 709–712.
14. Pietersen, A. M., van der Eb, M. M., Rademaker, H. J., et al. (1999) Specific tumor-cell killing with adenovirus vectors containing the apoptin gene. *Gene Ther.* **6,** 882–892.
15. Kay, M. A., Holterman, A. X., Meuse, L., Gown, A., Ochs, H. D., Linsley, P. S., and Wilson, C. B. (1995) Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration. *Nat. Genet.* **11,** 191–197.
16. Jooss, K., Yang, Y., and Wilson, J. M. (1996) Cyclophosphamide diminishes inflammation and prolongs transgene expression following delivery of adenoviral vectors to mouse liver and lung. *Hum. Gene Ther.* **7,** 1555–1566.
17. Engelhardt, J. F., Litzky, L., and Wilson, J. M. (1994). Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2a. *Hum. Gene Ther.* **5,** 1217–1229.
18. Yang, Y., Nunes, F. A., Berencsi, K., et al. (1994) Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. *Nat. Genet.* **7,** 362–369.
19. Parks, R. J., Chen, L., Anton, M., et al. (1996) A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc. Natl. Acad. Sci. USA* **93,** 13565–13570.
20. Morsy, M. A., Gu, M., Motzel, S., et al. (1998) An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. *Proc. Natl. Acad. Sci. USA* **95,** 7866–7871.
21. Greber, U. F., Willetts, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* **75,** 477–486.
22. Nakano, M. Y., Boucke, K., Suomalainen, M., Stidwill, R. P., and Greber, U. F. (2000). The first step of adenovirus type 2 disassembly occurs at the cell surface, independently of endocytosis and escape to the cytosol. *J. Virol.* **74,** 7085–7095.
23. Bergelson, J. M., Cunningham, J. A., Droguett, G., et al. (1997) Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275,** 1320–1323.

24. Krasnykh, V., Dmitriev, I., Mikheeva, G., et al. (1998) Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J. Virol.* **72,** 1844–1852.
25. Michael, S. I., Hong, J. S., Curiel, D. T., and Engler, J. A. (1995) Addition of a short peptide ligand to the adenovirus fiber protein. *Gene Ther.* **2,** 660–668.
26. Wickham, T. J., Roelvink, P. W., Brough, D. E., and Kovesdi, I. (1996) Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. *Nat. Biotechnol.* **14,** 1570–1573.
27. Vigne, E., Mahfouz, I., Dedieu, J. F., et al. (1999) RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J. Virol.* **73,** 5156–5161.
28. Miller, C. R., Buchsbaum, D. J., Reynolds, P. N., et al. (1998) Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res.* **58,** 5738–5748.
29. Douglas, J. T., Rogers, B. E., Rosenfeld, M. E., et al. (1996) Targeted gene delivery by tropism-modified adenoviral vectors. *Nat. Biotechnol.* **14,** 1574–1578.
30. Wickham, T. J., Segal, D. M., Roelvink, P. W., et al. (1996) Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol.* **70,** 6831–6838.
31. Stevenson, S. C., Rollence, M., White, B., Weaver, L., and McClelland, A. (1995) Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. *J. Virol.* **69,** 2850–2857.
32. Krasnykh, V. N., Mikheeva, G. V., Douglas, J. T., and Curiel, D. T. (1996) Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J. Virol.* **70,** 6839–6846.
33. Gall, J., Kass-Eisler, A., Leinwand, L., and Falck-Pedersen, E. (1996) Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J. Virol.* **70,** 2116–2123.
34. Wickham, T. J., Tzeng, E., Shears, L. L., 2nd, et al. (1997) Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J. Virol.* **71,** 8221–8229.
35. Krasnykh, V. N., Douglas, J. T., and van Beusechem, V. W. (2000). Genetic targeting of adenoviral vectors. *Mol. Ther.* **1,** 391–405.
36. Fallaux, F. J., Bout, A., van der Velde, I., et al. (1998) New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum. Gene Ther.* **9,** 1909–1917.
37. Schiedner, G., Hertel, S., and Kochanek, S. (2000) Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. *Hum. Gene Ther.* **11,** 2105–2116.

13

Random ENU Mutagenesis

Dian Soewarto, Véronique Blanquet, and Martin Hrabě de Angelis

1. Introduction

Mouse models play an important role in the elucidation of molecular pathways underlying human disease. Mutations in mouse can be generated by a variety of techniques including those using inducing agents such ionizing radiation or chemicals and those that involve genetic manipulations such as transgenic insertions or knockouts. Random mutagenesis by ionizing radiation or chemical agents has a long tradition in classical genetics and has allowed the generation of a large number of mutant phenotypes. Ionizing radiation causes breaks in the chromosome, leading to deletions, translocations, and other gross chromosomal rearrangements. Chemical mutagens, which have been shown to produce a large number of mutations, are characterised by a differential spermatogenic response.

The lesions produced by these agents can be divided in three groups: the first group consists of agents such as *N*-ethyl-*N*-nitrosourea (ENU) or methylnitrosourea (MNU), and the effect is on stem cell spermatogonia. The second category includes chemicals affecting the early spermatid. Chlorambucil (CHL) and melphalan belong to this group. The third group includes chemicals like ethyl-methanesulfonate (EMS) that have effects on spermatozoa and late spermatids ***(1–3)***.

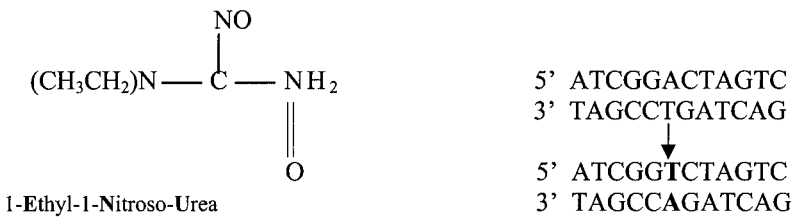
The frequency and types of mutations induced by chemical mutagens have been analyzed by employing the specific locus test (SLT) ***(4)***. The SLT consists of mating ENU males to untreated females, homozygous for seven recessive loci, and scoring in the first-generation offspring for mutations at any of the marked loci. The seven loci were chosen whose mutant phenotypes were easily recognized at weaning. These include loci controlling coat pigmentation color,

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

intensity, or pattern, as well as the size of the external ear (a, non-agouti; b, brown, cch, chinchilla, d, dilute, p, pink-eyed dilution, s, piebald, se, short ear). The SLT in the mouse is described in detail by Searle ***(5)***, and Ehling and Favor ***(6)***.

Because of their high potential for causing mutations, ENU and CHL have been the mutagens of choice in creating mouse mutants. Here we focus on ENU mutagenesis, which mainly induces point mutations, i.e., A-T base pair substitutions. ENU has been successfully used to obtain a variety of alleles of single genes. These include loss of function alleles, hypomorphs, and gain of function mutations. Allelic series at single loci are extremely powerful in further defining gene function. Furthermore, phenotype-driven ENU screens provide the tools to dissect developmental and biochemical pathways. Taking advantage of the mutagenecity of ENU and its ability to create allelic series of mutations, Chen et al. ***(7)*** have developed a complementary approach to the conventional whole-animal ENU mutagenesis strategies by generating mutations using mouse embryonic stem cells.

ENU is an alkylating agent, which can transfer its ethyl group to oxygen or nitrogen radicals in DNA, resulting in mispairing and base pair substitution if not repaired, as shown below:



The highest mutation rates occur in premeiotic spermatogonal stem cells, with an average frequency of 1 : 700/locus/gamete. ENU treatment can involve one or three weekly injections of ENU followed by a minimal waiting period of 7 weeks to allow the animals to recover their fertility, as ENU causes temporary depletion of spermatogonal stem cells ***(8,9)***. It is likely that ENU depletes multiple stem cell types, which can lead to higher infection risks and/or a decrease of life expectancy. Optimal conditions of ENU treatment vary considerably depending not only on dose and frequency of chemical treatment but also on the genetic background of the inbred strain used ***(10,11)***.

Different approaches can be carried out to recover induced ENU mutations in the mouse: genome-wide screens ***(12–15)*** or region-specific saturation mutagenesis, which exploits chromosomal deletions ***(16–18***; *see* **Note 1***)*. Some screens target dominant mutations, whereas others are designed to isolate recessive mutations ***(12,14,15,17,19,20)***. In the case of either the dominant or

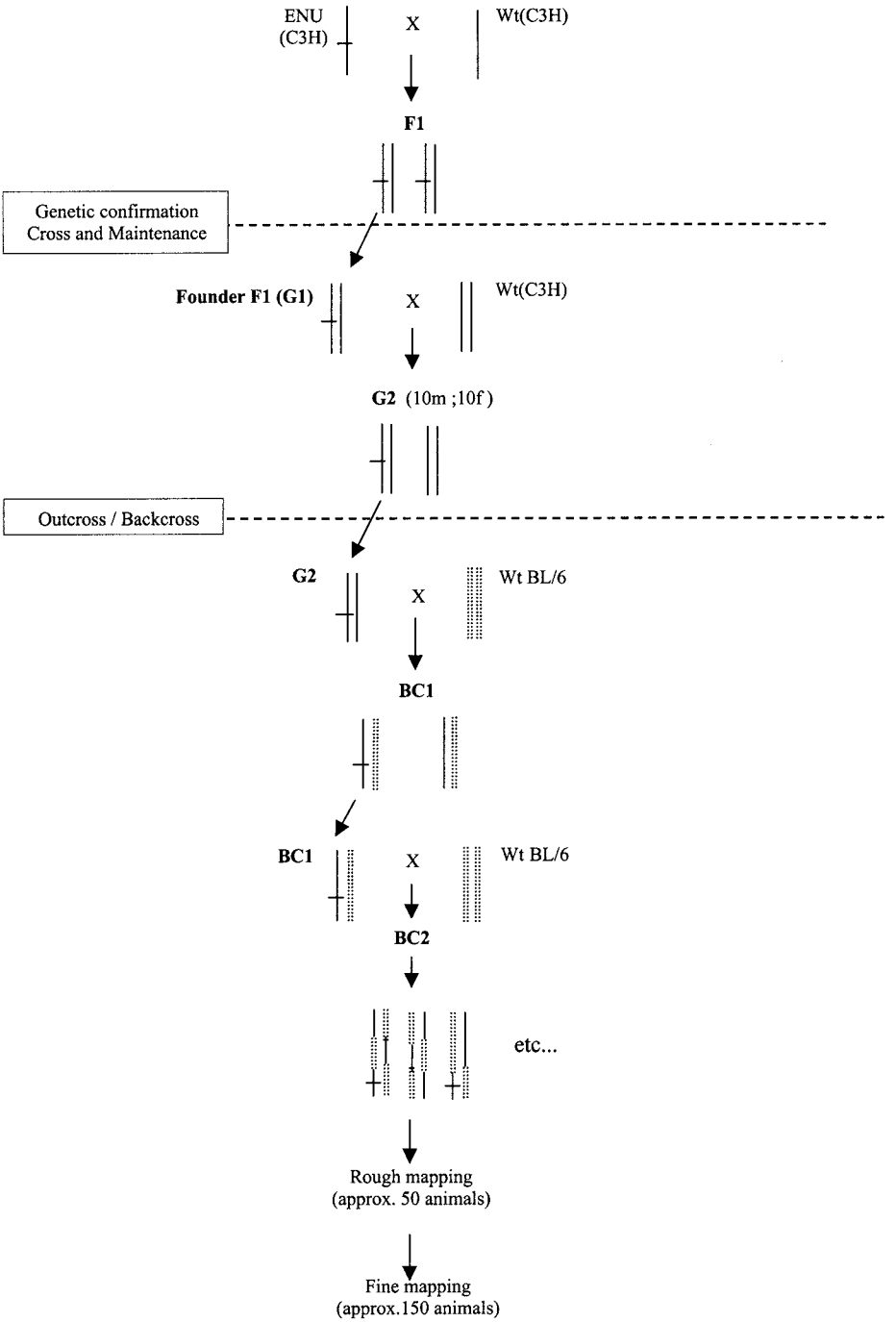
recessive screens, male mice are injected with ENU and then mated to females to produce F1 founders. These F1 mice can be either analyzed directly for dominant mutations or subsequently bred further to study recessive phenotypes.

It is possible to analyze very large numbers of mice in a dominant F1 (G1) screen. The screen for recessive mutations involves two generations of breeding. From F1 (G1) founder males, G2 female offspring are raised, half of which are heterozygous for the newly induced mutations. Then either G2 females are backcrossed to F1 (G1) founder males, or intercrossing is performed among the G2 to identify recessive mutant phenotypes among G3 offspring.

From the breeding scheme (**Figs. 1** and **2**), it is evident that performing a recessive screen requires more effort than performing a dominant F1 screen. However, dominant mutations are recovered at lower frequencies than recessive mutations. As shown for dominant and recessive cataract mutations, it is about 10 times lower ***(21)***. The main interest in a phenotype-driven strategy is the establishment of efficient and integrated screening systems, which is critical for the assessment of mutant phenotypes and essential to obtain animal models of human diseases and insight into functions of genes.

The main challenge lies in screening large numbers of progeny with comprehensive, systematic assays. Primary screens should be broad but rapid, easily applicable to many mice, and noninvasive ***(12,22)***. Then mice, who present an interesting phenotypic abnormality, are analyzed in detail with more sophisticated secondary or tertiary screens. Visible abnormalities that affect coat, sense organs, limbs, axial skeleton, or neurologic behavior are easily detectable, and a large number of mutations have already been identified ***(23)***. Clinical chemical screens ***(24)*** can lead to the identification of mouse mutants relevant to human clinical diseases such as diabetes mellitus, hypercholesterinemia, obesity, or coronary heart disease. The setup of this type of screen is based on routine clinical examinations used for checkups in human medicine (*see* www.gsf.de/ieg/groups/enu-mouse.htlm). Clinical chemistry tests can lead to the characterization of defects in different organs such as the liver, pancreas, heart, and kidneys. Electrospray-tandem mass spectrometry can detect metabolic alterations affecting lipids, fatty acids, or amino acids ***(25)***. Immunologic screens lead to the detection of mouse mutants as models for primary immune deficiencies and hypersensitivity disorders ***(26)***. Specific screens such as DNA repair using single-cell gel electrophoresis (COMET assay) can lead to the establishment of mouse models with inherited radiation sensitivity ***(27***; *see* **Note 2***)*.

Unlike gene targeting or insertional mutagenesis, mutant mice produced by ENU are not molecularly tagged. The identification of the mutated gene has to be performed by either the positional cloning or the candidate gene


ENU
(C3H)
X
Wt(C3H)
F1
Genetic confirmation
Cross and Maintenance
Founder F1 (G1)
X
Wt(C3H)
G2 (10m ;10f)
Outcross / Backcross
G2
X
Wt BL/6
BC1
BC1
X
Wt BL/6
BC2
etc...
Rough mapping
(approx. 50 animals)
Fine mapping
(approx.150 animals)

approach. These two approaches to cloning a gene are still not trivial, but new developments in mouse genomics, i.e., the complete sequence of the mouse genome or a high-throughput single nucleotide polymorphism detection system will lead to a faster identification of the underlying gene involved.

The overall strategy for ENU mutagenesis is presented in **Fig. 3**.

2. Materials

2.1. Reagents and Equipment

1. 1-Nitroso-1-ethylurea (ENU), solid (Serva, Heidelberg, Germany, cat. no. 30800). Since ENU powder is sensitive to humidity and light, it should be stored in a dry place at –20°C. The stability of ENU in solution is pH-dependent. The half-life of ENU decreases with increasing pH and decomposes to diazoethane in alkaline solution, with a half-life of 34 min at pH 7.0, 37°C ***(28)***. At pH 6.0, ENU has a half-life of 31 h. Every single ENU flask should only be used once for one experiment. No chemical lot of ENU should be stored longer than 1 year.
2. 66 m*M* Na_2HPO_4 (Merck, Germany, cat. no. 1.06580) stock solution for Soerensen buffer, pH 9.1–9.4 at 50 g/L, 20°C.
3. 66 m*M* KH_2PO_4 (Merck, cat. no. 1.04877) stock solution for Soerensen buffer, pH 4.2–4.5 at 50 g/L, 20°C).
4. H_2O dest.
5. pH meter/pH strips for pH 4–7/0.2 (Merck, cat. no. 1.09542).
6. 500-mL beaker (Schott, Germany).
7. 50-mL Falcon tubes, polypropylene (Becton Dickinson, Germany).
8. Crushed ice.
9. Disposal bags.
10. 10-mL sterile glass pipets.
11. Gloves.
12. Masks.
13. Goggles.
14. High-flow chemical hood.

Fig. 1. *(see opposite page)* Dominant breeding scheme. Progeny in the F1 generation are produced by mating an ENU-treated male with a wild-type untreated female. The possible outcomes are shown for one chromosome pair. (The ENU induced mutation on one parental chromosome is indicated by a bar.) The inheritance of the mutant phenotype is tested by mating the F1 founder to a wild-type animal producing G2 offspring. The mutation is confirmed if at least one of the offspring shows the mutant phenotype in the G2 generation. In the outcross/backcross mapping protocol, two inbred strains are used (for example, C3H and C57BL/6). The affected G2 animal (C3H background) is mated to a wild-type C57BL/6 female producing BC1 hybrids. Affected BC1 animals are then crossed to wild-type C57BL/6 females producing BC2 animals, which are required for mapping. BC, backcross; m, male; f, female.

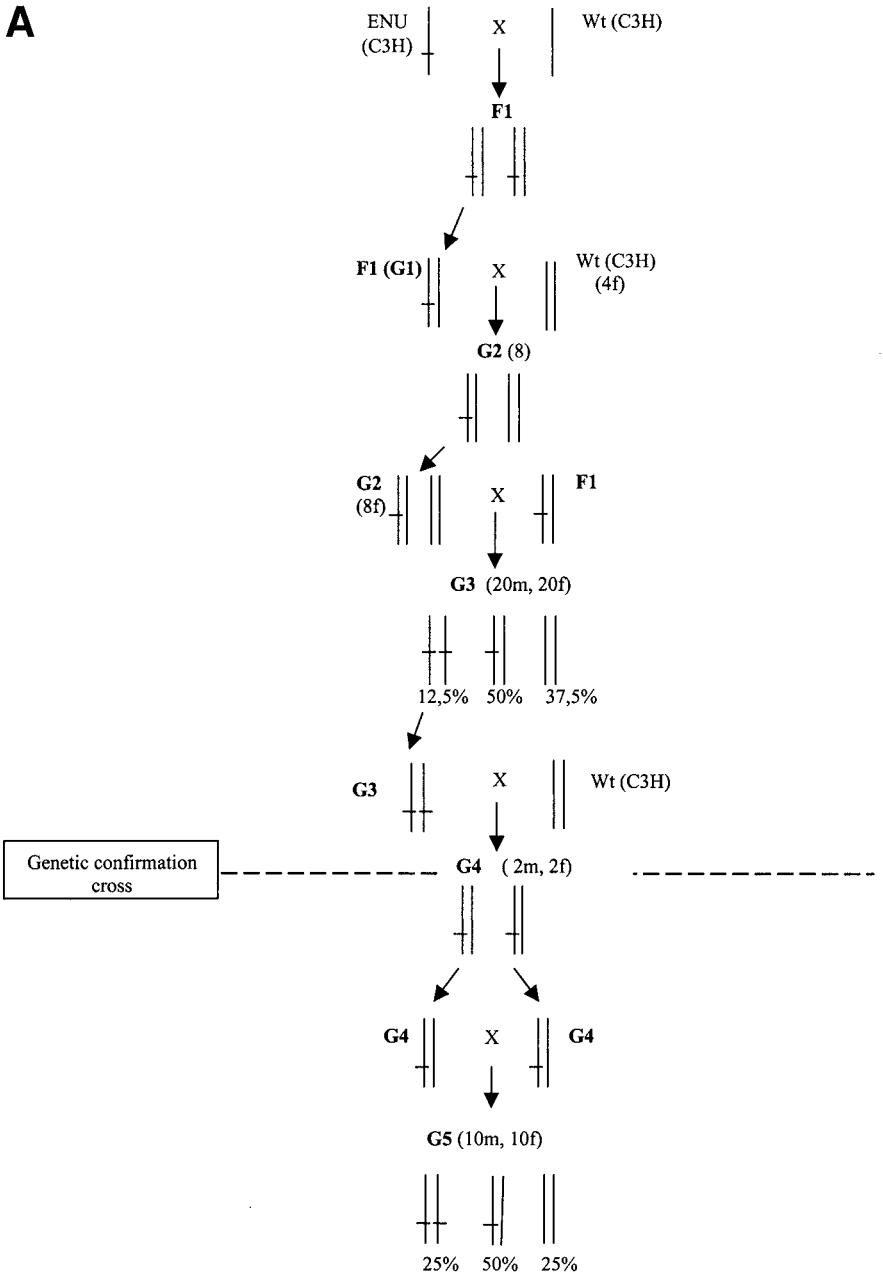


Fig. 2. **(A)** Recessive breeding scheme. Progeny in the F1 generation are produced by mating an ENU-treated male with a wild-type untreated female. A male F1 founder is crossed to a wild-type female producing G2 offspring. Only female G2 animals (+/– or +/+) are collected and crossed back to the F1 founder, producing G3 offspring, which

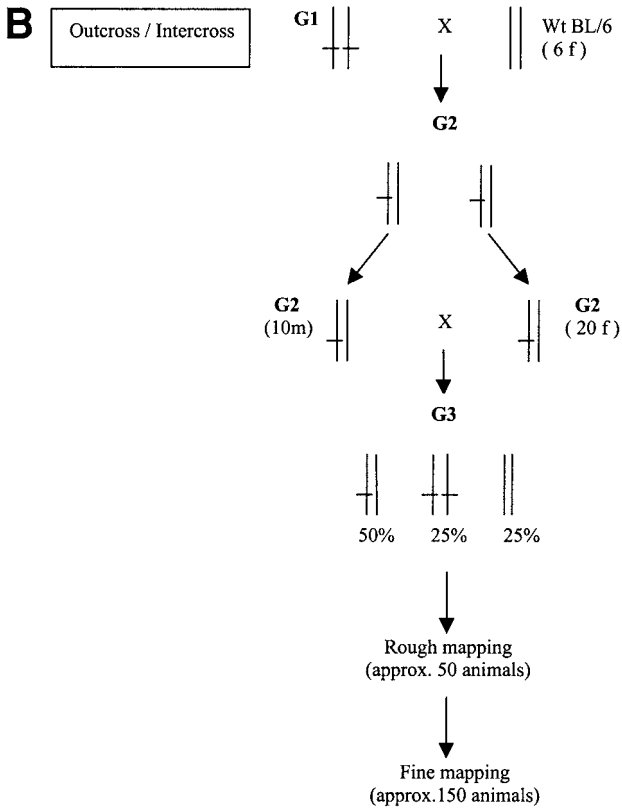


Fig. 2. *(continued)* are to be assessed for recessive mutations. The inheritance of the mutant phenotype is tested by two-step breeding; the potential, homozygous (–/–) G3 animals are crossed to wild-type animals producing heterozygous (+/–) F4 offspring. G4 × G4 intercrosses are performed to produce G5 offspring. At least 20 G5 animals are collected for phenotyping. m, male; f, female. **(B)** Recessive screen: outcross/intercross mapping protocol. Homozygous animals (G1; C3H background) are crossed to wild-type C57BL/6 females producing G2 offspring. Two heterozygous G2 animals are mated to produce progeny in the G3 generation. Animals from G3 are used for mapping. Numbers in parentheses indicate the numbers of mice required to produce 50–200 G3 animals.

15. Scale (with scale accuracy of 0.1 g) for weighing mice (IP65, Sartorius, Germany).
16. Alkaline solution: 0.1 *M* NaOH. Since stability of ENU is pH-dependent, it can be easily inactivated with 0.1 *M* sodium hydroxide (NaOH).
17. 1-mL syringes with 27-gage needle (Becton Dickinson).

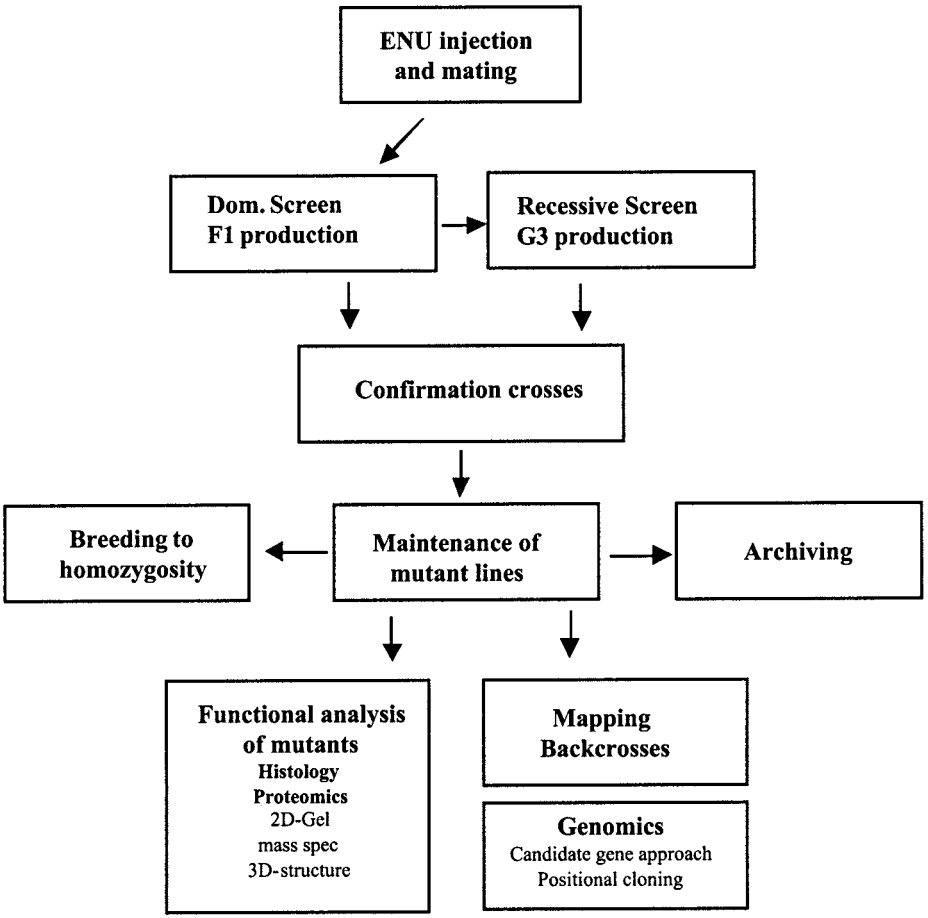


Fig. 3. Overview of the *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis procedure.

2.2. Soerensen Buffer

For a 1000-mL stock solution, stocks of 66 m*M* KH_2PO_4 (stock A) and 66 m*M* $Na_2HPO_2 \times 2H_2O$ (stock B) are made as shown in **Table 1**.

For a Soerensen buffer with a pH of 6.0, take 121 mL of stock A and fill up to 1000 mL with stock B. pH has to be tested after preparation and before use at 4°C (since pH is temperature-dependent), and the injection solvent will have a temperature of 4°C. Adjustment of pH can be performed with stock A (acid) and/or stock B (basic). After sterilization, the Soerensen buffer can be stored at 4°C for at least 6 months.

Table 1
Stock Preparation

Stock	Compound	Amount	Dissolved in
A	KH_2PO_4	9.078 g	1000 mL H_2O dest.
B	$Na_2HPO_2 \cdot 2H_2O$	11.876 g	1000 mL H_2O dest.

2.3. Precautions for Handling ENU

Persons who are dealing with ENU should be aware of its genotoxicity and should take some essential precautions beforehand:

1. ENU must be weighed in a high-flow chemical hood.
2. During weighing of ENU, the experimenter should wear gloves, lab coat, goggles, and a mask.
3. Prepare some alkaline solution (0.1 *M* NaOH) for the hood. If any ENU spills, pour the alkaline solution on the spill and let it stand for a few minutes before cleaning up.

2.4. ENU Preparation

1. Adjust Soerensen buffer at a temperature of 4°C to pH 6.0 with 66 m*M* KH_2PO_4 (stock A, acid) or 66 m*M* $Na_2HPO_2 \cdot 2H_2O$ (stock B, basic).
2. The amount of ENU is calculated according to the mean weight of a group of mice such that an injection volume of 500 µL gives the desired concentration (*see* **Subheading 3.1.**). In our lab we use three weekly injections of 90 mg/kg.
3. ENU is weighed under a flow hood. Dissolve ENU in ice-cold Soerensen buffer in a 50-mL Falcon tube.
4. Shake the solution thoroughly for 2–3 min until the ENU is completely dissolved.
5. The ENU solution should be clear and should have light yellow color. If this is not the case, the ENU is degraded and a new flask of ENU should be used.
6. Check the pH of the ENU solution again with a pH strip. Keep the ENU solution on ice after preparation and during the injection procedure.

2.5. Waste Removal

Soak all instruments and gloves that might have come in contact with ENU in 0.1 *M* NaOH before discarding. All utensils should be discarded in plastic bags even after inactivation. All ENU disposal bags should be designated as hazardous waste and removed by a central waste facility. All surfaces in the flow hood or workplaces used for ENU preparation or injection should also be cleaned with the alkaline solution.

2.6. Quality Control

Since mutagenesis screens are long-term experiments, one has to make sure from the start that the ENU preparation and injection have been performed correctly (*see* **Note 3**).

1. The ENU solution should be clear and have a light yellow color. If this is not the case, the ENU is degraded, and a new flask of ENU should be used.
2. Every single flask of ENU should be used only once for one experiment. No lot of ENU should be stored for longer than 1 year.
3. Check the quality of the ENU lot to be used by determining its melting point (melting point ≈ 90–95°C) and ultraviolet (UV) absorption analysis (can be sent to the QC department of the producer, Serva, Heidelberg, Germany).
4. Because of continuous degradation of ENU, the injection procedure should be finished within 60 min after the initial ENU preparation. Otherwise, the mutation ratio can turn out to be lower than desired.

3. Methods

3.1. Husbandry and Strain Differences

3.1.1. SPF Conditions

Optimal conditions for mutagenesis experiments are to be found under a specific pathogen-free (SPF) environment. Since ENU depletes multiple stem cell types, such as hematopoetic stem cells, it also causes temporary sensitivity to pathogens. Therefore, injected males not only undergo a period of sterility, but they are also more susceptible to infections than untreated males and tend to die at an earlier age. The question of the necessity of a SPF environment is also dependent on the phenotype, which is to be assessed. For example, if infectious animals (i.e., mouse hepatitis virus-positive animals) are analyzed for immunologic parameters, the phenotype cannot be clearly defined, since infections may result in an alteration of the immunologic response compared with noninfectious mice.

3.1.2. Strain Differences

One important prerequisite for effective mutagenesis screens is the choice of appropriate mouse strains. It is known that certain outbred and inbred strains have a different sensitivity to chemical mutagens, presumably owing to a variable capability for DNA repair in their germ cells ***(29,30)***. If an outbred strain is selected for a mutagenesis experiment ***(9,11,20)***, one has to consider the mixed genetic background, which may complicate further analysis of the mutant phenotype. When inbred strains are used for mutagenesis, one can avoid the difficulties of a polymorphic genetic background for phenotypic

analysis and characterization, since the mutant phenotype can be clearly defined compared with wild-type mice. Since a different inbred strain is used for backcrossing, one has to consider that different strain-dependent alleles at the same locus or modifying genes may still interfere with the mutagenized allele. This may cause more complexity in chromosomal mapping. Modifiers or modifying genes can be located on the same or on a completely different chromosome, where they can act as regulatory elements by alteration or compensation of the mutagenized allele.

A second issue is the choice of an appropriate strain for mutagenesis according to the category of phenotypes and parameters that have to be assessed ***(12,22,31)***. In phenotype-driven screens, inbred strains with a genetic predisposition might not be appropriate where this genetic predisposition represses the phenotype of interest. This could be possible, for example, for wild-type C57BL/6, which displays a genetic susceptibility to arteriosclerosis ***(32)*** or non-insulin-dependent diabetes mellitus and hypertension ***(33)*** when fed a high-fat diet. In addition, the C3H mouse strain should be used with care in behavioral studies, since these mice carry the *rd* (retinal degeneration) gene and are blind after about 6 weeks.

The third issue is the determination of the optimal dose of ENU for that (inbred) strain ***(10,11)*** in order to perform an effective mutagenesis screen. Effectiveness of ENU is measured by the highest possible dose considering 1) the period of sterility; 2) the percentage of males that regain fertility; 3) the mortality rate before or after regaining fertility; and 4) the mutation frequency in the F1 or G3 generation. Optimal doses of ENU were determined by Weber et al. ***(10)*** for several inbred strains. They came to the conclusion that a fractionated dose, which includes three weekly injections, results in a high number of fertile males and a satisfactory mutation frequency.

3.2. Preparation of Animals

For purposes of adaptation, animals should be moved into the injection room 1 week before injection. A maximum number of four males per cage should not be exceeded, since the mice are being stressed during and after ENU injection.

For ENU injection, we select C3H male mice between the ages of 10 to 14 weeks and between 24 and 36 g body weight on the day of injection. The optimal weight range can vary from strain to strain and has to be determined in preliminary experiments. Since the animals are under stress during and after ENU injection, male mice should be physically fully developed. Another important issue is the animal's body weight. One has to consider that the ENU solution cannot be absorbed by fat tissue and that the increase of body fat is not proportional to body weight. Therefore, there is a risk of exceeding the

ENU dose, since the concentration is calculated according to the animal's body weight. This is the main reason why animals with a body weight higher than 36 g (for C3H) should be excluded. If it is feasible, one can establish weight categories of mice such that the range of body weights does not deviate by more than 5% from the group mean. Another way is to establish injection groups consisting of mice with a body weight falling into intervals of 2.5-g steps. This procedure is easier than determining the mean body weight of each group.

The number of male mice to be used for a mutagenesis experiment is dependent on several factors, the most important of which are the operator's skills and experience in handling mice and the number of persons injecting ENU. In terms of security, a minimum of two persons should carry out the ENU injection.

3.3. ENU Injection

3.3.1. Precautions

The persons who perform the injection should also take precautions:

1. During ENU injection, wear gloves, lab coat, goggles, and a mask.
2. Prepare some alkaline solution (0.1 *M* NaOH) for the workplace. If any ENU spills, pour the alkaline solution on the spill and let it stand for a few minutes before cleaning up.

3.3.2. Injection Procedure

1. The mice are injected ip with 0.5 mL of ice-cold ENU solution with the concentration according to the mean body weight of each group. Injection is performed ip according to the procedure described in *Manipulating the Mouse Embryo* by Hogan et al. ***(34)***.
2. The injection procedure should be completed within 60 min after ENU preparation.
3. All surfaces and instruments have to be cleaned properly as described above (*see* **Subheading 2.5.**). If any ENU solution is left, it should be inactivated immediately with 0.1 *M* NaOH.
4. After ENU injection, quarantine the room for at least 24 h to minimize the risk of contact with the mutagen.

3.4. Sterility Testing

For sterility testing, each mutagenized male is mated to a wild-type female 7 weeks after the last injection to ensure that only those offspring derived from exposed spermatogonal stem cell stages are analyzed for mutations. The duration of spermatogenesis in mice from stem cell spermatogonia to mature spermatozoa was determined to be 49–51 days ***(35)***. The sterility period is

dependent on successful repopulation of spermatogonia in the seminiferous tubules. Since it is known that there is a correlation between the period of sterility and mutation frequency, we include only those F1 offspring for phenotyping that came from conceptions occurring more than 80 days after injection.

3.5. F1/G3 Breeding

3.5.1. Breeding for Dominant Screen

The breeding scheme for production of dominant phenotypes is shown in **Fig. 1**.

1. Fertile, mutagenized males with a tested sterility period of 80 days are mated to wild-type females producing F1 animals, which are assumed to carry heterozygous mutations.
2. Mating each male to a group of two females (or more) and moving the male into a new group of females each week can accelerate F1 production. After a 7-week period, the males are rotated back to the original group of females to begin the cycle over again.
3. The number of offspring per mutagenized male should be limited to 100 F1 animals, since clustered mutations (more than one mutant of the same type from the same ENU male) can appear owing to the low number of remaining spermatogonial stem cells.

3.5.1.1. Inheritance of Dominant Phenotypes

1. Mating the suspected F1 animal (variant) to a wild-type animal producing G2 offspring tests inheritance of the mutant phenotype.
2. Twenty G2 offspring animals are collected and assessed for the phenotype of interest. This number is valid for a probability of $p = 0.01$ for mutations with full penetrance.
3. The mutation is confirmed if at least one of the offspring displays the mutant phenotype.
4. Establish a new dominant mutant line by outcrossing affected (+/–) animals to the original inbred strain in order to produce an isogenic line. Since only 50% of the following generations can be expected to be mutants, the phenotypic assessment has to be performed for all offspring.
5. Semidominant mutations can be determined if two affected, heterozygous (+/–) animals are mated producing homozygous (–/–) animals. If there is a gradual, phenotypic difference between heterozygous and homozygous animals, a semidominant trait can be concluded.

3.5.2. Breeding for Recessive Screen

The breeding scheme for production of recessive phenotypes is shown in **Fig. 2A**. Since using male F1 for G3 animal production is much faster than using

female F1, we focus on the "fast" strategy in this chapter. The numbers shown are valid for obtaining recessive mutations with a probability of $p = 0.005$.

For selecting F1 founders for a recessive family, the following points have to be considered:

1. A male F1 animal is expected to have heterozygous mutations but not an obvious phenotype.
2. Each F1 founder should come from a different mutagenized male, to obtain a wide variety of recessive phenotypes.

3.5.2.1. Breeding Strategy with a Male F1

1. A male F1 founder (+/–) is crossed to four wild-type females producing G2 offspring (**Fig. 2A**). Only female G2 animals (+/– or +/+) are collected here.
2. Cross eight G2 females back to the F1 founder, producing 40 G3 offspring, which are to be assessed for recessive mutations.

3.5.2.2. Breeding Strategy with a Female F1 (Not Shown)

1. A female F1 founder is crossed to a wild-type male producing G2 offspring (+/– or +/+). Both sexes are collected here.
2. Set up at least four G2 x G2 intercrosses for producing G3 animals considering that only 8.3% (1/12) of the G3 offspring might have a recessive phenotype.

Remark: Alternatively, it is possible to mate fewer G2 females to F1 males, producing fewer G3 animals for the screening procedure considering that more pedigrees can be set up, but with a higher probability of missing potential mutant phenotypes (for example, 4 F2 females producing 20 G3 animals; $p < 0.1$).

3.5.2.3. Inheritance of Recessive Phenotypes

Test inheritance of the mutant phenotype by the following two-step breeding:

1. Cross the suspected, homozygous (–/–) G3 animal to a wild-type (+/+) animal producing heterozygous (+/–) G4 offspring.
2. Set up two G4 × G4 intercrosses to produce G5 offspring. At least 20 G5 animals are collected for phenotyping. This number is valid for a mutation with a full penetrance resulting in 25% of the expected phenotype.
3. The mutation is confirmed if at least one of the offspring displays the mutant phenotype.
4. Establish a new recessive mutant line by crossing two affected (–/–) animals. To test recessive mutants with respect to their fertility, cross affected (–/–) male and female mice to the original wild-type background in parallel. If they are homozygous, it can happen that one of the sexes is not fertile. Then it is necessary to switch to a (–/–) homozygote (+/–) heterozygote breeding strategy.

3.6. Mapping

The overall method of positional cloning or gene candidate approach is to map the chromosomal localization of the mutation by linkage analysis via outcross/backcross or outcross/intercross mapping strategies using inbred mouse strains (**Figs. 1**, **2B**). The mapped location of the chromosome is used to clone the gene or to test the already known genes. A backcross strategy is preferred if the mutant allele is fully dominant or if the mutant allele is recessive and homozygotes are fertile. An intercross strategy is preferred if the homozygous individuals are not fertile and the mutation is recessive and affected. Optimal conditions for linkage analysis is the use of two mouse strains having high polymorphism. Linkage analysis is performed using a panel of microsatellite markers for the whole genome ***(36)*** (www.informatics.jax.org) or, more recently, using single-nucleotide polymorphism (SNP) maps ***(37)*** (www.genome.wi.mit.edu/SNP/mouse/).

For a dominant mutation to be mapped, 50 offspring from the second backcross (**Fig. 1**) are collected and phenotyped initially. For a recessive mutation to be mapped, 50 offspring from the G3 (**Fig. 2B**) are collected and categorized for their mutation genotype. Linkage of the mutated allele with microsatellite markers is tested using the pooled DNA method. Computer programs are available for the analysis of mouse linkage data, for example, *Map manager*, written by Manly et al., or *Gene Link*, written by Montagutelli et al. *Mapmaker*, developed by Lander et al., is the most useful multilocus pedigree-based program. A map with a resolution of 10 cM requires the analysis of 100 meioses. From this stage onward, the strategy is then to search for candidate genes or to establish a high-resolution physical map for positional cloning. In the candidate gene approach, one picks a set of known genes, which might be important in the phenotype (for example, based on the biochemical functions of their products). The candidate genes are then analyzed in the mutant mice to search for alterations or for in vivo complementation using bacterial artificial chromosomes. In the positional cloning strategy, high-resolution physical maps are needed in an attempt to move closer and closer to the mutated gene of interest. Looking for features in the DNA fragments from the region known as DNA binding motifs, such as Sp1 sites or enhancer elements, which are commonly seen in active genes, allows the identification of a gene. One gene is proven to be the mutated gene when found in the DNA sequence from the affected animal's mutations that are excluded as a polymorphism.

3.7. Database

Data management is critical in a large-scale project. Therefore a database is needed that can combine the functions of a management and workflow

system for animal breeding with the features of a laboratory information and management system (LIMS). This database should be able to store and manage all data from the screening ***(15,38)***.

4. Notes

1. An alternative to genome-wide screens is to perform region-based screens (or sensitized screens). This approach provides a tool for identifying and characterizing genes in a defined chromosomal subregion in a two-generation breeding scheme ***(18,39)***.
2. In nearly all animal facilities in which large-scale screening programs are performed and mutant stocks have to be maintained, space is a limiting factor. Cryopreservation of spermatozoa from mutant male mice is an efficient and reliable way to archive mutants and should be included in the concept of a mutagenesis project. Cryopreservation technologies are described in more detail in Chapter 3.
3. In this chapter (**Subheading 2.6.**) quality control of ENU was restricted to visual control, melting point, and UV absorption analysis. A biologic QC can be established by using the specific locus test (SLT) to assess every single mutagenesis experiment for the mutation frequency expected. After regaining fertility, ENU-injected males are mated to SLT females producing F1 animals, which are assessed for the seven markers of interest. Approximately 2000–2500 F1 animals have to be checked to obtain reliable results.

References

1. Ashburner, M. (1989) *Drosophila. A Laboratory Handbook*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Russell, L. B., Hunsicker, P. R., Cacheiro, N. L., et al. (1989) Chlorambucil effectively induces deletion mutations in mouse germ cells. *Proc. Natl. Acad. Sci. USA* **86,** 3704–3708.
3. Russell, L. B., Hunsicker, P. R., and Shelby, M. D. (1992) Melphalan, a second chemical for which specific-locus mutation induction in the mouse is maximum in early spermatids. *Mutat. Res.* **282,** 151–158.
4. Russell, W. L. (1951) X-ray-induced mutations in mice. Cold Spring Harbor Symp. Quant. Biol. 327–336.
5. Searle, A. G. (1974) *Mutation Induction in Mice*. Academic, New York.
6. Ehling, U. H. and Favor, J. (1984) Recessive and dominant mutations in mice, in *Mutation, Cancer and Malformations* (Chu, E. H.Y. and Generoso, W.M., eds.), Plenum, New York, pp. 389–428.
7. Chen, Y., Yee, D., Dains, K., et al. (2000) Genotype-based screen for ENU-induced mutations in mouse embryonic stem cells. *Nat. Genet.* **24,** 314–317.
8. Russell, W. L., Kelly, E. M., Hunsicker, P. R., et al. (1979) Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. *Proc. Natl. Acad. Sci. USA* **76,** 5818–5819.

9. Hitotsumachi, S., Carpenter, D. A., and Russell, W. L. (1985) Dose-repetition increases the mutagenic effectiveness of N-ethyl-N-nitrosourea in mouse spermatogonia. *Proc. Natl. Acad. Sci. USA* **82,** 6619–6621.
10. Weber, J. S., Salinger, A., and Justice, M. J. (2000) Optimal N-ethyl-N-nitrosourea (ENU) doses for inbred mouse strains [letter]. *Genesis* **26,** 230–233.
11. Justice, M. J., Carpenter, D. A., Favor, J., et al. (2000) Effects of ENU dosage on mouse strains. *Mamm. Genome* **11,** 484–488.
12. Rogers, D. C., Fisher, E. M., Brown, S. D., et al. (1997) Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm. Genome* **8,** 711–713.
13. Brown, S. D. and Nolan, P. M. (1998) Mouse mutagenesis—systematic studies of mammalian gene function. *Hum. Mol. Genet.* **7,** 1627–1633.
14. Hrabe de Angelis, M. and Balling, R. (1998) Large scale ENU screens in the mouse: genetics meets genomics. *Mutat. Res.* **400,** 25–32.
15. Hrabe de Angelis, M., Flaswinkel, H., Fuchs, H., et al. (2000) Genome wide large scale production of mutant mice by ENU mutagenesis. *Nat. Genet.* **25,** 444–447.
16. Rinchik, E. M., Carpenter, D. A., and Long, C. L. (1993) Deletion mapping of four loci defined by N-ethyl-N-nitrosourea-induced postimplantation-lethal mutations within the pid-Hbb region of mouse chromosome 7. *Genetics* **135,** 1117–1123.
17. Justice, M. J., Zheng, B., Woychik, R. P., and Bradley, A. (1997) Using targeted large deletions and high-efficiency N-ethyl-N-nitrosourea mutagenesis for functional analyses of the mammalian genome. *Methods* **13,** 423–436.
18. Schimenti, J. and Bucan, M. (1998) Functional genomics in the mouse: phenotype-based mutagenesis screens. *Genome Res.* **8,** 698–710.
19. Rinchik, E. M. and Carpenter, D. A. (1999) N-ethyl-N-nitrosourea mutagenesis of a 6- to 11-cM subregion of the Fah-Hbb interval of mouse chromosome 7: Completed testing of 4557 gametes and deletion mapping and complementation analysis of 31 mutations. *Genetics* **152,** 373–83.
20. Justice, M. J., Noveroske, J. K., Weber, J. S., Zheng, B., and Bradley, A. (1999) Mouse ENU mutagenesis. *Hum. Mol. Genet.* **8,** 1955–1963.
21. Favor, J. (1983) A comparison of the dominant cataract and recessive specific-locus mutation rates induced by treatment of male mice with ethylnitrosourea. *Mutat. Res.* **110,** 367–382.
22. Soewarto, D., Fella, C., Teubner, A., et al. (2000) The large scale Munich ENU-mouse-mutagenesis screen. *Mamm. Genome* **11,** 507–510.
23. Fuchs, H., Schughart, K., Wolf, E., Balling, R., and Hrabe de Angelis, M. (2000) Screening for dysmorphological abnormalities—a powerful tool to isolate new mouse mutants. *Mamm. Genome* **11,** 528–530.
24. Rathkolb, B., Decker, T., Fuchs, E., et al. (2000) The clinical-chemical screen in the Munich ENU Mouse Mutagenesis Project: screening for clinically relevant phenotypes. *Mamm. Genome* **11,** 543–546.
25. Rolinski, B., Arnecke, R., Dame, T., et al. (2000) The biochemical metabolite screen in the Munich ENU Mouse Mutagenesis Project: determination of amino

acids and acylcarnitines by tandem mass spectrometry. *Mamm. Genome* **11,** 547–551.
26. Flaswinkel, H., Alessandrini, F., Rathkolb, B., et al. (2000) Identification of immunological relevant phenotypes in ENU mutagenized mice. *Mamm. Genome* **11,** 526–527.
27. Schindewolf, C., Lobenwein, K., Trinczek, K., et al. (2000) Comet assay as a tool to screen for mouse models with inherited radiation sensitivity. *Mamm. Genome* **11,** 552–554.
28. Shibuya, T. and Morimoto, K. (1993) A review of the genotoxicity of 1-ethyl-1-nitrosourea. *Mutat. Res.* **297,** 3–38.
29. van Zeeland, A. A. (1988) Molecular dosimetry of alkylating agents: quantitative comparison of genetic effects on the basis of DNA adduct formation. *Mutagenesis* **3,** 179–191.
30. Vogel, E. W., Nivard, M. J., Ballering, L. A., et al. (1996) DNA damage and repair in mutagenesis and carcinogenesis: implications of structure-activity relationships for cross-species extrapolation. *Mutat. Res.* **353,** 177–218.
31. Ehling, U. H., Favor, J., Kratochvilova, J., and Neuhauser-Klaus, A. (1982) Dominant cataract mutations and specific-locus mutations in mice induced by radiation or ethylnitrosourea. *Mutat. Res.* **92,** 181–192.
32. Nishina, P. M., Wang, J., Toyofuku, W., et al. (1993) Atherosclerosis and plasma and liver lipids in nine inbred strains of mice. *Lipids* **28,** 599–605.
33. Mills, E., Kuhn, C. M., Feinglos, M. N., and Surwit, R. (1993) Hypertension in CB57BL/6J mouse model of non-insulin-dependent diabetes mellitus. *Am. J. Physiol.* **264,** R73–78.
34. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Adler, I. D. (1996) Comparison of the duration of spermatogenesis between male rodents and humans. *Mutat. Res.* **352,** 169–172.
36. Schalkwyk, L. C., Jung, M., Daser, A., et al. (1999) Panel of microsatellite markers for whole-genome scans and radiation hybrid mapping and a mouse family tree. *Genome Res.* **9,** 878–887.
37. Lindblad-Toh, K., Winchester, E., Daly, M. J., et al. (2000) Large-scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. *Nat. Genet.* **24,** 381–386.
38. Pargent, W., Heffner, S., Schable, K. F., et al. (2000) MouseNet(c) database: digital management of a large-scale mutagenesis project. *Mamm. Genome* **11,** 590–593.
39. Anderson, K. V. (2000) Finding the genes that direct mammalian development: ENU mutagenesis in the mouse. *Trends Genet.* **16,** 99–102.

14

Autopsy and Histologic Analysis of the Transgenic Mouse

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1. Introduction

Ttransgenic and knockout mice often show phenotypes that differ from our expectations. Therefore, it is important to carry out a complete pathologic examination using both macroscopic and microscopic analysis. It should be noted that, apart from (un)expected phenotypes caused directly by expression of the transgene, the integration of the transgene may also silence genes, leading to phenotypic changes. For analyzing these phenomena, it is valuable to do routine histologic studies of the complete mouse, with all organs are examined in paraffin sections using a regular hematoxylin and eosin staining. If abnormalities are observed in tissues, immunohistochemistry can give extra information regarding the cell types involved.

This chapter gives protocols for autopsy of the mouse, decalcification of the bone, and trimming, embedding, sectioning, and staining of the tissues, as well as a general protocol for immunohistochemistry. Additional, very valuable information can be obtained from the pathology web site of the EULEP: http://www.eulep.org/Necropsy_of_the_Mouse/index.php

2. Materials

2.1. Autopsy of the Mouse

1. Scalpel.
2. Scissors (12 cm).
3. Forceps.
4. Pliers.
5. Pins.

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen © Humana Press Inc., Totowa, NJ

6. Dissection board.
7. 10-mL syringe with 25-gage needle.
8. Needleholder.
9. Spatula.
10. 70% ethanol.
11. Fixative: 10% buffered formalin: 108 mL 37% formaldehyde and 892 mL phosphate-buffered saline (PBS).

2.2. Trimming Procedures, Embedding, and Sectioning

1. 100% ethanol.
2. Toluene.
3. Paraffin.
4. Embedding molds.
5. Embedding cassettes.
6. Microtome.

2.3. Decalcification of the Bone

1. Decalcification solution: 40 g NaOH, 827 mL distilled water, 173 mL formic acid. **Caution:** dissolving NaOH releases heat.

2.4. Staining of the Slides

1. Mayer's hematoxylin: dissolve in 1 L aqua dest. at 80°C, 50 g potassium alum, 1 g hematoxylin, 200 mg sodium iodate, 25 g chloral hydrate, 500 mg citric acid.
2. Eosin: 1% eosin Y (yellowish) in H_2O. Add a crystal of thymol to prevent the growth of molds.

2.5. Immunohistochemistry

1. Mouse tissues.
2. 2-Methylbutane.
3. Tissue-tek (Sakura).
4. Dry ice.
5. Cryomolds.
6. Cryomicrotome.
7. Slide storage box.
8. Silica gel.
9. PBS.
10. Triton X-100
11. Bovine serum albumin.
12. Avidin/biotin block (Vector).
13. Biotinylated secondary antibody.
14. Normal serum (from the species in which the secondary antibody was raised).
15. Streptavidin-PO (DAKO).

16. Glycergel (DAKO).
17. 2% Parafix: Dissolve 2 g paraformaldehyde in 80 mL H_2O and heat (to approx 50°C, not higher!). Clear with approx. 10 drops of 0.1 *M* NaOH and then filter through a paper filter. Add 10 mL 10x PBS and 10 mL 1 *M* HEPES and cool on ice.
18. Glucose oxidase quench: To 50 mL PBS add 50 µL 1 *M* sodium azide and 90 mg glucose. Heat to 37°C and add 20 U glucose oxidase (Sigma) just before use.
19. APES (amino-propyl-triethoxy-silane)-coated slides: Clean slides very thoroughly (using DECON or other soap). Rinse with tapwater and then distilled water. Dry at 37°C. Dip slides for 10 s in APES solution in acetone. Dip slides 5 times in distilled water. Dry at 37°C. Store at room temperature.

3. Methods

3.1. Autopsy of the Mouse

3.1.1. Initiation and Superficial Organs

During the necropsy, all observations made must be described, preferably on an appropriate card. On this pathology card (**Fig. 1**), the information on the identification of the animal will be transcribed and all the macroscopic observations made during necropsy will be reported. All isolated tissues are immediately transferred to 10% formalin (*see* **Note 1**).

1. Sacrifice the mouse by CO_2 asphyxation (*see* **Note 2**). To avoid autolysis, carry out the necropsy as soon as possible. Fix the mouse on a dissection board with the limbs spread and held firmly with pins in the four paws.
2. First examine the mouse's general condition: state of nutrition and development of the skeletal muscular masses, presence of skin alterations, fur, and superficial lesions. Spray the fur with 70% ethanol.
3. Perform a median longitudinal cut superior to the jaw, taking care to separate the skin accurately from the underlying musculature. Now the superficial cervical, (above the salivary glands), axillary (present in the axillary fossa), brachial (in proximity to the angle of the scapula), and inguinal (close to the bifurcation of the superficial epigastric vein) lymph nodes are visible. They are grayish and shaped like a small bean. Dissect these first and use a small container for their collection.
4. Take part of the skin with the mammary glands.
5. Remove the salivary glands (ventral cervical region).
6. In the males, remove the preputial glands, which lie above the penis in the connective tissue, and are leaf-shaped and of a yellowish color.
7. Once the observation of the superficial organs is over, proceed with examination of the inner cavities of the mouse, starting from the abdomen, then the thorax, and finally the skull.

GROSS NECROPSY / BIOPSY REPORT

Project nr.:..... **Histology nr.:**.....

Animal nr.:..... Investigator:.....
Species:..... Prosector/biopteur:.....
Sex:..... Pathologist:.....
Date of birth:..... Date of necropsy/biopsy:.....
Fixation:.....
Title of Protocol:.....

NATURE OF DEATH: spontaneous/ill/euthanasia/sacrified/accident
MATERIAL: necropsy/biopsy (tissue:.....)

Immunohistochemistry	☐
Photo gross	☐
Photo micro	☐
Photo slide	☐

Previous treatment/disease:	none	☐	data enclosed	☐
Recent (experimental) treatment:	none	☐	data enclosed	☐
Clinical chemical data:	none	☐	data enclosed	☐
Hematology data:	none	☐	data enclosed	☐

Questions for pathology:

PATHOLOGY REPORT by pathologist:

Fig. 1. General outline of the first page of the pathology card. For describing gross findings, a table containing the following tissues should be present on the other side: skin, teeth, tongue, salivary glands, mammary tissue, lungs, pleural surface, pericard, heart, large vessels, peritoneal cavity, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, anus, liver, gallbladder, pancreas, adrenals, kidney, urinary bladder, testis, epididymis, seminal vesicles, prostate, preputial gland, ovary, uterus, cervix, vagina, clitoral gland, spleen, lymph nodes (mesenteric, cervical, axillary, inguinal, others), Peyer's patches, thymus, skeleton, bone marrow, joints, nose, trachea/larynx, thyroid/parathyroid, brain/meninges, spinal cord, pituitary, epiphysis, ears, and eyes.

3.1.2. Abdominal Cavity

1. Cut along the median axis of the muscular wall and observe the position of the organs and the presence of liquids in the cavity. The liver is a large glandular organ that occupies a large portion of the abdominal cavity of the mouse. It adheres to the diaphragm and is dark red. To remove the liver, cut the falciform and coronary ligaments that keep the organ intimately connected with the diaphragm. The gallbladder is visible on the inferior surface of the organ, where

it appears as a small bag a few millimeters in diameter. It has to be removed together with the liver.

2. The spleen is situated in the left superior abdominal quadrant and has a dark red color and a lengthened, oval, slightly curved shape. Take into account the volume, consistency, color, margins, and any evident lesions. During the extraction, the pancreas can also be easily removed because of its intimate connection with the spleen. The pancreas is not a compact organ but is embedded in the mesenteric adipose tissue.
3. The mesenteric lymph node in the mouse is found intimately connected to the ascending colon and is easily detectible when the cecum is lifted to the right. The color is yellowish, compared with the white mesentery.
4. With just one movement, the extraction of the whole intestine and stomach can be achieved, starting with a single cut at the level of the esophagus. The intestine is approximately 40 cm long and comprises the small and large intestine. The small intestine is divided into the duodenum, jejunum, and ileum. The duodenum starts from the stomach and is approximately a quarter of the length; the next part (approximately half of the small intestine) is the jejunum, and the last quarter is the ileum. The large intestine comprises the cecum, which consists of a small bag located in the right inferior quadrant of the abdomen, the colon, and the rectum, which is the final portion of the intestine. Take the stomach with a small portion of the duodenum, a small portion of the jejunum, together with the Peyer's patches (yellowish nodules on the outside of the intestine), and the cecum with a small portion of the ileum on one side and a small portion of the colon on the other; transfer these organs to formalin. Once the abdominal cavity is free of the organs previously removed, the investigator will be able to examine the urinary apparatus: kidneys, ureters, bladder, and urethra.
5. Remove the kidneys together with the adrenals (small glands in the superior pole of the kidneys, which are opaque and pale in females and rose-colored in males), cut the right kidney in two pieces, after very careful examination, and transfer one part together with the adrenal to the formalin as well as the intact left kidney.
6. In females, cut the bladder at the lowest part. In males, take the bladder out together with the genital apparatus. This includes the testes, epididymis, seminal vesicles, prostate (very difficult to detect macroscopically), penis, and preputial glands. The testes are situated at the side of the bladder, inside the scrotum, and adhere to fat pads. Remove the testes together with the epididymis by pulling the fat pads, so the testis comes out of the scrotum, and cutting the excretory ducts close to their outlet in the membranous urethra. Then remove the seminal vesicles and coagulating glands together with the prostate and the bladder. The female genital apparatus includes the vagina, cervix, uterus, oviducts, and ovaries. Remove the whole female genital apparatus by cutting the vagina near the anal opening and lifting it with forceps. From here, proceeding upward, cut the mesometrium ligaments, which fix the organs to the posterior wall of the

abdomen, up to the level of the ovaries at the back. Cut the ligaments by which the ovaries are attached to the inferior poles of the kidneys.

3.1.3. Thoracic Cavity

1. Open the thoracic cavity by cutting the ribs from the sternum and cut the front margins of the diaphragm. Keeping the sternal plate raised, separate the parietal pleura and the pericardium. The investigator must consider the position of the organs, the presence of adhesion (as a result of pleuritis or pericarditis), or hydro- and hemothorax. Then cut next to the esophagus, through the lower jaw between the incisors.
2. Lift up the tongue with forceps and remove with scissors all the thoracic organs to the level of the diaphragm. The thoracic organs consist of the thymus, lungs and esophagus, heart, and thyroid. The thymus consists of two lobes and lies on the median line of the vertebral column, close to the base of the heart. In young mice, the thymus is well developed; in the adult, the thymus is atrophic and macroscopically difficult to detect. The lungs are two large organs and are composed of lobes. The right lung consists of four lobes and the left lung shows a single lobe. Usually it is pale rose-colored. The heart has a pyramidal triangular shape, with its greater axis oriented obliquely to the left. The thyroid consists of two small lobes with an oval shape adherent to the lateral and dorsal surfaces of the trachea and has a yellow color. Leave the thoracic organs intact.
3. Grip the tongue with forceps and search for the opening of the esophagus. Inject gently approx 3 mL formalin in the esophagus using a syringe with a 25-gage needle until the lungs are nicely blown up and close the esophagus with the needleholder. Take care of the thyroid.

3.1.4. Cranial Cavity

For opening the skull, grip the head firmly with large forceps and remove the skin from the skull. Open the cranial cavity using scissors that must be used only for this procedure since they end up very blunt. Make a cross-section at the level of the nasal septum, which divides the two orbital cavities, and cut the occipital and parietal bones. Next, remove the skull; the brain and meninges can be seen. Separate the bulb from the spinal cord with a small spatula and remove the brain. Now the cranial base can be examined, with particular attention to the pituitary. This is an oblate spheroid with its greater axis perpendicular to the cranial base.

3.1.5. Head

Remove the lower jaw and cut off the head behind the skull. Remove most of the skin for good fixation. The head contains the nose, inner ear, eyes, harderian gland, and pituitary. The pituitary can be fixed *in situ* together with the base of the skull to avoid damage. Fix the entire head.

3.1.6. Spinal Cord

Prepare the cervical, thoracic, and lumbar spinal cord by removing the surrounding tissues with a scalpel, and transfer the spinal cord to formalin.

Fix all tissues in 10% formalin for at least 24 h.

3.2. Trimming Procedure in the Mouse

High-quality trimming is important, to be able to observe putative microscopic abnormalities with the highest probability. The more tissue that can be examined, the more accurate the examination will be. Trim the organs as high as the embedding cassette, about 3 mm.

The following terms are used for determination of the trimming directions:

1. Longitudinal: in the direction of the length axis of the body, an organ or part of an organ.
2. Transverse: across the length axis of an organ or part of an organ.
3. Vertical: in the direction of the dorsoventral axis of an organ or part of an organ.
4. Horizontal: in the direction perpendicular to the dorsoventral axis of an organ or part of an organ.

Prior to trimming, all tissues can be rinsed in tap water to remove the formalin.

3.2.1. Skin and Mammary Tissue

A section transverse to the axis of the hair shaft in the inguinal region close to the nipples is suggested for combined examination of the mammary gland, the skin, and the subcutaneous tissue.

3.2.2. Lymph Nodes

The lymph nodes are most often embedded untrimmed as a whole organ because of their small size. It is important that a section be taken from the middle area of the lymph node to be able to examine all major areas of the lymph node including the cortex, paracortex, and medulla.

3.2.3. Salivary Gland

The salivary gland consists of the mandibular, parotid, and sublingual gland. Furthermore, the cervical superficial lymph nodes are present immediately above the submandibular salivary glands. The three salivary glands and the mandibular lymphatic center, which consists of two or three lymph nodes, are embedded together. If necessary, remove a small piece of the salivary gland longitudionally.

3.2.4. Preputial Gland

Embed the whole preputial gland.

3.2.5. Liver and Gallbladder

Make one transverse section through the left lateral lobe, the gallbladder, and the right lateral lobe

3.2.6. Spleen

Make a transverse section through the middle of the organ. This plane of section guarantees the presence of all anatomic structures of this organ.

3.2.7. Pancreas

Embed the whole pancreas.

3.2.8. Small and Large Intestine

Take one transverse section from each part of the unopened bowel. Cut transversely through the Peyer's patches of the jejunum.

3.2.9. Adrenals

Cut the adrenals from the kidneys and embed the whole adrenals together with other small organs such as the lymph nodes. It is necessary to cut the sections until the medulla can be observed.

3.2.10. Kidneys

Cut the right kidney transversely. The transverse section of the kidney from the middle portion permits optimal representation of the renal papilla. The renal pelvis and the ureteropelvic junction can also be evaluated in the same section. Cut the right kidney longitudinally. The longitudinal section permits histologic evaluation of a relatively large area of tissue that includes both renal poles. This is advantageous for evaluation of any focal lesions. In addition, the regions of the renal pelvis close to the poles are of interest with respect to concretions and urothelial changes. It therefore seems optimal to have both planes of sections available for comprehensive evaluation of the kidneys.

3.2.11. Urinary Bladder

The bladder is cut vertically through the ventral knot to access the following regions:

1. Vertex and bottom: the vertex is the area probably most prone to development of neoplasms; deposition of sediments and calculi occurs mainly at the bottom, which can lead to urothelial alterations.

2. Dorsal part of the bladder.
3. Bladder neck with trigone: although in most of the urinary bladder the urothelium is of entodermal origin, in the trigone it is derived from the mesodermal epithelium of the Wolffian ducts.

3.2.12. Testis and Epididymis

Remove the fat pads but not the epididymis and make a longitudinal section through the middle of the testis. Do not cut the epididymis.

3.2.13. Seminal Vesicles, Coagulating Gland, and Prostate

Remove half of the seminal vesicles (transversely). Cut longitudinally through the seminal vesicles, coagulation gland, and prostate complex, including dorsolateral and ventral lobes, urethra, and, optionally, ureter and ductus deferens.

3.2.14. Ovaries

Remove most of the fat around the ovaries and embed the whole ovaries.

3.2.15. Uterus, Cervix, and Vagina

Remove half of the uterine horns. Make a longitudinal section through part of the uterus, the cervix, and part of the vagina.

3.2.16. Thyroid

One transverse section through both lobes of the thyroid gland including the parathyroid gland with the underlying trachea and esophagus is required. To include the parathyroid consistently, more than one section may be cut.

3.2.17. Thymus

Embed the whole thymus.

3.2.18. Heart

Make a longitudinal section through both ventricles from the base to the apex of the heart. The two halves with the main vessel trunks are blocked to get a section through the opened ventricles and atria with the auricles as well as through the base, septum, apex, papillary muscle, and main vessels of the heart.

3.2.19. Lungs

Cut the left lobe and the right caudal lobe vertically through the lobar bronchus and its main branches.

3.2.20. Brain

Obtain three transverse sections: the first section at the level of the optic chiasma including the basal ganglia, septum, cortex, and anterior hypothalamus,

the second section at the level of the hippocampus containing the cortex and brainstem at the transition of the diencephalon to mesencephalon, and the third section containing the cerebellum and pons in transition to the medulla oblongata.

3.2.21. Pituitary

Remove the pituitary from the skull. The pituitary consists of three parts: pars distalis, pars intermedia, and pars nervosa. All three parts should be present in one section with the largest possible area. This is best achieved by embedding the whole pituitary with its caudodorsal part toward the cut surface of the block.

3.2.22. Ears, Nasal Cavity, and Eyes

The sections can only be made of decalcified tissue (*see* **Subheading 3.3.**). Make three transverse sections through the head:

1. At the level of the second palatine crest.
2. At the level of the eyes.
3. At the level of the inner ears.

3.2.23. Spinal Cord

The sections can only be made of decalcified tissue (*see* **Subheading 3.3.**). Make three transverse sections:

1. Cervical cord at the upper cervical segment.
2. Thoracic cord at the midthoracic segment.
3. Lumbar cord at the fourth lumbar segment close to the last rib.

The remaining parts can be longitudinally trimmed.

3.3. Decalcification of Bone

After fixation for more than 24 h in 10% buffered formalin, the spinal cord and the skull have to be decalcified for 2 weeks, by submerging them in the decalcification solution.

3.4. Embedding and Sectioning of the Tissues

Take the tissues through the following series:

1. 70% alcohol, 1 h.
2. 70% alcohol, 1 h.
3. 70% alcohol, 1 h.
4. 96% alcohol, 1 h.
5. 96% alcohol, 1 h.
6. 100% alcohol, 1-1/2 h.

7. 100% alcohol, 1-1/2 h.
8. 100% alcohol, 1-1/2 h.
9. Toluene, 1 h.
10. Toluene, 1 h.
11. Paraffin, 1 h, 60°C.
12. Paraffin, 60°C, until the tissues are removed (but remove them the same day).
13. Embed the tissues in a cassette (*see* **Notes 3** and **4**).
14. Cut 4-μm sections with a microtome.

3.5. Staining of the Sections (Hematoxylin and Eosin)

1. Xylene, 5 min.
2. Xylene, 5 min.
3. 100% alcohol, 10 s.
4. 96% alcohol, 10 s.
5. 70 % alcohol, 10 s.
6. Aqua dest, 10 s.
7. Hematoxylin, 10 min.
8. Wash in running tap water, 5 min.
9. Eosin, 4 min.
10. Wash in tap water.
11. 70% alcohol, 10 s.
12. 96% alcohol, 10 s.
13. 100% alcohol, 10 s.
14. Xylene, 5 min.
15. Xylene, 5 min.

After staining is complete, the section is usually permanently sealed under a thin glass cover slip, the mountant being a natural resin or a synthetic one, almost invariably not mixable with water or ethanol. Clear and mount. Nuclei stain blue and other tissue components shades of red and pink.

3.6. Immunohistochemistry

The following protocols describe the detection of proteins in mouse tissue sections. It is possible to perform immunohistochemistry on paraffin-embedded, formalin-fixed tissue sections; however, antigens are much better preserved in frozen tissues. Therefore cryosections are preferred. For isolation and identification of different tissues, *see* **Subheading 3.1.** Instead of fixing the tissues in 10% formalin, they are frozen.

3.6.1. Tissue Preparation and Cryosectioning

1. Cool 2-methylbutane in a beaker on dry ice.
2. Take the tissues from the mice and trim them to the right size and plane. Work quickly.

3. Put the tissues you want to combine in a cryomold appropriate for your particular cryomicrotome.
4. Cover the tissues in the cryomold with Tissue-tek, making sure not to enclose any air bubbles and that the tissues are not touching each other.
5. Submerge the mold in the precooled 2-methylbutane.
6. After the Tissue-tek is frozen, remove from the 2-methylbutane and store at –70°C until further use.

3.6.2. Sectioning

1. Take the tissue blocks from –70°C to the cryomicrotome.
2. Make sure that the tissue blocks are not warmed at any time; transport can be done best on dry ice!
3. Section the tissues at 7 μm.
4. Collect the sections on APES-coated slides.
5. After sectioning, dry the slides for at least 2 h at room temperature.
6. After this period, put the slides in a plastic container.
7. Add a small amount (in a small paper box with pinholes) of silica gel to the container.
8. Freeze the slides at –20°C until further use.

3.6.3. Immunostaining

Unless stated otherwise, all steps are performed at room temperature.

1. Thaw slides (for at least 30 min at room temperature, outside the storage box).
2. Fix sections in 2% Parafix on ice for 10 min.
3. Wash in PBS-T (PBS + 0.1% Triton X-100), 10 min.
4. Quench endogenous peroxidase in glucose oxidase quench for 15 min at 37°C.
5. Wash in PBS-T, 5 min.
6. Avidin block, 15 min.
7. Wash in PBS-T, 5 min.
8. Biotin block, 15 min.
9. Block with 5% normal serum (from the species in which the secondary antibody was raised) in PBS/1% BSA for 30 min.
10. Add primary antibody, diluted in 5% normal serum/PBS/1% BSA, 60 min.
11. Wash in PBS-T, 5 min.
12. Add biotinylated secondary antibody (*see* **Note 5**) in PBS/1% BSA (optionally with 2% normal mouse serum, and let stand for 10 min before adding to section (*see* **Note 6**), 30 min.
13. Wash in PBS-T, 5 min.
14. Add streptavidin–PO in PBS/1% BSA, 30 min.
15. Wash in PBS-T, 5 min.
16. Develop with 3-amino-g-ethylcarbazole (AEC) according to the manufacturer's instructions.

17. Wash in PBS, distilled water, and finally tap water.
18. Counterstain with hematoxylin for 10 s, wash in tap water, and coverslip with Glycergel®.

4. Notes

1. Do not transfer the small organs such as lymph nodes together with other organs in the 10% formalin but transfer them to a small container.
2. Do not sacrifice the mice by cervical dislocation since this will cause hemorrhages in the thoracic cavity.
3. Embed the small organs, such as lymph nodes, ovaries, and adrenals together, and embed the skin alone.
4. Embed more organs together in one cassette to save time with sectioning.
5. We have very good results, with low backgrounds, using secondary antibodies raised in donkey.
6. If the primary antibodies are rat monoclonals, some background may occur through crossreaction of the secondary antibody. To overcome this, preincubation of the diluted secondary antibody with 2% normal mouse serum for 10 min may be tried.

Useful Websites

http://www.ita.fhg.de/reni/trimming/TR_F.htm. Information on the trimming procedure of the tissues of the mouse.

http://www.eulep.org/Necropsy_of_the_Mouse/index.php. Very good website on autopsy of the mouse.

http://www.ncifcrf.gov/vetpath/. Information on autopsy of the mice and additional information on immunohistochemical stainings.

Suggested Readings for Pathologic Evaluation

Maronpot, R. R. (1999) *Pathology of the Mouse*, Cache River Press, Vienna, IL.

Ward, J. M. Mahler, J. F., Maronpot, R. R., and Sundberg, J. P. (2000) *Pathobiology of the Genetically Engineered Mice.* Iowa State University Press, Ames, IA.

15

Bone Marrow Transplantations to Study Gene Function in Hematopoietic Cells

Menno P. J. de Winther and Peter Heeringa

1. Introduction

1.1. General

All cellular components of the blood originate from hematopoietic stem cells in the bone marrow. These pluripotent stem cells give rise to two lineages of specialized cells (**Fig. 1**). The lymphoid lineage produces B- and T-cells; the myeloid lineage gives rise to leukocytes, erythroblasts (the precursors of erythrocytes), and megakaryocytes (which produce platelets) ***(1)***.

Transplantation of the hematopoietic systems offers the unique opportunity to replace genes in cells originating from the bone marrow. Murine recipients can be provided with a completely new, permanently functioning hematopoietic system after transplantation with donor bone marrow. The donor marrow can be derived from any transgenic or knockout mice, making it easy to manipulate gene function in the hematopoietic system. The transplantation results in a gradual replacement of the recipient bone marrow cells by cells from a donor of choice, establishing a new hematopoietic system, including new monocytes and tissue macrophages.

The bone marrow microenvironment is a complex structure in which hematopoietic stem cells proliferate, mature, differentiate, migrate into the sinusoidal space, and enter the circulation in a strictly regulated fashion. After entering the circulation, bone marrow cells keep their capacity to repopulate the bone marrow. This feature is called *homing* and is essential for bone marrow transplantation ***(2)***. Upon transplantation, the stem cells must home themselves to the extravascular compartment of the bone marrow. This can only be achieved if the endogenous stem cells are eradicated, which is usually

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

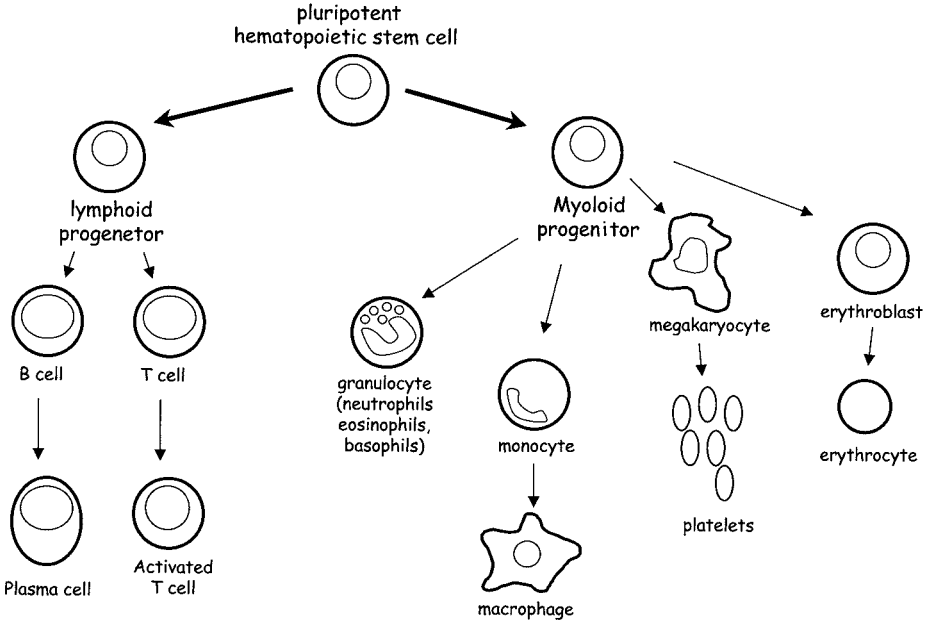

Fig. 1. Hematopoietic stem cell-derived lineages.

done by a lethal dose of total body irradiation (TBI) of the recipient. Without donor bone marrow cell transplantation after TBI, the recipient will die within 2 weeks. Bone marrow aplasia will develop in about 2–3 days, and atrophy can be observed in the lymphatic tissues of the lymph nodes, spleen, intestinal tract, and thymus. In 2 weeks' time, a strong depression of lymphocytes, granulocytes, thrombocytes, and finally erythrocytes will develop. In the second week after irradiation without transplantation, depletion of the thrombocytes and leukocytes usually results in the death of the mouse caused by hemorrhages and septicemia ***(3)***.

However, following administration of donor bone marrow after TBI, repopulation of the marrow will occur and the recipient can survive. Two distinct phases of engraftment can be distinguished. The initial rescue of the hematopoietic system is mediated by committed progenitors; the final long-term recovery occurs through establishment of pluripotent stem cells in the marrow ***(4)***. Four days after administration of the donor cells, repopulation starts through homing of the cells to the marrow cavity. The mechanisms for this homing are not well understood but probably involve integrins ***(2)***. By the 7th day after transplantation, the bone marrow cell population is completely restored. Peripheral blood cell counts will return to normal levels after 4 weeks, with the number of mononuclear cells being depressed for the longest.

One of the major complications of bone marrow transplantations is graft-versus-host disease (GVHD), in which allogenic bone marrow recognizes the tissues of the recipient as foreign, causing a severe inflammatory disease, often resulting in death. These complications are mediated by donor T-cells. The occurrence of GVHD can be depressed by in vitro elimination of mature T-cells from the donor bone marrow. Those T-cells that will develop newly in the recipient after transplantation will be tolerant to the recipient's antigens. In addition, immunosuppressive treatments can overcome GVHD. The easiest way to prevent GVHD is to match the most relevant tissue antigens (MHC) of the donor with that of the recipient. This is usually established by matching the genetic background of the donor and recipient mice.

This chapter discusses two approaches toward adaptation of the hematopoietic system. First, complete bone marrow transplantations are discussed. This method is especially useful if monocyte or macrophage functions are studied. Conventional bone marrow transplantation will result in replacement of the monocyte/macrophage system in the mice. The second part of the protocols discusses T-cell transfers since replacement of this cell lineage requires additional measurements.

1.2. T-Cell Transfers

T-cells develop and mature in the thymus. One of the major functions of T-cells is to provide "help" (helper T-cells) to B-cells and other T-cells through the production of cytokines. However, some T-cells can kill cells expressing foreign antigens and are termed cytotoxic T-cells. The T-cell population can be further subdivided into two major subpopulations based on the differential expression of the cell surface molecules CD4 and CD8. The major difference between these two subsets of T-cells is that CD4 T-cells recognize antigens in association with MHC II proteins, whereas CD8 T-cells mostly recognize antigens in association with MHC class I proteins.

Many assays have been developed for testing the functional properties of T- and B-cells in vitro ***(5)***. Among others, these include methods of measuring cytotoxic activity, proliferation, and antibody production that are usually performed on isolated lymphocyte subsets. Although these tests can provide important information on the function of lymphocytes in a particular experimental setting, they do not take into account the complex interplay between (immune) cells that occurs in vivo. Therefore, investigation of the biologic consequences of altering genes in lymphocytes still relies on experiments in living animals. To study the role of T-cells in immune responses in vivo, methods have been developed in which circulating T-cells or T-cell subsets are eliminated by treatment with specific depleting monoclonal antibodies. This method, however, requires large amounts of monoclonal antibodies, especially

when experiments require long-term depletion. An alternative to the depletion method is adoptive T-cell transfer. In this method, T-cells or subsets of T-cells are isolated from lymphoid organs from donor mice and subsequently transferred into histocompatible mice in which no T-cells are present. Thus, using adoptive transfer, mice can be created with a particular set or subset of T-cells, making it possible to analyze the effects of these cells on immune responses.

2. Materials

2.1. Bone Marrow Transplantation

1. Cross-flow cabinet.
2. Filter-top cages (or other semisterile housing).
3. Autoclaved acidified water, pH 2.0 with HCl.
4. Neomycin solution (Gibco).
5. Polymyxin B sulphate solution (Gibco).
6. X-ray apparatus.
7. Mouse container (in which to irradiate the mice).
8. Scissors.
9. Forceps.
10. Flow cabinet.
11. Ethanol.
12. Phosphate-buffered saline (PBS).
13. 19-, 25-, and 27-gage needles.
14. RPMI + 2% fetal calf serum + 5 U/mL heparin.

2.2. T-Cell Transfers

2.2.1. Preparation of Single Cell Suspensions from Spleen

1. Complete RPMI or Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS).
2. 60 × 15-mm Petri dishes.
3. Scissors and forceps.
4. 6-mL syringes with 19-gage needles.
5. 100-μm nylon screen.
6. Red blood cell lysing buffer: 8.29 g NH_4Cl (0.15 *M*), 1 g $KHCO_3$ (10.0 m*M*), 37.2 mg Na_2EDTA (0.1 m*M*); add 800 mL H_2O and adjust to pH 7.4 with 1 N HCL. Add H_2O to 1 L and filter-sterilize through a 0.2-μm filter; store at room temperature.

2.2.2. Isolation of T-Cells from Splenocyte Suspensions by Negative Selection Using Antibody-Coated Magnetic Beads

1. Monoclonal antibodies directed against I-A, I-E, CD3, CD4, CD8, and B220.
2. Isolated splenocytes (*see* **Subheading 3.2.1.**).

3. Coating medium: Hank's balanced salt solution (HBSS) without Ca^{2+}, Mg^{2+}, and phenol red containing 10% heat-inactivated FCS and 20 m*M* HEPES.
4. Goat anti-mouse IgG-coated magnetic beads (Dynabeads M-450, Dynal cat. nos. 11005, 11006) or sheep anti-rat IgG-coated magnetic beads (Dynabeads M-450, Dynal, cat. nos. 11007, 11008).
5. Magnetic separation device (Dynal MPC-I, cat. no. 12001).
6. 15-mL polypropylene tubes.
7. Flow cytometer.

2.2.3. Thymectomy

1. Mouse (> 3 weeks old).
2. 70% ethanol.
3. Dissecting board.
4. Scissors.
5. Forceps (2 pairs of 10-cm half-curved).
6. 9-mm wound clips and clip applier.
7. Pentobarbital.
8. 1-mL syringe with 27-gage needle.

3. Methods

3.1. Bone Marrow Transplantation

3.1.1. Preparation of Acceptor Mice

Seven days before the bone marrow transfer:

1. Transfer recipient mice to semisterile conditions (e.g., filter-top cages).
2. Feed mice a regular chow diet.
3. Put mice on acidified water to which neomycin (100 mg/L) and polymyxin B sulphate (60,000 U/L) have been added, until 4 weeks after the transfer.

One day before the bone marrow transfer:

4. In a cross-flow, transfer mice from their filter-top cages to an irradiation box (which can be a plastic box suitable for 5–10 mice).
5. Subject mice to a lethal dose of total body irradiation using a röntgen source (10 Gy) (*see* **Note 1**).
6. After irradiation, transfer mice, in the cross-flow, back to their filter-top cages.

3.1.2. Isolation of Bone Marrow Cells (on the Day of the Transfer)

1. Sacrifice the donor mice by cervical dislocation or CO_2 asphyxiation (*see* **Note 2**).
2. Cut off the hind legs after removing the skin.
3. Isolate femurs and tibia from the mice and remove most of the skeletal muscles (*see* **Note 3**).
4. Put the bones in PBS, keep on ice, and take to flow cabinet.

5. Sterilize the bones by submerging them for 30 s in 70% ethanol in a Petri dish.
6. Transfer to sterile PBS in a Petri dish.
7. Take a bone between forceps, cut off the ends, and flush the bone using PBS by inserting a 25-gage needle at one end.
8. Collect bone marrow in a 50-mL tube.
9. Repeat previous steps for all the bones.
10. Make the bone marrow cell suspension single cell by first passing it through a 19-gage needle, transfer it to a new tube, and then pass it through a 25-gage needle.
11. Count the cells.
12. Spin the cells down at 250*g*, 5 min at 4°C.
13. Resuspend the cells in RPMI-1640/2% FCS/5 U/mL heparin at a density of 5×10^7 cells/mL and keep on ice.

3.1.3. Transplantation of the Bone Marrow Cells

1. Take the cells to the cross-flow and inject the irradiated mice intravenously with 100 µL of cell suspension using a 27-gage needle (*see* **Note 4**).
2. Four weeks after transplantation, mice may be removed from the filter-top cages, drinking water can be regular again, and experiments may start (*see* **Note 5**).

3.2. T-Cell Transfers

3.2.1. Preparation of Single Cell Suspensions from Spleen

Following is a basic protocol for the preparation of single cell suspensions from murine spleens. The same protocol can be used for making cell suspension of lymph nodes and thymus (*see* **Note 6**).

1. Sacrifice the donor mice by cervical dislocation or CO_2 asphyxation.
2. Remove the spleen and place it in a Petri dish containing 3 mL of medium. Cut the spleen into small pieces.
3. Using a plunger or a 6-mL syringe, press the pieces against the bottom of the Petri dish until mostly fibrous tissue remains.
4. Disperse clumps further by drawing up and expelling the suspension through a 6-mL syringe with a 19-gage needle.
5. Expel suspension into a 50-mL polypropylene centrifuge tube through a 100-µm nylon sieve. Wash the Petri dish with 4 mL of medium.
6. Centrifuge for 10 min at 200*g*. Discard supernatant.
7. Resuspend pellet in red blood cell lysing buffer, 5 mL per spleen.
8. Incubate for 5 min at room temperature with occasional shaking.
9. Add 5 mL of medium, centrifuge at 200*g*, and discard supernatant.
10. Resuspend pellet in medium, centrifuge at 200*g*, discard supernatant.
11. Resuspend in medium.

3.2.2. Isolation of T-Cells from Splenocyte Suspensions by Negative Selection Using Antibody-Coated Magnetic Beads

Cell suspensions isolated from spleens are a heterogeneous mixture of cell types consisting of T-cells, B-cells, and accessory cells. In many cases, effects or functions of lymphocyte populations or subpopulations need to be studied separately (e.g., B-cells versus T-cells or CD4 T-cells versus CD8 T-cells). This requires techniques for the purification of these cell populations, preferably without affecting their activation state. For this purpose, a variety of techniques have been developed, most of which are based on antibody-mediated selection or depletions.

Immunomagnetic isolation of lymphocyte subpopulations uses antibody-mediated selection, magnetic polymer particles, and a magnet device to separate coated cells. This technique has several advantages over other methods including purity of the final cell preparation, reproducibility, and ease of handling small to large cell numbers. Using magnetic beads, both direct and indirect methods can be employed. In the direct method, magnetic beads coated with antibodies specific for a lymphocyte subset are applied to the cell suspension. After binding, the target cells can be recovered or removed from the cell suspension using a magnet. In the indirect method, subset-specific antibodies are first added to the cell suspension, followed by magnetic beads coated with an antibody specific for the primary subset specific antibody. The magnetic beads will bind the cells to which the primary antibody has bound, and these cells can then be recovered or removed using a magnet. Using magnetic cell separation, both positive isolation and negative selections (i.e., removing cell subsets from the cell suspension using specific antibodies and magnetic beads) of cell populations can be performed. If subsequent functional studies need to be performed, a negative selection procedure is recommended because the binding of antibody to cells as used in positive selection methods may affect functional properties of the cells. Here, a basic protocol is described for the negative selection of T-cells from splenocytes. Apart from immunomagnetic selection, other isolation procedures are possible. These techniques include antibody/complement-mediated lysis, cell sorting of fluorescence-labeled cells, and panning with immobilized antibodies.

1. Before starting the separation procedure, choose the appropriate combination of antibodies for negative selection of T-cells and determine the saturating concentration by flow cytometry. For purification of T-cells from murine splenocyte suspensions, B-cells and accessory cells can be removed by using monoclonal antibodies directed against I-A/I-E (*see* **Table 1**). Once the optimum antibody concentration has been determined, prepare a 10X antibody stock solution.

Table 1
Monoclonal Antibodies Against Mouse I-A/I-E *(5)*

Primary specificity	Crossreactive specificity	Hybridoma	Isotype	ATCC no.
I-A^k	I-$A^{f,r,s}$	10-2.16	IgG1	TIB 93
I-A^d	None	MK-D6	IgG2a	HB3
I-A^b	I-A^d, H-$2^{p,q}$	25-9-17	IgG2a	HB26
I-E^k	I-E^r	17-3-3	IgG2a	HB6
I-E^k	H-$2^{k,d,p,r}$	14-4-4	IgG2a	HB32
I-A	I-$A^{b,d,q}$, I-$E^{d,k}$	M5/114.15.2	Rat IgG2b	TIB 120
I-A	I-$A^{b,d}$	B21-2	Rat IgG2b	TIB 229

2. Resuspend splenocytes in coating medium at a concentration of 2×10^7 cells/mL.
3. From the 10X antibody stock solution add appropriate volume (i.e., 1/10 of the final cell suspension volume) to the cell suspension. Incubate on an end-over-end rotator for 30 min at 4°C.
4. Wash cell suspension to remove unbound antibody by spinning for 10 min at 150*g*. Resuspend in cold coating buffer and repeat washing. After second wash, resuspend cells in cold coating buffer at 2×10^7 cells/mL.
5. Wash goat anti-mouse IgG-coated magnetic beads by resuspending the beads in the vial. Transfer appropriate amount of beads to a 15-mL polypropylene tube. (Typically the amount of beads needed can be calculated by estimating the number of target cells and keeping the ratio of bead to target cell between 4 and 10.) Fill up with coating buffer and agitate. Pull beads to the side of the tube with a magnetic device. Incubate for approx. 5 min. Aspirate washing fluid. Repeat this procedure once.
6. Resuspend washed beads in an equal volume of coating buffer that was originally pipeted from the vial.
7. Add washed beads to the antibody-treated splenocytes from **step 4**. Rotate suspension for 1 h at 4°C.
8. Separate the cells labeled with monoclonal antibodies and magnetic beads using the magnetic apparatus. Incubate for 5 min. Transfer the unbound cells to a new tube and repeat the magnetic separation.
9. Count the cells and resuspend the cells. (Medium and volume depend on subsequent assays.)
10. Repeat **steps 5–8** if complete depletion is necessary.
11. Analyze the cell preparation for purity by flow cytometry using antibodies directed against MHC class II (I-A or I-E, accessory cells, and B-cells) B220 (B-cells) CD3 (all T-cells), CD 4, and CD8 (*see* **Notes 7** and **8**).

3.2.3. Thymectomy

Development and maturation of T-cells is dependent on the thymus. Therefore, removal of the thymus creates a mouse devoid of T-cells and can be used to study the role of these cells in immune response. However, this is only true when thymectomy is performed in neonatal mice from birth to day 3. Thymectomy in the adult mouse (>3 weeks) does not create a fully T-cell-depleted mouse but merely prevents generation of any new T-cells. In these mice, T-cells generated until the time of thymectomy will be present and can persist for a long time (*see* **Note 9**).

1. Anesthetize mouse by intraperitoneal injection of pentobarbital (40–60 mg/kg).
2. Position the mouse on the dissecting board in the dorsal position with the head facing the operator. Place a rolled-up gauze pad under the shoulders. Restrain arms and legs. Extend the neck by placing a rubber band in the mouth and securing the head.
3. Clean neck and chest with 70% ethanol. Using scissors, make a longitudinal midline incision over the suprasternal notch extending 2–3 cm down the chest.
4. Using the blunt end of a forceps, loosen the skin from the muscle layer. Retract the skin to expose the thoracic cage.
5. Insert scissors under the sternum and cut to the third rib.
6. Turn the dissecting board 90°C.
7. Hold forceps in both hands. Insert the tips of the closed caudal forceps (i.e., the forceps closest to the tail) into the incision and expose the chest by allowing the forceps to open.
8. With the cranial forceps (i.e., the forceps that is closest to the head), retract the strap muscles by inserting the closed forceps through the muscle layer and allowing the forceps to open. Insert the caudal forceps and open. The strap as well as the chest should now be held open, and the thymus should be visible as two thin white lobes overlying the heart.
9. Using both forceps, dissect the both lobes of the thymus.
10. Hold the skin closed to seal the chest.
11. Secure the skin with one or two 9-mm wound clips.
12. Clean the wound and warm the mouse with a heat lamp.
13. The mouse should recover from surgery and anesthesia in about 60 min.

3.2.4. Adoptive Transfer of T-Cells

Once the T-cells of interest are isolated and the appropriate recipient mouse strain has been chosen, adoptive transfer of T-cells can be performed. For this purpose, adjust cell number to 5×10^7 cells in PBS and inject 0.2 mL intravenously into recipient mice.

4. Notes

1. The dose for irradiation should be determined empirically; currently we are using a dose of 10 Gy. Testing several doses of irradiation followed either by no-transplantation and analysis of survival or by transplantation and analysis of chimerism (*see* **Note 5**) after 4 weeks will be essential.
2. On average, we inject 5×10^6 cells/recipient mouse. We obtain $2–4 \times 10^7$ cells/donor mouse.
3. Skeletal muscle from the bones is easily removed by rubbing them with a paper tissue.
4. Warming the mice using a heated mat or infrared lights enhances the visibility of the tail vein, highly facilitating the intravenous injections.
5. Ideally, complete chimerism is established, meaning that all hematopoietic cells of the recipient are of donor origin. However, sometimes chimerism is incomplete or donor cells have only replaced specific lineages of the hematopoietic system. Therefore, it is often important to establish the amount of chimerism in the recipients. This can be done, for example, by transplanting female recipient mice with male donor bone marrow and quantitatively genotyping the bone marrow after transplantation for the presence of Y-markers (by quantitative RT-PCR or Southern blotting). Other genetic differences between donors and recipients can also be used to quantify chimerism.
6. Various factors influence lymphocyte recoveries including gender, mouse strain, and, age. The number of recovered lymphocytes also varies between organs. As a reference, a 6-week-old mouse should give approximately $10–15 \times 10^7$ from the spleen, $5–10 \times 10^7$ from collected lymph nodes, and $10–20 \times 10^7$ from the thymus of recovered lymphocytes. In addition, the percentage of T-cells varies between these organs: 20–30% in the spleen, 70–80% in lymph nodes, and more than 90% in the thymus.
7. T-cells from mouse spleen and lymph nodes do not express cell surface MHC class II proteins, whereas B-cells and accessory cells do. This feature makes it possible to purify T-cells from these organs using anti-MHC class-specific monoclonal antibodies and negative selection magnetic bead separation. In the mouse, MHC class II proteins are encoded by the genes I-A and I-E. Therefore, it is necessary to establish which of these genes are expressed in the mouse strain used. Table 1 gives a list of hybridomas available from the ATCC, with antigen specificities.
8. The protocol can be easily adjusted to purify T-cell subsets (i.e., CD4 or CD8). In that case, saturating concentrations of antibodies specific for mouse CD4 or CD8 are added to the antibody mixture (**step 1**). The hybridomas are available from the ATCC (CD4: ATCC cat. no. TIB 207; CD8: ATCC cat. no. TIB 105).
9. The most stringently T-cell-depleted mouse can be created by thymectomizing mice at 4–6 weeks of age in conjunction with lethal irradiation to remove remaining T-cells followed by reconstitution with (T-cell) depleted bone marrow from syngeneic mice as described above. As an alternative to the thymectomy, immunodeficient strains of mice can be used as acceptor mice. Strains commonly

used for this purpose are nude mice and severe combined immunodeficiency (SCID) mice ***(6,7)***. In the nude mouse, a genetic defect results in failure to develop a thymus. However, these mice also appear to have a defect in epithelial development. In SCID mice, a recombinase necessary for rearrangement of T-cell receptor and immunoglobulin genes is lacking, resulting in the absence of cellular and humoral immune responses. Some mice are known as "leaky" SCID mice and can develop a low level of lymphocyte function. More recently, immune-deficient mice have been created by knockout technology. These include the recombinase activating gene (RAG) 1 or RAG-2 deficient mice ***(8,9)***. Like SCID mice, these mice lack functional recombinases necessary for rearrangement of T-cell receptor and immunoglobulin genes. Consequently, they do not have mature T- and B-cells and do not develop cellular and humoral immune responses. The maintenance and handling of immune-deficient mice require special accommodations. These mice should be kept separate from other mice and housed in filter-top cages. All procedures using these mice should be performed in laminar flow cabinets.

References

1. Janeway, C. A., Travers, P., Walport, M., and Capra, D. J. (eds.) (1999) *Immunobiology*, 4th ed., Elsevier Science/Garland, New York.
2. Vermeulen, M., Le Pesteur, F., Gagnerault, M. C., et al. (1998) Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. *Blood* **92,** 894–900.
3. Van Bekkum, D. W. and Lowenberg, B. (eds.) (1985) *Bone Marrow Transplantation*, Marcel Dekker, New York.
4. Jones, R. J., Celano, P., Sharkis, S. J., and Sensenbrenner, L. L. (1989) Two phases of engraftment established by serial bone marrow transplantation in mice. *Blood* **73,** 397–401.
5. Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W. (eds.) (1991) *Current Protocols in Immunology*, John Wiley, New York.

   (An extensive collection of in vitro and in vivo protocols used in immunologic research. It also provides detailed protocols for the T-cell isolation and adoptive transfer experiments described here including alternatives and extensive background information).
6. Miyawaki, S., Nakamura, Y., Suzuka, H., et al. (1994) A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur. J. Immunol.* **24,** 429–434.
7. Yoshikai, Y., Reis, M. D., and Mak, T. W. (1986) Athymic mice express a high level of functional gamma-chain but greatly reduced levels of alpha- and beta-chain T-cell receptor messages. *Nature* **10,** 482–485.
8. Mombaerts, P., Iacomini, J., Johnson, R. S., et al. (1992) RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68,** 869–877.
9. Shinkai, Y., Rathbun, G., Lam, K. P., et al. (1992) RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68,** 855–867.

Useful Websites

http://www.protocol-online.net/index.htm. A collection of protocols for biomedical research including immunonologic research.

http://www.ncifcrf.gov/vetpath/necropsy.html. A virtual mouse necropsy on the web including a schematic overview of the location of the various lymph nodes.

http://www.rodentia.com/wmc/. Everything about (mutant) mice.

http://www.dynal.no/. Vendor of immunomagnetic beads.

http://www.jax.org/.Vendor of (mutant) mouse strains.

http://www.atcc.org/. American Type Culture Collection.

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Quantification of Atherosclerosis in Mice

Alan Daugherty and Stewart C. Whitman

1. Introduction

Traditionally, studies on the development of atherosclerosis have been performed in animals larger than mice, with a particular preponderance of studies in rabbits. Studies in the mid-1980s began to introduce the mouse as a model for the development of atherosclerosis. The extensive genomic information that was available on inbred mice proved to be attractive in identifying genetic links to atherosclerosis susceptibility. However, it was not until the availability of genetically engineered mice that this species became more widely accepted as a model that mimicked several aspects of the human disease.

This chapter briefly overviews the inbred and genetically engineered strains of mice that are available, discusses aspects of experimental design, and provides a detailed technical description of the most common methods of quantifying the extent of atherosclerosis.

1.1. Strains of Mice Available for Atherosclerosis Research

One selection criterion for using a specific model should be the similarity between the atherosclerotic lesions that develop in mice compared with humans. A brief overview of the current thinking of the progression of human lesions is that the initial cellular event in the development of atherosclerosis is the adhesion of monocytes to an intact endothelial monolayer at specific regions in the arterial tree. A chemoattractant gradient induces diapedesis of monocytes, which enables them to migrate into the subendothelial space, where they become differentiated into macrophages. Macrophages become progressively engorged with lipid and form fatty streaks. At this stage of

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen © Humana Press Inc., Totowa, NJ

progression, the lipid deposition in the subintimal space is predominantly in the intracellular space. The next major stage of progression is characterized by an acellular lipid-rich core covered by a fibrous cap consisting of smooth muscle cells and extracellular matrix ***(1)***. A final stage of progression involves a rupture or erosion of lesions and the formation of a thrombus. Although several strains of mice are used for atherosclerosis research, none of the presently available mouse models of atherosclerotic disease cover the complete sequence of human lesions.

Early atherosclerosis studies in mice were performed with inbred strains. These demonstrated that the most susceptible strain was C57BL/6, BALB/c mice were of intermediate susceptibility, and C3H mice were totally resistant ***(2)***. However, even in the C57BL/6 strain, lesions are restricted to developing in the aortic arch, are diminutive, and contain few cells.

Several genetically modified mouse models of atherosclerotic diseases are now becoming widely used. A list of the most frequently used models and some of their characteristics is given in **Table 1**. The most commonly used model is the apoE$^{-/-}$ mouse. This genotype was created in three independent laboratories, all of which seem to have the same general phenotypic characteristics ***(3–5)***.

A further consideration is the strain of the mouse that has been genetically engineered. As mentioned earlier, differences in atherosclerotic lesion susceptibility have been described in inbred strains. Recent evidence has also shown that strain has a marked effect on atherogenesis occurring under conditions of apoE deficiency. As with the inbred strains, apoE deficiency has the most striking effect on atherosclerosis in a C57BL/6 background. In contrast, apoE deficiency has a more modest effect on atherogenesis in FVB ***(6)*** and C3H ***(7)*** strains.

1.2. Protocol Considerations

Many of the experimental design decisions have to be based on empirical information. When initiating a study on the development of atherosclerosis, the following questions need to be asked.

1.2.1. What Is the Most Appropriate Type of Mouse?

As noted above, none of the currently available mouse models of atherosclerosis develop the full spectrum of lesions present in humans ***(1)***. Many mouse models develop lesions that resemble American Heart Association (AHA) type I and II; a more limited number have lesions that evolve into AHA type IV. No mouse models are currently available that develop the ruptured and eroded lesions that precipitate acute cardiovascular events in humans. Therefore, investigations that study lipid deposition and monocyte adhesions may be

Table 1
Selected Genetically Manipulated Mice That Have Been Used in Atherosclerosis Research

Mouse	Characteristics of atherosclerosis	Area of lesion characterization	Modified diet required	Commercial availability
Targeted Deletions				
ApoE$^{-/-}$ ***(3,5,23)***	Progress from predominantly foam cell lesion to lesions with necrotic cores and fibrous caps	Aortic root, aorta, carotid	No	Jackson Taconic
ApoE$^{-/+}$ ***(5,24)***	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	
LDL receptor$^{-/-}$ ***(25,26)***	Lesions composed predominantly of lipid-laden foam cells	Aortic root, aorta	Yes	Jackson
Trangenics				
ApoB***(20)***	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	Taconic
Apo (a) ***(27)***	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	
Cholesterol ester transfer	Lipid-rich lesions of undefined cellularity protein ***(28)***	Aortic root	Yes	Jackson
ApoE(arg112,Cys142) ***(29)***	Lesions composed predominantly of lipid-laden foam cells	Aortic root	Yes	
ApoE3Leiden ***(30)***	Lesions composed predominantly of lipid-laden foamcells	Aortic root	Yes	
apoC-III ***(31)***	Not defined	Aortic root	Yes	
Compound				
Apobec$^{-/-}$ × LDL receptor$^{-/-}$ ***(32)***	A range of lesions from lipid-laden foam cells to those containing fibrosis and smooth muscle hyperplasia	Aortic root, aorta	No	

performed in mice that develop lesions of simple morphology. However, the study of mechanisms of more complex tissue remodeling may require models that have mature aspects of the disease process (*see* details in **Table 1**).

Early studies used inbred mice, primarily of the C57BL/6 strain. However, as mentioned above, these mice develop small lesions, even when fed diets that have been modified to induce hyperlipidemia. Therefore, most contemporary studies have used a genetically modified variant, of which the most commonly used have been low-density lipoprotein (LDL) receptor$^{-/-}$ and apoE$^{-/-}$ mice. LDL receptor$^{-/-}$ mice require a modified diet to generate lesions. These lesions tend to have a simple morphology, in which lipid-laden macrophages are the predominant cell type throughout development. LDL receptor$^{-/-}$ mice are preferred by some investigators because the distribution of cholesterol within plasma lipoproteins bears more resemblance to humans in that most of the sterol is present in LDL. Lesions in apoE$^{-/-}$ mice start with a simple morphology of lipid-laden contained macrophages. As they mature, they will acquire a more complex morphology that includes acellular lipid cores containing cholesterol clefts and fibrous caps ***(8,9)***. Much of the cholesterol in apoE$^{-/-}$ mice is transported in the very low-density lipoprotein (VLDL) fraction. A characteristic of the LDL fraction is that it contains a more complex array of apolipoproteins other than apoB.

Therefore, deciding which mouse model to use will depend in part on the specific pathogenic process under investigation that is most accurately mimicked compared with humans.

1.2.2. Should a Normal or Modified Diet Be Used?

Early studies using inbred mice required the use of a modified diet to generate a hyperlipidemic state, with subsequent development of atherosclerotic lesions. The most common modification was to have mice consume a diet highly enriched in saturated fat, cholesterol, and cholate ***(10)***. This diet is often referred to as the *Paigen diet*, after the investigator who popularized it. However, in the era in which genetically modified mice are available, such pronounced dietary manipulations are not needed. The apoE$^{-/-}$ mouse develops hypercholesterolemia and atherosclerosis when maintained on a normal diet. However, many studies have been performed on these mice during feeding of a diet that is primarily enriched with saturated fat and cholesterol to a level present in the diet of western industrial nations. This diet contains 40% of calories from fat (21% by weight) and is colloquially referred to as the *western diet*. It has been demonstrated that feeding this diet accelerates the development of atherosclerosis in apoE$^{-/-}$ mice without promoting gross changes in morphologic characteristics of lesions ***(8)***. However, feeding a saturated fat diet may influence the mechanism of the disease process. This is illustrated by

the study of total lymphocyte deficiency in apoE$^{-/-}$ mice. Total lymphocyte deficiency was produced by the deletion of either recombinant activator genes 1 or 2, which are required to produce mature B- and T-lymphocytes. Total lymphocyte deficiency had no effect on the extent of atherosclerosis development in mice that were fed a western diet. In contrast, there was a 42% decrease in lesion size in the aortic root when mice were fed a normal diet ***(11,12)***. Therefore, it is possible that the regulation of atherosclerosis by some mechanisms may be overridden by the production of severe hypercholesterolemia.

1.2.3. What Duration of Study Is Needed?

There are evolving technologies in the noninvasive quantification of atherosclerosis in mice by modalities such as magnetic resonance imaging ***(13)***. However, such techniques are in a developmental phase, and lesion quantification presently requires termination of the mouse to acquire the vascular tissue. Therefore, decisions have to be made on the interval of tissue acquisitions. These decisions are facilitated by knowledge of the extent of atherosclerosis under specific circumstances. However, there appears to be substantial variation in the extent of atherosclerosis generated between investigators, even using the same strain of mice and the same diet. Therefore, each individual laboratory needs to define the extent of lesion formation in that environment.

The duration of the study is partially dictated by the underlying hypothesis being studied and the mode of analysis. If an intervention is being studied that inhibits the development of atherosclerosis, then a robust response needs to be generated in the control mice. Lesions form more rapidly in the aortic root, and therefore studies of shorter duration are permissible in this region compared with *en face* analysis of the entire aortic intima.

In the vast majority of studies, the extent of atherosclerosis is quantified at a single interval. There are many reasons for constraining studies to one interval, including the expense of the studies and the considerable work needed to complete the analysis. However, for some studies it may be important to define the effect of an intervention on the temporal characteristics of atherosclerosis.

1.2.4. How Many Mice Should Be Used per Group?

The number of mice needed per group can be determined from power calculations. This requires prior knowledge of the variance within a control group. Given the differences in variance that are noted between investigators' laboratories, it is not possible to provide these estimates from literature values. Therefore, this information needs to be derived for each environment.

Studies that quantify atherosclerotic lesions are frequently characterized by wide variances in the data. These wide variances occur even in mouse

studies performed with inbred strains, which adds genetic equivalence to a standardized environment of such variables as feeding and housing. The wide variance, combined with a common lack of normal distribution for the data, frequently leads to an inability to perform the most commonly used parametric statistical tests. Although parametric tests have a considerably enhanced sensitivity over nonparametric tests, their inappropriate use can lead to a type I statistical error. In our studies, we use the SigmaStat (SPSS) statistical package, which provides information on the appropriate use of a specific statistical test. (*See* **Note 1**.)

1.2.5. Which Arteries Should Be Used for Quantifying Atherosclerosis?

The majority of atherosclerosis determinations in mice are performed in the aortic root or in the entire aortic tree. This emphasis is due to the early experience with wild-type C57BL/6 mice fed modified diets in which lesions only formed in these regions. Subsequent experiments used *en face* quantification of the entire aorta ***(14)***, in a process that had been used in several other species, particularly rabbits ***(15,16)***. Use of this technique requires genetically modified mice since these are the only strains in which significant disease occurs throughout the aorta. In our hands, the quantification of atherosclerosis by the *en face* technique is considerably more rapid than using the aortic root.

The carotid artery has been used extensively in vascular damage and transplant studies ***(17,18)***, but has had scant use in atherosclerosis studies. The coronary bed is of obvious interest, although there is no publication demonstrating a formal quantification process in this region.

2. Materials

1. Paraformaldehyde solution (4% w/v) for tissue fixation. Dissolve paraformaldehyde in phosphate-buffered saline (PBS). This will require boiling the solution in a loosely covered flask, which must be performed in a fume hood. The solution should be made on the day of the experiment.
2. Tissue molds (Fisher Scientific, cat. no. 22-038217).
3. OCT for embedding tissue (Fisher Scientific, cat. no. NC9509852).
4. Dissection equipment. In addition to general dissection equipment, small equipment is needed including small spring scissors (Fine Science Tools, Foster City, CA, cat. no. 1500-02); small forceps (Fine Science Tools, cat. no. 11065-07); and 0.2-mm minutien pins (Fine Science Tools; cat. no. 26002-20).
5. 1-mL syringes with 23-gage needles.
6. Hardware needed includes a dissection microscope such as a Nikon SMZ, an upright microscope, and a camera for acquiring images on a computer. For image analysis, we use ImagePro (Media Cybernetics).
7. Wako cholesterol kit (Wako, Richmond VA, cat. no. 276-64909).

3. Methods

3.1. Preparation of the Mouse

1. Anesthetize mouse with either a parental injection (ketamine/xylazine; 100 mg/kg and 1 mg/kg, respectively) or by inhalation with metafane. Place mouse on the dorsal side and cut the skin on the ventral side from the base of the abdomen to the underside of the chin. Cut the abdominal skin until the xyphoid process is in view. Lift the xyphoid process with hemostats, make cuts on either side of the rib cage, and cut the diaphragm carefully. Then make two cuts down either side of the rib cage to reveal the heart. Now displace the ribs to provide clear access to the heart.
2. Exsanguinate the mouse by placing a 23-gage needle through the apex of the ventricle. We find that it is generally easiest to use the right ventricle since the position of the needle tip within the chamber is more readily apparent than in the left ventricle. It is best to enter the ventricle at an angle parallel to the septum of the heart. During drawing of the blood, periodically rotate the needle 360°: this will prevent blocking of the needle opening by the walls of the ventricle. For a 25-g mice, it should be possible to acquire approx. 0.8–1 mL of blood.
3. Perfusion of the heart and aorta is performed via a cannula placed in the left ventricle. Remove the right atrium to allow fluid to escape from the body. Again, a 23-gage needle works well for introduction of the perfusate. As with blood collection, try to enter the left ventricle at an angle parallel to the septum of the heart. First perfuse the mouse with approx. 20 mL of PBS to remove blood. Removal of the blood is a great assistance to the dissection of the aorta, especially in the abdominal region.
4. There are some circumstances in which it will be preferable to fix the tissue. This should be performed at arterial pressure of 80–100 mmHg. We use 4% paraformaldehyde dissolved in PBS. The perfusion is performed in a fume hood with an appropriate method of recapturing the fluid. Perfusion of the mouse for 30 min is sufficient to achieve reasonable fixation.

3.2. Dissection of the Heart and Aorta

1. For quantification of atherosclerosis in the aortic tree, it is imperative that the entire tissue from heart to ileal bifurcation be removed without damage. We initially dissect the aorta free in the abdominal region. The intestinal tissue is displaced, and the most accessible region of the aorta, distal to the renal branches, is dissected free. It is easy to continue this dissection down to the ileal bifurcation. Probably the most difficult part of the dissection is between the renal branches and the diaphragm. The ease with which this region of the aorta can be seen varies considerably between strains and is also dependent on variables such as diet. Generally, until sufficient experience has been obtained, it is best to perform conservative cuts at some distance from the aorta.

   Once the abdominal region has been dissected free, we subsequently move to the proximal regions. The lungs and esophagus are removed, and the heart is

held at the apex with forceps. Small spring scissors are used to cut toward the spine to sever the left and right carotid arteries and the left subclavian artery. The ease of visualizing these branches is largely dependent on the size of the thymus, which is related to factors such as the strain and age of the mouse. Once the arch region has been dissected free, the thoracic section is readily visible and can be removed by carefully cutting along the spine. The aorta and attached heart are dissected free by a final cut of the iliac arteries.

2. To separate the heart from the aorta, the anterior aspect of the heart is placed toward the operator, and the aorta is cut at the point where it emerges from the ventricular tissue.
3. The aorta is placed in a vial containing approx. 5 mL of freshly prepared 4% paraformaldehyde solution. We find that an overnight fixation facilitates the removal of the adventitial tissue. However, prolonged fixation can hinder this process. Therefore, the tissues are incubated overnight and then the fluid is exchanged for PBS.
4. For the heart, a scalpel is used to cut away approximately the lower 70% of the ventricular mass, as depicted in **Fig. 1A**. The upper cardiac portion is placed into a tissue mold and covered with OCT. The tissue is manipulated to ensure that the aortic root is filled with OCT. The aortic root needs to be oriented so that it is perpendicular to the bottom surface of the tissue mold. The mold is then placed on the Peltier stage of the cryostat, for rapid freezing.
5. Each mold should be individually wrapped in parafilm and stored in a freezer at –20°C in an air-tight container. OCT becomes rubbery and difficult to cut if it desicates in a freezer.

3.3. Quantification of Lesions in the Aortic Root

1. The frozen tissue blocks are mounted in the cryostat with the ventricular tissue facing outward. Provided the tissue was placed correctly into the mold, placement of the block on the cryostat chuck will ensure that the aortic root is positioned perpendicular to the knife blade. The ventricular tissue is sectioned and discarded until the aortic sinus is reached. This is identified by the appearance of aortic valves or cusps. A diagrammatic scheme of the cutting is shown in **Fig. 1B** and an example of this region is shown in **Fig. 2A** (*see* Color Plate 2 following p. 144).
2. Slides are subsequently sectioned at 8-μm intervals. There is no generally accepted standard for the number of sections that are cut or the length of aorta that is sectioned. In our laboratory we usually retain every section for approx. 700 μm. From the start of section acquisition at the aortic sinus, we collect every section and sequentially place them on 10 slides. Therefore, each slide has approx. 9 sections that are 80 μm apart. (*See* **Note 2**.) The length of aorta that is sectioned is partially dependent on the severity of the disease. For investigations of aortic root atherosclerosis in mouse strains in which there is only a minor presence of disease, there may be no lesions present in distal portions of the sinus. Therefore, the length of aortic root that is sectioned could be reduced.

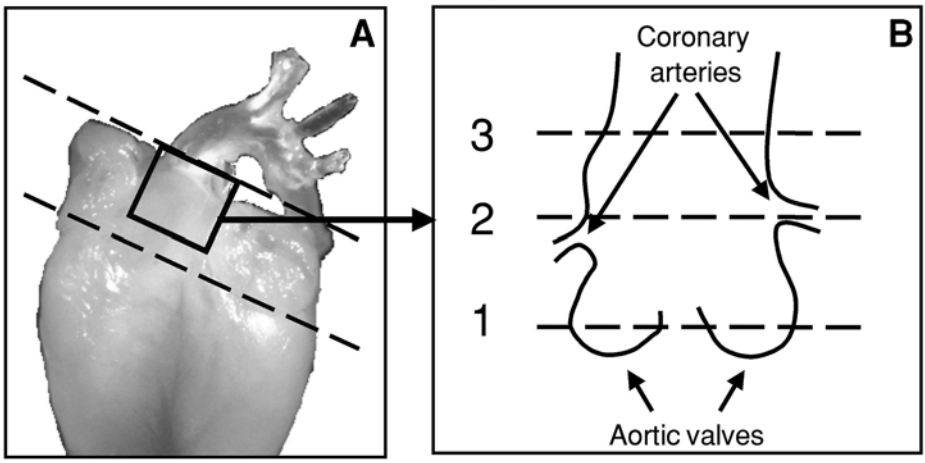

Fig. 1. A guide to the sectioning of the aortic root for quantification of atherosclerosis. **(A)** Tissue is cut in the regions represented by dotted lines to remove ventricular tissue and aorta. **(B)**Diagrammatic representation of the aortic root. The dotted lines indicate the regions used for examples in **Fig. 2**.

3. It should be noted that in the original description by Paigen et al. ***(19)*** of quantification of aortic atherosclerosis, the segment between the start of the aortic sinus and the orifices of the coronary artery was not used (*see* **Fig. 2**). However, most studies now include this region.
4. Sections are stained with hematoxylin to assist in tissue visualization. To quantify lesions, a stained slide is placed under a microscope, and images are captured to a computer at a magnification that permits clear definition of the lesion boundaries. Using the area quantification feature of the image analysis software, in each section, the area of lesion is defined by the internal elastic lamina and the luminal boundary. Data are commonly presented as the total lesion area in the number of sections quantified. Another useful presentation is to provide the total lesion area for sections at the specific regions of the aortic root, as described by Purcell-Huynh et al. ***(20)***.
5. Oil Red O staining is commonly used to assist in quantification of atherosclerotic lesions in mice. The color of the neutral lipid staining may enable the use of automated area determinations that are available in many image analysis packages. The usefulness of this approach is dependent on the composition of the lesions. It has utility in simple lesions that are uniformly stained for neutral lipid. However, in lesions that contain significant non-lipid-laden cells such as smooth muscle cells, extracellular matrix, and free cholesterol, then the extent of Oil Red O staining will not be representative of the entire area of the lesion.

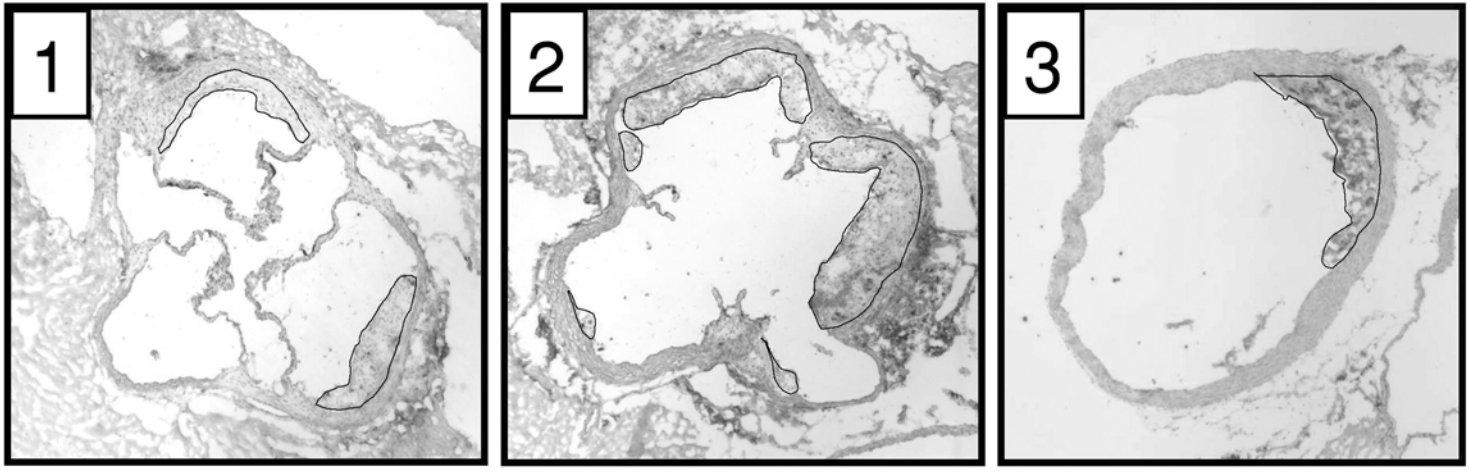

Fig. 2. Examples of sections in specific regions of aortic roots as indicated diagrammatically in **Fig. 1**. Sections are represented as follows: **1**, the start of the aortic sinus; **2**, the orifices of the coronary arteries marking the start of the ascending arch; **3**, the ascending aorta. The atherosclerotic lesions have been outlined for clarity. These examples are from aortic tissue of an apoE$^{-/-}$ mouse.

3.4. Preparation of Tissue for Quantification of Intimal Lesions on Aortic Intima

1. Place the aorta on a dark background and use small forceps to gently pull adventitial tissue away from the aorta. This removal process needs to be completed without excessive manipulation of the aorta, and the tissue needs to be kept moist with PBS.
2. Following removal of the adventitial tissue, one of the arbitrary decisions that needs to be made is how much of the branches of the left and right carotids and left subclavian arteries should be retained. The branch points of these vessels from the aorta tend to be locations of predilection for lesion formation. Therefore, inconsistency in standardizing the tissue would adversely influence the results. Routinely we cut the carotid arteries 1 mm from the branch, whereas the left subclavian artery is removed at its point of origin.
3. Following preparation of the outside of the aorta, the tissue needs to be cut open to expose the intimal surface. This procedure involves introducing small spring scissors into the lumen of the vessel. One potential problem is that atherosclerotic lesions in the mouse can be relatively easily dislodged from the intimal surface (*see* **Note 3**). Fortunately, the mouse aorta is virtually transparent and lesions can be seen from the exterior of the vessel. Therefore, the cutting of the aorta should be performed under a dissecting microscope so that any dislodgement can be documented.
4. The first cut is through the lesser curvature of the aortic arch. The cut then continues down the entire length of the blood vessel.
5. Next, cut at the top of the aortic arch on the outer curvature from the ascending arch to the left subclavian artery.

3.5. Lipid Staining of Aortas

Lipid staining of aortas is not necessary for quantification of the intimal area covered by atherosclerotic lesions. However, it can be of assistance in contrasting the lesioned area against normal tissue. Also, if lesions are very small, lipid staining will be needed to visualize the disease.

1. Preheat a water bath to 37°C.
2. Prepare fixed aorta samples by cutting to expose the intima as described above (*see* **Subheading 3.4.**).
3. Place each tissue into a 0.5-mL Eppendorf tube and label appropriately.
4. Fill each tube with 400 μL of Sudan IV (5 mg/mL in 70% isopropanol).
5. Place in water bath for 40 min at 37°C.
6. Remove sections of aorta and check extent of staining under a dissecting microscope. Periadventitial adipose tissue will stain very brightly with Sudan IV and should be removed before images of the aorta are collected. Keep tissues wet in PBS while cleaning.
7. Place the tissue in another 0.5-mL Eppendorf tube and fill with 400 μL of the 70% isopropanol wash.

Fig. 3. An example of the arch region of an aorta pinned out for acquisition of an image that is used to quantify atherosclerosis by the *en face* technique described in **Subheading 4.5.3.** The bright white material is unstained atherosclerotic lesions.

8. Agitate by hand for 15 s and return to water bath for 5 min.
9. Check the extent of lipid staining under the dissecting microscope. If additional destaining is required, incubation with 70% ispopropanol should be repeated until there is optimal contrast between lesion and the normal segments of aorta.

3.6. En Face *Determination of Percent of the Intimal Area Covered by Atherosclerotic Lesions*

1. The aorta is pinned on black paper placed over dental wax using minutien pins, as shown in **Fig. 3**. We find that the use of paper provides some modest adhesion of the aorta and minimizes the number of pins required to obtain flat aortic tissues. A ruler is placed next to each image to assist in calibration of the image. Tissues need to be maintained under moist conditions.
2. An image is captured to the computer. We collect this image using a digital camera connected to a dissecting microscope that has a reducing and polarizing lens to obtain an appropriate field and reduce glare, respectively.
3. In the case of unstained aortas, sometimes glare will provide the appearance of a lesion on the image. For lipid-stained lesions, any residual adipose tissue on

the adventitial surface will be clearly seen, since the mouse aorta is translucent. Therefore, we verify the location of the lesions by comparing the image to the pinned tissue.

4. Image analysis software is used to determine the area of the intima and lesions. We manually trace the outline of the intima and lesions. We have not been able to obtain images with sufficient contrast to perform these measurements reliably in an automated mode with image analysis software. Even with stained lesions, it is our experience that the staining is so variable that it is difficult to set color thresholds to provide data that are reliably the same as a manual measurement.
5. A second observer should verify the data generated by the primary operator. (*See* **Note 4**.)
6. We represent the data for the three major regions of the aorta: arch, thoracic, and abdominal. We define these regions as follows: Arch, root to 3 mm beyond the left subclavian; thoracic, end of arch segment to the last intercostal branch; and abdominal, end of thoracic segment to the ileal bifurcation.

3.7. Tissue Sterol Analysis

There are several reasons to determine tissue sterol content. One is that it acts as an independent parameter of atherosclerosis. Second, we have observed examples in which the lesion area is not changed, but visual inspection shows that the lesions are thicker ***(21,22***; *see* **Note 5*)***. In these cases, assuming the lesions are primarily lipid-containing, this measurement provides an index of lesion volume. Finally, the determination of the ratio of esterified to unesterified cholesterol can provide mechanistic insight into the mode of lipid deposition. It is important to note that tissue sterol analysis cannot be performed on aortas that have been stained with Sudan IV.

1. Determine the intimal area of the aortic segment from the captured image, as described in **Subheading 3.4.**
2. Transfer each sample into a Kimax tube.
3. Add 3 mL of 95% methanol and allow to sit for 1 h at room temperature in capped tubes. For short incubations (<2–4 h), we use marbles in place of tube caps because the marbles are much faster to place and remove.
4. Add 6 mL of chloroform and mix, ensuring that all tissues are floating and solvents have mixed in the capped tubes.
5. Allow to sit overnight at room temperature in capped tubes.
6. Add 2 mL of saline. Vortex well (approx. 1 min) and allow phases to separate for 1 h. The separation of phases can be enhanced by low-speed centrifugation (200*g*, 10 min).
7. Transfer bottom chloroform phase into a 16 × 100-mm disposable glass tube using a glass pipet.
8. Using a stock cholesterol solution (200 mg/dL; generally provided in the Wako assay kit), a standard curve is generated by pipeting duplicate aliquots into 16 × 100-mm disposable glass tubes, as shown below:

Final cholesterol concentration (μg)	μL to pipet
blank	0
1	5
2	10
5	25
10	50
20	100

9. Evaporate standards and samples to dryness under a gentle flow of N_2 with mild heating (at 37°C). For standards, add 1 mL of chloroform plus 1 mL of 1% Triton X-100 in chloroform, and mix by vortexing. For samples, add 450 μL of chloroform plus 450 μL of 1% Triton X-100 in chloroform, and mix by vortexing.
10. Evaporate the standards and samples again to dryness under N_2 with heating (37°C). For standards, add 500 μL of deionized water and for samples add 225 μL. Roll or vortex until the Triton X-100 goes into solution and incubate at 37°C for 15 min in a water bath.
11. Pipette duplicate 50-μL aliquots of each standard and each sample into individual wells of two microtiter plates. One of the microtiter plates will be used to determine the total cholesterol content of each sample, and the second microtiter plate will be used to determine the unesterifed cholesterol content of each sample. Note the number of plates you will need before you prepare the standards; 500 μL of each standard should be enough for two total cholesterol and two unesterified cholesterol plates.
4. Add 150 μL of either total cholesterol or unesterified cholesterol reagent per well and mix. Incubate for 10 min at 37°C in a dry oven and then for 50 min at room temperature prior to reading absorbance on a plate reader using the correct wavelength as specified by the manufacturer of the cholesterol assay. The dilution correction factor will be 4.5 (225 μL of sample/50 μL of sample/well).

3.8. Summary

At present there are no standards for the assessment of atherosclerosis in mice. Therefore, the methods detailed here should be taken as examples. In addition to quantitating the size of lesions, the composition of the lesion may also be an important parameter. Therefore, although a detailed description is beyond the scope of this chapter, investigators are encouraged to perform standard histologic and immunocytochemical analysis of lesions.

4. Notes

1. The quantification of atherosclerosis is inherently variable, and data are frequently not normally distributed. Data must be tested to define whether the application of either parametric or nonparametric analysis is appropriate.
2. For aortic root analysis, care needs to be taken in defining landmarks so that lesion sizes are compared in sections from the same region. Furthermore, lesions

in the sinus may not be reflective of changes in the ascending aorta, and it is preferable to acquire sections from all of the root region.

3. Atherosclerotic lesions in mice are relatively easily displaced from the luminal surface. To minimize lesion loss, tissues should be handled with care, and cutting to expose the intimal surface should be performed under conditions in which visualization of the lesions is possible.
4. The determination of the extent of atherosclerosis in mice frequently requires some subjective judgments to define the boundaries of lesions. Therefore, lesions should be quantified by more than one observer. Blinding of studies is preferable.
5. For *en face* analysis, a similar area of lesion coverage of the intima does not necessarily mean that lesion size is the same. Frequently, careful visual inspection of the tissue illustrates that lesion thickness may be different in the absence of changes in area. For lipid-rich lesions, this difference can be defined using the measurement of tissue sterols.

Acknowledgments

We thank members of the laboratory (Melissa Drawdy, Victoria King, Ninetta Kosswig, Michael Manning, Debra Rateri, and Punnaivanam Ravisankar) for providing feedback on this manuscript. Work in the authors' laboratory is supported by NIH grants (R01-HL55487 and RO1-HL62846), the Heart and Stroke Foundation of Canada, and the American Heart Association.

References

1. Stary, H. C., Chandler, A. B., Dinsmore, R. E., et al. (1995) A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis—a report from The Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler. Thromb. Vasc. Biol.* **15,** 1512–1531.
2. Paigen, B., Holmes, P. A., Mitchell, D., and Albee, D. (1987) Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c, and C3H. *Atherosclerosis* **64,** 215–221.
3. Plump, A. S., Smith, J. D., Hayek, T., et al. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein-E-deficient mice created by homologous recombination in ES cells. *Cell* **71,** 343–353.
4. Piedrahita, J. A., Zhang, S. H., Hagaman, J. R., Oliver, P. M., and Maeda, N. (1992) Generation of mice carrying a mutant apolipoprotein-E gene inactivated by gene targeting in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **89,** 4471–4475.
5. van Ree, J. H., van den Broek, W. J. A. A., Dahlmans, V. E. H., et al. (1994) Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis* **111,** 25–37.

6. Dansky, H. M., Charlton, S. A., Sikes, J. L., et al. (1999) Genetic background determines the extent of atherosclerosis in ApoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **19,** 1960–1968.
7. Shi, W., Wang, N. J., Shih, D. M., et al. (2000) Determinants of atherosclerosis susceptibility in the C3H and C57BL/6 mouse model: evidence for involvement of endothelial cells but not blood cells or cholesterol metabolism. *Circ. Res.* **86,** 1078–1084.
8. Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L., and Ross, R. (1994) ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* **14,** 133–140.
9. Reddick, R. L., Zhang, S. H., and Maeda, N. (1994) Atherosclerosis in mice lacking apo E—evaluation of lesional development and progression. *Arteriosclero. Thromb.* **14,** 141–147.
10. Paigen, B., Ishida, B. Y., Verstuyft, J., Winters, R. B., and Albee, D. (1990) Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. *Arteriosclerosis* **10,** 316–323.
11. Daugherty, A., Pure, E., Delfel-Butteiger, D., et al. (1997) The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E-/-mice. *J. Clin. Invest.* **100,** 1575–1580.
12. Dansky, H. M., Charlton, S. A., Harper, M. M., and Smith, J. D. (1997) T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc. Natl. Acad. Sci. USA* **94,** 4642–4646.
13. Fayad, Z. A., Fallon, J. T., Shinnar, M., et al. (1998) Noninvasive in vivo high-resolution magnetic resonance imaging of atherosclerotic lesions in genetically engineered mice. *Circulation* **98,** 1541–1547.
14. Tangirala, R. K., Rubin, E. M., and Palinski, W. (1995) Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* **36,** 2320–2328.
15. Daugherty, A., Zweifel, B. S., and Schonfeld, G. (1989) Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Br. J. Pharmacol.* **98,** 612–618.
16. Sendobry, S. M., Cornicelli, J. A., Welch, K., et al. (1997) Attenuation of diet-induced atherosclerosis in rabbits with a highly selective 15-lipoxygenase inhibitor lacking significant antioxidant properties. *Br. J. Pharmacol.* **120,** 1199–1206.
17. Iafrati, M. D., Karas, R. H., Aronovitz, M., et al. (1997) Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice. *Nature Med.* **3,** 545–548.
18. Shi, C. W., Lee, W. S., He, Q., et al. (1996) Immunologic basis of transplant-associated arteriosclerosis. *Proc. Natl. Acad. Sci. USA* **93,** 4051–4056.
19. Paigen, B., Morrow. A., Holmes, P., Mitchell, D., and Williams, R. (1987) Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* **68,** 231–240.

20. Purcell-Huynh, D. A., Farese, R. V., Johnson, D. F., et al. (1995) Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet. *J. Clin. Invest.* **95,** 2246–2257.
21. Daugherty, A., Zweifel, B. S., and Schonfeld, G. (1991) The effects of probucol on the progression of atherosclerosis in mature Watanabe heritable hyperlipidaemic rabbits. *Br. J. Pharmacol.* **103,** 1013–1018.
22. Cassis, L. A. and Daugherty, A. (1999) Chronic angiotensin II infusion promotes atherogenesis in low density lipoprotein receptor –/– mice. *Proc. N.Y. Acad. Sci.* **892,** 108–118.
23. Zhang, S. H., Reddick, R. L., Piedrahita, J. A., and Maeda, N. (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258,** 468–471.
24. Zhang, S. H., Reddick, R. L., Burkey, B., and Maeda, N. (1994) Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. *J. Clin. Invest.* **94,** 937–945.
25. Ishibashi, S., Goldstein, J. L., Brown, M. S., Herz, J., and Burns, D. K. (1994) Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J. Clin. Invest.* **93,** 1885–1893.
26. Roselaar, S. E., Kakkanathu, P. X., and Daugherty, A. (1996) Lymphocyte populations in atherosclerotic lesions of apoE –/– and LDL receptor –/– mice. Decreasing density with disease progression. *Arterioscler. Thromb. Vasc. Biol.* **16,** 1013–1018.
27. Lawn, R. M., Wade, D. P., Hammer, R. E., et al. (1992) Atherogenesis in transgenic mice expressing human apolipoprotein(a). *Nature* **360,** 670–672.
28. Marotti, K. R., Castle, C. K., Boyle, T. P., et al. (1993) Severe Atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature* **364,** 73–75.
29. Fazio, S., Sanan, D. A., Lee, Y. L., et al. (1994) Susceptibility to diet-induced atherosclerosis in transgenic mice expressing a dysfunctional human apolipoprotein E(Arg 112,Cys142). *Arterioscler. Thromb.* **14,** 1873–1879.
30. van Vlijmen, B. J. M., van den Maagdenberg, A. M. J. M., Gijbels, M. J. J., et al. (1994) Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3 Leiden transgenic mice. *J. Clin. Invest.* **93,** 1403–1410.
31. Masucci-Magoulas, L., Goldberg, I. J., Bisgaier, C. L., et al. (1997) A mouse model with features of familial combined hyperlipidemia. *Science* **275,** 391–394.
32. Powell-Braxton, L., Veniant, M., Latvala, R. D., et al. (1998) A mouse model of human familial hypercholesterolemia: Markedly elevated low density lipoprotein cholesterol levels and severe atherosclerosis on a low-fat chow diet. *Nat. Med.* **4,** 934–938.

17

Genetically Modified Mice in Cancer Research

David A. Largaespada

1. Introduction

The laboratory mouse offers tremendous opportunities for studies on the genetic basis of cancer. No other model system is so easily and widely used to test the effect of potentially oncogenic mutations in a living animal. Mouse strains differ extraordinarily in their innate susceptibilty to various forms of cancer (see Jackson Laboratories website: http://tumor.informatics.jax.org/straingrid.html). The reasons for these differences are thought to lie in allelic variation in cancer susceptibility genes between strains. Breeding experiments have allowed researchers to localize some of these susceptibility genes regionally ***(1,2)***. The molecular identification of these susceptibility genes has proved elusive. However, cancer research has yielded many other types of cancer genes whose function has been tested using the power to manipulate the mouse germline. A large number of oncogenes have been discovered by studying oncogenic retroviruses, from transfection of tumor DNAs into mouse embryo fibroblast cell lines, and by molecular analyses of human tumors. Tumor suppressor genes have been discovered from linkage studies and positional cloning in human families segregating highly penetrant predisposition to specific tumor types ***(3)***. Mouse transgenesis has proved to be the most powerful and widely used system for the in vivo analysis of the function of cancer genes discovered by these methods. Several excellent reviews have been written on this subject ***(4–6)***. In this chapter, I briefly describe the history of mouse transgenesis in cancer research, some widely utilized models, new technologies, and future directions for mouse genetics in cancer research.

The laboratory mouse has been used for cancer studies since the generation of the first inbred strains. In fact, Clarence Cook Little at the Jackson Laboratories

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

generated the first strain of inbred mice for laboratory research, DBA, in order to study cancer, which he hypothesized had a genetic basis ***(7)***. Indeed, it was discovered that the common inbred laboratory strains of mice differ tremendously in their susceptibility to spontaneous and induced cancer. However, it was with the advent of mouse transgenesis by pronuclear injection that the molecular genetic basis of oncogenesis using in vivo studies began. The ability to introduce DNA into the germline of mice offered the ability to test these new potential oncogenes in vivo. Among the earliest examples of published transgenic mice were cancer-prone transgenic strains carrying *Ras* or *Myc* transgenes. These early experiments represent a milestone in cancer research because they proved for the first time that what were then newly discovered, putative cancer genes could actually initiate *bona fide* cancer in a living organism. A classic example is the generation of lymphoma-prone mice, by the expression of the *CMYC* protooncogene from a B-cell-specific promoter, by Adams et al. in 1985 ***(8)***. The human *CMYC* gene had been shown to be dysregulated by translocation in human B-cell Burkitt's lymphomas ***(9,10)***. This paper provided an early convincing model of a specific human cancer. Many transgenic experiments on *CMYC*-related genes, and on the combination of *MYC* transgenes with other oncogenes, have followed this initial study. These studies show how the generation of a good model leads to the ability to ask many subsequent questions.

Cancer geneticists are now utilizing genetically modified mice in many interesting ways to extend the power of these early studies. For example, many tissue-specific promoters have been employed for these studies. In vivo regulatable, transgene promoters have also been created that work reliably, and genes can now be inactivated in specific tissues (so-called tissue-specific knockouts). Many obstacles for the study of cancer genes in mice have been overcome, and strain differences in susceptibility are now amenable to more sophisticated analyses using techniques and resources generated by the mouse and human genome projects.

2. General Considerations

It is important when working with mouse models of cancer to be aware of certain specific issues. Foremost, perhaps, is the increasing need for scientists trained in mouse genetics to learn the basics of tissue pathology and physiology and have collaborators trained in pathology. This is especially true as more attention is paid to perineoplastic disorders, specific cancer traits, and other subtle differences with a genetic basis. Second, because progress in cancer genetics using the mouse now depends in many instances on combining mutations, it is important to have specific pathogen-free (SPF) facilities so that strains can be easily shared between labs and institutions. Furthermore, many

cancer studies involve aging mice for considerable lengths of time. There is good evidence to suggest that the status of the normal flora or pathogens in mice influences oncogenesis. In certain cases, some degree of inflammation, such as that caused by subacute infections common in conventional animal facilities, is a required component of the cancer process ***(11–13)***. Such a consideration would also be important in experiments in cancer immunology using mice.

Finally, careful attention to strain background differences is critical when combining mutations. It is especially important in such experiments to control for strain background when comparing experimental groups. Clearly the innate susceptibility to various forms of cancer varies incredibly among inbred strains (see Jackson Laboratories website given above). When combining transgenes or gene mutations from differing strains, it is possible to observe differences that actually relate to the inheritance of genes linked to the transgene or knockout gene in question, rather than those genes themselves. Therefore, spurious conclusions about the cooperativity or requirement for specific genes during oncogenesis could be made.

3. Transgenesis by Pronuclear Injection in Cancer Research

Many cancer-prone mouse strains have been created by pronuclear injection of transgenes. Indeed, this type of study was central to verifying the oncogene hypothesis, which says that certain mutant or inappropriately expressed genes can trigger cancer. Over 2000 entries are listed in Medline with both "mouse transgenesis" and "cancer" as keywords. Over 80 different transgenes have been shown to cause cancer in mice. Nearly 25% of these are actually viral oncogenes. A few oncogenic transgenes truly dominate the literature: those involving *Ras*, *Myc*, or the *Neu* oncogenes. Also quite commonly used are transgenes derived from the large T-antigen gene, Tag, from simian virus 40 (SV40). Each of these genes has been expressed from a variety of promoter constructs in transgenic mice.

Certain promoter elements have stood out in cancer studies as highly successful in giving reproducibly strong expression. For ubiquitous expression, the cytomegalovirus (CMV) immediate early promoter has been widely used ***(14,15)***. Good examples of widely used and successful lineage-specific promoters are the Eμ-immunoglobulin heavy chain gene enhancer/promoter for the B-cell lineage ***(8)***, the mouse mammary tumor virus long terminal repeat and whey acid protein gene promoter for the mammary gland ***(16)***, and the human keratin 14 promoter for skin ***(17)***. It is likely that an increasing number of studies will involve the use of in vivo regulatable promoters. One successful example of such a system is the "Tet-off" regulated promoter series. In the presence of tetracycline, or its analog doxycyclin, a mutant form of

the tetracyclin transactivator protein can suppress expression of transgenes from chimeric promoters containing transactivator binding sites ***(18)*** (*see* Chapter 5). This system has been used to express the *BCR-ABL* transgene after tetracycline is withdrawn from the drinking water available to transgenic mice ***(19)***. These studies showed that continued expression of the *BCR-ABL* transgene is required for the growth of induced acute lymphoblastic leukemia, except in very advanced cases. Similar studies will allow determination of the requirements for specific gene mutations during early versus late stages of oncogenesis.

MYC family genes have been used in transgenic models of cancer by many investigators. One of the first, and still widely used, models of transgenic oncogenesis was the overexpression of *Cmyc* in B-lineage cells using the immunoglobulin heavy chain gene (IgH) enhancer/promoter ***(8)***. The Myc proteins are a family of basic-helix-loop-helix transcription (bHLH) factors ***(20)***. The basic domain binds to a DNA site—specifically on E-box sequences. Myc proteins are thought to bind to DNA as heterodimers with other bHLH proteins. Cmyc protein levels generally increase during the transition from G1 to the S phase of the cell cycle. Dysregulation of *CMYC* expression or activity seems to be a major end point of many oncogenic mutations. *CMYC* expression is dysregulated by chromosomal translocations involving the IgH or T-cell receptor gene (TcR) loci in human and mouse lymphoid malignancies. The *CMYC* locus is also amplified in some breast tumors ***(21)***. The related *NMYC* gene is amplified in a high percentage of neuroblastomas ***(22)***. The *MYC* oncogenes are primarily dysregulated at the level of transcriptional control and so are usually studied in transgenic mouse models of cancer by overexpression from tissue-specific promoters.

Cmyc overexpression can initiate mammary cancer in transgenic mice in cooperation with an activated *Ras* gene ***(23)***. *Nmyc*, *Lmyc*, or *Cmyc* enforced expression can contribute to lymphomagenesis in transgenic mice ***(8,24,25).*** Targeted expression of *NMYC* in transgenic mice causes neuroblastoma ***(26)***. A number of studies have been performed to determine which other oncogenes are capable of cooperating with *CMYC* dysregulation to achieve cancer ***(23)***. Also, *MYC* transgenic mice have allowed formal tests of the roles that *p53* gene-regulated pathways play during lymphomagenesis, mammary tumor develoment, and hepatocellular carcinogenesis ***(27–29)***.

Ras proteins are encoded by three different *RAS* genes: *NRAS*, *HRAS*, and *KRAS* (for review, *see* **ref. *30***). *RAS* genes are members of a large superfamily that encode small GTPase proteins. These proteins function as bimolecular switches in signal transduction pathways. They switch from an inactive GDP-bound state to an active GTP-bound state by the action of guanine nucleotide exchange factors. In the GTP-bound state, Ras interacts with downstream

effector molecules. Two of the major downstream Ras effectors thought to be important for oncogenesis are the Raf kinases and phosphotidyl inositol 3′ kinase. *RAS* genes are mutated in 30% of all human cancers, and the large number of transgenic strains made using *RAS* genes is thus justified.

Certain human cancers have very high frequencies of *RAS* gene mutations. These include lung, pancreatic, breast, and gastrointestinal tract cancers ***(31)***. Some of these cancers have been modeled in mice using tissue-specific promoters ***(32–34)***. The generation of these mice has allowed preclinical studies using new anticancer compounds ***(35,36)***, attempts to define the specific effects of hyperactive Ras signaling in the absence of other mutations ***(37)***, tests of specific oncogenes or TSG mutations that can collaborate with *RAS* oncogenes in cancer ***(38,39)***, and determination of important downstream effectors of Ras action for tumorigenicity ***(40,41)***.

Many models of cancer generated in transgenic mice involve expression of the SV40 large T-antigen, Tag (reviewed in **ref. *42***). SV40 Tag is a complicated viral protein derived from a circular DNA virus. It was known from the early 1970s that SV40 replicates well in primate cells, but is nonreplicative in rodent cells. Instead, SV40 spontaneously integrates into the chromosomes of rodent cells, resulting in their transformation (reviewed in **ref. *43***). SV40 large T-antigen was one of several proteins recognized by antisera from mice that had mounted antitumor immune response. SV40 large T-antigen binds to the viral origin of replication and is required for replication of the viral genome. To prevent host cell apoptosis or cell cycle arrest, SV40 large T-antigen has evolved other functions. These include binding to and inactivating p53, Rb, and perhaps other tumor suppressor proteins ***(43)***. The use of SV40 large T-antigen as a transgene has allowed development of immortalized cell lines from a variety of mouse tissues ***(44,45)***, definition of minimal oncogenic changes required for brain tumor development ***(46)***, the creation of mouse models of cancer in which tumor progression could be easily studied ***(47)***, and a simple way to create tumors in mice that mimic specific types of human cancers for which no mouse model had existed ***(42)***.

Among many cancer-prone transgenic models, there are few that are perfect mimics of a human counterpart, but many bear striking similarities. Good examples are those that have been described for acute promyelocytic leukemia (APL) ***(48–50)***. APL is one subtype of human acute myeloid leukemia. In the vast majority of APL patients, a balanced reciprocal translocation, t(15;17)(q22;q11.2), results in the generation of the *PML-RARα* fusion oncogene ***(51)***. The chimeric fusion oncoprotein PML-RARα is composed of PML and retinoic acid receptor-α (RARα) protein sequences. The *PML-RARα* fusion oncogene has been expressed in mice from a human cathepsin G gene promoter, or the myeloid-restricted protein 8 (Mrp8) gene promoter, to create

mouse models of human APL ***(48,49)***. In many ways these models have been useful, from accurately mimicking the therapeutic responses of APL to retinoic acid therapy to allowing researchers to test hypotheses about the reasons that *PML-RARα* is oncogenic and why some *RARα* fusions respond to retinoic acid therapy or arsenic and some do not ***(52)***. For example, the resistance of *PLZF-RARα*-positive APL to retinoic acid therapy is maintained in the transgenic mouse model of this disease ***(53)***. This has been linked to the ability of the PZLF-RARα protein to suppress transciption at target genes via two independent domains, which can recruit histone deacetylase complexes ***(53)***. Only the RARα domain is senstive to retionoic acid treatment, which results in the release of the histone deacetylase complex. The capacities of different forms of *RARα* and *PML-RARα* to induce APL in transgenic mice have been compared. These studies showed that the PML domain of PML-RARα provides a crucial role in causing APL and that the capacity of the RARα domain to mediate transcriptional transactivation is dispensible for leukemogenicity ***(54)***.

4. Embryonic Stem Cell Technology in Cancer Research

The use of embryonic stem (ES) cell technology has had a major impact on the study of cancer using genetically modified mice. This is most apparent in the ability to generate models of familial cancer in humans. In most of these cases, inheritance of one inactive copy of a tumor suppressor gene (TSG) occurs. Individuals who inherit one mutant, inactive copy of a TSG are then prone to cancer development owing to somatic loss of the remaining wild-type allele.

The first such TSG gene knockout was also the first TSG cloned, the *RB1* gene (reviewed in **ref. *55***). In many ways the evaluation of this knockout mouse illustrates many of the challenges in the tumor suppressor gene field. Unlike $RB1^{+/-}$ patients, $Rb1^{+/-}$ mice do not develop retinoblastomas ***(56)***. The reason for this may be that, given the number of retinoblast cells at risk and their fleeting appearance in the early life of mice, there is simply not enough time, nor are there enough target cells at risk for acquiring somatic mutation of the wild-type allele. This could be called the "target cell number and time" dilemma. The natural history of the mouse (which is over two logs less massive than the average adult human and lives 2 years instead of 70) does not recapitulate that of the human. As it turns out, the fact that $Rb1^{+/-}$ mice do not develop retinoblastoma was the impetus to pursue the idea that multiple genetic events, such as p53 pathway inactivation, may be required to induce retinoblastoma with high efficiency ***(57)***. Expression of the SV40 large T-antigen (which can bind to and inactivate both Rb and p53 protein) in the retina of transgenic mice results in retinoblastoma ***(58)***. Coexpression of bovine papilloma virus (BPV) E6 and E7 proteins, which inactivate p53 and Rb,

respectivelty, in the lens causes tumor development, whereas either oncogene alone does not ***(59)***. If $p53^{-/-}$ mice are crossed with BPV E7 gene transgenic mice expressing photoreceptor cells, the result is the development of retinoblastoma ***(60)***. These studies showed that inactivation of Rb protein in these cells normally causes apoptosis. However, as the apoptosis is p53-dependent, loss of p53 allows the cells to remain viable and to progress to retinoblastoma. Thus, p53 functional impairment was implicated as a requirement for retinoblastoma development.

One method to overcome the "target cell and time" problem with mouse TSG knockouts is the use of tissue-specific activation of the site-specific recombinase, Cre, to achieve simultaneous inactivation of both TSG alleles in somatic cells. This process will be described in more detail below. However, not all TSG knockouts are lethal in the homozygous state. For example, homozygous null $p53^{-/-}$ mice are fertile and viable and so have been used extensively by cancer researchers.

Much has been learned about the function of the p53 protein using transgenesis and gene knockout mice (reviewed in **ref. *61***). The fact that $p53^{-/-}$ mice are viable is consistent with the guardian of the genome hypothesis about the function of the p53 protein. The p53 protein signals apoptosis or cell cycle arrest in response to DNA damage and so might not be expected to be an essential gene for viability. However, consistent with a role in tumor suppression, both $p53^{+/-}$ and $p53^{-/-}$ are cancer prone, developing primarily lymphomas and sarcomas ***(62,63)***. $p53^{-/-}$ null cells from these mice have been used extensively to study the cellular roles of p53 in cultured cells ***(64,65)***. $p53^{-/-}$ or $p53^{+/-}$ mice are sensitized to cancer development and have thus found a place in carcinogenesis studies, which can be done using smaller groups than would be needed using normal laboratory mice ***(66)***.

Neurofibromatosis type 1 (NF1) syndrome is a common, autosomal, dominant cancer-predisposition disorder caused by inheritance of one mutant copy of the *NF1* gene ***(67)***. The study of *Nf1* gene knockout mice illustrates many of the problems and opportunities that are faced in modeling TSG loss in human cancer (**Fig. 1**). NF1 syndrome is characterized by the development of benign neurofibromasarcomas, which are composed of Schwann cells, perineural fibroblasts, and mast cells. In addition, NF1 patients have café au lait spots and Lisch nodules, which are glial cell growth on the iris. NF1 patients are predisposed to a variety of specific malignancies including pheochromocytoma, malignant peripheral nerve shealth tumor (MPNST), astrocytoma, and childhood forms of myeloid leukemia. However, $Nf1^{-/-}$ embryos die between days 11 and 14 of gestation and, unlike $NF1^{+/-}$ human counterparts, $Nf1^{+/-}$ mice do not display the benign manifestations of NF1 syndrome ***(68)***. $Nf1^{+/-}$ mice, if aged, do develop specific tumors, including fibrosarcomas, myeloid leukemia, and

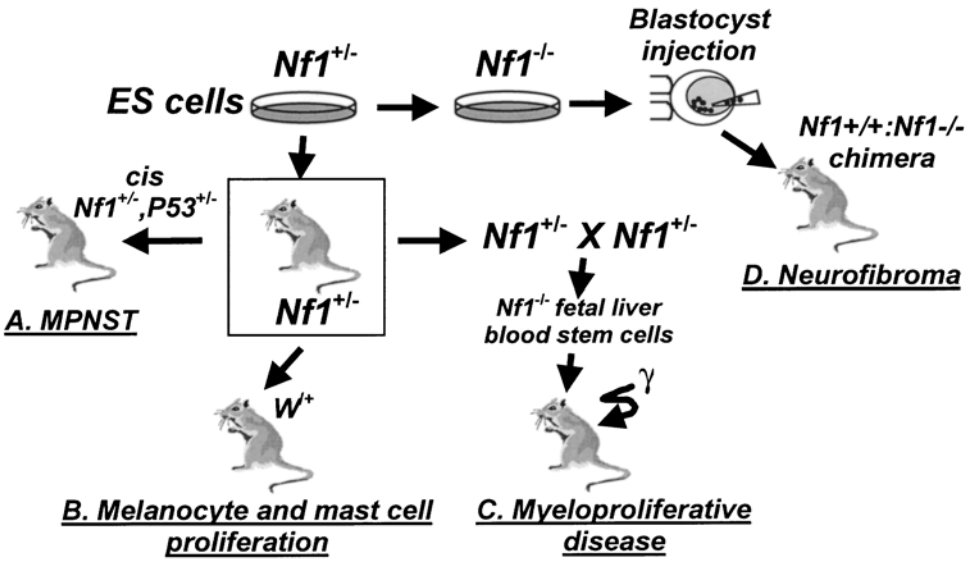

Fig. 1. Study of cell growth control and oncogenesis using mice with targeted inactivation of the *Nf1* gene. The neurofibromatosis type 1 gene (*Nf1*) has been inactivated by homologous recombination in ES cells. **(A)** Mice heterozygous for both the *Nf1* and *p53* genes, in the *cis* configuration (i.e., on the same chromosome 11 homolog), develop malignant peripheral nerve shealth tumors (MPNSTs), which occur in 3–5% of NF1 patients. **(B)** A role for *Nf1* in promoting mast cell and melanocyte proliferation, analogous to café au lait spots in NF1 patients, is revealed in strains carrying hypomorphic alleles of the *Ckit* or *W* gene. **(C)** Intercrossing $Nf1^{+/-}$ mice results in embryonic lethality of resultant $Nf1^{-/-}$ embryos. However, prior to their death *in utero*, such $Nf1^{-/-}$ embryos can be used as a source of fetal liver blood stem cells. Reconstitution of lethally irradiated mice with $Nf1^{-/-}$ fetal liver blood stem cells results in a chronic myeloproliferative disease similar to NF1 syndrome-associated leukemias such as juvenile myelomonocytic leukemia. **(D)** ES cells have been taken through two rounds of gene targeting to create $Nf1^{-/-}$ cells. If these are injected into wild-type blastocysts, the resultant chimeric animals develop lesions along nerves much like the benign neurofibromas that occur in all NF1 patients.

pheochromcytomas, which show loss of the $Nf1^{+}$ allele, but they do not develop abnormally pigmented areas, nor do they develop benign neurofibromas ***(69)***.

Two different approaches have been taken to develop models of neurofibroma and MPNST development. In one approach, ES cells were taken through two rounds of gene targeting to inactivate both copies of the *Nf1* gene. In addition, the cells were marked with the ROSA26 LacZ marker, allowing tissues derived from these ES cells in chimeric mice to be visualized using the X-gal substrate

(70). The *Nf1*$^{-/-}$ ES cells were injected into wild-type LacZ-negative blastocysts to generate *Nf1*$^{+/+}$:*Nf1*$^{-/-}$ chimeras. If the proper number of ES cells is injected, then the resultant chimeric mice can survive to birth. In such cases, neurofibroma-like lesions develop that are composed entirely of ES-derived cells and consist of abnormally differentiated Schwann cells ***(70)***. In the second approach, mice heterozygous for both the *Nf1* and *p53* genes were generated ***(70,71)***. These genes are tightly linked on mouse chromosome 11, and so the null *p53* and *Nf1* alleles were placed in either the *cis* or *trans* configuration (**Fig. 1**). Doubly heterozygous mice, in the *cis* configuration but not the *trans* configuration, developed MPNSTs, presumably because one nondisjunction event or mitotic crossover could result in simultaneous loss of both the wild-type *p53* and *Nf1* alleles. These data are consistent with studies on human MPNSTs showing that in some cases both the *NF1* and *p53* genes are mutated ***(72)***, and they provide the first mouse model for NF1-associated MPNST development. Interestingly, other unknown genetic modifiers of glial tumor development must exist because the *p53*$^{+/-}$, *Nf1*$^{+/-}$ *cis*-heterozygous mice develop astrocytomas on a different inbred strain background ***(73)***.

Neurofibromatosis type 1 syndrome involves dysfunction of many cell types derived from the neural crest including the melanocytes and glial cells. The stem cell factor (SCF) and its receptor, C-kit, also play a pivotal role in the survival, migration, and proliferation of neural crest-derived cells. The role of *Nf1* in regulating signals important to melanocyte and mast cell growth was revealed in mice carrying hypomorphic weak alleles of the *Ckit* gene. Heterozygosity for *Nf1* partially rescued deficiencies in mast cell and melanocyte numbers caused by *Ckit* mutation ***(74)***. These observations show that *Nf1* gene dosage in mice can influence the fate of melanocytes in vivo, perhaps analogous to the development of café au lait spots in NF1 patients.

Children with NF1 syndrome are predisposed to the development of certain rare forms of chronic myeloid leukemia (CML), especially juvenile myelomonocytic leukemia (JMML) ***(75)***. Although homozygous inactivation of the *Nf1* gene causes embryonic lethality, a myeloproliferative disease can be induced in lethally irradiated, adult mice by transplant of *Nf1*$^{-/-}$ fetal liver blood stem cells obtained from embryos prior to their death *in utero* ***(76)***. It has also been shown that like JMML cells, *Nf1*$^{-/-}$ hematopoietic precursors, are hypersensitive to the growth-promoting effects of granulocyte macrophage colony-stimulating factor (GM-CSF) ***(76,77)***. Birnbaum et al. ***(78)*** investigated the requirement for GM-CSF in myeloproliferative disease induced by transplant of *Nf1*$^{-/-}$ blood stem cells. The myeloproliferative disease was greatly attenuated if *Nf1*$^{-/-}$, *Gmcsf*$^{-/-}$ stem cells were transplanted into *Gmcsf*$^{-/-}$ recipients. This result suggests that GM-CSF signaling is required to drive myeloproliferative disease after *NF1* gene loss. Similar studies have shown

that the TEL-platelet-derived growth factor receptor (PDGFR)β fusion oncoprotein requires STAT5 signaling to induce CML in mice. STAT5 is one member of a family of transcription factors that are tyrosine-phosphorylated and activated by janus kinases, which are themselves activated by ligation of cytokine receptors such as the GM-CSF receptor. An aggressive and lethal myeloproliferative disease develops in irradiated mice transplanted with bone marrow stem cells infected with a *TEL-PDGFR*β retroviral vector. However, this virus cannot induce disease in *STAT5a/b*$^{-/-}$ mice, which lack all STAT5 activity ***(79)***.

5. Cooperativity and Gene Dependence in Oncogenesis

Certainly one of the great strengths of a reverse genetic approach in the mouse is the ability to test specific combinations of mutations for evidence of cooperativity in oncogenesis. One such early demonstration is transgenic mice expressing *Cmyc* and infected with a retrovirus expressing an activated *Ras* oncogene. These mice developed lymphomas much faster than mice carrying just the *Cmyc* transgene or nontransgenic mice infected with the retrovirus ***(80)***. This result had been predicted because of the ability of these two oncogenes to cooperate in the transformation of primary rat fibroblasts in tissue culture ***(81)***. Cancer-prone transgenic mouse strains have also been used to discover cooperating oncogenic events by accelerating cancer using oncogenic retroviruses. *Cmyc* oncogene transgenic mice have been used extensively in this type of study (reviewed in **ref.** ***82***).

When these transgenic mice are infected with the Moloney murine leukemia virus (M-MuLV), which can cause leukemia by acting as an insertional mutagen, the disease latency is greatly reduced. These faster developing lymphomas harbor multiple, clonal, M-MuLV proviral insertions. Several oncogenes have been discovered that are recurrently activated by proviral insertion in M-MuLV-infected *Cmyc*-transgenic lymphomas and thus are excellent candidates for cooperation with the *Cmyc* oncogene. Among these cooperating oncogenes are the serine/threonine kinase *Pim1* and the polycomb-group transcription factor *Bmi1* ***(83)***.

Similar experiments have been carried out in mice transgenic for the *NEU* oncogene infected with the Mouse mammary tumor virus (MMTV), which causes mammary tumors in susceptible strains of mice by acting, as does M-MuLV, as an insertional mutagen ***(84)***. In a very innovative approach, M-MuLV acceleration experiments were carried out on a *Pim1*$^{-/-}$ strain background, created by gene knockout ***(85)***. In this way, it was possible to determine whether the activation of other oncogenes could substitute for *Pim1* activation. The prediction was verified that, in such a system, genes downstream of *Pim1* or in pathways parallel to *Pim1* would be activated by

proviral insertion. The *Myc* transgenic/*Pim1*$^{-/-}$ animals infected with M-MuLV developed lymphomas with proviral insertions in a *Pim1* homolog, which was designated *Pim2* ***(85)***.

It is possible to determine whether cancer, induced by oncogene or TSG mutations, depends on the expression of specific normal genes. This type of study is very important because it allows the identification of potential drug targets for human cancers with specific gene mutations. One example, mentioned above, was the demonstration that GM-CSF signals drive myeloproliferative disease in the absence of *Nf1* expression ***(78)***. Similar studies have been done using the *Apc*^{Min} mouse combined with other gene mutation. Mice and individuals that are heterozygous for inactivating mutations in the *APC* gene are predisposed to the development of multiple intestinal polyps, the precursors to adenomas and carcinoma (reviewed in **ref.** ***86***). These polyps result from somatic loss of the *APC*$^{+}$ allele or its expression.

However, other genes influence polyp formation. It has been possible to test candidate genes by combining them with the *Apc*^{Min} or *Apc* knockout mutations. The nonsteroidal antiinflammatory drugs (NSAIDs) target cyclooxygenase (Cox) enzymes and have been shown to inhibit polyp formation in *Apc*$^{Min/+}$ mice ***(87)***. By combining both the *Cox2* knockout mutation with an *Apc* mutant allele, called *Apc*$^{\Delta 716}$, it was possible to demonstrate that the action of NSAIDs probably involves suppression of Cox-2 activity, since this knockout combined with *Apc*$^{\Delta 716}$ yielded substantially fewer polyps than were seen in control *Apc*$^{\Delta 716/+}$ *Cox2*$^{+/+}$ mice ***(88)***. These sorts of data have allowed drug design efforts to focus on Cox proteins as a target for colorectal cancer prevention or treatment as well as for other malignancies ***(66,89)***.

6. Emerging Technologies

Several newer technologies in mouse genetics are likely to have major impacts on how cancer is modeled and studied in mice. Many of these technologies involve the ability to turn transgenic oncogenes on and off in vivo or to inactivate tumor suppressor genes in specific tissues. Several promoters have been described that can be induced in transgenic mice. These include the ecdysone-inducible promoter ***(90)***, tetracycline transactivator-responsive promoters ***(19,91)*** (*see* Chapter 5), and the interferon-inducible promoter *Mx1* ***(92,93)***. In addition, it is possible to keep transgenes from being expressed by separating them from a promoter with a so-called recombination-activated gene expression (RAGE) cassette, which contains stop codons in all three reading frames and a site for polyadenylation, and is flanked by LoxP sites. If the Cre recombinase is induced in vivo, or introduced by a virus, then the RAGE cassette will be removed by site-specific recombination, and transgene expression will occur ***(94)***. The use of Cre recombinase and LoxP sites are the

basis for several other important advances in gene targeting and transgenesis that are of relevance to cancer studies (*see* also Chapter 9).

It has been shown using tetracycline-regulated transgenes, continued expression of the Bcr-Abl oncoprotein and mutant H-ras are each required for maintenance of the transformed phenotype in vivo ***(19,91)***. In the former case, a version of the Tet transactivator that is repressed by antibiotic treatment (doxycycline is usually used rather than tetracycline) was introduced into mice by transgenesis. Independently, a construct expressing mutant Bcr-Abl from a tet operator-MMTV long terminal repeat (LTR) promoter was introduced into mice by transgenesis. Thus, in doubly transgenic mice, treatment with doxycycline could suppress expression of the transgene. Tumor regression involving massive apoptosis was seen when transgene expression was suppressed in tumor-bearing animals. Interestingly, Bcr-Abl-induced tumors that had progressed or developed over a long time became independent from continued transgene expression, suggesting that the tumor clone had acquired other oncogenic mutations allowing it to persist ***(19)***. In the case of *Hras*, the mutant gene was induced by addition of doxycyclin in mice nullizygous for the *INK4a* tumor suppressor gene, resulting in melanoma development ***(91)***. Even large melanoma tumors regressed after doxycyclin was withdrawn from the drinking water. As in the other model, the tumor cells showed massive apoptosis after downregulation of oncogene expression ***(91)***.

Loci modified by homologous recombination in ES cells can also now be regulated by removal of sequences using the Cre recombinase. In the simplest version of such a system, an endogenous gene can be "floxed" such that a critical exon or region of the gene is removed upon expression of the Cre gene (*see* Chapter 9). This can be done by tissue-restricted expression of the Cre transgene or upon induction of a Cre transgene. In this way, biallelic inactivation of TSGs that are embryonically lethal can be achieved in a tissue-specific manner. This is one useful approach for overcoming the limited number of target cells and the short life span a mouse has in which to incur loss of the wild-type TSG allele on a heterozygous background. For example, this approach has been successfully used to generate schwannoma tumors in the mouse that are associated with inactivation of the neurofibromatosis type 2, *Nf2*, tumor suppressor gene ***(95)***. $Nf2^{+/-}$ mice do not develop schwannomas, but instead develop osteosarcomas, presumably because of an insufficient rate of $Nf2^{+}$ allele loss in mouse Schwann cells.

Another use of LoxP sites and Cre recombinase is the RAGE cassette, which can interrupt the expression of a dominant-oncogenic and possibly lethal transgene or knockin allele until such time as the Cre recombinase is expressed. Several knockin alleles, which mimic the result of chromosomal translocation, have been shown to result in embryonic lethality when passed

through the germline of mice ***(96,97)***. Therefore, an approach such as this may need to be taken for these cases.

Human tumors are characterized by frequent unbalanced chromosomal rearrangements ***(98)***. In some cases entire chromosomes can be lost (resulting in monosomy) or gained (resulting in trisomy). Smaller gains and losses such as deletions, duplications, and nonreciprocal translocations are also common. Many of these events occur recurrently in human cancers and are thought to be the result of selective pressure; that is, these changes give the tumor clone a growth or survival advantage. Unbalanced chromosomal rearrangements can now be modeled in the mouse germline using ES cell technology. In order for deletions to be generated in ES cells, two different approaches have been developed. One approach, pioneered by Dr. John Schimenti and colleagues, involves introducing a herpes simplex virus thymidine kinase (TK) gene into the ES cell genome, irradiating the ES, and selecting the irradiated cells against TK expression using gancyclovir ***(99)***. A significant percentage of the resultant gancyclovir-resistant clones have acquired deletions ranging in size from several hundred kilobase pairs to several centimorgans. If the TK gene is inserted at a specific site in the ES cell genome by homologous recombination, then a series of clones can be obtained with deletion end points that vary in their distance from the site of initial TK gene insertion.

A second approach involves placing LoxP sites and the 3′ and 5′ halves of the *HPRT* minigene at specific sites, on different parts of the same chromosome, using homologous recombination ***(100)***. Upon introduction of the Cre recombinase and selection for *HPRT* expression, clones can be obtained that have undergone deletion, inversions, or duplication of the region between the two LoxP sites. The outcome of Cre-mediated recombination depends on whether the LoxP sites had been placed in the same or opposite orientations with respect to the centromere and whether they had been placed in *cis* (same homolog) or *trans* (opposite homologs) with respect to each other. This technique is powerful because duplications and deletions can be created, thereby mimicking deletion or duplication that can occur as the result of nonreciprocal translocations in human tumors. If these deletion chromosomes were passed through the germline, mice carrying them could be used to determine whether such deletions confer a growth advantage to tumors by haploinsufficiency or, in addition, might be used to uncover the presence of a TSG in the region. Because the human and mouse genomes share extensive linkage homology, it is possible to model specific recurrent deletions in human cancer by choosing the appropriate regions of the mouse genome to delete.

Another technique has opened the possibility of inducing monosomy at a high rate in mouse somatic cells. In this technique an array of LoxP sites whose orientations differ is placed on a specific mouse chromosome. In the presence

of Cre expression, such chromosomes are unstable and are often lost, resulting in monosomy ***(101)***. This loss is presumably due to Cre-mediated homologous recombination, between chromatids, that utilizes LoxP sites in inverted orientations. The results of this recombination are unstable dicentric or acentric chromosomes. Such chromosomes are lost at high frequency during mitosis.

New genomic technologies for human cancer research are likely to be even more useful for cancer studies using mouse models. Among these technologies is spectral karyotyping, which is especially useful in the mouse, in which traditional cytogenetics using G-banding has been more difficult than with human cancer samples ***(102–104)***. This use of mouse models, where cancer can easily be studied from early to late stages, or in the presence of very specific gene mutations, is also very likely to give valuable information from massive gene expression analyses using cDNA microarray hybridization, sequential analysis of gene expression (SAGE), or similar techniques.

Finally, mouse models of human cancer can be used to identify cancer susceptibility loci, which may be common in human populations. Crosses involving TSG mutations or transgenes onto different strain backgrounds have shown that they can differ tremendously in their susceptibility to cancer in these models ***(63,73)***. The mouse has many advantages over human genetic studies for the identification of what may be low penetrance, complex or multigenic effects. Included among these advantages are controlled crosses, the ability to control for environmental variables, the use of recombinant inbred and congenic strains, and the ability to generate large numbers of genetically identical animals. Several examples have been obtained demonstrating the presence of such genes in mouse crosses. The *Plgpa* gene stands out as one major modifier of risk from *Apc* loss-induced polyp formation in mice ***(105)***. It may be possible to identify more modifiers using the powerful germline mutagen ethyl nitrosourea (ENU).

7. Meeting the Goal: *Translation to Human Oncology*

One of the ultimate goals of cancer research is to improve the care and treatment of patients with cancer. How can mouse models of human cancer help us achieve this goal? Are mice useful, valid models for human cancer? How can they be better exploited and improved in this regard? These are some of the questions that the National Cancer Institute has asked the community of mouse cancer researchers via a new opportunity called the Mouse Models of Human Cancer Consortium (MMHCC). This consortium has been created by way of awarding research grants to research teams, composed of multiple investigators all of whom have decided to focus their different approaches and areas of expertise on a single type of cancer or cancer problem.

All the teams have in common the goal of a multidisciplinary approach to technology development, model building, and new avenues of exploration. Some of the areas of emphasis are pathology, genomics, imaging, diagnosis, mouse genome engineering, and generating a shared "tool kit" for investigators to use for manipulating the mouse genome. These shared tools will include useful vectors, protocols, and mouse strains. Another area of emphasis will be preclinical trials of new therapies. Collectively, these efforts should answer many of these questions that the MMHCC poses for the future of mouse cancer studies. In summary, the advantages mouse models have for meeting these goals are shared synteny, the ability to map genetic modifiers, the forthcoming availability of the whole genome sequence, many polymorphic markers, and a large number of inbred strains. Finally, paramount in cancer studies to date has been the ability to manipulate the mouse genome in so many ways.

References

1. Fijneman, R. J., de Vries, S. S., Jansen, R. C., and Demant, P. (1996) Complex interactions of new quantitative trait loci, Sluc1, Sluc2, Sluc3, and Sluc4, that influence the susceptibility to lung cancer in the mouse. *Nat. Genet.* **14,** 465–467.
2. Nagase, H., Bryson, S., Cordell, H., et al. (1995) Distinct genetic loci control development of benign and malignant skin tumours in mice. *Nat. Genet.* **10,** 424–429.
3. Fearon, E. R. (1997) Human cancer syndromes: clues to the origin and nature of cancer. *Science* **278,** 1043–1050.
4. Clarke, A. R. (2000) Manipulating the germline: its impact on the study of carcinogenesis. *Carcinogenesis* **21,** 435–441.
5. Klausner, R. D. (1999) Studying cancer in the mouse. *Oncogene* **18,** 5249–5252.
6. Macleod, K. F. and Jacks, T. (1999) Insights into cancer from transgenic mouse models. *J. Pathol.* **187,** 43–60.
7. Russell, E. S. (1978) Origins and history of mouse inbred strains: contributions of Clarence Cook Little, in *Origins of Inbred Mice*, 3rd ed. (Morse, H. C., ed.), Academic, New York, pp. 33–44.
8. Adams, J. M., Harris, A. W., Pinkert, C. A., et al. (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318,** 533–538.
9. Adams, J. M., Gerondakis, S., Webb, E., Corcoran, L. M., and Cory, S. (1983) Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. *Proc. Natl. Acad. Sci. USA* **80,** 1982–1986.
10. Taub, R., Kirsch, I., Morton, C., et al. (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc. Natl. Acad. Sci. USA* **79,** 7837–7841.

11. Byrd, L. G., McDonald, A. H., Gold, L. G., and Potter, M. (1991) Specific pathogen-free BALB/cAn mice are refractory to plasmacytoma induction by pristane. *J. Immunol.* **147,** 3632–3637.
12. Enno, A., O'Rourke, J. L., Howlett, C. R., et al. (1995) MALToma-like lesions in the murine gastric mucosa after long-term infection with *Helicobacter felis*. A mouse model of *Helicobacter pylori*-induced gastric lymphoma. *Am. J. Pathol.* **147,** 217–222.
13. Cooper, H. S., Murthy, S., Kido, K., Yoshitake, H., and Flanigan, A. (2000) Dysplasia and cancer in the dextran sulfate sodium mouse colitis model. Relevance to colitis-associated neoplasia in the human: a study of histopathology, B-catenin and p53 expression and the role of inflammation. *Carcinogenesis* **21,** 757–768.
14. Furth, P. A., Hennighausen, L., Baker, C., Beatty, B., and Woychick, R. (1991) The variability in activity of the universally expressed human cytomegalovirus immediate early gene 1 enhancer/promoter in transgenic mice. *Nucleic Acids Res.* **19,** 6205–6208.
15. Schmidt, E. V., Christoph, G., Zeller, R., and Leder, P. (1990) The cytomegalovirus enhancer: a pan-active control element in transgenic mice. *Mol. Cell. Biol.* **10,** 4406–4411.
16. Mangues, R., Symmans, W. F., Lu, S., Schwartz, S., and Pellicer, A. (1996) Activated N-ras oncogene and N-ras proto-oncogene act through the same pathway for in vivo tumorigenesis. *Oncogene* **13,** 1053–1063.
17. Young, M. R., Li, J. J., Rincon, M., et al. (1999) Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. *Proc. Natl. Acad. Sci. USA* ***96,*** 9827–9832.
18. Furth, P. A., St. Onge, L., Boger, H., et al. (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306.
19. Huettner, C. S., Zhang, P., Van Etten, R. A., and Tenen, D. G. (2000) Reversibility of acute B-cell leukaemia induced by BCR-ABL1. *Nat. Genet.* **24,** 57–60.
20. Grandori, C., Cowley, S. M., James, L. P., and Eisenman, R. N. (2000) The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu. Rev. Cell Dev. Biol.* **16,** 653–699.
21. Liao, D. J. and Dickson, R. B. (2000) c-Myc in breast cancer. *Endocr. Relat. Cancer* **7,** 143–164.
22. Maris, J. M. and Matthay, K. K. (1999) Molecular biology of neuroblastoma. *J. Clin. Oncol.* **17,** 2264–2279.
23. Sinn, E., Muller, W., Pattengale, P., et al. (1987) Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell* **49,** 465–475.
24. Moroy, T., Fisher, P., Guidos, C., et al. (1990) IgH enhancer deregulated expression of L-myc: abnormal T lymphocyte development and T cell lymphomagenesis. *EMBO J.* **9,** 3659–3666.
25. Sheppard, R. D., Samant, S. A., Rosenberg, M., Silver, L. M., and Cole, M. D. (1998) Transgenic N-myc mouse model for indolent B cell lymphoma: tumor

characterization and analysis of genetic alterations in spontaneous and retrovirally accelerated tumors. *Oncogene* **17,** 2073–2085.
26. Weiss, W. A., Aldape, K., Mohapatra, G., Feuerstein, B. G., and Bishop, J. M. (1997) Targeted expression of MYCN causes neuroblastoma in transgenic mice. *EMBO J.* **16,** 2985–2995.
27. Schmitt, C. A., McCurrach, M. E., de Stanchina, E., Wallace-Brodeur, R. R., and Lowe, S. W. (1999) INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev.* **13,** 2670–2677.
28. Renard, C.A., Fourel, G., Bralet, M.P., et al. (2000) Hepatocellular carcinoma in WHV/N-myc2 transgenic mice: oncogenic mutations of beta-catenin and synergistic effect of p53 null alleles. *Oncogene* **19,** 2678–2686.
29. McCormack, S. J., Weaver, Z., Deming, S., et al. (1998) Myc/p53 interactions in transgenic mouse mammary development, tumorigenesis and chromosomal instability. *Oncogene* **16,** 2755–2766.
30. Wittinghofer, A. (1998) Signal transduction via Ras. *Biol. Chem.* **379,** 933–937.
31. Rodenhuis, S. (1992) ras and human tumors. *Semin. Cancer Biol.* **3,** 241–247.
32. Quaife, C. J., Pinkert, C. A., Ornitz, D. M., Palmiter, R. D., and Brinster, R. L. (1987) Pancreatic neoplasia induced by ras expression in acinar cells of transgenic mice. *Cell* **48,** 1023–1034.
33. Hundley, J. E., Koester, S. K., Troyer, D. A., et al. (1997) Increased tumor proliferation and genomic instability without decreased apoptosis in MMTV-ras mice deficient in p53. *Mol. Cell. Biol.* **17,** 723–731.
34. Maronpot, R. R., Palmiter, R. D., Brinster, R. L., and Sandgren, E. P. (1991) Pulmonary carcinogenesis in transgenic mice. *Exp. Lung Res.* **17,** 305–320.
35. Shi, B., Yaremko, B., Hajian, G., et al. (2000) The farnesyl protein transferase inhibitor SCH66336 synergizes with taxanes in vitro and enhances their antitumor activity in vivo. *Cancer Chemother. Pharmacol.* **46,** 387–393.
36. Nielsen, L. L. (2000) Combination therapy with SCH58500 (p53 adenovirus) and cyclophosphamide in preclinical cancer models. *Oncol. Rep.* **7,** 1191–1196.
37. Kalkuhl, A., Troppmair, J., Buchmann, A., et al. (1998) p21Ras downstream effectors are increased in activity or expression in mouse liver tumors but do not differ between ras-mutated and ras-wild-type lesions. *Hepatology* **27,** 1081–1088.
38. Adnane, J., Jackson, R. J., Nicosia, S. V., et al. (2000) Loss of p21WAF1/CIP1 accelerates Ras oncogenesis in a transgenic/knockout mammary cancer model. *Oncogene* **19,** 5338–5347.
39. Mangues, R., Corral, T., Lu, S., et al. (1998) NF1 inactivation cooperates with N-ras in in vivo lymphogenesis activating Erk by a mechanism independent of its Ras-GTPase accelerating activity. *Oncogene* **17,** 1705–1716.
40. Saez, E., Rutberg, S. E., Mueller, E., et al. (1995) c-fos is required for malignant progression of skin tumors. *Cell* **82,** 721–732.
41. Trempus, C. S., Haseman, J. K., and Tennant, R. W. (1997) Decreases in phorbol ester-induced papilloma development in v-Ha-ras transgenic TG.AC mice during reduced gene dosage of bcl-2. *Mol. Carcinog.* **20,** 68–77.

42. Furth, P. A. (1998) SV40 rodent tumour models as paradigms of human disease: transgenic mouse models. *Dev. Biol. Stand.* **94,** 281–287.
43. Ozer, H. L. (2000) SV40-mediated immortalization. *Prog. Mol. Subcell. Biol.* **24,** 121–153.
44. Noble, M., Groves, A. K., Ataliotis, P., Ikram, Z., and Jat, P. S. (1995) The H-2KbtsA58 transgenic mouse: a new tool for the rapid generation of novel cell lines. *Transgenic Res.* **4,** 215–225.
45. Obinata, M. (1997) Conditionally immortalized cell lines with differentiated functions established from temperature-sensitive T-antigen transgenic mice. *Genes Cells* **2,** 235–244.
46. Chen, J. D. and Van Dyke, T. (1991) Uniform cell-autonomous tumorigenesis of the choroid plexus by papovavirus large T antigens. *Mol. Cell. Biol.* **11,** 5968–5976.
47. Hager, J. H. and Hanahan, D. (1999) Tumor cells utilize multiple pathways to down-modulate apoptosis. Lessons from a mouse model of islet cell carcinogenesis. *Ann. NY Acad. Sci.* **887,** 150–163.
48. Grisolano, J. L., Wesselschmidt, R. L., Pelicci, P. G., and Ley, T. J. (1997) Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. *Blood* **89,** 376–387.
49. He, L.-Z., Tribioli, C., Rivi, R., et al. (1997) Acute leukemia with promelocytic features in PML/RARalpha trnasgenic mice. *Proc. Natl. Acad. Sci. USA* **94,** 5302–5307.
50. Brown, D., Kogan, S., Lagasse, E., et al. (1997) A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. *Proc. Natl. Acad. Sci. USA* **94,** 2551–2556.
51. Grimwade, D., Biondi, A., Mozziconacci, M. J., et al. (2000) Characterization of acute promyelocytic leukemia cases lacking the classic t(15;17): results of the European Working Party. Groupe Français de Cytogenetique Hematologique, Groupe Français d'Hematologie Cellulaire, UK Cancer Cytogenetics Group and BIOMED 1 European Community-Concerted Action "Molecular Cytogenetic Diagnosis in Haematological Malignancies". *Blood* **96,** 1297–1308.
52. Rego, E. M., He, L. Z., Warrell, R. P., Wang, Z. G., and Pandolfi, P. P. (2000) Retinoic acid (RA) and As2O3 treatment in transgenic models of acute promyelocytic leukemia (APL) unravel the distinct nature of the leukemogenic process induced by the PML-RARalpha and PLZF-RARalpha oncoproteins. *Proc. Natl. Acad. Sci. USA* **97,** 10173–10178.
53. He, L. Z., Guidez, F., Tribioli, C., et al. (1998) Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat. Genet.* **18,** 126–135.
54. Kogan, S. C., Hong, S. H., Shultz, D. B., Privalsky, M. L., and Bishop, J. M. (2000) Leukemia initiated by PMLRARalpha: the PML domain plays a critical role while retinoic acid-mediated transactivation is dispensable. *Blood* **95,** 1541–1550.
55. Lipinski, M. M. and Jacks, T. (1999) The retinoblastoma gene family in differentiation and development. *Oncogene* **18,** 7873–7882.

56. Jacks, T., Fazeli, A., Schmitt, E. M., et al. (1992) Effects of an Rb mutation in the mouse. *Nature* **359,** 295–300.
57. Kaye, F. J. (1997) Can p53 status resolve paradoxes between human and non-human retinoblastoma models? *J. Natl. Cancer Inst.* **89,** 1476–1477.
58. Windle, J. J., Albert, D. M., O'Brien, J. M., et al. (1990) Retinoblastoma in transgenic mice. *Nature* **343,** 665–669.
59. Pan, H. and Griep, A. E. (1994) Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. *Genes Dev.* **8,** 1285–1299.
60. Howes, K.A., Ransom, N., Papermaster, D.S., et al. (1994) Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. *Genes Dev.* **8,** 1300–1310.
61. Sands, A., Donehower, L. A., and Bradley, A. (1994) Gene-targeting and the p53 tumor-suppressor gene. *Mutat. Res.* **307,** 557–572.
62. Donehower, L. A., Harvey, M., Slagle, B. L., et al. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356,** 215–221.
63. Harvey, M., McArthur, M. J., Montgomery, C. A., Bradley, A., and Donehower, L. A. (1993) Genetic background alters the spectrum of tumors that develop in p53-deficient mice. *FASEB J.* **7,** 938–943.
64. Attardi, L. D., Lowe, S. W., Brugarolas, J., and Jacks, T. (1996) Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis. *EMBO J.* **15,** 3693–3701.
65. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362,** 847–849.
66. Alexander, J. (2000) Use of transgenic mice in identifying chemopreventive agents. *Toxicol. Lett.* **112–113,** 507–512.
67. Parada, L. F. (2000) Neurofibromatosis type 1. *Biochim. Biophys. Acta* **1471,** M13–19.
68. Brannan, C. I., Perkins, A. S., Vogel, K. S., et al. (1994) Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. *Genes Dev.* **8,** 1019–1029.
69. Jacks, T., Shih, T. S., Schmitt, E. M., et al. (1994) Tumor predisposition in mice heterozygous for a targeted mutation in Nf1. *Nat. Genet.* **7,** 353–361.
70. Cichowski, K., Shih, T. S., Schmitt, E., et al. (1999) Mouse models of tumor development in neurofibromatosis type 1. *Science* **286,** 2172–2176.
71. Vogel, K. S., Klesse, L. J., Velasco-Miguel, S., et al. (1999) Mouse tumor model for neurofibromatosis type 1. *Science* **286,** 2176–2179.
72. Legius, E., Dierick, H., Wu, R., et al. (1994) TP53 mutations are frequent in malignant NF1 tumors. *Genes Chromosomes Cancer* **10,** 250–255.
73. Reilly, K. M., Loisel, D. A., Bronson, R. T., McLaughlin, M. E., and Jacks, T. (2000) Nf1;Trp53 mutant mice develop glioblastoma with evidence of strain-specific effects. *Nat. Genet.* **26,** 109–113.

74. Ingram, D. A., Yang, F. C., Travers, J. B., et al. (2000) Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumor suppressor gene modulates melanocyte and mast cell fates in vivo. *J. Exp. Med.* **191,** 181–188.
75. O'Marcaigh, A. S. and Shannon, K. M. (1997) Role of the NF1 gene in leukemogenesis and myeloid growth control. *J. Pediatr. Hematol. Oncol.* **19,** 551–554.
76. Largaespada, D. A., Brannan, C. I., Jenkins, N. A., and Copeland, N. G. (1996) Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukaemia. *Nat. Genet.* **12,** 137–143.
77. Emanuel, P. D., Bates, L. J., Castleberry, R. P., Gualtieri, R. J., and Zuckerman, K. S. (1991) Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. *Blood* **77,** 925–929.
78. Birnbaum, R. A., O'Marcaigh, A., Wardak, Z., et al. (2000) Nf1 and Gmcsf interact in myeloid leukemogenesis. *Mol. Cell* **5,** 189–195.
79. Schwaller, J., Parganas, E., Wang, D., et al. (2000) Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. *Mol. Cell.* **6,** 693–704.
80. Langdon, W. Y., Harris, A. W., and Cory, S. (1989) Acceleration of B-lymphoid tumorigenesis in E mu-myc transgenic mice by v-H-ras and v-raf but not v-abl. *Oncogene Res.* **4,** 253–8.
81. Land, H., Parada, L. F., and Weinberg, R. A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304,** 596–602.
82. Berns, A., Mikkers, H., Krimpenfort, P., et al. (1999) Identification and characterization of collaborating oncogenes in compound mutant mice. *Cancer Res.* **59,** 1773s–1777s.
83. van Lohuizen, M., Verbeek, S., Scheijen, B., et al. (1991) Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. *Cell* **65,** 737–752.
84. Shackleford, G. M., MacArthur, C. A., Kwan, H. C., and Varmus, H. E. (1993) Mouse mammary tumor virus infection accelerates mammary carcinogenesis in Wnt-1 transgenic mice by insertional activation of int-2/Fgf-3 and hst/Fgf-4. *Proc. Natl. Acad. Sci. USA* **90,** 740–744.
85. van der Lugt, N. M., Domen, J., Verhoeven, E., et al. (1995) Proviral tagging in E mu-myc transgenic mice lacking the Pim-1 proto-oncogene leads to compensatory activation of Pim-2. *EMBO J.* **14,** 2536–2544.
86. Goss, K. H. and Groden, J. (2000) Biology of the adenomatous polyposis coli tumor suppressor. *J. Clin. Oncol.* **18,** 1967–1979.
87. Jacoby, R. F., Seibert, K., Cole, C. E., Kelloff, G., and Lubet, R. A. (2000) The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. *Cancer Res.* **60,** 5040–5044.
88. Oshima, M., Dinchuk, J. E., Kargman, S. L., et al. (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* **87,** 803–809.

89. Langenbach, R., Loftin, C. D., Lee, C., and Tiano, H. (1999) Cyclooxygenase-deficient mice. A summary of their characteristics and susceptibilities to inflammation and carcinogenesis. *Ann. NY Acad. Sci.* **889,** 52–61.
90. Saez, E., Nelson, M. C., Eshelman, B., et al. (2000) Identification of ligands and coligands for the ecdysone-regulated gene switch. *Proc. Natl. Acad. Sci. USA* **97,** 14512–14517.
91. Chin, L., Tam, A., Pomerantz, J., et al. (1999) Essential role for oncogenic Ras in tumour maintenance. *Nature* **400,** 468–472.
92. Tomita, S., Sinal, C. J., Yim, S. H., and Gonzalez, F. J. (2000) Conditional disruption of the aryl hydrocarbon receptor nuclear translocator (Arnt) gene leads to loss of target gene induction by the aryl hydrocarbon receptor and hypoxia-inducible factor 1alpha. *Mol. Endocrinol.* **14,** 1674–1681.
93. Lam, K. P., Kuhn, R., and Rajewsky, K. (1997) In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* **90,** 1073–1083.
94. Utomo, A. R., Nikitin, A. Y., and Lee, W. H. (1999) Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat. Biotechnol.* **17,** 1091–1096.
95. Giovannini, M., Robanus-Maandag, E., van der Valk, M., et al. (2000) Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2. *Genes Dev.* **14,** 1617–30.
96. Castilla, L. H., Wijmenga, C., Wang, Q., et al. (1996) Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. *Cell* **87,** 687–696.
97. Yergeau, D. A., Hetherington, C. J., Wang, Q., et al. (1997) Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. *Nat. Genet.* **15,** 303–306.
98. Mitelman, F., Mertens, F., and Johanson, B. (1997) A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. *Nat. Genet.* **Special Issue,** 417–474.
99. You, Y., Bergstrom, R., Klemm, M., et al. (1997) Chromosomal deletion complexes in mice by radiation of embryonic stem cells. *Nat. Genet.* **15,** 285–288.
100. Ramiro, R.-S., Liu, P., and Bradley, A. (1995) Chromosome engineering in mice. *Nature* **378,** 720–724.
101. Lewandoski, M. and Martin, G. R. (1997) Cre-mediated chromosome loss in mice. *Nat. Genet.* **17,** 223–225.
102. Zimonjic, D. B., Pollock, J. L., Westervelt, P., Popescu, N. C.,and Ley, T. J. (2000) Acquired, nonrandom chromosomal abnormalities associated with the development of acute promyelocytic leukemia in transgenic mice. *Proc. Natl. Acad. Sci. USA* **97,** 13306–13311.
103. Schrock, E. and Padilla-Nash, H. (2000) Spectral karyotyping and multicolor fluorescence in situ hybridization reveal new tumor-specific chromosomal aberrations. *Semin. Hematol.* **37,** 334–347.

104. Coleman, A. E., Ried, T., and Janz, S. (2000) Chromosomes 1 and 5 harbor plasmacytoma progressor genes in mice. *Genes Chromosomes Cancer* **29,** 70–74.
105. MacPhee, M., Chepenik, K. P., Liddell, R. A., et al. (1995) The secretory phospholipase A2 gene is a candidate for the Mom1 locus, a major modifier of ApcMin-induced intestinal neoplasia. *Cell* **81,** 957–966.

18

Neuropathobiology in Transgenic Mice

The Case of Alzheimer's Disease

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1. Introduction

The last decade has witnessed a quantum leap in our technical abilities to generate transgenic mice, up to the point that transgenic mouse models for any aspect of normal and pathologic physiology are within reach. Most difficult to attain, and impossible in humans, are invasive approaches in vivo, to define the molecular, biochemical and cellular functioning of the adult central nervous system (CNS) in normal conditions. However, even more desired, at least by some, are animal models for those neurodegenerative diseases that become increasingly devastating in the elderly. The most evident case in point is Alzheimer's disease (AD).

Generating transgenic models obviously requires and is based on clinical and molecular genetics, and on the identification of genes and mutations linked to genetic diseases. All useful transgenic mouse models available today, e.g., those for amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, prion disease, and AD, are based on mutated genes that were identified by genetic studies in families.

It should be remembered that "linkage" of a gene or a mutation to an inherited disease, however tight, remains tentative and cannot be considered foolproof. Its experimental verification, preferably in vivo, is needed to prove that a given mutant gene is the direct cause of a given clinical symptom or syndrome. Needless to say, in the absence of natural models, genetic manipulation, particularly in mice, has proved to be useful and powerful in this respect.

From: *Methods in Molecular Biology, vol. 209: Transgenic Mouse Methods and Protocols*
Edited by: M. H. Hofker and J. van Deursen

It should also not be forgotten that initial mishaps were both numerous and embarrassing; however, they have been overcome faster and more successfully than anticipated by most critics a decade ago.

The present contribution is not intended to review the history or the entire field of transgenic model building for AD. This chapter is restricted to an account of our efforts to characterize phenotypically several different transgenic mouse strains that are proving useful or that have potential as experimental models for fundamental and applied research into the many different aspects of AD. The models were generated by overexpression of wild-type and mutant human genes that are genetically implicated in AD, i.e., amyloid precursor protein (APP), presenilin (PS1 and PS2), protein tau, and, most recently, apolipoprotein E (ApoE4) ***(1,2)***. In addition to neuron-specific overexpression using the mouse thy1 gene promoter, we are also implementing neuron-specific modification of some of these genes. To this end we have generated a strain of transgenic mice with neuron-specific expression of Cre-recombinase, hoping to gain an additional tool for answering fundamental questions that are being raised in the field of AD.

First we briefly describe the relevant pathologic hallmarks of humans suffering from AD, as well as the questions and problems (fundamental and applied) that are being asked or addressed. This is followed by an overview of the different transgenic mouse strains currently under study, in different stages of phenotypic characterization; we try to highlight the relevance for the human situation. The focus is on two major aspects: the successful APP London mutant (APP/Ld) transgenic mouse model for amyloid pathology, and the problematic aspects of modeling tau pathology in transgenic mice. Finally, we discuss hypotheses and the prospects and potentials of these animal models for future research into the neurobiology of the proteins involved in normal and in disease conditions, especially, but not only in AD.

2. Major Pathologic Features of Alzheimer's Disease

A major distinction is to be made between the clinical and pathologic characteristics of dementia of the Alzheimer's type. This chapter concentrates mainly on brain pathology. The postmortem human AD brain is characterized by two types of lesions: extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). In addition, practically all AD patients have amyloid deposits in the walls of blood vessels of the brain, termed cerebral amyloid angiopathy, which is also evident in about 30% of the elderly. In this and the following paragraphs we concentrate on the amyloid pathology and its successful recapitulation in the brain of APP/Ld transgenic mice. Then we discuss the completely different situation and the problems encountered in developing transgenic mice with NFT pathology.

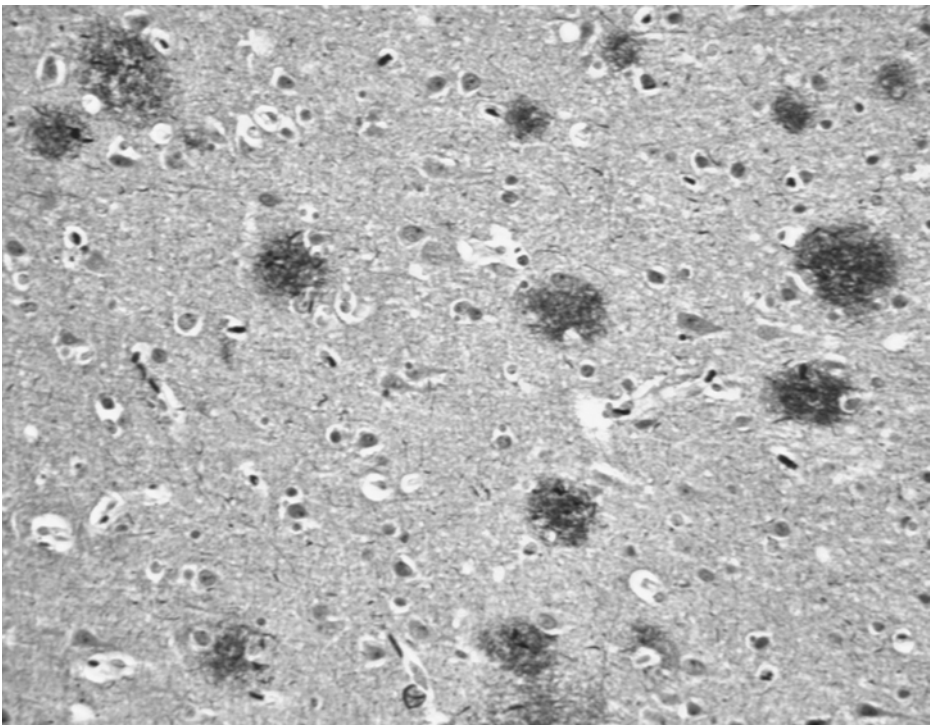

Fig. 1. Neocortex from Alzheimer's disease patient with numerous argyrophilic plaques. (Bielschowsky's silver method; ×90.)

2.1. Senile Plaques

Two types of amyloid deposits are evident in the parenchym of the human brain: neuritic and nonneuritic (diffuse) senile plaques. Neuritic plaques are diagnostic for AD. They are 20–150 μm across and consist of a central core of amyloid, surrounded by abnormal neuronal processes, i.e., swollen neurites. These dystrophic neurites are distended and contain degenerating cellular organelles, mitochondria, and lysosomes. Interspersed among the neurites are also processes of activated microglia and astrocytes.

Neuritic plaques are visualized in paraffin sections by silver impregnation, essentially as done by A. Alzheimer (1907) ***(3)*** and modified by Bielschowsky (**Fig. 1**; *see* Color Plate 3 following p. 144) and others. "Amyloid" is a misnomer since the central amyloid core consists of protein and is best demonstrated by classical "amyloid" stains, i.e., Congo Red or ThioflavinS. Ultrastructurally, the central amyloid core consists of amyloid fibers with a diameter of 8–11 nm. Biochemically, these are composed of the 4-kDa amyloid peptides, derived

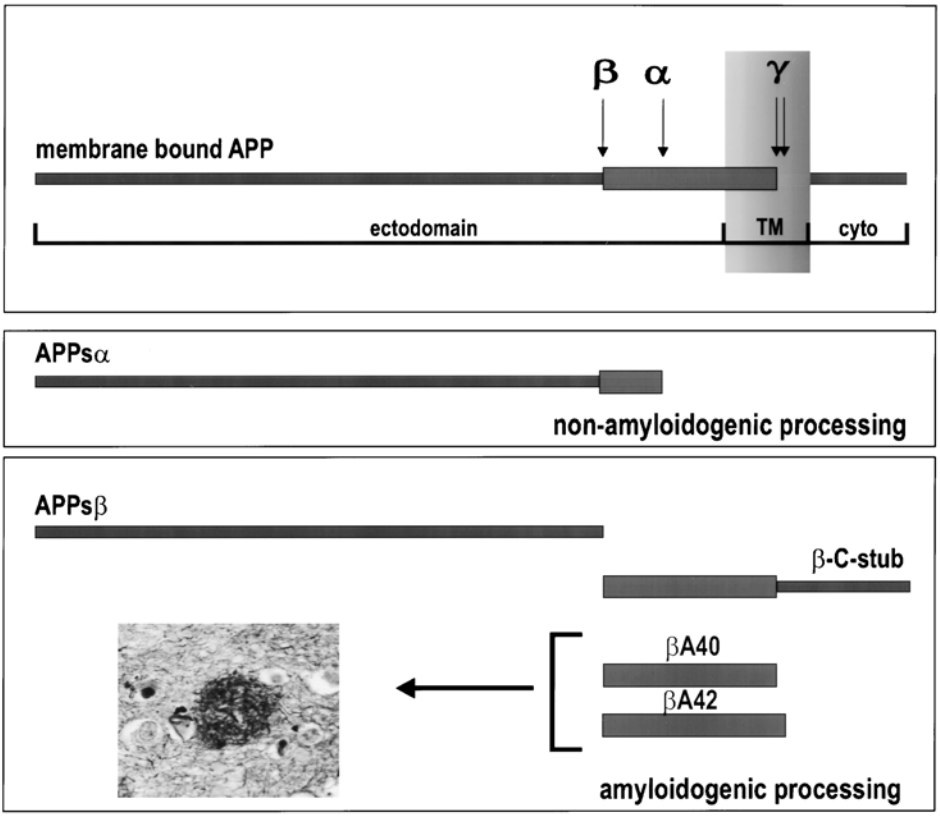

Fig. 2. Schematic representation of amyloid precursor protein (APP) processing intermediates generated by endoproteolytic processing of APP. In the non-amyloidogenic processing pathway, APP is cleaved inside the Aβ sequence by α-secretase (α). This liberates a soluble derivative (APPsα). In the amyloidogenic processing pathway, APP is cleaved by β-secretase (β), generating a C-terminally truncated form of APPs (APPsβ) and a β-C-stub, bearing the Aβ sequence. The latter is further processed by γ-secretase (γ), leading to the formation of Aβ peptides of varying length, Aβ40 and Aβ42. TM, transmembrane domain; cyto, cytoplasmatic domain.

from the 110–130 kDa APP, which is cleaved by proteinases known as β- and γ-secretase (**Fig. 2**) ***(4)***. These peptides are produced in two main forms of 40 or 42 amino acids, named Aβ40 and Aβ42. The two extra amino acids at the C-terminus of Aβ42 are part of the transmembrane domain of APP, making Aβ42 the least soluble amyloid peptide; this characteristic is probably responsible for initiation and promotion of amyloid plaque formation. Senile plaques are particularly abundant in the neocortex, hippocampus, and amygdala;

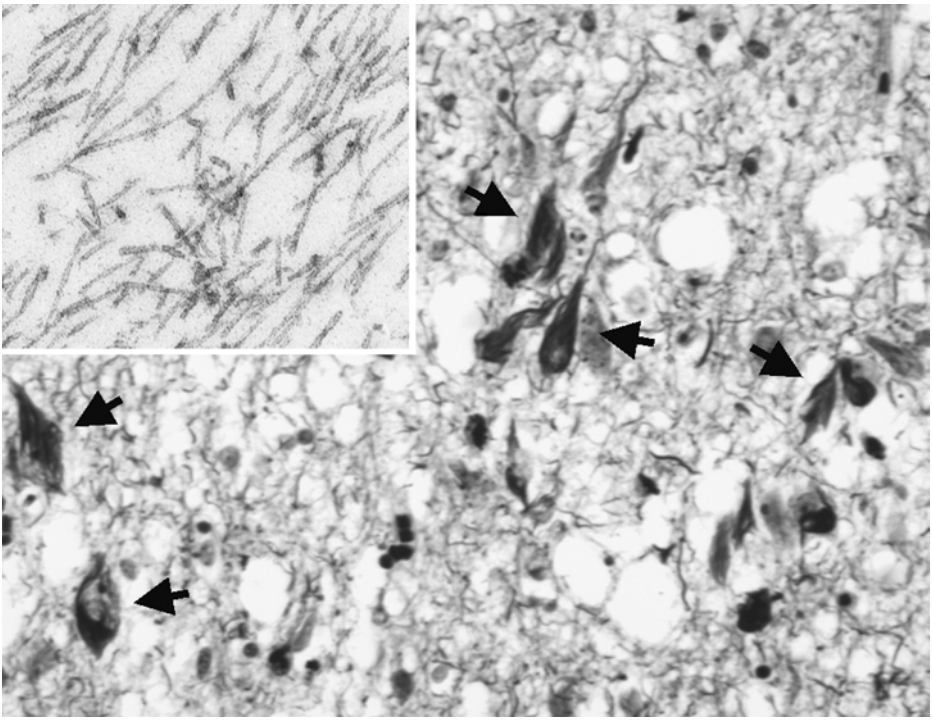

Fig. 3. Numerous neurofibrillary tangles (arrows) in the hippocampus of an Alzheimer's disease patient. (Bielschowsky's silver method; ×200.) Inset: electron microscopy reveals that the tangles are composed of 20-nm-thick filaments. (×25,000.)

these are absent or rare in brains of nondemented old people and are pathologically diagnostic for AD.

Diffuse plaques contain neither an amyloid core nor abnormal neuritic processes. They are demonstrated preferably by immunohistochemical staining for Aβ. The widely accepted hypothesis is that diffuse plaques, which are also present in the brain of many nondemented elderly, eventually and for unknown reasons evolve into neuritic plaques in the brain of AD patients. The original and fiercely debated question of whether the neuritic plaques are "the cause or the effect" of AD can finally be settled in the transgenic mouse models, but it remains a major challenge.

2.2. Neurofibrillary Tangles

NFTs are abnormal fibrillary deposits that form as intraneuronal inclusions (**Fig. 3**; *see* Color Plate 4 following p. 144), but they can become externalized as the neuron degenerates to yield "ghost tangles." Tangles are located mainly

in the neuronal cell bodies and in the apical dendrites, but they are also present in the dystrophic neurites that surround neuritic plaques. Like senile plaques, they become clearly visible by Bielschowsky's silver impregnation and Congo Red and ThioflavinS staining, which make them appear as thickened or tortuous fibrils within neurons. By electron microscopy, they appear as dense bundles of unbranched filaments with a diameter of about 20 nm. In the AD brain, most are in the form of paired helical filaments, although occasionally straight filaments have been observed. Biochemically, the filaments consist mainly, if not exclusively, of highly phosphorylated forms of the microtubule-associated protein tau. Neurofibrillary tangles are neither exclusive nor specific for AD but occur also in many other neurodegenerative diseases. They are rare and restricted in distribution in the brain of nondemented elderly subjects. In the AD brain, NFTs are most numerous in the transentorhinal and entorhinal cortex, the hippocampus, and the associative cortex. The progression of the severity of the dementia appears to correlate more closely with NFTs, both in numbers and in the cortical areas involved, than with senile plaques. Nevertheless, the same question of "cause and effect" applies here, or even more, since mutations in the tau gene do not cause AD but another type of dementia (see further).

2.3. Cerebral Amyloid Angiopathy

In practically all AD patients, amyloid deposition in the wall of blood vessels of the brain is an inherent diagnostic element of the pathology; it also occurs sporadically in about a third of elderly people over 60 years old ***(5)***. Cerebrovascular amyloid is most commonly deposited in meningeal and cortical arteries and arterioles and less frequently in veins and capillaries. Vascular amyloid causes degeneration of the vessel wall, leading to aneurysms, and is thereby thought to be responsible for up to 15% of all hemorrhagic strokes in the elderly. Cerebral amyloid angiopathy in AD constitutes a direct or indirect link to the related clinical entity known as vascular dementia, the etiology of which is unknown; it does involve defects in the vascular wall. In daily clinical practice, the differential diagnosis of AD and vascular dementia is a difficult problem, leading some to suggest that they are two extremes of the same pathology.

3. Major Questions Regarding AD Pathology: *Models Badly Needed*

We will not touch further upon the clinical features or symptoms that are less typical or not common for AD patients and that have led to the suggestion to use the terminology of syndrome, rather than disease, when referring to AD. In the absence of objective biochemical, molecular, or clinical methods to diagnose AD early and reliably, the clinical diagnosis is only 60–80% certain. It is evident that early diagnosis is absolutely necessary, to allow development

of effective therapy. The many problems encountered in this respect are beyond the scope of this chapter, but readers should be aware of their utmost importance.

We concentrate here on the transgenic mice that recapitulate the human neuropathology as seen in AD. This is in itself a major contribution toward understanding the biochemistry of APP and presenilin. It is generally believed that mishaps in the processing of APP, in which presenilins are intimately involved ***(6)***, eventually lead to amyloid peptide production as the central problem in AD ***(7,8)***.

Many aspects of the pathogenesis and the role of neuritic plaques and of cerebral amyloid angiopathy in AD remain in large part unclear. The fact that senile plaques and vascular amyloid are present in nearly all AD patients, and that they consist mainly of amyloid peptides, is more than suggestive of the essential and central role of the process of "amyloidogenesis" in AD. All other nonspecific clinical and pathologic features of AD are thought to be secondary to or caused by amyloid peptides, i.e., synaptic loss, gliosis, neuronal loss, brain shrinkage, and even NFT formation.

This latter point is fiercely debated in particular, and a primary role for protein tau, because of its evident "hyper"-phosphorylation and tangle formation and the ensuing tauopathy, is claimed by many researchers (known as "tau-ists," as opposed to "β-aptists." Nevertheless, an objective observer has to take into account the duality of the pathology, i.e., plaques and tangles, and try to understand and to model in transgenic mice not only amyloid pathology but also hyperphosphorylation of protein tau and NFT formation. It is to be expected that the actual mechanism will be somewhere at the intersection of these two extreme points; the mechanism need not even be "unified" but could be different in individuals, in unrelated families, or in ethnic groups. In this respect, geneticists have identified or proposed many different and diverse genes as potentially linked or associated with AD. Let it suffice to state here that only the ApoE4 allele is accepted as a risk gene or allele for sporadic, late-onset AD. The ApoE4 connection is therefore a major target for fundamental studies that need to model a brain-specific ApoE4 effect in transgenic mice.

4. APP/London Transgenic Mice: Robust Models for Amyloid Pathology

We have generated and characterized a transgenic mouse strain that shows all aspects of the amyloid pathology of AD patients robustly and reproducibly: diffuse and senile amyloid plaques as well as cerebral amyloid angiopathy. This strain was obtained by overexpression of the London mutant of human APP (APP/Ld), or APP/V717I, using a construct based on the neuron-specific elements of the murine thy1 gene (**Fig. 4**; *see* Color Plate 5 following p. 303)

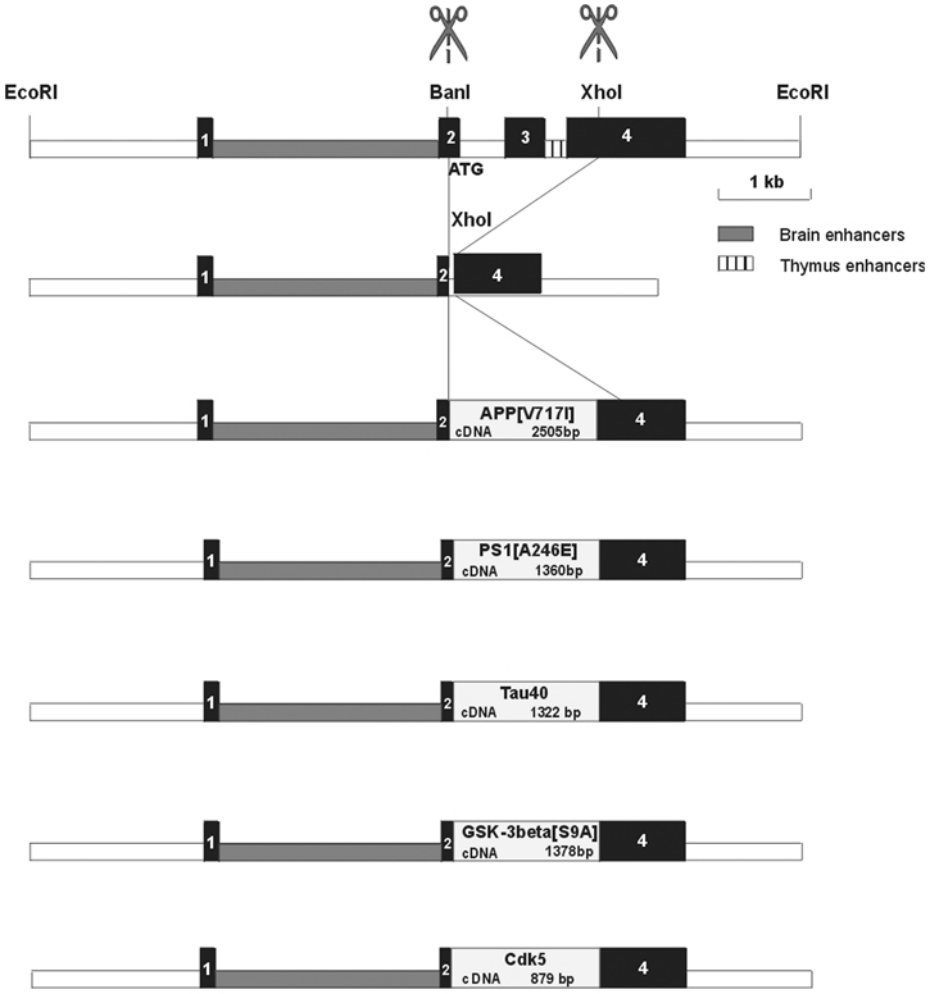

Fig. 4. Structure of the mouse thy1-gene and of the modified minigene constructs yielding neuron-specific expression of the embedded cDNA.

(9,10). In the brain of heterozygous APP/Ld transgenic mice, the level of overexpression of human APP is about 3–5 times higher than endogenous murine APP. Expression of the human trans-gene is exclusive in and restricted to neurons in the brain and spinal cord, as demonstrated by *in situ* hybridization and confirmed by immunohistochemistry *(9–11)*.

4.1. Senile Plaques

With aging, the APP/Ld mice develop pathologic features that exhibit striking similarity to those observed in AD patients. From the age of 1 year, the

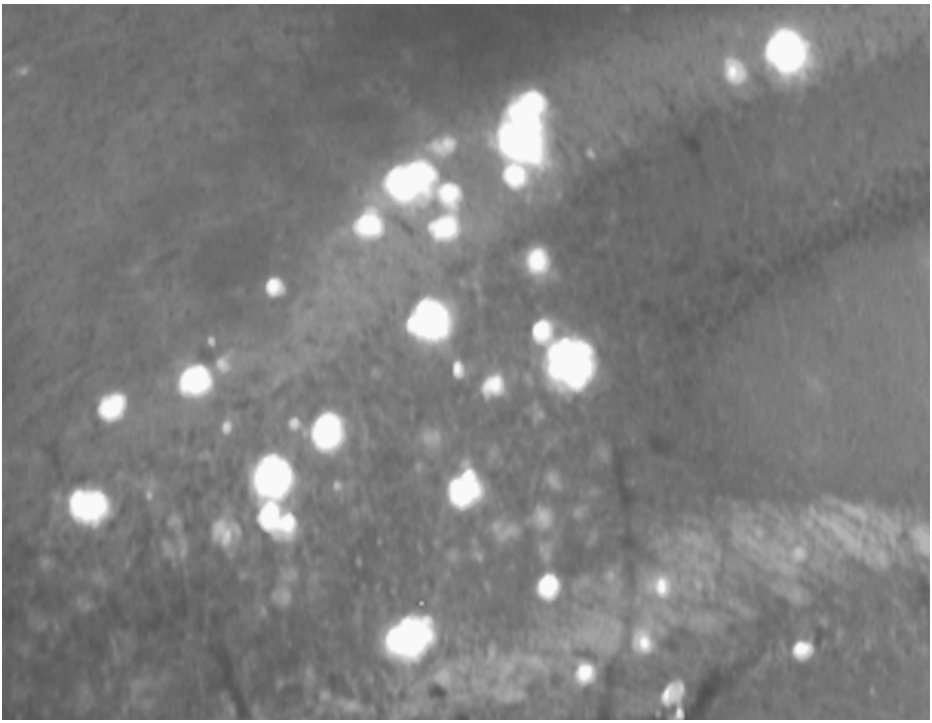

Fig. 5. Senile plaques in the subiculum of a 20-month-old APP/Ld transgenic mouse. (Thioflavine-S staining; ×85.) *See* Color Plate 5 following p. 144.

APP/Ld mice develop neuritic plaques with a morphology and characteristics practically identical to those observed at autopsy in AD brain. The neuritic plaques contain a core of amyloid that stains intensely with ThioflavinS (**Fig. 5**) and Congo Red, and they are surrounded by numerous dystrophic neurites. Ultrastructurally, the core consists of bundles of amyloid fibers, 8–11 nm in diameter (**Fig. 6**); immunogold staining shows that the amyloid fibers contain both Aβ40 and Aβ42. Diffuse plaques in APP/Ld mice are detected immunohistochemically with specific antibodies against Aβ40/42. As in AD patients, they are quantitatively the more important component of the total amyloid load in old APP/Ld transgenic mice. The senile plaques develop preponderantly in the hippocampus and neocortex but are also evident in thalamus and other mouse brain regions, except the cerebellum ***(11)***.

4.2. Cerebral Amyloid Angiopathy

In addition to senile plaques, aging APP/Ld mice progressively develop significant amyloid deposits in cerebral blood vessels. This phenomenon of

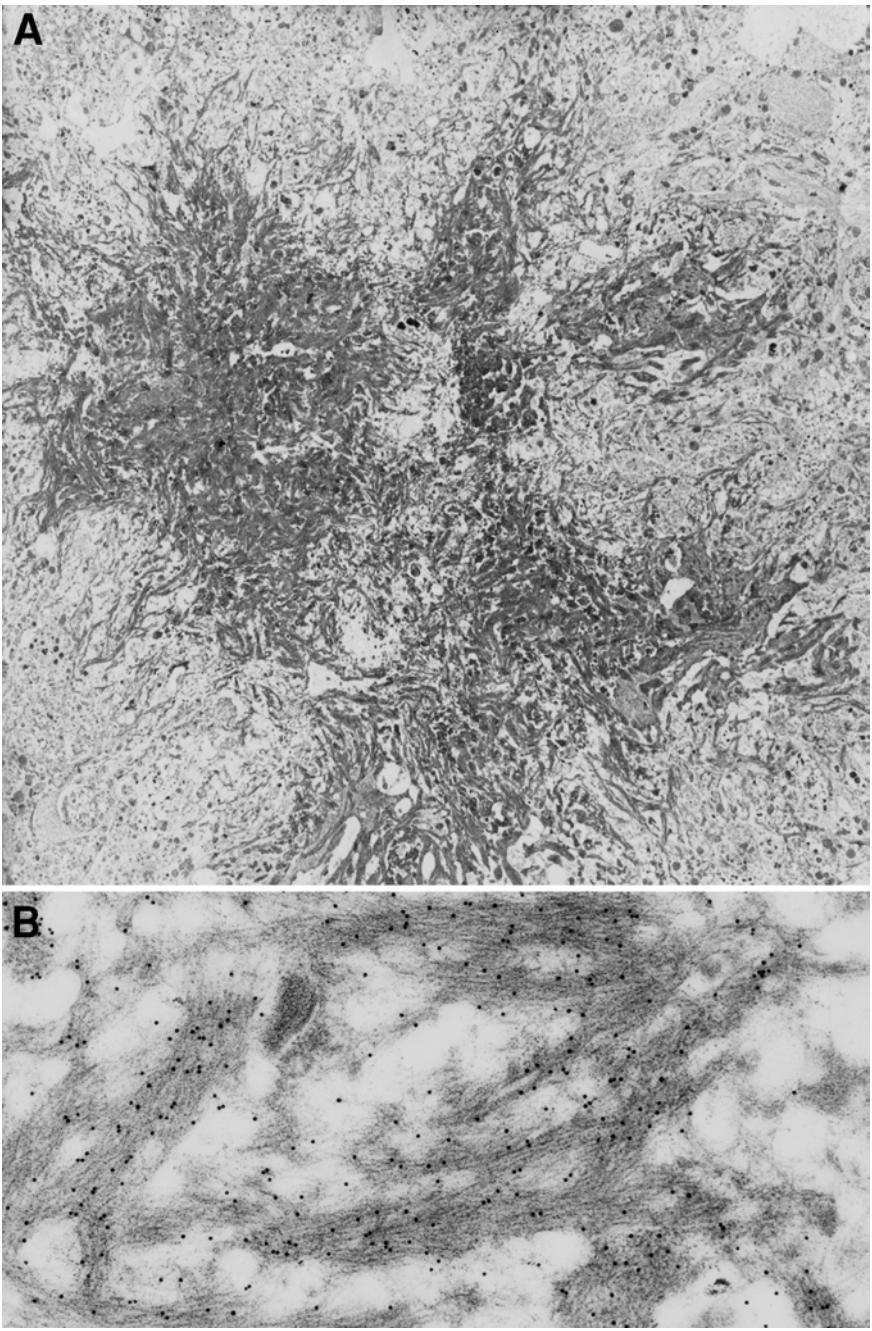

Fig. 6. **(A)** Electron micrograph showing electron-dense amyloid surrounded by altered neuropil (neuritic plaque; ×2650.) **(B)** High magnification of plaque amyloid showing 8–10-nm-thick amyloid fibers. (Immunogold staining for Aβ42; ×50,000.)

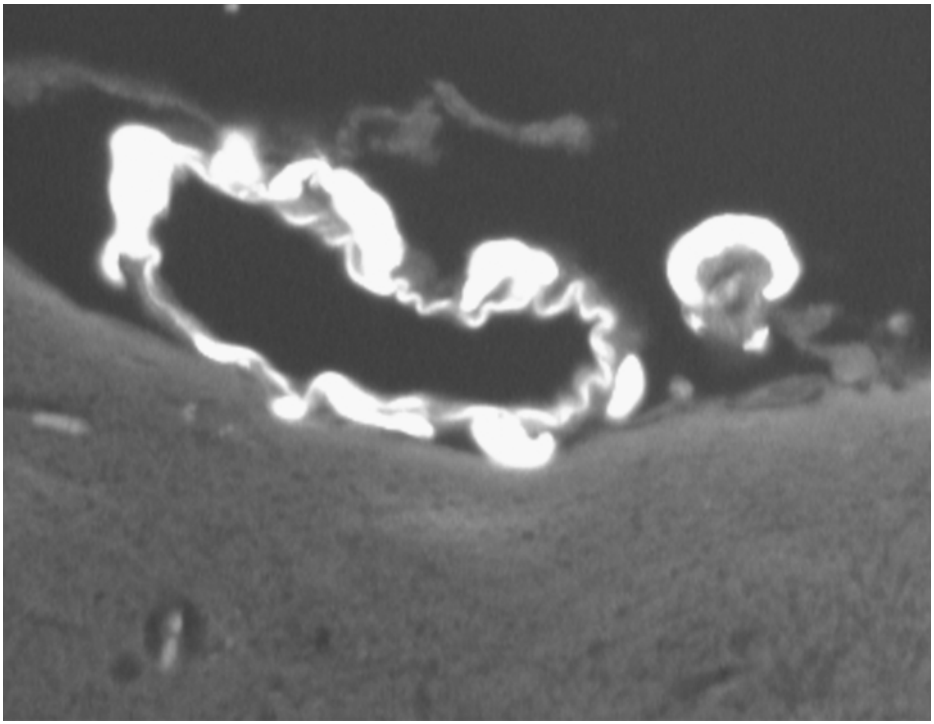

Fig. 7. Leptomeningeal vessels of 20-month-old APP/Ld transgenic mouse with cerebral amyloid angiopathy. (Thioflavine-S staining; ×600.)

cerebral amyloid angiopathy becomes primarily evident in the leptomeningeal blood vessels (**Fig. 7**; *see* Color Plate 6 following p. 144) but also in cortical, thalamic, and hippocampal vessels. Amyloid accumulation in blood vessels becomes apparent slightly later than senile plaques. As in AD patients, individual variation is evident in the distribution of senile plaques and cerebral amyloid angiopathy in old APP/Ld mice. A minor difference is that plaques and vascular amyloid deposits are never observed in the cerebellum or the leptomeninges surrounding the cerebellum of APP/Ld transgenic mice. This is caused by the much lower expression of the human transgene in the cerebellum relative to the cerebrum, a difference of magnitude that is also evident in our other transgenic mouse strains using the mouse thy1 gene promoter. Therefore this appears to be an intrinsic characteristic of this gene promoter construct and not typical of the APP/Ld transgenic mice. Since in AD patients the cerebellar pathology is not well developed and/or not well studied, this minor aspect is not a problem for the current APP/Ld transgenic model.

By ThioflavinS staining, the most severely affected arteries exhibit fluorescence in a pattern of concentric rings, whereas less affected vessels show more focal accumulations, very similar to human cerebral amyloid angiopathy. Arteries are more affected than veins, and these show only small focal accumulations. Capillaries are rarely affected, but some are observed to have amyloid penetrating into the neuropil, resembling the dyshoric amyloid in AD patients. As in human cerebral amyloid angiopathy, neither the large arteries at the base of the transgenic mouse brain, i.e., the arterial circle of Willis, nor the extracranial blood vessels are affected ***(11)***. Vessels with important amyloid deposition show loss of smooth muscle cells; only rarely is the internal elastic lamina damaged. The vessel wall damage leads to dilation and aneurysm formation, again very similar to human patients. Hemorrhages have never been noted in the brain of APP/Ld mice, and the cerebral blood flow, measured by laser-Doppler flowmetry, appears to be unaffected even in APP/Ld mice as old as 2 years.

4.3. Amyloid Structure and Composition

Ultrastructurally, focal amyloid depositions in the blood vessels are situated in the outermost (abluminal) part of the media around smooth muscle cells, often interrupting the external elastic lamina (**Fig. 8**). In larger amyloid depositions, amyloid fibers spread toward the internal elastic lamina and interrupt the smooth muscle layer. Vessels with important amyloid deposition show loss of smooth muscle cells, and rare vessels have a damaged internal elastic lamina. The vascular deposits consist of randomly oriented, slightly curved proteinaceous fibers with a diameter comparable to that of amyloid fibers in plaques.

Immunochemically, both Aβ40 and Aβ42 are present in vascular amyloid depositions, and some rare depositions react positively only for Aβ42. In common with AD patients, APP/Ld mice also have the typical calculated ratio of insoluble Aβ42/Aβ40, based on specific enzyme-linked immunosorbent assay (ELISA) for each isoform. The Aβ42/40 ratio is much higher in the neocortical plaques than in the leptomeningeal blood vessels, i.e., relative to Aβ42 there is about 8 times more Aβ40 in the amyloid deposits of leptomeningeal blood vessels than in the neocortical plaques.

4.4. Aging and Amyloid Pathology: A Paradox of Pathology versus Biochemistry

In APP/Ld transgenic mice, both vascular and plaque amyloid load progressively increase with age, and the plaque core load correlates well with the number of vessels containing vascular amyloid ***(11)***. At the age of 24 months, APP/Ld transgenic mice have about 90% of the leptomeningeal arterioles over the cerebrum loaded with amyloid.

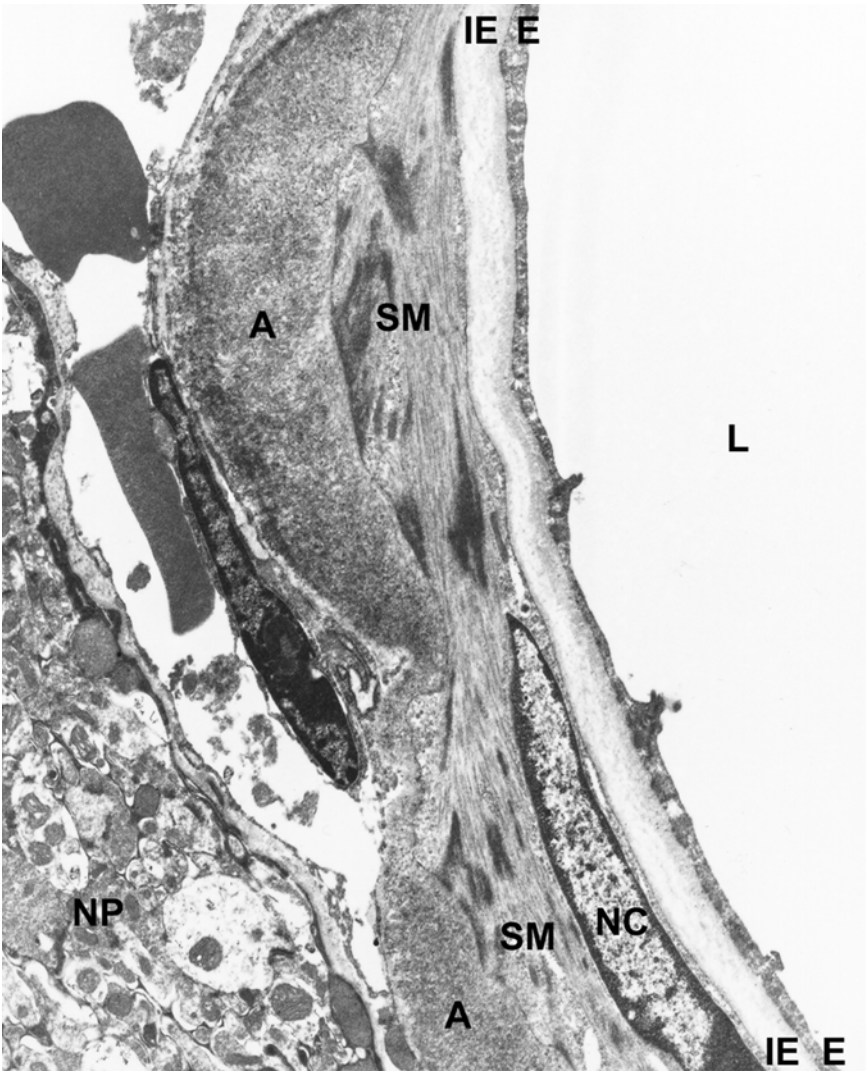

Fig. 8. Electron micrograph of vessel with amyloid deposition in the outer part of the smooth muscle layer. A, amyloid deposition; E, endothelium; IE, internal elastic lamina; L, lumen; NC, nucleus of smooth muscle cell; NP, neuropil; SM, smooth muscle cell. (×7000.)

Aging remains the most important and effective, but least understood, parameter or risk factor for dementia and AD. Clinical mutations of the APP and PS genes that cause early-onset familial AD (EOFAD), only account for less than 5% of all AD cases, but they have been very informative for the study

of AD. The clinical EOFAD mutations were shown to affect the processing of APP, causing a shift to the amyloidogenic processing pathway, i.e., production of more and/or longer amyloid peptides. To study the effect of aging on APP processing, we analyzed APP/Ld transgenic mice at the age of 3 months, at 6–9 months, and at 15 months and measured the intermediate and end products of APP processing, i.e., the membrane-bound precursor (APPm), the soluble and plaque-associated amyloid peptides Aβ40 and Aβ42, the secreted ectodomain of APP processed by α- or β-secretase (APPsα and APPsβ), and the C-terminal transmembrane and cytoplasmatic domain resulting from β-secretase cleavage, referred to as β-*C-stubs* ***(4,12)***. Comparative analysis revealed that aging does not appreciably affect the normalized levels of either the α-secretase-cleaved ectodomain (APPsα) or the residual β-secretase-cleaved C-terminal stubs of APP (**Fig. 2**). Between the age of 3 and 15 months, the most obvious effect was a dramatic increase in the soluble and plaque-associated amyloid peptides Aβ40 and Aβ42, as well as an increased Aβ42/40 ratio.

Aging of APP/Ld transgenic mice causes precipitation of the amyloid peptides in the physical form of amyloid plaques, concomitant with or (more likely) following increased levels of amyloid peptides, especially Aβ42. Both phenomena do not occur before the age of 12 months in APP/Ld transgenic mice. Our data confirm and extend findings in two unrelated APP transgenic mouse models in which Aβ increased with age ***(13,14)***.

The combined data demonstrate that in the brain of the APP/Ld transgenic mouse, aging *per se* does not markedly affect normal processing of APP as mediated by α- and β-secretase. Therefore, the marked increase in both soluble and plaque-associated amyloid peptides, essentially occurring at age 12–15 months, is not a direct consequence of a disturbed or tilted balance in the two competing proteolytic events that govern APP processing. Amyloid deposition is unequivocal and increases with age, but this is not caused by increased production of amyloid peptides. The biochemical analysis of aging thus reveals a paradox between pathology and biochemistry in the brain of the APP/Ld transgenic mouse. This paradox can be resolved by the hypothesis that accumulation of amyloid peptides with aging is caused by decreased clearance of amyloid peptides rather than increased production, which will be a major point for further study in APP/Ld transgenic mice.

4.5. Pathogenesis of Cerebral Amyloid Angiopathy: QED?

Another long-standing debate is on the origin of vascular amyloid. In human and mouse brain, as well as in other tissues, endogenous APP is ubiquitously expressed by many cell types, including those of the vasculature. It has been hypothesized that Aβ in the vessel walls is derived from vascular smooth muscle cells and/or pericytes ***(15)***. The observation that pial vessels (which are

apart from the neuropil) are more often affected was interpreted by some to suggest that local production could be an important source. On the other hand, these hypotheses fail to explain the specific neuroanatomic pattern of cerebral amyloid angiopathy and its exclusive localization in intracranial vessels.

As demonstrated by Western blotting, blood plasma of aged APP/Ld mice does not contain amyloid peptides, as opposed to cerebrospinal fluid (CSF), which contains Aβ in the range of 10–20 ng/mL. This further confirms the already strong indications (taken from the exclusive neuronal expression of the transgene and from the abluminal site of deposition of vascular amyloid) ***(11)*** that the amyloid peptides originate from the neurons within the brain.

The APP/Ld transgenic mice show that a neuronal source of amyloid peptides is sufficient to produce amyloid deposits as a perfect recapitulation of human cerebral amyloid angiopathy. Evidently, cerebral amyloid angiopathy in vessels that do not express the transgene requires and implies a means of transport of amyloid peptides to this deposition site. Amyloid peptides are present in the CSF of normal and AD individuals, but this cannot explain the presence of CAA in pial vessels of the cerebrum and its absence in those of the cerebellum. In addition, in the APP/Ld mice, the small leptomeningeal branches of the cerebral arteries are affected considerably more than the large main branches, while all are bathed in the same CSF. Small intracortical arterioles are also affected, which are at greater distance from the CSF and separated from it by the pia mater. Clearly, specific drainage pathways must be involved, situated in or along the perivascular space around intracortical and leptomeningeal arteries. These channels eventually connect with nasal lymphatics draining to the cervical lymph nodes. The suggestion that significant amounts of Aβ drain along this pathway in humans ***(16)*** agrees with the analysis of the data in APP/Ld transgenic mice ***(11)***.

4.6. Aβ42 Causes Amyloid Pathology

In the brain of AD patients, the ratio of Aβ42 to Aβ40 is higher in plaques than in the vascular amyloid deposits. This formed the basis for the hypothesis that neurons produce mainly Aβ42, whereas cells in the vasculature produce mainly Aβ40. This hypothesis is undermined, however, by the observation of a much higher Aβ42/Aβ40 ratio in plaque than in vascular amyloid in APP/Ld mice. In addition, cells in or around blood vessels, which do not express the transgene, cannot be the source of amyloid peptides of any length.

Patients with cerebral amyloid angiopathy have vascular amyloid deposits that immunohistochemically do not contain Aβ40 and therefore appear to consist solely of Aβ42, whereas the opposite has never been observed. Despite much higher levels of Aβ40 in vascular amyloid, we observed some vascular deposits that consisted only of Aβ42 in APP/Ld mice. Similar to senile plaque

formation, Aβ42 appears to be the first amyloid peptide to become deposited in the vessel walls (nucleation), whereas the more soluble Aβ40 is subsequently entrapped (growth). This is supported by the earlier and increased formation of senile plaques and cerebral amyloid angiopathy in double transgenic APP/Ld × PS1[A246E] mice, obtained by crossing the respective single transgenic mice. In these double transgenic mice, the extra incorporation of the human mutant PS1 transgene causes a selective increase in production of Aβ42 over Aβ40 ***(12)***. Although APP/Ld mice express APP at higher levels than humans, the striking similarity to human cerebral amyloid angiopathy is a strong suggestion that similar mechanisms are causing the vascular amyloid deposition in both patients and mice.

4.7. Conclusion: Amyloid Pathology Modeled Well?

The senile plaques and cerebral amyloid angiopathy in the APP/Ld mouse exhibit a striking similarity to those observed in AD patients and aged individuals. The morphologic pattern, the ultrastructural aspects, and the biochemical composition of the senile plaques and vascular amyloid deposits in humans are recapitulated very closely in the APP/Ld transgenic mice (**Table 1**).

In humans, AD displays a protracted clinical course covering 10–20 years. The life span of laboratory mice does not exceed much more than approx. 2 years, and it is surprising that the entire pathologic history of the disease can be compressed into 6–12 months. It is evident that this shorter time span presents a great opportunity and considerable advantages for investigations into the pathogenesis of the devastating neurodegeneration of AD, particularly to identify new methods for early and objective diagnosis and to test novel therapeutic drugs or strategies. Experiments and trials could be measured in months in transgenic mice, as opposed to years in humans. As these and other transgenic models are available, there is every reason to believe that they will help to accelerate the pace of drug discovery, leading to the recognition of therapeutic agents that are effective in postponing the onset or slowing the progression of neurodegenerative diseases, not only Alzheimer's disease, but also amyotrophic lateral sclerosis and Parkinson's disease, as well as other neurobiologic conditions.

5. Protein Tau in Neurodegeneration and Alzheimer's Disease

5.1. Introduction: Tauopathy

The other major pathologic hallmark in the postmortem AD brain, i.e., NFTs, are made of highly phosphorylated protein tau. Tau assembles first in paired helical filaments (PHFs) that, with other proteins, form tangles inside neurons in the brain of patients suffering from AD or other tauopathies. Protein

Table 1
Comparison of Pathology in AD Patients and APP/Ld Transgenic Mice

	AD patients	APP/Ld mice
Senile plaques	+++	++
Amyloid fibers (diameter)	8–11 nm	8–11 nm
Dystrophic neurites	++	++
Neuron loss	++	Around plaques
Synaptic loss	++	Around plaques
Gliosis	++	+
Hyperphosphorylation of tau	Somatodendritic Dystrophic neurites	Dystrophic neurites
Cerebral amyloid angiopathy	+++	++
Neurofibrillary tangles	+++	Absent

+, Common characteristics in mice and AD patients.

tau represents a family of isoforms of phosphoproteins that copurify with microtubuli and can assemble tubulin dimers into microtubules ***(17)***. Six isoforms of the low molecular weight protein tau are derived by alternative RNA splicing, containing either none, or one, or two N-terminal inserts of unknown function; in the C-terminal domain, three or four microtubule binding repeats are essential for binding to microtubuli (**Fig. 9**). Phosphorylation of serine and threonine residues within these repeat regions, but also in the flanking regions, alter the interaction with microtubules considerably ***(18–20)***. Many kinases and phosphatases have been implicated in the control of phosphorylation of protein tau, but in PHF tau, many phosphorylated amino acids are immediately followed by a proline. Proline-directed kinases, e.g., microtubule-associated protein (MAP)-kinase, cyclin-dependent kinase 5 (cdk5), and glycogen synthase kinase 3β (GSK-3β), have therefore received the most attention (and also because they are associated with microtubules and can phosphorylate protein tau to generate PHF-specific epitopes, at least in vitro).

It is not known which kinases normally phosphorylate protein tau in neurons in vivo, nor is it known whether hyperphosphorylation is a prerequisite or a consequence for PHF formation. Last, but by no means least, it is not known whether the formation of NFTs *per se* causes (part of) the neuropathology or is rather a phenomenon that is correlated with or even a consequence of the amyloid pathology. This problem is very similar to the formation of amyloid plaques and amyloid angiopathy, as outlined above.

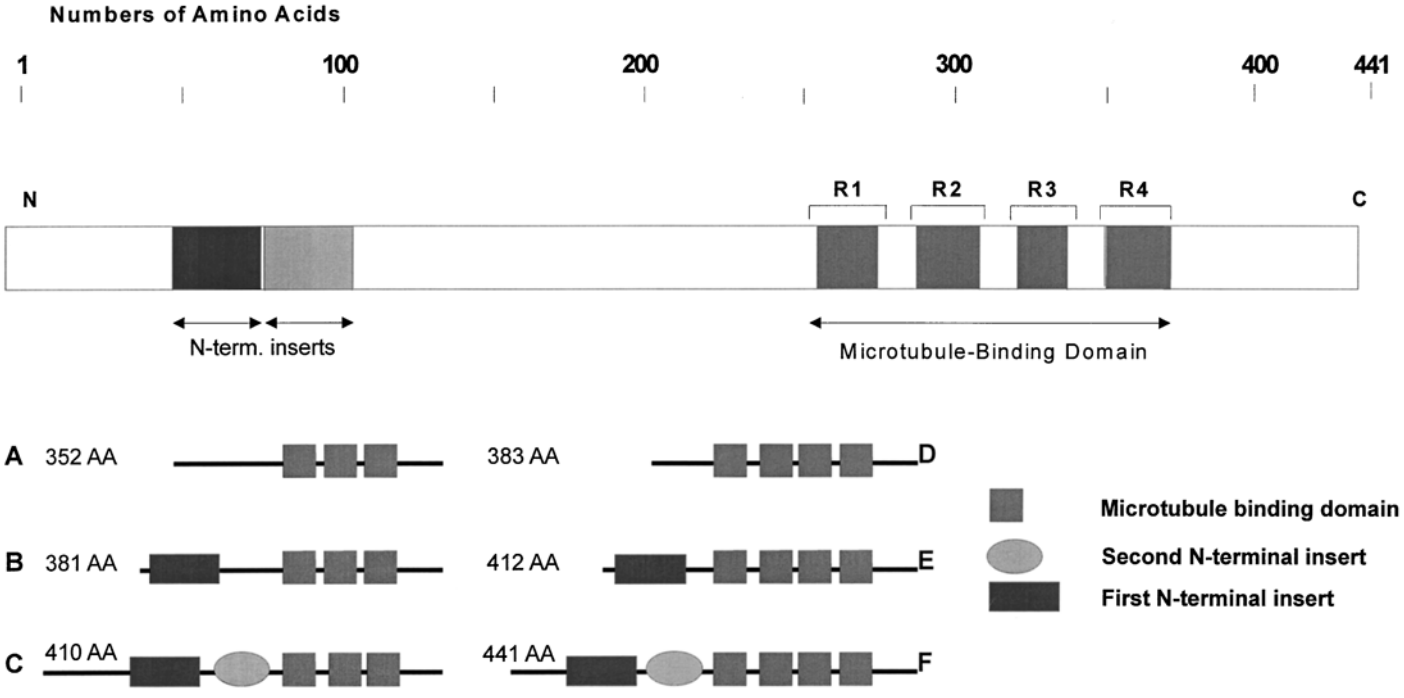

Fig. 9. The protein tau family. In adult human neurons, protein tau is present as six isoforms, with the largest isoform containing four microtubule binding repeats and two N-terminal inserts.

The hypothesis prevails that tau pathology develops because "improper" intracellular signaling causes hyperphosphorylation of protein tau, thus preventing it from binding to microtubules and forming aggregates or polymers known as PHFs. The resulting defective microtubular transport and/or the accumulating aggregates would then negatively affect the synaptic and neuronal functions, and ultimately the neurons degenerate.

Currently, major attention in the field is devoted to the nature and enzymology of the posttranslational modifications of protein tau, relating to functional repercussions as well as to the mechanism of PHF formation and neurodegeneration. Needless to say, modeling in transgenic mice is therefore almost a must, particularly in light of the success of the amyloid transgenic models.

5.2. Protein Tau and FTDP Neuropathology

The most direct indication of the pathophysiology caused by protein tau is the mutations in the tau gene that are linked to neurodegeneration and dementia, known as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) ***(2)***. In vitro, most clinical missense mutations located in the microtubule binding domains reduce the association of protein tau with microtubuli ***(21)***. In addition, several intronic mutations as well as two missense mutations induce a preponderance of four-repeat over three-repeat protein tau isoforms ***(2)***. All patients carrying these mutations develop protein tau filaments in their brain consisting of hyperphosphorylated protein tau. Although mutations in tau do not segregate with AD, the resemblance of clinical symptoms (i.e., dementia) and pathology (i.e., NFTs) in AD and FTDP-17 patients will help to elucidate the etiology of the tauopathy in AD.

5.3. A Proven Strategy: Neuronal Overexpression in Transgenic Mice

To address the many questions, transgenic mice were generated that overexpress human protein tau. In addition, we generated mice transgenic for the putative human tau kinases GSK-3β and cdk5 (and its neuron-specific regulatory subunit p35). All the constructs were based on the mouse thy1 gene promoter, with its proven high and exclusive expression in neurons of the CNS (**Fig. 4**). This strategy further allowed us to derive double and triple transgenic mice, in which the use of the same promoter construct warrants coexpression of the different transgenes in the same neurons. This is evidently a prerequisite for enzyme-substrate interaction.

Transgenic mice were generated by microinjection of linearized constructs into 0.5-day-old mouse prenuclear embryos that were reimplanted into pseudopregnant females ***(9,10)***. The different mouse thy-1 gene constructs were shown to drive expression effectively and exclusively to neurons, with high

expression levels especially in the hippocampus and cortex ***(9–11,22,23)***. The expression cassette is randomly integrated in the mouse genome in a variable number of copies, which required that different founders for each transgene be generated and characterized. On the other hand, this allowed observation of important transgene-dosage effects, which is essential for interpretation of the phenotypic outcome of the transgenic strains, as discussed.

Analysis of the brain of the transgenic mice made it possible to define in vivo phosphorylation as well as alterations in structure or cellular location of protein tau, by standard immunohistochemical and biochemical methods. We therefore made use of the many antibodies available that are directed to phosphorylated or conformation-dependent epitopes of human protein tau.

5.4. Prominent Axonopathy in Tau Transgenic Mice

Three founder transgenic strains, i.e., htau40-1, htau40-2, and htau40-5, with different expression levels of four-repeat human tau were selected, transmitting the transgene stably and in a mendelian fashion. All strains expressed human protein tau exclusively in neurons, with the highest expression in strain htau40-1.

Rather unexpectedly, the neuronal excess of human protein tau caused an axonopathy evidenced by proximal axonal dilations with accumulation of neurofilaments, microtubuli, mitochondria, and vesicles and by a wallerian type of degeneration of the distal parts of the axons. The grossly dilated axons (axonal spheroids) in the brain and spinal cord could be demonstrated by Bielschowsky's silver impregnation (**Fig. 10**) and immunohistochemistry with anti-tau and anti-neurofilament antibodies. The dilations had mostly a rounded contour and were often as large as neighboring neuronal cell bodies. Although no neuron loss was established, accumulation of ubiquitinated protein conjugates in some of the dilated axons and astrogliosis in both the brain and spinal cord of homozygous htau40-1 transgenic mice reflected the onset of neurodegeneration. This model proves that even an excess of normal protein tau is sufficient to cause neuronal injury in the absence of intraneuronal NFTs. This is important since many of the mutations in the tau gene that cause FTDP-17 produce normal protein tau but have a disturbed balance of three- to four-repeat protein tau. The degree of axonopathy, of motoric impairment, and of muscle atrophy are all directly related to the level of overexpression of the transgene (**Fig. 11**) ***(11)***.

Fig. 10. *(See facing page)* Bielschowsky's silver staining of a brain section of an htau40 transgenic mouse. The axon of a pyramidal neuron with the cell body (arrowhead) in cortical layer III is dilated over the entire proximal part and shows an axonal spheroid (arrow). III, cortical layer III; CC, corpus callosum.

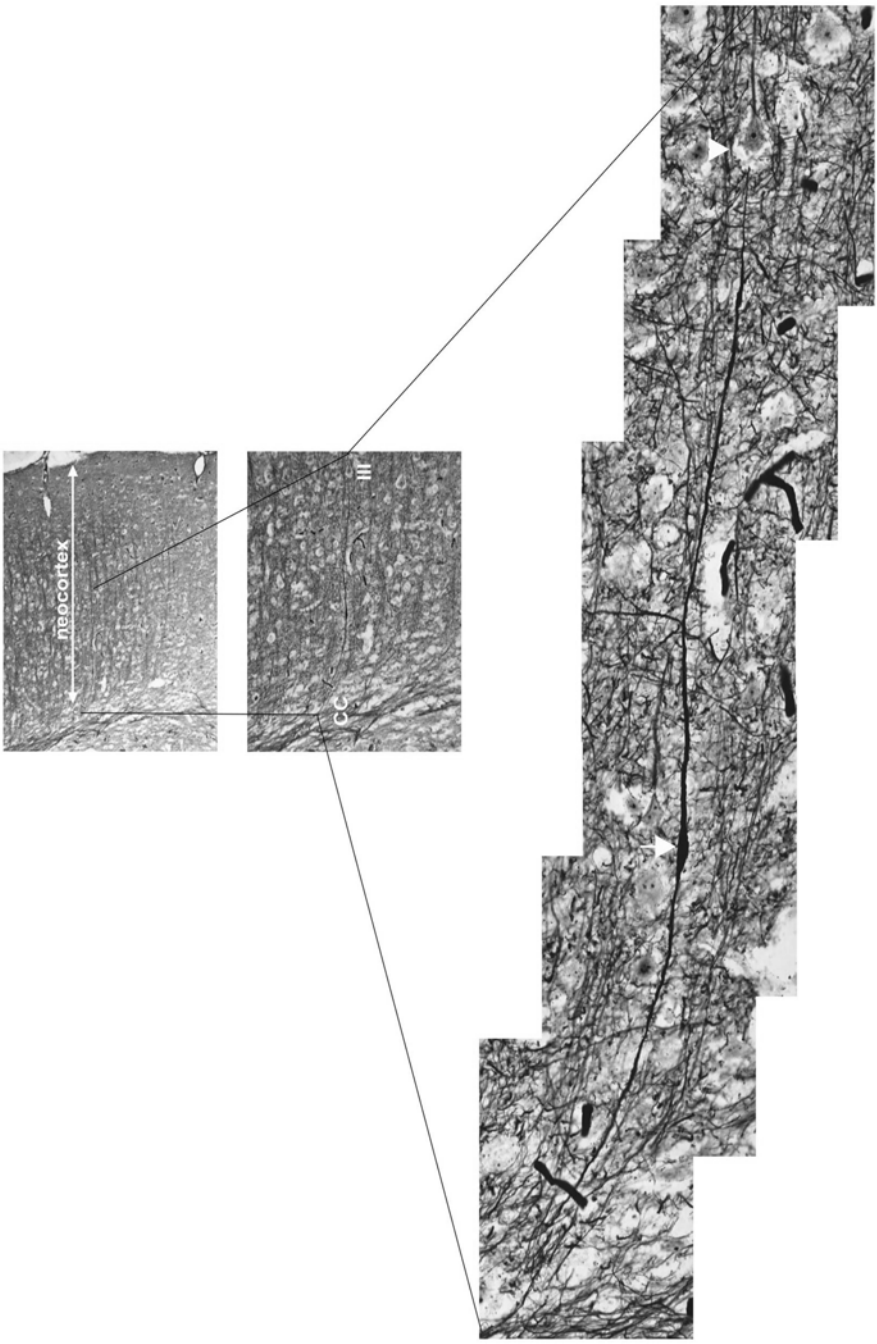

Fig. 10.

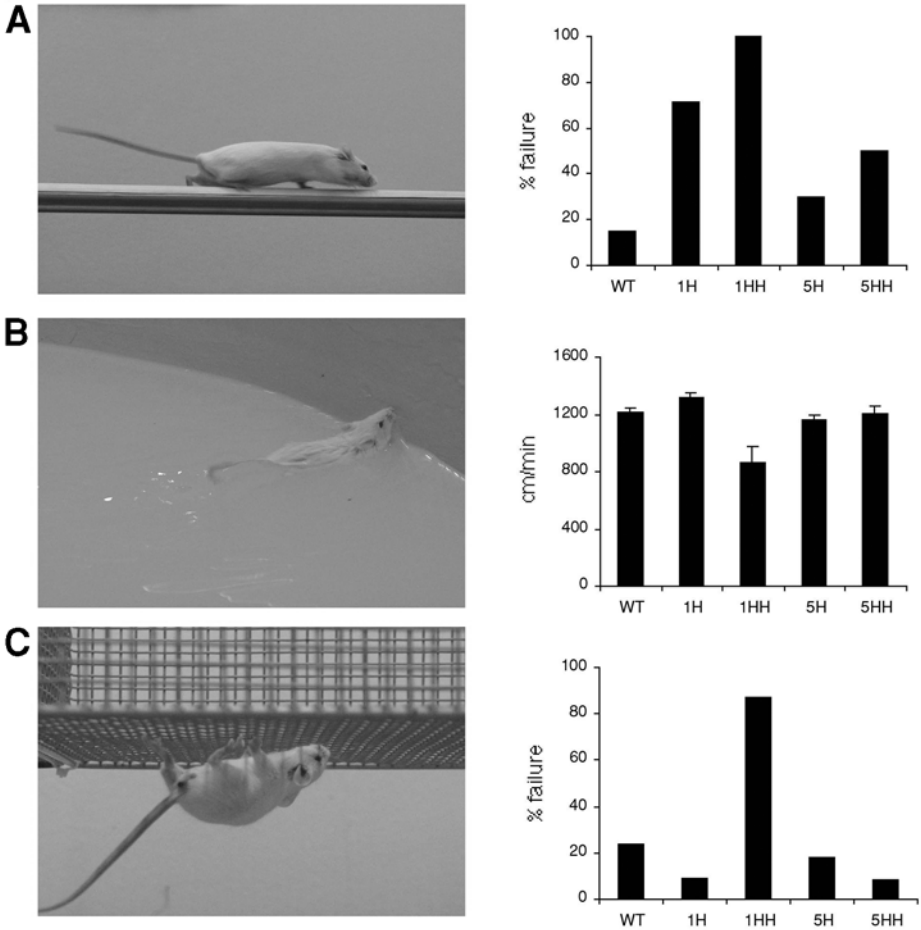

Fig. 11. Analysis of motor problems of htau40 transgenic mice. Rod walking **(A)**, forced swimming **(B)**, and inversed grid-hanging **(C)** are significantly compromised in homozygous htau40-1 transgenic mice relative to wild-type (WT) and htau40-5 mice. The htau40 transgenic mice display reduced endurance, postural instability, loss of motor coordination, loss of equilibrium, and loss of muscular strength. 1, htau40-1; 5, htau40-5; H, heterozygous; HH, homozygous.

Axonal spheroids, reminiscent of the dilated axons in the htau40 transgenic mice, have been documented in neurologic disorders such as amyotrophic lateral sclerosis, in which disturbed axonal transport has been documented. This finding lends support to the hypothesis that excess of four-repeat protein tau would saturate binding sites on the microtubuli, thus interfering with kinesin-dependent transport, as observed in cellular systems ***(24)***.

Dystrophic neurites, defined as thickened or irregular neuronal processes immunoreactive for protein tau, are a well-known feature of neuropathologic disorders and are considered to mark widespread alteration of the neuronal cytoskeleton. In the AD brain, dystrophic axons appear to be prominent and widespread and are particularly abundant in the hippocampal fiber tracts originating from the subiculum, CA1, and entorhinal cortex and may represent one of the main regional pathologic lesions in AD. The morphologic, immunohistochemic, and ultrastructural features of the dystrophic axons seen in the brain of the htau40 transgenic mice resemble those in AD.

Similar tau pathology is evident in conditions known as familial multiple system tauopathy with presenile dementia (MSTD) ***(25)*** and disinhibition-dementia- parkinsonism-amyotrophy complex (DDPAC) ***(21,26)***, two tauopathies with an intronic mutation in the tau gene. In MSTD, the established preponderance of four-repeat protein tau isoforms causes axonal swellings in the spinal cord, as observed in the htau40 mice, as well as NFTs in the brain and spinal cord. In DDPAC, excess of four-repeat protein tau provokes anterior horn pathology with muscle wasting, in the absence of NFTs. Subtle regional differences in expression and sensitivity of neurons to different isoforms of protein tau are thought to be involved in the differential aspects of pathology. This subtlety needs further experimental clarification in vivo, probably by making use of other gene promoters or control elements.

Nevertheless, the prominent axonal swellings in the spinal cord, the neurogenic muscle atrophy, and, in addition, the direct causal relationship between the pathologic characteristics and the genotype, make the htau40 transgenic mice interesting experimental models that recapitulate important pathologic features of certain tauopathies like MSTD and DDPAC, as well as AD.

5.5. Protein Tau Phosphorylation In Vivo: Which Kinases?

The protein tau filaments in the brain of patients suffering from AD (or FTDP-17 and other tauopathies) consist entirely of protein tau that is highly phosphorylated. The neuronal kinases operating in vivo, their triggers, and their control mechanisms in neurons all need to be identified. In a direct approach, we have generated transgenic mice that overexpress either of two putative tau kinases, GSK-3β or cdk5/p35.

The GSK-3β and cdk5/p35 transgenic mice were further crossed with the htau40 transgenic mice described above, and since all recombinant transgenic constructs were based on the adapted mouse thy1 gene promoter, the needed coexpression in neurons of the CNS is warranted. Another reason for this strategy is that human protein tau is molecularly and structurally far better characterized than mouse protein tau, allowing us to use the many monoclonal antibodies directed against specific epitopes of human protein tau.

These aspects of protein tau and the tauopathies are largely work in progress, and the following discussion illustrates the problems and strategies being deployed, rather than offering final answers.

5.5.1. Mice Transgenic for Glycogen Synthase Kinase 3β

Interest in GSK-3β in the AD field was fuelled by the finding that GSK-3β is identical to the elusive "tau protein kinase-I" ***(27)***. This kinase was shown to generate the typical epitopes on protein tau that are detected in PHFs isolated from brain of AD patients; antibodies directed to GSK-3β decorate PHFs, indicating a direct association ***(28)***.

GSK-3β is a potent proline-dependent protein tau kinase in cell-free systems, thereby reducing the ability of protein tau to initiate microtubule nucleation ***(29)***. Cotransfection of GSK-3β with protein tau in CHO cells increases phosphorylation concomitant with a loss of prominent microtubule bundles ***(30)***. Despite a wealth of in vitro data, convincing evidence for any functional repercussion of the phosphorylation of protein tau by GSK-3β in vivo is still lacking. Therefore, transgenic mice were generated overexpressing a constitutively active form of the human kinase, i.e., GSK-3β[S9A], in which serine at position 9 is replaced by alanine to prevent inactivation by phosphorylation. In-depth characterization of the GSK-3β[S9A] transgenic mice is in progress, but some aspects are already evident. An expression level of less than twice the endogenous level is sufficient for both endogenous and human transgenic protein tau to become extra phosphorylated. Interestingly, preliminary characterization of htau40 × GSK-3β double transgenic mice indicates that the tauopathy of single tau transgenic mice is considerably reduced. This "restoration" comprises 1) the reduction by an order of magnitude of the number of axonal dilations in brain and spinal cord; 2) the reduction of axonal and muscular degeneration; and 3) alleviation of practically all the motor problems. Evidence for tangles or PHFs in the brain of GSK-3β transgenic mice is at the moment still lacking. Such evidence is being pursued by "aging" of the single and double transgenic mice to increase stress provoked by age on the CNS.

5.5.2. Mice Double Transgenic for Cyclin-Dependent Kinase 5 and Its Activator p35

In mammalian brain, cdk5 mRNA is highly transcribed in differentiated postmitotic neurons of the brain, spinal cord, and peripheral ganglia. Different activators, e.g., p39ncdk5ai, p67, p35, and its proteolytic derivative p25, regulate the enzymatic activity of cdk5. The activator p35 is exclusively expressed in neurons, appears first in young migrating postmitotic neurons, and remains abundant in areas of the adult brain characterized by high neuronal plasticity. This combination of characteristics makes cdk5/p35 an attractive

candidate as tau-kinase. In isolated systems, cdk5 phosphorylates protein tau on residues that are also phosphorylated in PHF tau. Interestingly, cdk5 immunoreactivity is more prominent in pretangle neurons or neurons bearing early stages of NFTs in the AD brain, implying a role for cdk5 at a relative early stage ***(31)***. The direct association of cdk5 with NFTs and the increased cdk5 activity in the brain of AD patients ***(32)*** could be ascribed to increased generation of p25, a proteolytic fragment of p35 ***(33)***.

To investigate the effect of increased cdk5/p35 activity on phosphorylation of protein tau in neurons in vivo, we have generated transgenic mice that are homozygous double transgenic for human cdk5 and p35. The recombinant constructs made use of the engineered mouse thy1 gene promoter to specifically direct expression of the transgene to the same neurons, as outlined above. These transgenic mice proved to be normal (even when 2 years old) for all characteristics and aspects of behavior analyzed.

5.5.3. Mice Triple Transgenic for Human Cdk5, Its Activator p35, and Human Protein Tau

Whereas increased cdk5 kinase activity is demonstrated in vitro and in vivo, neither murine nor human protein tau are appreciably phosphorylated in the brain of triple transgenic mice, containing human protein tau and cdk5/p35. These mice behave and reproduce normally. Silver impregnation and immunohistochemistry of brain sections demonstrate that neurofilament proteins are redistributed in apical dendrites of cortical neurons. This finding suggests some cytoskeletal alteration, but no other relevant brain pathology became apparent. These observations therefore do not support the claim that cdk5/p35 would be a major protein tau-kinase in vivo, at least not in transgenic mouse brain. The difference in neurodegeneration in p25 transgenic mice, as recently reported ***(34)***, shifts the focus to the problem of the proteinase that converts p35 into p25 and its control in the CNS.

6. Status and Prospects of AD Models: A Case for Multiple Transgenic Mice?

Whereas amyloid pathology in all its aspects in the brain of AD patients has been copied well by overexpression of mutant forms of APP, a similar strategy failed to yield NFTs in the brain of transgenic mice—thus far. Generating an animal model for AD that contains or develops all the neuropathologic features was and is an enormous challenge (**Table 2**). Following the current success with amyloid, animal models for AD are needed that will include progression with age as in patients, with matching topology and regional distribution of lesions and including quantitative characteristcs that recapitulate the human disease. These aspects were unheard of 5–10 years ago.

Table 2
Comparison of Pathologic Features in htau and/or Kinase Overexpressing Mice and in AD Patients

	Transgenic mice				
	htau	GSK	htauxGSK	cdk5xp35 (xhtau)	AD Patients
Pathologic feature					
Axonal dilations	+[a]	–	±	–	+
Dystrophic axons	+	–	±	–	+
Hyperphosphorylated tau	+	+	++	–	+
Insoluble tau	+[a]				+
Neurodegeneration	±	–	–	–	+
Tangles in neurons	–	–	–	–	+

+, Common characteristics in mice and AD patients.
[a]Date from **refs. *38*** and ***39***.

The successful generation of mice with plaques and angiopathy (which only lack NFTs to show complete AD pathology) is very encouraging. The ongoing cross-breeding of the APP/Ld and tau transgenic mice might be the first step toward the inclusion of AD related tauopathy. In addition, APP/Ld and APP/Ld × PS1 mice yield evidence for dystrophic neurites that contain hyperphosphorylated protein tau. The ongoing generation of triple transgenic mice (i.e., APP/Ld × PS1 × tau) is therefore expected to result in NFT pathology.

The mechanism(s) by which neurons are "executed" in AD still needs to be understood in detail. Recently reported findings further emphasize the role and importance of degradation and clearance pathways of amyloid peptides ***(35)*** and of the conversion of p35 to p25 for or during neurodegeneration in AD ***(33,36)***. The involvement of ApoE lipoproteins and its receptors in brain pathology also remains enigmatic ***(23,37)***. It is clear that in the continued effort to understand the pathology of AD, many fundamental questions are still to be tackled. In this major effort, transgenesis and mouse models have already been proved of great value and will continue to play a pivotal role, particularly through combinations of human genes in multiple transgenic mice.

References

1. Lendon, C. L., Ashall, F., and Goate, A. M. (1997) Exploring the genetics of Alzheimer's disease using molecular genetics. *JAMA* **277,** 825–831.
2. Hutton, M., Lendon, C. L., Rizzu, P., et al. (1998) Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* **393,** 702–705.

3. Alzheimer, A. (1907) Uber eine eigenartige Erkrankung der Hirnrinde. *Algemeine Z. Psychiatry Phychisch-Gerichtliche Med. (Berl.)* **64,** 146–148.
4. Selkoe, D. J. (1998) The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell Biol.* **8,** 447–453.
5. Vinters, H. V. (1987) Cerebral amyloid angiopathy: a critical review. *Stroke* **18,** 311–324.
6. De Strooper, B., Saftig, P., Craessaerts, K., et al. (1998) Deficiency of presinilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391,** 387–390.
7. Hardy, J. (1997) Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci.* **20,** 154–159.
8. Selkoe, D. J. (1999) The origin of Alzheimer's disease: a is for amyloid. *JAMA* **282,** 1615–1617.
9. Moechars, D., Lorent, K., De Strooper, B., Dewachter, I., and Van Leuven, F. (1996) Expression in brain of amyloid precursor protein mutated in the α-secretase site causes disturbed behavior, neuronal degeneration and premature death in transgenic mice. *EMBO J.* **15,** 1265–1274.
10. Moechars, D., Dewachter, I., Lorent, K., et al. (1999) Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. *J. Biol. Chem.* **274,** 6483–6492.
11. Van Dorpe, J., Smeijers, L., Dewachter, I., et al. (2000) Prominent cerebral amyloid angiopathy in transgenic mice overexpressing the London mutant of human APP in neurons. *Am. J. Pathol.* **157,** 1283–1298.
12. Dewachter, I., Van Dorpe, J., Smeijers, L., et al. (2000) Aging increased amyloid peptide and caused amyloid plaques in brain of old APP/V717I transgenic mice by a different mechanism than mutant Presenilin 1. *J. Neurosci.* **20,** 6452–6458.
13. Hsiao, K., Chapman, P., Nilsen, S., et al. (1996) Correlative memory deficits, Abeta elevation and amyloid plaques in transgenic mice. *Science* **274,** 99–102.
14. Johnson-Wood, K., Lee, M., Motter, R., et al. (1997) Amyloid precursor protein processing and A beta42 deposition in a transgenic mouse model for Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* **94,** 1550–1555.
15. Natte, R., de Boer, W. I., Maat-Schieman, M. L., et al. (1999) Amyloid beta precursor protein-mRNA is expressed throughout cerebral vessel walls. *Brain Res.* **828,** 179–183.
16. Weller, R. O., Massey, A., Newman, T. A., et al. (1998) Cerebral amyloid angiopathy: amyloid β accumulates in putative interstitial fluid drainage pathways in Alzheimer's disease. *Am. J. Pathol.* **153,** 725–733.
17. Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., and Kirschner, M. W. (1975) A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* **72,** 1858–1862.
18. Goedert, M. and Jakes, R. (1990) Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. *EMBO J.* **9,** 4225–4230.
19. Mandelkow, E. M., Biernat, J., Drewes, G., et al. (1995) Tau domains, phosphorylation and interactions with microtubules. *Neurobiol. Aging* **16,** 355–363.

20. Billingsley, M. L. and Kincaid, R. L. (1997) Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. *Biochem. J.* **323,** 577–591.
21. Hong, M., Zhukareva, V., Vogelsberg-Ragaglia, V., et al. (1998) Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. *Science* **282,** 1914–1917.
22. Spittaels, K., Van den Haute, C., Van Dorpe, J., et al. (1999) Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. *Am. J. Pathol.* **155,** 2153–2165.
23. Tesseur, I., Van Dorpe, J., Spittaels, K., et al. (2000) Expression of human apolipoprotein E4 in neurons causes hyperphosphorylation of protein tau in the brains of transgenic mice. *Am. J. Pathol.* **156,** 951–964.
24. Ebneth, A., Godemann, R., Stamer, K., et al. (1998) Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmatic reticulum: implications for Alzheimer's disease. *J. Cell. Biol.* **143,** 777–794.
25. Spillantini, M. G., Murrell, J. R., Goedert, M., et al. (1998) Mutations in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc. Natl. Acad. Sci. USA* **95,** 7737–7741.
26. Lynch, T., Sano, M., Marder, K. S., et al. (1994) Clinical characteristics of a family with chromosome 17-linked disinhibition-dementia-parkinsonism-amyotrophy complex. *Neurology* **44,** 1878–1884.
27. Ishiguro, K., Shiratsuchi, A., Sato, S., et al. (1993) Glycogen synthase kinase 3β is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.* **325,** 167–172.
28. Hanger, D. P., Betts, J. C., Loviny, T. L. F., Blackstock, W. P., and Anderton, B. H. (1998) New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry. *J. Neurochem.* **7,** 2465–2476.
29. Mandelkow, E. M., Drewes, G., Biernat, J., et al. (1992) Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett.* **314,** 315–321.
30. Lovestone, S., Hartley, C. L., Pearce, J., and Anderton, B. H. (1996) Phosphorylation of tau by glycogen synthase kinase-3β in intact mammalian cells: the effects on the organization and the stability of microtubules. *Neurosci.* **73,** 1145–1157.
31. Pei, J.-J., Grundke-Iqbal, I., Iqbal, K., et al. (1998) Accumulation of cyclin-dependent kinase 5 (cdk5) in neurons with early stages of Alzheimer's disease neurofibrillary degeneration. *Brain Res.* **797,** 267–277.
32. Lee, K. Y., Clark, A. W., Rosales, J. L., et al. (1999) Elevated neuronal Cdc-2-like kinase activity in the Alzheimer disease brain. *Neurosci. Res.* **34,** 21–29.
33. Patrick, G. N., Lawrence, Z., Nikolic, M., et al. (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* **402,** 6154–622.
34. Ahlijanian, M. K., Barrezueta, N. X., Williams, R. D., et al. (2000) Hyperphosphorylated tau and neurofilament and cytoskeletal disruptions in mice overexpressing human p25, an activator of cdk5. *Proc. Natl. Acad. Sci. USA* **97,** 2910–2915.

35. Vekrellis, K., Ye, Z., Qiu, W. Q., et al. (2000) Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. *J. Neurosci.* **20,** 1657–65.
36. Lee, M. S., Kwon, Y. T., Li, M., et al. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* **405,** 360–364.
37. Hyman, B. T., Strickland, D., and Rebeck, G. W. (2000) Role of the low-density lipoprotein receptor-related protein in beta-amyloid metabolism and Alzheimer disease. *Arch. Neurol.* **57,** 64–50.
38. Ishihara, T., Hong, M., Zhang, B., et al. (1999) Age-dependent emergence and progression of a tauopathy in transgenic mice overexpressing the shortest human tau isoform. *Neuron* **24,** 751–762.
39. Probst, A., Götz, J., Wiederhold, K. H., et al. (2000) Axonopathy and amyotrophy in mice transgenic for human four-repeat tau protein. *Acta Neuropathol.* **99,** 469–481.