Transgenic Mouse Methods and Protocols

Second Edition

Edited by
Marten H. Hofker
Jan M. van Deursen

METHODS IN MOLECULAR BIOLOGY™

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Preface

Modern biomedical research is gradually tightening its grip on the genetic basis of common diseases by studying complete genomes, transcriptomes, and other complex biological components. Pivotal to this progress in building complex networks is the detailed knowledge of the individual components. Hence, functional studies of individual genes will remain crucial. To obtain this functional information, genetically modified mice are likely to stay at the center stage for the years to come. An important reason for this role is that mice are genetically very similar to man. Moreover, gene function studies in mice are in the context of a whole organism, and therefore provide information of gene–gene and gene–environment interaction. This information offers excellent insight in the contribution of individual genes to the system. Moreover, virtually all human genes are conserved in the mouse. The second edition of "Transgenic Mouse Methods and Protocols" covers the production and analysis of transgenic and knockout mice. Much progress has been made to facilitate the generation of genetically modified mice, and also to make the mouse models more precise. The latter improvement involves a superior control over the timing, level, and location of gene expression or gene disruption.

Many researchers played a crucial role in developing mouse technology to the excellent state of art that has now been achieved. Landmarks include the generation of (1) transgenic mice, (2) pluripotent embryonic stem (ES) cell cultures, (3) gene knockout mice, (4) tissue-specific knockouts, and (5) systems for inducible gene expression mice. Most of these landmarks have not been achieved in other mammalian systems with a comparable efficiency. In part, this is attributable to the availability of hundreds of different inbred mouse strains, which allowed researchers to choose from a wide range of strains while establishing these technologies. Transgenic Mouse Methods and Protocols have essentially the same format as previous volumes of the series Methods in Molecular Biology. Since mouse technology offers a wide range of possibilities, most chapters provide the rationale for choosing the given protocol, which is then described in step-by-step detail. The book can be roughly divided into three parts: a general introduction describing how to deal with mice and how to generate transgenic mouse models; a part describing the generation of conditional and induced knockout and transgenic mice, and a final section offering alternative routes to study gene function in mice. We would like to thank the authors for their excellent contributions and Ingrid van der Strate and Marijke Schreurs for editorial assistance. We are very grateful to Be Wieringa, Anton Berns, and Robin Lovell Badge for leading us into the world of gene-targeting and ES cell technology.

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Chapter 1

Introduction: Strategies for Developing Genetically Modified Mice

Marten H. Hofker

Abstract

Advancements in transgenic technologies have made the mouse one of the most useful animal models for biomedical research. Several technological breakthroughs have allowed the generation transgenic and knockout mouse models some 25 years ago. Subsequently, the technology has undergone many improvements, advancing our ability to control the expression of the genes and determine the cell types where the genetic modification should take place. Hence, the mouse is unique in offering the possibility to understand genotype–phenotype relationships that are relevant for unraveling the biological role of these genes in the human. This chapter provides an introductory overview.

Key words: Knockout mouse, Disease model, Gene-function

1. Introduction

Advancements in transgenic technologies 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 with respect to organ systems, tissues, physiological 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 possibility to understand genotype–phenotype relationships that are relevant for unraveling the biological 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 germ line. 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 follow the "candidate gene approach." Such studies start upon 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 gene and will help to unravel the underlying molecular and biochemical mechanisms. In addition, the ensuing disease model will help in designing novel 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 for diseases (see http://jaxmice.jax.org) that emerged from transgenic studies, or were obtained after spontaneous or induced random mutation events. Additional resources have also proven to be important, including the Mutant Mouse Regional Resource Centers (http://www.mmrrc.org), and the European Mutant Mouse Archive (http://www.emmanet.org).

Transgenic studies can also initiate 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. Homeobox genes were first discovered in *D. melanogaster*. Subsequently, their evolutionarily conserved biological function was shown in the mouse.

Following 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 approaches. By crossing the novel mouse model onto a sensitized strain, increased

genetic pressure can be achieved. An excellent example of this strategy involves the role of putative "atherosclerosis" genes. Novel mutant mouse strains can be crossed with a known model for atherosclerosis. This breeding is crucial because common inbred mouse strains are generally resistant to atherosclerosis. Many genes with a role in atherosclerosis will not be recognized because mice have a very healthy lipoprotein profile offering a strong protection against the development of fatty streaks and subsequent atherosclerotic plaques. Therefore, it is important to make use of an athero-susceptible background. The apolipoprotein E-deficient mouse shows very high levels of atherogenic lipoproteins and 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 containing cholesterol are commonly used to induce atherosclerosis, thereby exposing the role of genetic factors.

2. Mouse–Human Differences

The differences between mouse and human are huge, but looking through the eyes of a molecular geneticist some differences are difficult to appreciate. For instance, virtually all genes are highly conserved between mouse and human. Larger differences exist at the DNA sequence level in noncoding regions. However, it is in most cases possible to substitute entire mouse gene regions with a full-length human gene and observe no differences in gene regulation and gene function. It can even be observed that a human-specific tissue-specific expression is faithfully reproduced in the mouse. Interestingly, mice and man show considerable metabolic differences when examining lipoprotein metabolism. Due to these differences, the mouse is virtually resistant to diet-induced atherosclerosis. However, only very few mechanistic differences in lipid metabolism between mouse and human have been discovered. When these differences are being "repaired" or compensated for, the lipoprotein metabolism changes into a "human-like" metabolism.

From a medical perspective, disease processes in mice are likely to be different from man. First, 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 lifespan 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. In the heterozygous state, this tumor suppressor gene

leads in almost all cases to tumors in humans. However, in general, the short time frame is not a major problem in mouse studies on cancer, neurodegenerative, and cardiovascular disease. Second, the physiological properties of a mouse are tuned to its small size. With an average weight of only 40 g, it 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 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. This similarity provides a unique opportunity to study human diseases in a small and quickly reproducing mammal. Therefore, one should accept the fact that a mouse will never develop a disease exactly as it occurs in a human.

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 micro-injection of fertilized oocytes has not altered much. Because many constructs at that time were based on intronless cDNA clones and not much insight was available regarding gene regulation, it has been difficult to create good quality mouse models. It was difficult to express transgenes at the right moment, level, and location. At present, however, knowledge of regulation of gene expression has dramatically improved, leading to many innovative methods that is introduced below.

From a geneticist's viewpoint, conventional transgenic mice represent gain-of-function models because one could only add a gene and thereby gain a function. However, human genetic disease is often characterized by the loss of function or by homozygous recessive gene mutations, which were both beyond the reach of conventional transgenesis. More recently, transgenesis using constructs based on RNA-interference (RNAi) has changed this situation. This technology was invented in the late 1990s (3) and allows reducing expression levels of a target gene. However, it is difficult to completely silence genes. Hence, the RNAi technology should be regarded as complementary to the knockout approach described below.

The advancements in the late 1980s that allowed generating the loss of function models were an enormous leap forward.

For this seminal work, the Nobel Price of Medicine was awarded to Martin Evans, Mario Capecchi, and Oliver Smithies in 2007. This technology was made possible due to progress in mouse embryonic stemcell (ES) biology (4) and genetic engineering using homologous recombination in mammalian cells (5). Then, in 1988, an *arbitrary* gene was targeted in mouse ES cells (6) demonstrating the wide applicability of the technology. 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 successful 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 (7). Hence, the mouse became the species of choice for biomedical research. At present, it is generally 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

Although the practices and principles of generating genetically modified mice have been well established during the last two decades, many innovations have been introduced. In this book, we provide the background and the detailed protocols for mouse technology, engineering constructs for generating transgenic and knockout mice and providing alternative approaches for establishing mouse models for the study of gene function. The 19 chapters are briefly introduced below.

4.1. General 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. Alternatively, Chapter 3 demonstrates how embryonic stem cells can be used to generate aggregation embryos. The advantage of this approach is that chimeric mice, which can be heterogeneous with respect to the cellular distribution of a specific mutation, can be efficiently generated. This technology is also very useful to study mutations that interfere with reproduction. In Chapter 4, protocols are provided on the preservation of mouse strains by cryopreservation of sperm and subsequent in vitro fertilization. These reliable protocols for cryopreservation are suitable for a wide application. Cryopreservation is crucial for preventing overcrowding of the mouse house. Further, the exchange of sperm may also turn out to be more convenient than shipping live mice to other laboratories. 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. Chapter 5 provides the protocols for autopsy and histology. Often, novel mouse models are being studied for only some key organs. The more general approach for studying the entire animal described here is justified, as it is crucial to identify phenotypic changes that may fall outside of the scope of the scientist.

4.2. Conventional Transgenesis

Chapter 6 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 microinjection.

Chapters 7 and 8 elaborate on several different strategies for transgene design and generating transgenic mice. Chapter 7 presents the use of tetracycline-inducible transgenes, in particular in combination with Cre recombinase. The tetracycline system is currently the best system for inducible gene expression available. Other systems exist as well. The applications of inducible promoters (metallothionein, interferon) have 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.

A recent strategy for the efficient generation of transgenic mice is the use of lentiviral gene transduction (see Chapter 8). This approach circumvents the need for laborious microinjections into the pronucleus of fertilized oocytes. Lentivirus can be injected in the zona pellucida, which is technically less demanding, and therefore leading to a higher success rate.

Chapter 9 addresses the caveat of conventional transgenesis that concerns the limited control over gene expression levels. This problem forces the characterization of many founder lines. Alternatively, transgenic mice can be generated upon the transfection of ES cells, which can be tested in vitro prior to generating the transgenic mice. Two methods are being described. The first allows for the induction of tissue-specific transgene expression based on LoxP-Cre recombinase. The second approach makes use of doxycycline-mediated induction of transgene expression.

4.3. Gene Targeting

Chapter 9 also summarizes the most essential protocols for culturing 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 Cell can be produced with high efficiency.

Furthermore, Chapter 10 describes the procedures for generating ES-lines with transgenic constructs for the generation of genetically modified mice. This approach addresses a major caveat

of conventional transgenics. The caveat is that initially a set of founder mice are being generated, that will differ among each other with respect to the expression level and pattern of the transgene. Hence, it is necessary to carry out a large breeding program for the selection of suitable transgenic lines. When generating a "transgenic ES line" the ES cells can be tested prior to the generation of the mouse model and only a few promising cell-lines can be used for embryo generation. This approach facilitates an important application, which is the use of a vector allowing to generate a "conditional transgenic mouse." In combination with mouse strains with tissue-specific expression of Cre recombinase, this approach offers generating a series of different inducible transgenic mouse models.

Chapter 10 and 11 explain the use of large bacterial artificial chromosome (BAC) clones for the generation of gene-targeting constructs and constructs for the generation of conventional transgenic mice. For generating transgenic mice, large-insert clones offer the best guarantee that most regulatory sequences are included in the construct and native expression pattern can be reproduced in the mouse. For the generation of gene-targeting clones, the approaches provide an efficient cloning system circumventing laborious cloning procedures, which are usually depended on the location of restriction enzyme cleavage sites. The approaches make use of homologous recombination in *Escherichia coli*. This strategy is required because long-insert clones cannot be handled in more conventional cloning procedures. Two methods are provided. Chapter 10 describes the use of the RecA system in *E. coli*. A complementary approach based on phage λ is provided in Chapter 11.

Despite the enormous advances provided by the knockout mice, there are also some caveats. One is the fact that the knockout mutations are transmitted via germ line cells. Hence, all cells of the body inevitably carry the induced mutation. In some cases, however, it is desirable to study cell type-specific knockout mice. The Cre-LoxP system (see Chapter 12) has been developed, allowing cell type-specific knockouts. In fact, LoxP-mediated recombination upon the induction of 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.

Chapter 13 describes a method to generate hypomorphic alleles. Such alleles are of high interest in case the complete knockout induces a lethal phenotype and in particular, when the knockout mutation reduces cell viability. In that case, generating a hypomorphic allele allows one to disrupt the function of genes to a

lesser degree and bypass the lethality caused by the knockout gene mutations. This chapter complements the preceding chapters by offering an approach to engineer a type of mutation that may be very common in predisposition to common diseases in man.

Vector generation is a time consuming step toward developing mouse models. Chapter 14 describes a resource named "MICER" after the Mutagenic Insertion and Chromosome Engineering Resource. MICER consists of an extremely large series targeting vectors. These vectors are a feature of the ENSEMBL mouse genome browser, which facilitates the selection of these clones. The chapter provides the necessary background information and methods that greatly facilitate the utilization of MICER. Chapters 15 and 16 describe a procedure to efficiently generate an allelic series of targeted mutations. As most genes are currently studied in knockout mice, it becomes more interesting to study some key genes in greater detail. For instance, one would like to carry out structure–function studies in vivo, by producing different mutations in a single gene. This chapter elaborates on the generation of knockin mice and focuses on recombinase-mediated cassette exchange (RMCE), which is highly suitable to repeatedly target a specific gene with high efficiency.

4.4. Other Genetic Approaches

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. There are many strategies for generating large series of mutations, ranging from the random insertion of DNA fragments to point mutations induced by N-ethyl-N-nitrosourea (ENU).

Chapter 17 provides a method using randomly targeted ES cells for the generation of mouse models. Key is to make use of a vector that allows unbiased integration in the mouse genome to obtain randomly mutated genes. Using pooling strategies and sequencing, the appropriate clones can be identified and used for the generation of mouse models for the gene of interest. Although the initial steps require a relatively large investment, resources have been established that ultimately lead to a wealth of different targeted mutations that can be archived and stored in biobanks until these clones are needed.

Chapter 18 describes the use of ENU-induced mutations. The ENU method is unique in generating truly random mutations. The only disadvantage of ENU is that the affected genes more difficult to find because ENU induces point mutations. By comparison, other mutations induced by transposons, (pro)viral insertions, or other DNA fragments have larger changes that are easier to identify. The protocol involves ENU-mutated male animals that are mated with untreated females. The subsequent F1

population carries ENU-induced mutations. One can make use of a phenotypic or a genotype driven screen. Due to the recent advancements in genetic technologies, the identification of mutations in a genotype driven screen has become more convenient and is frequently used to identify a set of mutations within a specific gene.

Chapter 19 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. The replacement of the hematopoietic system is of great interest, for instance, for research into the role of the innate immune system in cardiovascular and metabolic disease. The main draw to use this technology is the fact that in order to generate genetic deficiencies specifically in the hematopoietic system, the bone marrow transplantation is convenient. Typically, bone marrow using cells from a donor with a particular knockout mutation is transplanted to another mouse strain. This experiment is relatively easy to organize. Alternative protocols using conditional knockout mice are usually much more time-consuming, especially if the background strain is another type of knockout mice, the mouse breeding may take around 1 year to complete.

Chapter 20 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.

5. Closing Remarks

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, the APOE3Leiden mouse model developed for lipoprotein metabolism was central to our studies on atherogenesis in the mouse, and the APOE3Leiden model also plays a role in studying diabetes and obesity. To appreciate the full potential of mouse models, it is advised to embark on these studies with a multidisciplinary team. It should be remembered that the actual generation of the model takes much less effort than the subsequent research that may originate from it.

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Chapter 2

Genetic Modification of the Mouse: General Technology – Pronuclear and Blastocyst Injection

Jan Willem Voncken

Abstract

Introduction of germ line mutations in mice via genetic engineering involves alterations of the structure and characteristics of genes. These alterations are mostly introduced via molecular genetic technology either in embryonal stem cells or in one-cell stage embryos. This chapter describes classic biotechnological methods used to generate mice from modified pre-implantation embryos.

Key words: Transgenic, Gene expression, Promoter, DNA construct

1. Introduction

Transgenesis, one of the first techniques specifically aimed at germ line alterations, makes use of introduction of exogenous DNA sequences, *transgenes*, into an organisms' genome. A transgenic animal is by definition an organism that has had extra, often times foreign DNA, artificially introduced in its genome. Transgenesis is applicable to not only a wide range of mammalian species including mice, rats, rabbits but also livestock such as sheep, pigs, cattle, and recently even primates. The focus of this chapter, however, will be on mice, because these are the most widely used laboratory animals for transgenic studies in experimental research. In addition to conventional transgenic technology described in detail below, mice are, as of yet, the only mammalian species suitable for gene targeting by homologous recombination in embryonic stem cells. Gene targeting allows for genetic germ line manipulation at predetermined genomic loci and is a very important and powerful extension of the current molecular genetic tools to generate experimental animal models to study gene function and disease.

This topic and some of its exciting applications are described in Chapters 8–10. In contrast to embryonic stem cell technology, there is no need for homology between the injected DNA and the host genome in this technique. Transgenic animals are generated by (retro)viral transduction of early embryos, introduction of transgenes in embryonic stem 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 multi-copy insertion), arranged in a head-to-tail fashion.

Transgenesis may be used to study overexpression or ectopic expression of a gene of interest. Alternatively, the effect of mutation of a gene may be the subject of studies. 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 be limited to distinct cell types or particular developmental stages. Alternatively, transgene expression may occur in cell types where 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 Figure 1 below, an overview 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) is presented. 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 equipment use for embryonic stem cell injection into blastocysts are in essence very similar to microinjection of fertilized eggs, blastocyst injection, and uterine transfer are discussed in this chapter as well (see Figure 1 and Subheadings 3.2–3.4). For basic molecular cloning techniques and strategies, and molecular detection methods, such as polymerase chain reaction (PCR)-based or Southern blot analysis, we recommend additional reading in "Molecular Cloning" by Sambrook et al. (6). Transgenic construct design is discussed in Chapter 4.
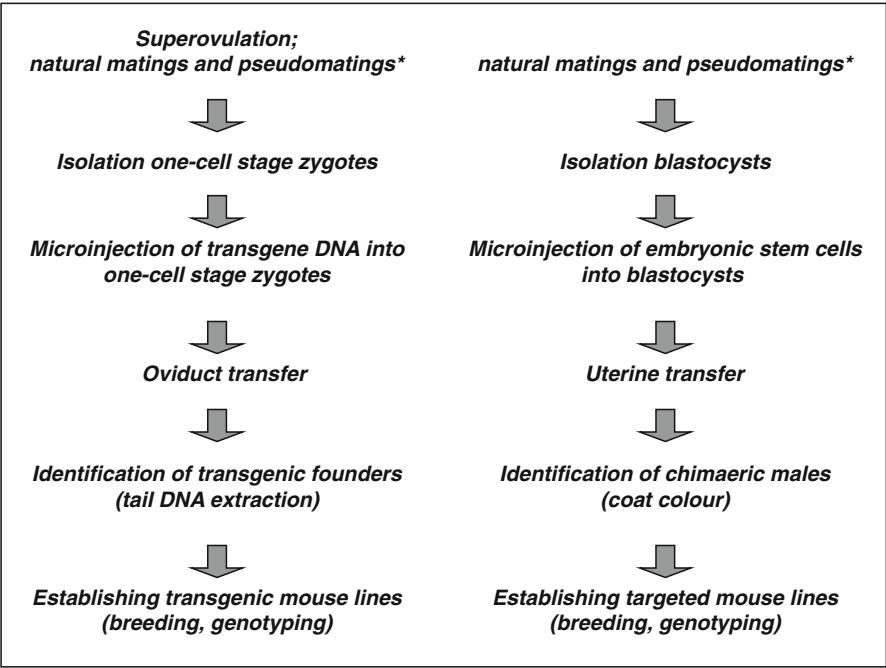
Fig. 1. Stepwise overview of the procedures for generating genetically modified mice by pronuclear injection of DNA (*left column*) and by gene targeting in embryonic stem (ES) cells, and subsequent injection of these ES cells into blastocysts (*right column*). *: Matings between foster females and vasectomized males.

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 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 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 to reintroduce genetically modified embryonic stem cells into. 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 out-bred strains, on mouse embryology, and dissection of specific developmental stages.

The need for genetic standardization of experimental animal models in experimental and applied research has been historically 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 immunological 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), while 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, 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 used frequently in immunological and behavioral studies as well. 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 to that in 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 often 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-G½″ needles.

8. Sterile 0.85% (w/v) sodium chloride solution or sterile water.

9. FSH analog: Pregnant mare serum gonadotrophin (PMSG); 1,000 IU.

10. LH analog: human chorionic gonadotrophin (hCG); 1,500 IU.

Hormones are available from Intervet, Boxmeer, the Netherlands: Folligon; Chorulon, or Sigma: PMSG: (1,000 IU); hGC: (2,500 IU).

2.2. Isolation of One-Cell Stage Zygotes; Isolation of Blastocysts

1. Mineral oil (Sigma).

2. M2 medium (Sigma).

3. M16 medium (Sigma).

4. Blastocyst isolation medium: 10% FBS, 10-mM HEPES in DMEM medium (+penicillin/streptomycin; see Note 13).

5. Blastocyst culture medium: 10% FBS in DMEM medium (+penicillin/streptomycin; see Note 13).

6. Penicillin and Streptomycin solution, 10,000 U/ml.

7. Pyruvate (Sigma).

8. Hyaluronidase type IV-S (Sigma).

9. Bovine serum albumin, fraction V (Sigma).

10. Phosphate-buffered saline (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 mm).

16. Mouth pipet/tubing.

17. Syringes (1 ml)/needles (27 G; see Note 13).

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.

2.2.1. Preparation of 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 on storage conditions tend to vary. For the sake of simplicity, preparations below use prefabricated solutions. It is of importance to avoid dust collection in media and other liquids. For this reason, we adhere to not wearing powdered gloves, or at least washing off the powder and dust thoroughly before use.

(a) M2 medium: to 50-ml of M2 medium, add 0.5 ml of penicillin/streptomycin solution. If necessary, adjust pH to 7.3–7.4 with 5 N NaOH. Add BSA to a final concentration of 4–5 mg/ml (see Note 3). Filter sterilize (0.22-mm filter). Pre-wash 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.

(b) M16 medium: to 50 ml of M16 medium, add 0.5 ml of penicillin/streptomycin solution. Filter sterilize (0.22-mm 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 ml) under paraffin oil overnight at 100% humidity, 2.5% CO2 (see Note 3).

(c) Hyaluronidase solution: dissolve 50 mg of hyaluronidase in 50 ml of M2-Medium (Sigma). If necessary, adjust pH to 7.3–7.4 with 5 N NaOH. Filter sterilize (0.22-mm filter). Discard the first few milliliters. Make 0.5-ml aliquots and store at –20°C.

(d) Pyruvate: dissolve 36 mg of pyruvate into 10 ml of water. Filter sterilize (0.22-mm filter). Discard the first few milliliters. Make 0.5-ml aliquots and store at –20°C.

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

2.3. Microinjection of One-Cell Stage Zygotes: In Vitro Culture of Injected Zygotes – Injection of Blastocysts with Embryonic Stem Cells

1. Mineral Oil.

2. M2 medium.

3. M16 medium.

4. Blastocyst isolation medium: 10% FBS, 10-mM HEPES in DMEM (+penicillin/streptomycin; see Note 13).

5. Blastocyst culture medium: 10% FBS in DMEM (+penicillin/streptomycin; see Note 13).

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 (A > 520119; Leitz) for holding pipets.

13. Glass capillary tubes (G-1; Narishige) for blastocyst injection needles (see Note 13) or glass capillaries with inner filament (GD-1; Narishige).

14. Sigmacote (Sigma).

15. 96% Ethanol (analytical grade).

16. Pipet puller (e.g., PB-7; Narishige).

17. Microforge (e.g., MF-9; Narishige).

18. Microgrinder (e.g., E-40; Narishige; see Note 13).

19. Inverted microscope system (e.g., Nikon, Olympus, Leitz, and Zeiss).

20. Micromanipulators (e.g., Narishige).

21. Dissection microscope.

22. Diamond pencil.

23. Stage micrometer.

24. Fluorinert (Sigma).

25. Read-out type (see Note 13), motor-driven microinjector, or large 50-ml glass injection syringe.

2.4. Oviduct Transfer: Uterine Transfer

1. Mineral Oil.

2. Clean M2.

3. Blastocyst isolation medium: 10% FBS, 10-mM HEPES in DMEM (+penicillin/streptomycin; see Note 13).

4. Blastocyst culture medium: 10% FBS in DMEM (+penicillin/streptomycin; see Note 13).

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 5).

14. Small desiccator.

15. Injection sedative (see Note 5).

16. Syringes (1 ml)/needles (27 G; see Note 13)

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-mM Tris–HCl, pH 8.5, 5-mM EDTA, 0.2% SDS, and 200-mM NaCl.

2. Proteinase K: fresh stock solution: 20 mg/ml in TE buffer.

3. TE buffer: 10-mM Tris–HCl, pH 7.2–7.6, 1-mM EDTA.

4. Pasteur pipets, flame-polished.

5. Reaction tubes, 1.5 ml.

6. 55°C oven.

7. Rotator.

2.5.2. Standard Procedure Tail-Tip DNA Extraction

1. Tail mix: 50-mM Tris–HCl, pH 8.6, 100-mM EDTA, 1% SDS, 100-mM NaCl.

2. Protease K: fresh stock solution: 10-mg/ml TE buffer

3. RNase: 10 mg/ml (heat inactivated; 10 min at 100°C).

4. Phenol–chloroform–isoamylalcohol (24:24:1; Phenol: saturated with demiwater (autoclaved) adjusted to pH 7.0 with 1-M Tris–HCl, pH 8.0.

5. TE buffer:10-mM Tris–HCl, pH 7.2–7.6, 1-mM EDTA.

6. Pasteur pipets, flame-polished.

7. Reaction tubes, 1.5 ml.

8. 55°C oven.

9. Rotator.

10. Bench or wrist shaker.

11. 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 time regimen, most often comprising a 6 a.m. to 6 p.m. light period (see Note 1). We have

good experience using 4–6 weeks old B6CBA/F$_1$ females (Jackson labs) for superovulation. Tables 1 and 2 present a summary of the different actions in the procedures.

3.1.1. Superovulation: Natural Matings

1. Lyophilized PMSG and hCG are dissolved in 0.85% NaCl solution or sterile water to a final concentration of 50 IU/ml. PMSG may be stored frozen at –20 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 and hCG (200 IU/ml) may be stored at 4°C for a period of 2 months, but need to be diluted properly prior to use.

Table 1
Time table of superovulation and matings to obtain one-cell stage embryos for microinjection

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

[a]Day/night rhythm: 6.00 a.m.: light, 6.00 p.m.: dark
[b]Matings between foster females and vasectomized males

Table 2
Time table of natural matings to obtain blastocysts for injection

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

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

2. Thaw a vial of PMSG and inject 4–6 weeks old females with 100-ml of PMSG (5 IU) between 1.00 and 2.00 p.m. on the first day.

3. At day 3, 46–48 h after PMSG injection, thaw a vial of hCG and inject the same females with 100 ml of hCG (5 IU; 12.00–1.00 p.m.).

4. The females are either transferred directly to fertile studs or in the afternoon (see Note 2). Use one male per female. To ensure maximum number of fertilized eggs, these male mice are used only once a week. At 8 months of age, or when the plugging ratio drops below 70%, the male mice are replaced.

5. Check for copulation plugs the next morning.

If natural matings are carried out for blastocyst production, use females of 2–4 months of age. In terms of numbers of mice used, the same guidelines apply as for "pseudomatings," i.e., 2–3 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 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 required for re-implantation 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) as those 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 5–6 females per intended oviduct transfer. To increase the chances of mating a female in estrus, several females (2–4) are placed in with one vasectomized male, in contrast to 1-on-1 matings between superovulated females and fertile studs. Experience in judging whether females are in estrus can be helpful in obtaining sufficient plugged pseudopregnant females. Females are placed in a cage with a resident male, *not vice versa*. Vasectomized males are housed separately for at least 1–2 weeks (see Note 2). Oviduct transfers take place on the day of copulation plug detection. The surplus of plugged, pseudopregnant fosters can be re-used 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.

Table 3
Time table of preparations for pronuclear microinjection

Action	Time
Preparation of media	Day before, morning
Check plugs, kill superovulated females	9.00 a.m.
Isolation of one-cell stage zygotes	9.30 a.m.
Set up microinjection stage; holding pipet	10.30 a.m.
Injection needles	11.00 a.m.
Set up microinjection needle	1.00 p.m.
Pronuclear injection of one-cell stage zygotes	1.00 p.m. onward

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

Depending on the strain, the litter will be delivered around 19–21 days after the oviduct transfer.

3.2. Isolation of One-Cell Stage Zygotes: Isolation of Blastocysts

When microinjections are planned, the order of procedures should have some (chrono)logic to it. Although the planning of a microinjection session is highly personal, some suggestions are presented below in Table 3. Day/night rhythm is as in Table 1(see Note 4).

3.2.1. Isolation of Fertilized Oocytes

1. On the day of isolation, add 20 µl of the pyruvate stock to 2 ml of M2 and M16 aliquots and mix by swirling gently.

2. Prepare the depression slides: one with 0.2 ml of M2, two depression slides with 0.2 ml of hyaluronidase solution, and three depression slides with 0.2 ml of 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.

3. Collect females with a clear copulation plug. Kill the mice by CO_2 asphyxiation or cervical dislocation and transfer the animals to the location where zygotes are isolated.

4. Place the females' abdomen upward on a paper towel, wet the abdomen with 70% ethanol, and make an incision over the xiphoid (see Figure 2).

5. Grasp the skin with both hands and firmly pull back in opposite directions (rostrally and caudally), essentially skinning the mouse completely (see Figure 2).

6. Make incisions in body wall by lifting it with tweezers and cutting it with scissors, and expose complete body cavity.

7. Move intestines aside gently and grab ovaries by 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.
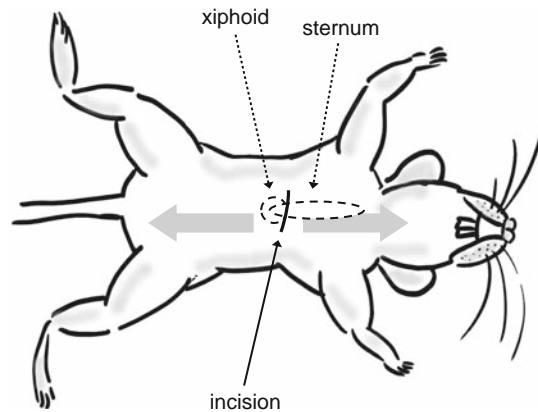
Fig. 2. Dissection of donor females and isolation of fertilized eggs. An incision is made over the xiphoid, after which the skin is firmly pulled back in the indicated direction (*gray arrows*).

8. Swollen ampullae (1) are ruptured 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 (I.D. 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 the easiest on top of the lid of a 10-cm dish, depression slides are best placed inside a 10-cm dish.

3.2.2. 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. One uterus is transferred to a dish with clean isolation medium. Disconnect the uterus horns from the cervix by making a cut just above the bifurcation point.

4. Carefully insert a short 27-G needle at the opening (closest to the cervix) and flush the blastocysts out using 0.5–1.0 ml of medium.

5. Collect the blastocysts with a wide-bore pipet (200–250 μm) and transfer to a drop culture with clean medium, wash, and transfer to culture medium at 37°C and 5% CO_2.

6. Fully expanded blastocysts are the easiest to use for injection; culturing blastocysts for some hours at 37°C and 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

3.3.1. Preparations

The availability of an operational microinjection set-up is considered a prerequisite to apply transgenic technology successfully. 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, micro-forges, and grinders, are commercially available (Leitz, Narishige, Nikon, Olympus, Sutter, and Zeiss). We refer to Hogan et al. (1) for a detailed description of a microinjection set-up. Microinjection of one-cell stage zygotes and subsequent transplantation are essentially carried out as described in ref. (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. Holding pipets and depression slides can be siliconized and rinsed extensively with clean, dust-free 96% ethanol; siliconized holding pipets can be re-used. Also, injection needles can be treated similarly: dip the needle tip into 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 be used alternately. Figure 3 depicts an example of a microinjection set-up.

3.3.2. Pronuclear Injection

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

2. Just before injection, a number of one-cell stage zygotes (20–40) are transferred 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
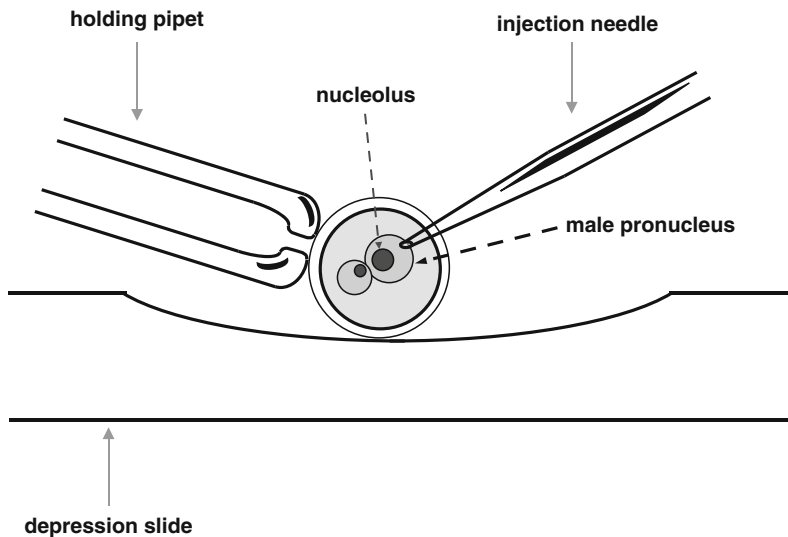
Fig. 3. Schematic representation of a microinjection chamber (*side view*). The basis of the injection chamber in this figure is a depression slide. Microinjection is carried out in a droplet of M2 medium.

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 inner filament), an opening is created in the injection needles 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. The injection needle is gently pushed through the plasma membrane and brought close to the nuclear membrane of one of the pronuclei. In most cases, the male pronucleus is the best accessible, because it is larger. If at this stage a small burst of DNA invaginates the plasma membrane further, this indicates that 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. The zygotes are placed within the optic field strategically, so that yet to be injected, abnormal and lysed zygotes, and
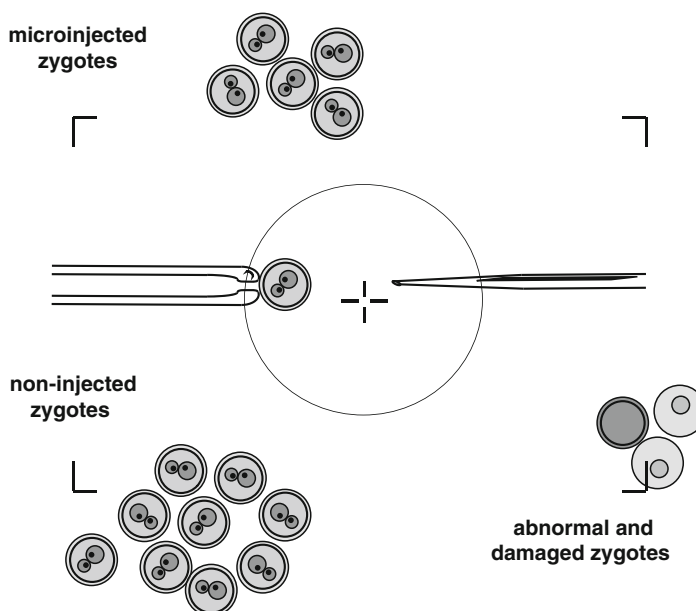
Fig. 4. Overview of the microinjection field (*top view*): non-injected, successfully injected, and abnormal zygotes, or zygotes that were damaged during microinjection are all kept apart throughout the injection procedure.

successfully injected zygotes are conveniently separated (see Figure 4).

9. Successfully injected zygotes are washed free of M2 medium, transferred to a 35-mm dish containing a few CO2-buffered M16 droplets, and kept in an incubator at 37°C and 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 and 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 application.

Positioning the holding pipet and injection needle is carried out at a magnification of 100×. Optimal needles are bevelled to an opening of 12–14 mm at 30–35°C. Injection needles may be siliconized and re-used.

2. Just before injection, a number of blastocysts (20–25) are transferred into the injection droplet. Injection is carried out in isolation medium or HEPES-buffered ES cell culturing medium (see Chapter 8).

3. ES cell suspensions are made 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 at hand to be able to repeat this procedure throughout the injection day. Wash ES culture twice with Ca2+/Mg2+m-free PBS and trypsinize the feeders plus ES cells. Sediment cells (5 min, at 1,000–1,200 rmp 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 so desired, the preplating procedure may be repeated. Collect the ES cells, sediment, suspend well in a small volume of Ca2+/Mg2+-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 ES cells). Typically, 12–15 ES cells are injected into one blastocyst.

5. Fix a blastocyst in position with the holding pipet, with the inner cell mass (ICM) located away from the injection site (see Figure 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. The injection needle is gently removed from the blastocoel, the blastocyst will visibly collapse shortly afterwards; move the injected blastocyst aside (as in Figure 4) and inject the next one.

7. The successfully injected blastocysts are transferred to a 35-mm dish containing a few CO2-buffered culture medium and kept in an incubator at 37°C and 5% CO2.

8. Repeat the injection procedure until all blastocysts are injected. ES cell suspension will tend to aggregate; repeat the preplating procedure with fresh suspension.

The successfully injected blastocysts are either transferred to pseudopregnant females the same day or cultured overnight at 37°C and 5% CO_2.
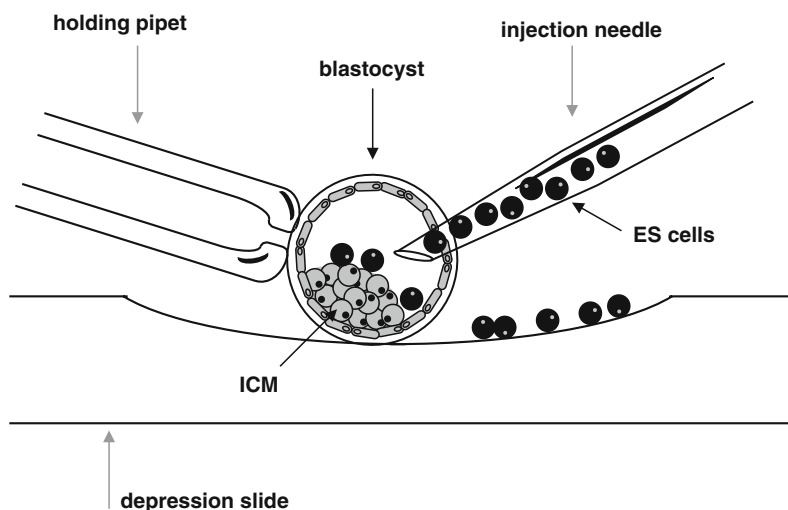
Fig. 5. Schematic representation of a blastocyst-injection chamber (*side view*). Genetically manipulated ES cells (*black*) are injected into a recipient blastocyst (*gray*). ES cells will adhere and become part if the inner cell mass (ICM) and partake in development. The resulting newborn will be a coat color chimera.

3.4. Oviduct Transfer: Uterine Transfer

3.4.1. Preparations for Oviduct Transfer

Injected zygotes are transferred either on the same day of microinjection, or the next day. If the oviduct transfer is carried out the same day, about 2×20 one-cell stage zygotes are transferred bilaterally to one recipient pseudopregnant female (i.e., 20 on each side). If two-cell stage embryos are transferred 1 day after mircroinjection, usually a smaller number of embryos suffice ($2 \times$ 12–14; see Notes 5 and 6).

1. Load transfer pipets as depicted in Figure 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 – $20 \times$ 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. Pre-anesthetize mouse using an inhalation anesthetic in a small desiccator (see Notes 7 and 8) and subsequently inject the female with a sufficient dose of sedative to anesthetize the animal for at least 30 min (see Notes 7 and 8).

4. The mouse is placed on an operation platform (10-cm dish with a plastic elevation positioned at the center).

5. Wet one flank with 96% 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 (see Figure 7a); grasp and lift skin with forceps and make a small
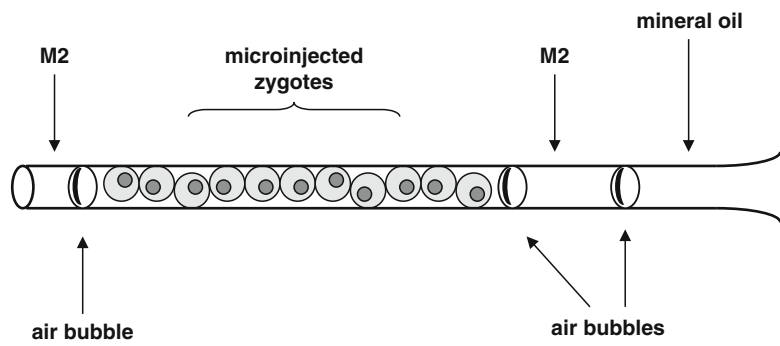
Fig. 6. Transfer pipet: microinjected zygotes are surrounded by small air bubbles, which function as markers during the oviduct transfer.
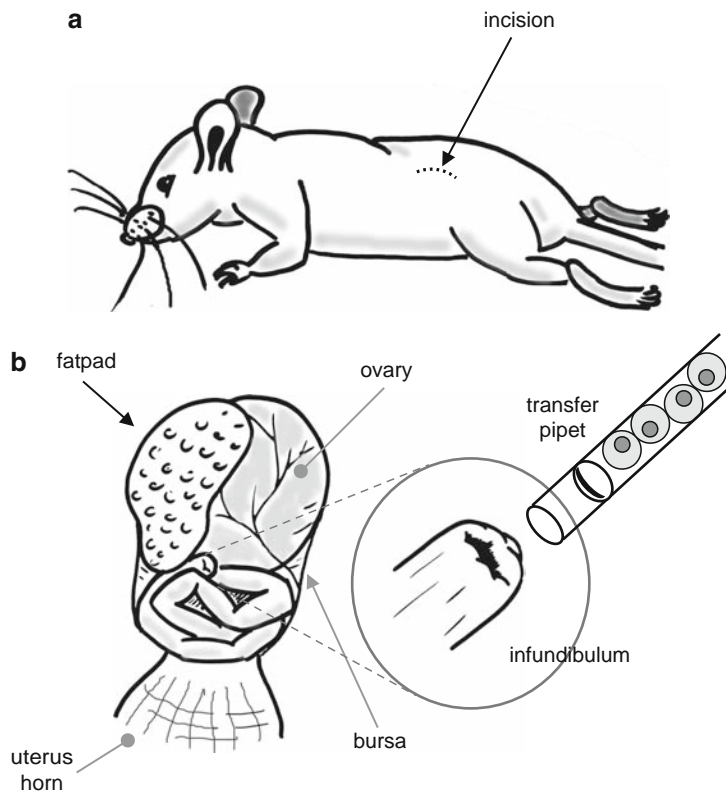
Fig. 7. Oviduct transfer. (**a**) An incision is made in the flank of the pseudopregnant female, just below the rib cage. (**b**) The ovary and oviduct are surrounded by the bursa, which needs to be ruptured for oviduct transfers. Inset: infundibulum and transfer pipet (see Fig. 5) magnified.

incision (scissors); insert the scissors into the incision and tear open the skin along the "parting" in the fur until an opening of approximately 6–8 mm is generated.

6. Grasp and lift body wall with forceps and make an incision with the scissors avoiding blood vessels; tear open in same

direction as the previous tear and attach suture to the upper "lip" of the wound (body wall).

7. Pull out ovary and oviduct gently 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 (i.e., the entrance to the fallopian tube), still covered by bursa, is visible (see Figure 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 mouth-piece of the transfer pipet in your mouth, while 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 2× 5-tweezers, avoid tearing blood vessels; if bleeding is caused inadvertently, use kimwipe tips to soak up excessive fluids.

11. Locate the infundibulum, pick up transfer pipet, and bring into position (i.e., line-up with the position of the infundibulum and fallopian tube). Probe the infundibulum carefully with the transfer pipet and when certain of deep enough insertion, gently expel the injected embryos by blowing.

12. While expelling, the air bubbles surrounding the embryos 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 uterus horn back into body cavity using the blunt tip of the serafine clamp, close the body wall with one stitch and a tight knot, and use suture or a wound clip to close the wound in the skin; proceed with the other side (see Notes 5 and 6).

14. When completed, ear-mark the female, if so desired (see Note 9), and return the animal to a cage.

15. Clean all equipment used with water and soap, and 70% ethanol, and sterilize.

3.4.2. Uterine Transfer   A uterine transfer is very similar to an oviduct transfer, with the exception that injected blastocysts are transferred directly into the uterus lumen. To this end, gently pull out the uterus horn a little further compared to that in an oviduct transfer (see Figure 7b); carefully insert a sterile 27-G 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 entrance through the hole will be blocked. Remove the needle and carefully insert a glass transfer pipet containing the injected blastocyst through into the uterus lumen. Gently aspirate the blastocysts into the uterus lumen; push back the uterus into the abdominal cavity and close the animal (see Subheading 3.4.1).

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 a lot more straightforward in case of germ line chimeras (ES cell manipulation and blastocyst injection) since this can be done visually (i.e., by coat color mozaicism). The analysis of chimeras and their offspring is discussed in Chapter 8. Transgenic founders will have to be genotyped by DNA analysis.

If expression of transgenic constructs in vivo 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 PCR analysis of purified mouse-tail DNA. Southern blot analysis, as opposed to PCR, offers the possibility to study actually 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 happens after the first cell division. In essence, this results in mice mosaic for transgene integration and expression. In such instance, the F0 animal will have a lower copy number than its F1 offspring. In case mosaic animals do transmit the transgene in a sub-Mendelian ratio to their F1 offspring, their F2 should of course show a 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. Sporadically, unstable integrations may present similar copy number discrepancies between F0 and F1 mice. Transgene rearrangements and sub-haploid copy numbers may be indicative for poor or absent expression and germ line 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 transgenic founders have been identified, subsequent identification of transgenic offspring does not require Southern analysis, but can be done by PCR. Southern blot analysis and PCR analysis are described in detail elsewhere (6). Here, a number of procedures for tail-tip DNA extraction are provided, described elsewhere. In addition to DNA analysis, it is wise to include a transgene

expression analysis on several tissues of F1 animals from different founder lines. This may include both RNA and protein analyses, siuch as for instance the detection of apolipoproteins in circulation, provided that immunological detection reagents and/or biochemical assays are available.

3.5.1. Rapid Procedure for 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 was even 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 quick method for DNA isolation is described below; in the next section (see Subheading 3.5.2), a tail DNA purification method is outlined employing phenol extraction. Mice are genotyped by tail DNA analysis (see Notes 10 and 11). Genotyping can be done from 10 days postpartum (pp) 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 pp) 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 pp) to 1.0 cm (3 weeks pp), in 1.5-ml reaction tubes on ice and either store dry at –80°C or in a 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 of tail mix and 10-μl of proteinase K (20 mg/ml). Incubate overnight at 55°C, preferably while rotating. This will greatly help dissolving and subsequently isolating the tail DNA.

3. Pellet the remaining debris (hairs and bone) in a microfuge at 14,000 rpm for 10–15 min at ambient temperature. Transfer the supernatant to a fresh microfuge tube.

4. Add 0.6× volume (i.e., ±400 μl) of isopropanol and mix by inverting the tubes *gently* (25×), allowing the DNA to precipitate in visible threads (vigorous shaking will hamper visible precipitation).

5. Either pellet the DNA briefly (15–20 s, 14,000 rpm) or fish the DNA clump out using a flame-polished glass Pasteur pipet.

6. Rinse the DNA sticking to the glass tip in 70% ethanol, let the DNA briefly air dry, and transfer to 500 μl of 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 an ambient temperature to dissolve the DNA overnight.

8. Store the DNA samples at 4°C until needed.

9. A volume of 1.5 µl of DNA suffices for a PCR. If a Southern analysis is attempted at this stage, 30–50 µl can be used.

3.5.2. Standard Procedure for Tail-Tip DNA Extraction

1. Collect mouse tails and add 700 µl of tail mix, or store (see Subheading 3.5.1); add 25-µl Proteinase K to each tube and incubate at 55°C overnight (in a rotator).

2. Add 10 µl of RNase and incubate for 1–2 h at 37°C.

3. Transfer the content of the tubes to new tubes[1] containing 400 µl of phenol–chloroform–isoamylalcohol using a cut blue tip and shake firmly for 15 min (bench or wrist shaker) at an ambient temperature.

4. Centrifuge at 14,000 rpm for 30 min, at an ambient temperature.

5. Transfer the supernatant to tubes containing 400 µl of phenol–chloroform–isoamylalcohol and shake (bench or wrist shaker) for 15 min at an ambient temperature.

6. Centrifuge at 14,000 rpm for 30 min at an ambient temperature.

7. Transfer the supernatant to tubes containing 400 µl of chloroform to remove phenol traces and shake for 5 min at an ambient temperature.

8. Centrifuge at 14,000 rpm for 5–10 min at an ambient temperature.

9. Transfer the supernatant to tubes containing 0.6× vol (±440 µl) of isopropanol.

10. Mix by inverting the tubes *gently* (25×), allowing the DNA to visibly precipitate (see Subheading 3.5.1).

11. Fish out DNA with (flame-polished, slightly bent at the tip) Pasteur pipet and wash in 1 ml of 70% ethanol. If the expected amount of DNA is too low, to fish out, pellet at 14,000 rpm for 10 min at an ambient temperature and wash the pellet with 70% ethanol (2×).

12. Let the DNA air dry for a while, then place the pipet in tube (50–100 µl of TE) until DNA comes off; DNA is dissolved by incubating for 10–15 min at 55–65°C, and subsequent incubation at 37°C, mixing firmly and repeatedly by hand until DNA is fully dissolved.

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[1]Tubes that have been incubated o/n at 55°C with SDS inside tend to leak when phenol–chloroform–isoamylalcohol is added.

13. Determine the DNA concentration: dilute 5 μl of DNA sample in 600 μl of water. Concentration (in μg/μl) is: OD_{260nm} (OD_{260} DNA = 50 μg/ml) × 6; typical yield: 50–200 μg of DNA

14. Perform a quantitative Southern analysis (see Notes 10 and 11).

15. DNA samples may be stored at 4°C or –20°C.

3.6. Establishing Transgenic Lines: Breeding and Analysis

Following 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 pp; 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 pp 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 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 ten backcrosses of a transgenic line to an existing inbred strain, although not inbred by definition (see Subheading 1.1 (1), produce 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 consequences: 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 contrary, 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, 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 mentioned clearly points out 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).

4. Notes

1. Although the light period is most often 6.00–7.00 a.m. to 6.00–7.00 p.m., the day–night rhythm at which animals are kept may be adjusted to local needs/convenience. A light period from 2.00–3.00 a.m. to 2.00–3.00 p.m. 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 the onset of the first cleavage, this methodological variation may be an option mainly for the experienced. Since mouse ovulation, 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.

2. For matings, female mice are always placed in with a male, not vice versa. Introducing a male into an unfamiliar environment will significantly affect their performance. 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 as well.

3. 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.

4. In order to keep animals SPF, all equipment and working surfaces should be sterilized before isolation of fertilized oocytes, microinjection, and oviduct transfer. If the aforementioned experimental procedures are not carried out within the confines of the animal facility, foster mice should be transported in filter-top cages.

5. 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 (O.D.) maximum. Ruler slides (slides with an engraved millimeter/micrometer scale) are very useful to estimate the bore size of transfer pipets.

6. 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 are possible

if carried out extremely gently. Some laboratories prefer unilateral oviduct transfers, other adhere to bilateral transfers, since sometimes unilateral transfers will not result in pregnancy.

7. 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 inhalation anesthetic and 2.5% avertin, described in Ref. (1), as the sedative during microsurgery. A volume of 20 μl of avertin/g body weight suffices, although less is preferred and possible if proficient in fallopian tube transfers; on the whole, 250–300 μl per average-sized female will do. To stop bleeding, epinephrin can be used (1).

8. An indicator for full sedation is the absence of a blinking reflex when blown (puffed) on the eyes softly. Care should be taken to exert only minimal peri-operative 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, etc.) at all times. We also record success rate of superovulations, natural matings, pseudomatings and oviduct transfers, litter sizes, and % 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) of DNA is fragmented by restriction endonucleases (REN) 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 (cat. # S0266; Sigma) 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 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 and 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 knock-out and transgenic mice and related topics is "TheTransgenic/Targeted Mutation DatabaseTBASE" 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 USA. Important information regarding the status of the mouse genome map, including available 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 by The European Collaborative Interspecific Mouse Backcross (EUCIB) consortium at http://www.hgmp.mrc.ac.uk/MBx/MBxHomepage.html.

13. Methods and procedures described are specifically used for blastocyst and ES cell manipulation, not for pronuclear injection and oviduct transfer.

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Chapter 3

Generation of Chimeras by Aggregation of Embryonic Stem Cells with Diploid or Tetraploid Mouse Embryos

Jérôme Artus and Anna-Katerina Hadjantonakis

Abstract

From the hybrid creatures of the Greek and Egyptian mythologies, the concept of the chimera has evolved and, in modern day biology, refers to an organism comprises of at least two populations of genetically distinct cells. Mouse chimeras have proven an invaluable tool for the generation of genetically modified strains. In addition, chimeras have been extensively used in developmental biology as a powerful tool to analyze the phenotype of specific mutations, to attribute function to gene products and to address the question of cell autonomy versus noncell autonomy of gene function. This chapter describes a simple and economical technique used to generate mouse chimeras by embryo aggregation. Multiple aggregation combinations are described each of which can be tailored to answer particular biological questions.

Key words: Mouse, Embryo, Chimera, Aggregation, Blastocyst, Morula, Development, ES cells, Diploid, Tetraploid, Developmental potential, Lineage contribution

1. Introduction

The mouse is the premier genetically tractable mammalian model organism with a sequenced genome and the ability to generate modifications at base pair resolution. The mouse is considered as a mammalian model not only to study human diseases, but also to understand the functions of genes under normal physiological conditions and during the embryonic development. So far, genome sequence annotation has revealed that the murine genome is composed of at least 30,000 genes. In the challenge to ascribe a function to each gene, a large collection of spontaneous and induced mutations have been generated. In the early 1980s, the development of the transgenesis technique by microinjection of DNA into zygotes contributed to the development of genetic modifications in mouse. However, this technique has some limitations

due to the random insertion of transgenes in the recipient genome. In 2007, the Nobel Prize of Medicine and Physiology was awarded to Mario Capecchi, Martin Evans, and Oliver Smithies for their contribution to the development of the targeted mutagenesis in the mouse by homologous recombination in embryonic stem (ES) cells. To date, the Mouse Genome Informatics database (http://www.informatics.jax.org) references 2,686 genes containing spontaneous and/or induced mutations, 3,757 genes targeted by transgenesis and 7,221 genes that have been targeted by targeted mutagenesis. Saturation mutagenesis of the mouse genome should be largely implemented within the next years due to large-scale mutagenesis efforts coordinated by the International Knockout Mouse Consortium (IKMC) (1). The IKMC coordinates the EUCOMM (*Eu*ropean *Co*nditional *Mo*use *Mu*tagenesis), KOMP (*K*nock*O*ut *M*ouse *P*roject), and NorCOMM (*Nor*th American *Co*nditional *Mo*use *Mu*tagenesis project) programs. It aims to generate a complete library of ES cells lines carrying null mutations in all genes. This is achieved using two complementary approaches based on gene-trap and gene-targeted mutagenesis. Such collection would provide a tremendous resource toward the goal of elucidating the functions of thousands of genes.

For decades, mouse embryologists have developed a series of embryo manipulation techniques, including the generation of chimeric mice, which are made by combining a recipient embryo with an additional cell component having a different genetic origin. The first mouse chimeras were been generated between two diploid morula (8-cell) stage embryos (2, 3). Later, chimeras were born from the injection of cells into the cavity of a blastocyst stage embryo (4).

To gain a solid grasp of the principles of the mouse, chimera production and interpretation, including the various combinations that can be set up by the aggregation method described in this chapter, a brief introduction to the mouse development is necessary. Early mammalian development is mainly devoted to the production of extraembryonic tissues that are necessary to sustain the embryo after implantation (see for reviews (5–8)). During the first cleavages, cells (referred to as blastomeres) remain totipotent, meaning they can give rise to all the embryonic and extraembryonic tissues. Totipotency is progressively lost as cleavages proceed. Cell fates are determined relatively late, at the blastocyst stage just prior to implantation. The blastocyst, comprises a fluid-filled cavity with a group of cells, the inner cell mass (ICM) at one end. The trophectoderm (TE), an epithelium surrounding the blastocelic cavity and the ICM which by the late blastocyst stage is composed of a single epithelial layer of primitive endoderm (PrE) at its surface lining the blastocele and surrounding the pluripotent epiblast (EPI) (Figure 1). This first three cell lineages possess restricted developmental potential. The EPI will
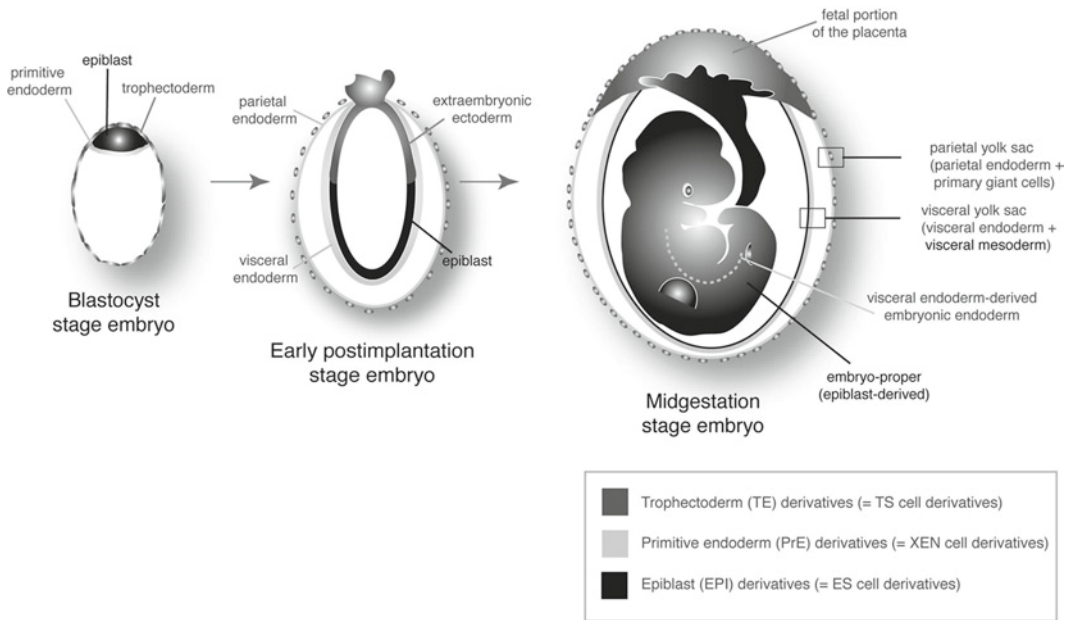
Fig. 1. Schematic representation of the emergence and lineage contribution of the first three cell lineages of the mammalian embryo. The TE, PrE, and EPI lineages that composed the blastocyst stage embryo at the end of the preimplantation period and their derivatives after implantation.

give rise to the embryo-proper and the extraembryonic mesoderm. The TE will give rise to the fetal portion of the placenta and the primary giant cells of the parietal yolk sac. The PrE will differentiate into the parietal endoderm that will contribute to the parietal yolk sac and the visceral endoderm that will form the visceral layer of the visceral yolk sac. Recent work has also suggested that derivatives of the VE might contribute to the gut endoderm of the fetus, the tissue that will give rise to the lining of the respiratory and digestive tracts and associated organs, such as lung, liver, and pancreas (9) (Figure 1).

The isolation and the characterization of ES cells, which are derived from and represent the epiblast lineage, have largely contributed to the development of new techniques in mouse embryology (10, 11). ES cells can be propagated and maintained indefinitely in defined culture conditions (12–15). In vitro, they can be directed to differentiate into derivatives of the epiblast lineage. When reintroduced into recipient morula or blastocyst stage embryos, they are able to contribute to the entire embryo in chimeras, including the germline (16–18). Interestingly, ES cells exhibit a restricted development potential that is limited to epiblast derivatives. In other words, they do not contribute to TE or PrE derivatives. This is also the case when ES cells are aggregated with 8-cell stage embryos. More recently, stem cells from the two extraembryonic lineages present at the blastocyst stage

have been isolated and characterized. Extraembryonic endoderm (XEN) cells represent the PrE (19), whereas trophoblast stem (TS) cells represent the TE (20). Like ES cells, these stem cells possess a restricted developmental potential that reflects their lineage of origin.

Two commons techniques are routinely used to generate chimeras from genetically modified ES cells: (1) the injection of ES cells into blastocyst embryo and (2) the aggregation between ES cell with early embryos. The first method consists of injecting cells into the blastocele cavity of a blastocyst stage embryo. This is achieved by micromanipulation, which requires expensive specialized equipment and is technically demanding. An advantage is the ability to readily screen cells to be injected based on morphological criteria. In the second aggregation method, ES cells clumps are aggregated with morula (uncompacted 8-cell) stage embryos. The latter method is relatively simple to master, and inexpensive to perform, as it only requires a stereo dissecting microscope. However, as clumps of cells are used, selection is less stringent. Even so, the aggregation technique facilitates the production of a large number of chimeras in a relative short period of time (Table 1 illustrates the timeline for an aggregation experiment).

Aggregation between ES cells and morulae, as well as the injection of ES cells into blastocyst embryos will result in a chimeric embryo composed of both ES cell and recipient embryo derivatives. However, extraembryonic tissues will be derived from the recipient embryo reflecting the restricted developmental potential and lineage contribution of ES cells which will not generate TE or PrE derivatives in chimeras. The contribution of ES cells to the embryonic compartment can be increased by the use of tetraploid embryos. Tetraploidy can be induced by different techniques (reviewed in (21)), including cleavage inhibition by cytochalasin B treatment (22, 23) and electrofusion of 2-cell stage embryos (24–26). Tetraploid embryos fail to progress to term (reviewed in (21)). When aggregated with ES cells, tetraploid cells have a developmental potential restricted to the TE and PrE lineages and thus contribute almost exclusively to extraembryonic tissues (24, 25). This polarized, but complementary, developmental potential of ES cells and tetraploid embryos has been experimentally exploited. In this way, the chimeric embryos and the animals generated by aggregation with, or injection into, tetraploid embryos are almost entirely ES cell-derived. This approach is considered the method of choice in animal cloning. Indeed, cloning from an ES cell intermediate is more efficient than from somatic cells. By combining tetraploid embryos with ES cells derived from a cloned blastocyst (referred as two-step nuclear transfer procedure), it is possible to readily generate cloned animals (26–28).

Table 1

Timeline for an aggregation chimera experiment. The exact timetable for ES cell preparation is determined empirically and will largely depend on their growth rate. For the aggregation experiments, animal matings are planned so as to generate 2.5 dpc embryos for diploid aggregation and 1.5 dpc for tetraploid aggregation. In addition, females mated with vasectomized males and will serve as recipients for aggregated chimeric embryos

	Day 1	Day 2	Day 3	Day 4	Day 5
1. ES cell	Thaw ES cell	Change medium	Plate w/o feeder	Prepare ES cell clumps	
2. Embryo	Set up matings	Check plugged females	Collect 2-cell stage (1.5 dpc) embryos → Electrofusion →	Collect 8-cell stage (2.5 dpc) embryos / Collect tetraploid 4-cell stage embryos	
3. Aggregation				Aggregation	
4. Embryo transfer		Set up matings	Check plugged females		Transfer aggregated embryos

Various combinations of diploid, tetraploid embryos, and ES cells can be set up to make aggregates which can be used as an assay to answer various biological questions, including the establishment of mouse strains from genetically modified ES cell lines, address the cell autonomy of a mutation, analyze the phenotype of a mutation in embryonic and/or extraembryonic tissues. These aspects are discussed at the end of this chapter.

2. Materials

2.1. ES Cell Culture

1. ES cell medium: High glucose Dulbecco's modified Eagle's media (D-MEM), 15% fetal bovine serum (FBS) ES cell culture and batch tested (see Note 1), 10^3 units/mL ESGRO® (LIF) (Chemicon) (see Note 2), 0.1 mM 2-mercaptoethanol, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin.

2. 0.25% Trypsin-EDTA.

3. PBS without Ca^{2+}, Mg^{2+}.

4. 0.1% (w/v) gelatin diluted into distilled H_2O and autoclaved to dissolve.

5. 35-, 60- and 100-mm sterile tissue culture dishes.

6. Humidified incubator, 5% CO_2 and 37°C.

2.2. Production and Isolation of 2-Cell and 8-Cell Stage Embryos

1. Stereo dissecting microscope with transmitted light source.

2. Flushing needle. 1 mL-syringe is connected by a 30 G needle with a sharp tip cut off and the resulting end rounded with a sharpening stone or sandpaper.

3. M2 medium (Millipore, Specialty Media).

4. KSOM medium (Millipore, Specialty Media).

M2 and KSOM media are usually aliquoted and stored at −20°C. When thawed, media should be kept at 4°C for no longer than 2 weeks. Before using, M2 and KSOM media should be incubated at 37°C. KSOM medium needs to be pH-equilibrated at 5% CO_2 and 37°C. M2 and KSOM can be alternatively prepared following the composition provided in Table 2. In this case, the media should be batch tested. For example, if 2-cell stage embryos are collected in M2 and put in KSOM medium, they should reach the blastocyst stage after 2–3 days in a tissue culture incubator.

5. Embryo culture dish. Put several ~5 μL-drops of KSOM medium into a 35-mm sterile culture dish and cover with embryo culture tested mineral oil (Sigma).

6. Mouth pipette. Amber natural rubber latex tubing (Fisherbrand) is connected at one extremity with a red mouth-piece (HPI hospital Products Med. Tech.). At the other one, a drawn outglass Pasteur pipette pulled on the flame is connected via a P1000 plastic pipette tip (see Note 3).

7. Humidified incubator, 5% CO_2 and 37°C. ES cells and embryos are cultured in separate incubators.

2.3. Preparation of Tetraploid Embryos by Electrofusion

1. 70% (v/v) ethanol.

2. Stereo dissecting microscope.

3. 0.3 M mannitol. D-Mannitol is prepared in water containing 0.3% BSA, filter-sterilized using a 0.22 μm Millipore filter, then aliquoted and stored at −20°C.

4. Mouth pipette.

5. CF-150/B pulse generator (BLS, http://www.bls-ltd.com) equipped with a GSS-250 electrode. Alternative models are available from BTX (http://www.btxonline.com/).

Table 2
Composition of M2 and KSOM media for ex utero mainte-nance of preimplantation mouse embryos

Component	Concentration (g/L)	
	KSOM	M2
NaCl	5.550	5.533
KCl	0.186	0.356
KH_2PO_4	0.048	0.162
$MgSO_4 \cdot 7H_2O$	0.049	0.293
Sodium lactate	1.12 or 1.87 of 60% syrup	2.61 or 4.349 of 60% syrup
D(+) glucose	0.036	1.000
Sodium pyruvate	0.022	0.036
$NaHCO_3$	2.100	0.349
$CaCl_2\ 2H_2O$	0.251	0.252
L-Glutamine	0.146	–
EDTA (tetrasodium salt)	0.0038	–
BSA	1.000	4.000
Penicillin-G	0.060	0.060
Streptomycinsulfate	0.050	0.050
Phenol red	–	0.010
HEPES buffer	–	4.969

2.4. Aggregation

2.4.1. Preparation of the Aggregation Plate

1. 35-mm sterile tissue culture dishes.
2. KSOM medium.
3. Embryo culture tested mineral oil.
4. 70% (v/v) ethanol.
5. Aggregation needle DN-09 (BLS, http://bls-ltd.com).
6. Humidified incubator, 5% CO_2 and 37°C.

2.4.2. Removal of the Zona Pellucida

1. Dissecting microscope.
2. Mouth pipette.
3. M2 and KSOM medium.
4. 100-mm sterile tissue culture dishes.
5. Acid Tyrode's solution (Sigma). Tyrode's solution can also be prepared using the following composition: 0.24 g/L $CaCl_2 \cdot 2H_2O$, 0.10 g/L $MgCl_2 \cdot 6H_2O$, 0.20 g/L KCl,

8.00 g/L NaCl, 1.00 g/L D-Glucose, 4.00 g/L Polyvinylpyrrolidone (PVP).

Adjust to a pH Equivalent of 2.5.

Filter-sterilize using a 0.22 μm Millipore filter, aliquot and store at –20°C.

3. Methods

3.1. Preparation of ES Cells for Aggregation

In the methods overviewed in this chapter, ES cells are used in combination with diploid or tetraploid embryos to generate chimeras using the aggregation method. Aggregation experiments require that ES cells retain their developmental potential and thus are pluripotent. Therefore, particular attention should be paid to the culture conditions and how ES cells are propagated. Any sudden changes in morphology should be noted and could serve as indicators of ES cell differentiation and loss of pluripotency. Detailed protocols for the optimum maintenance of ES cells in culture are beyond the scope of this chapter but can be found in various other book chapters (29, 30). As a general recommendation, we suggest the use of ES cell lines that have been propagated for a limited number of passages, are well characterized, come from a reliable source, and have been shown to retain pluripotency both by markers expression and/or contribution in chimeras. ES cell lines should be routinely karyotyped to assay that most of the cells retain a normal number of chromosomes. When possible, the gold standard to assay the competency of the ES cell line is to test its ability to contribute to the germline. ES cells should be cultured in fresh media (generally not more than 2 weeks) containing FBS that has been tested specifically on the ES cell lines used. ES cells exhibit a rapid proliferation and are usually passaged every 2 days. The daily observation of the cells under an inverted microscope should help to detect any sudden change of morphology and/or cell death, diagnostic features of cells that are differentiating.

Preparation of ES cells for aggregations requires the formation of small clumps of loosely connected cells, generally comprising 8–15 cells. The following protocol should be adapted to the ES cell line used.

3.1.1. Day 1

Thaw the ES cells on inactivated Mouse Embryo Fibroblast (MEF) feeder layer (see Note 4). We usually freeze ES cells at a density equivalent to 5×10^6 cells per cryovial and thaw a vial into a 60-mm culture dish.

3.1.2. Day 2

Change the medium.

3.1.3. Day 3

1. Remove the medium and wash the cells once with Ca^{2+}, Mg^{2+}-free PBS.

2. Add 1 mL of trypsin and incubate 5 min at 37°C.

3. Resuspend the cells by gently pipetting.

4. Add 4 mL of ES cell medium.

5. Incubate at 37°C for 15–20 min. By this time, MEF cells should be loosely attached to the dish while ES cells remain in suspension.

6. Gently collect all the medium containing the ES cells.

7. Spin at $111 \times G$ or 1,000 rpm for 5 min.

8. Plate at a 1:20 dilution on gelatinized dish (see Note 5).

3.1.4. Day 4
(Day of Aggregation)

1. Remove the medium and wash the cells once with Ca^{2+}, Mg^{2+}-free PBS.

2. Add a minimal amount of trypsin to cover the plate (0.5 mL for a 60-mm dish) and incubate at room temperature for 2–3 min. Watch under a microscope that the clumps of ES cells detached to the dish (Fig. 3c).

3. Inactivate the trypsin by adding 4 mL of ES cell medium. Cells are now ready to be aggregated with diploid or tetraploid embryos (see Note 6).

3.2. Production and Isolation of 2- and 8-Cell Stage Embryos

We preferentially use the outbred strain ICR to generate embryos for chimeras by aggregation, but other strains give good results, such as the outbred strain CD-1 or F1 hybrids $C57 \times CBA$ or $C57 \times DBA$. The advantages of these strains are the low costs and good yields of embryos after superovulation or spontaneous matings. We usually use spontaneous mating which gives rise to 8–12 embryos per ICR female.

3.2.1. Day 1

Set up matings by putting 2–3 females per stud male.

3.2.2. Day 2

Check the successful matings in the early morning by checking the presence of a vaginal plug.

3.2.3. Day 3

Collect the 2-cell stage embryos at 1.5 dpc for tetraploid embryos.

3.2.4. Day 4

Collect the 8-cell stage embryos at 2.5 dpc for diploid embryos. The procedure for collecting 2-cell and 8-cell stage embryos is essentially the same and consists of flushing the oviducts. Careful manipulation of the embryos is important in order to maintain them in the healthiest state. The time during which embryos stay at room temperature should be minimized. In addition, extended manipulations at room temperature may lead to evaporation of

the drops of media in which embryos are successively transferred and thus to a dramatic change in their chemical composition.

1. Collect the oviducts and transfer them individually into small drops of M2 medium.

2. Insert a flushing needle in the infundibulum and flush with M2 medium. Successful flushing is visualized by the expansion of the size of the oviduct and by the release of the embryos within the medium.

3. Collect the embryos with a mouth pipette and wash them by sequentially transferring them through several drops of M2 medium.

4. Transfer and wash the embryos into several drops of KSOM medium. Then, transfer them into an embryo culture dish and place it in the incubator.

3.3. Preparation of Tetraploid Embryos by Electrofusion

On day 3, tetraploid embryos are generated from 2-cell stage embryos fused by electrofusion. The electrofusion protocol is shown in Figure 2. Under optimal conditions, the rate of fusion should be around 80–90%.

1. Set up the electrode chamber.

    (a) Sterilize the electrode with 70% ethanol.

    (b) Place the electrode containing slide in a 100-mm tissue culture dish. Tape the slide to the dish so as to avoid it moving. The most recent series of electrodes available from BLS have specially designed clips for securing onto the dish.

    (c) Connect the cables to the pulse generator and adjust all the parameters. First, select the "non-electrolyte" position in the back of the generator. Using a CF-150/B pulse generator and an electrode with a 250 μm of gap, we routinely use two pulses ("repeat" button is set to 2) of 35 V ("amplitude" is set up to 35) and a 35 μs duration ("width" button is set to 35). The A/C voltage is set to 0.5–1 V (click first on button "enable" then you can set the voltage using button "amplitude", click again on button "enable" to shut off the A/C voltage). The pulse generator should be left on 1–2 h before the fusion.

    (d) Place two large drops of M2 medium and one drop of mannitol in the dish.

    (e) Cover the electrode with mannitol solution.

2. Transfer the Embryos to one drop of M2 Medium

3. From this step, work with no more than 10–15 embryos at a time. Place the embryos in the drop of mannitol.
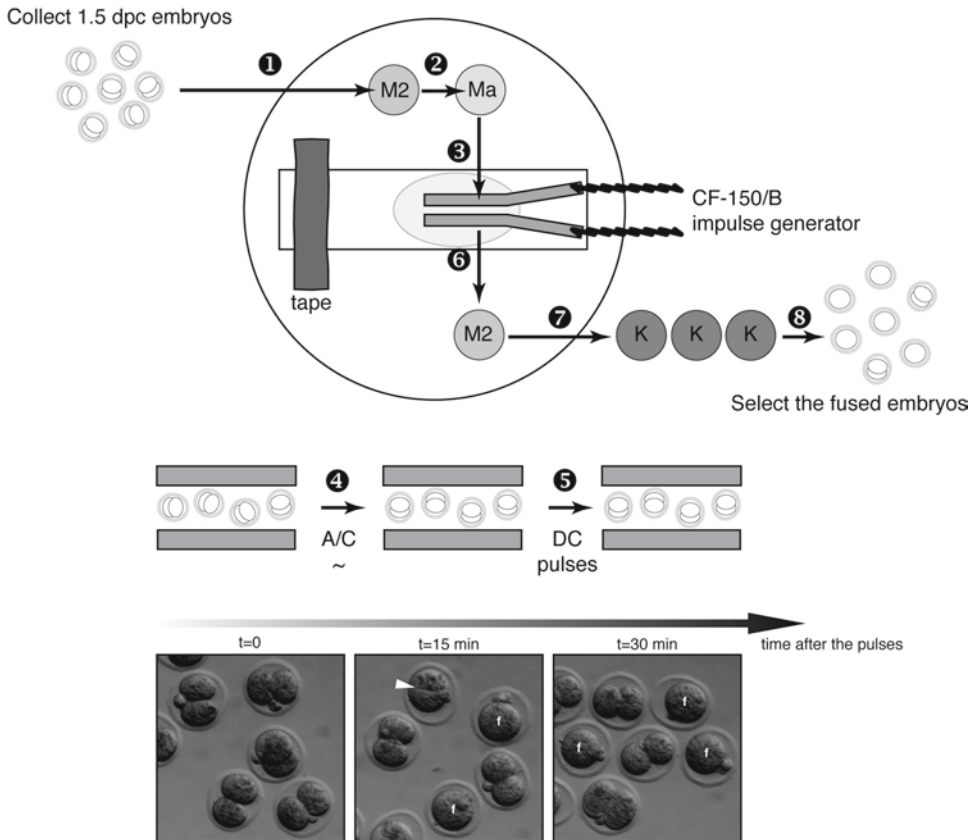
Fig. 2. The generation of tetraploid embryos by electrofusion. Two-cell stage embryos are transferred successively into (1) a drop of M2 medium and then (2) a drop of mannitol. Once the embryos sink to the bottom of the dish, they are transferred to the drop of mannitol placed on the slide and positioned (3) between the electrodes. (4) Embryos are oriented using an A/C voltage. (5) Pulses are then applied to induce cell fusion. (6) Embryos are immediately transferred into M2 medium, (7) successively washed into drops of KSOM medium and then (8) placed in an embryo culture dish and observed at regular intervals in order to select and isolate the embryos which successfully fused. The lower panel depicts a time series of 2-cell stage embryos after the electrofusion ($t=0$). Within 15 min ($t=15$ min), two embryos have fused (f); one embryo is undergoing the fusion between its two blastomeres (*white arrowhead*). After 30 min ($t=30$ min), three embryos fused. *f* fused embryos, *K* KSOM medium, *M2* M2 medium, and *Ma* mannitol.

4. Once they have settled to the bottom of the dish, transfer them between the electrodes.

5. Apply the A/C orientating electric field by clicking on the "enable" button. Embryos will orientate in such a way that the cleavage plane will be parallel to the electrodes. Correct manually the misaligned embryos using a mouth pipette. Click on the "enable" button to turn off the A/C (see Note 7).

6. When all the embryos are correctly oriented, apply the two pulses. A beep is heard after the pulses have been delivered.

7. Immediately transfer the embryos to the second drop of M2.

8. Repeat the steps 3–7 with the remaining embryos (see Note 8).

9. Wash the embryos through several drops of KSOM, transfer them into a culture dish containing KSOM drops under oil and return the dish to the incubator.

10. Carefully check the embryos every 10–15 min to monitor fusion. Move fused embryos into a new drop labeled "fused." It is crucial to follow the embryos that fused and separate them from the unfused embryos as the embryos will start to divide again and 2-cell embryos that have failed to fuse will be indistinguishable from 2-cell embryos that fused and then divided (see Note 9).

11. Culture embryos overnight.

12. On day 4, tetraploid embryos should contain three to four blastomeres.

3.4. Aggregation

3.4.1. Preparation of the Aggregation Plate

The aggregation plate, which consists of depressions that bring into contact the embryos and cells, is made to aggregate diploid or tetraploid embryos with ES cells. Alternatively, handing drops can be used. The aggregation plate is generally prepared on day 4 and put in the incubator for at least 1 h. Alternatively, the aggregation plate can be set up on day 3 and let in the incubator overnight. Preparation of the aggregation plate is illustrated in Figure 4.

1. Place four rows of ~3-mm drops of KSOM medium. Three drops are made on the first and fourth rows and serve as embryo reservoirs and to select and store the ES cell clumps. The second and third rows are composed of four to five drops in which the aggregations will be set up.

2. Cover the drops with mineral oil.

3. Sterilize the aggregation needle with 70% ethanol.

4. Make six to eight depressions per drop of the second and third rows. A depression is made by pressing the aggregation needle firmly into the plastic in a circular movement. The goal is to make a depression deep and large enough to receive an embryo. Size and shape of the depressions is important. Larger drops will affect the aggregation efficiency because components of the aggregate (e.g., embryos and cells) will not stay in place in close proximity (vibration, movement). Deeper drops will make the collection of the aggregated embryos on day 5 difficult.

5. Place the dish in the incubator for at least 1 h.

3.4.2. Removal of the Zona Pellucida

Since the cells and embryos need to mix, the zona pellucida that surrounds early embryos needs to be removed before the aggregation is set up. This step can be achieved by a chemical treatment using acid Tyrode's solution as illustrated in Figure 3.
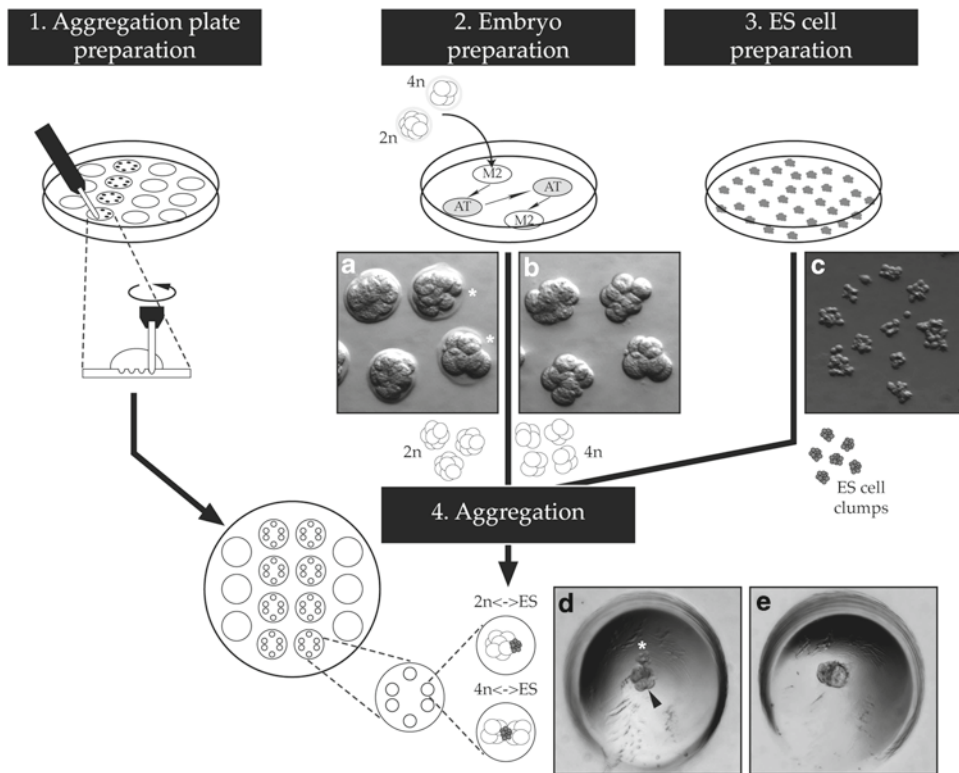
Fig. 3. The aggregation protocol. Prior to setting up the aggregates (4), it is necessary to prepare: (1) the aggregation plate, (2) the embryos, and (3) the ES cells. (1) The aggregation plate is made by placing four to five rows of drops of KSOM medium covered by mineral oil. In the middle rows, six to eight depressions are generating by applying a circular movement on the aggregation needle while pushing down on the bottom of the dish. (2) To facilitate aggregation of cells, the zona pellucida of embryos is removed using Acid Tyrode's (AT) solution from diploid ($2n$) 8-cell stage or tetraploid ($4n$) 4-cell stage embryos. (**a**) Eight-cell stage embryos in AT drop. Zona pellucida is starting to dissolve (asterisks). (**b**) After a couple of minutes, 8-cell stage embryos are stripped of their zona pellucida, are then rinsed into M2 medium, and are ready for aggregation. (3) ES cells are briefly trypsinized to generate small clumps containing 8–15 cells (**c**). (4) The aggregate is set up in the depressions by first placing one diploid embryo or two tetraploid embryos. Then, an ES cell clump is placed into the depression and care must be taken to make sure that it is in contact with the embryo(s). For tetraploid chimeras, the "sandwich" arrangement is commonly used and consists in placing an ES cell clump between two tetraploid embryos. (**d**) An ES cell clump (*asterisk*) is in contact with an 8-cell stage embryo (*black arrowhead*). (**e**) Aggregated embryo has reached the blastocyst stage 24 h after in culture. *2n* diploid, *4n* tetraploid, *ES* embryonic stem, *M2* M2 medium, *AT* acid Tyrode's.

1. Prepare in a 10-cm dish two large drops of M2 and two drops of acid Tyrode's solution.

2. Transfer the diploid or the tetraploid embryos in the first drop of M2.

3. Transfer five to ten embryos to the first drop of Tyrode's solution to wash off the M2. Transfer them immediately to the second drop of acid Tyrode's solution. It is important to avoid the transfer of too much M2 media in acid Tyrode's solution.

4. Gently move the embryos by pipetting them up and down using a mouth pipette. Do not let the embryos sit on the bottom of the dish as they may have a tendency to be sticky ones their zona is removed. Once the zona pellucida disappears (Fig. 3a, b), transfer them immediately into the second drop of M2 medium (see Note 10).

5. Repeat steps 3 and 4 with the remaining embryos.

6. Wash the embryos to several drops of KSOM and transfer them to the aggregation plate in a drop without depressions (embryo reservoir). Make sure the embryos are well spread out, and that there are not too many in any one drop, as if they are touching they may aggregate with one another (see Note 11).

3.4.3. Aggregation Between Diploid/Tetraploid Embryos and ES Cells

Key to the success of the aggregation experiment is the quality of embryos. Diploid embryos should be at the 8-cell to uncompacted morula stage. Tetraploid embryos should be at the 3- or 4-cell stage.

1. Zona-free embryos have been transferred to a drop without depression in the aggregation plate.

2. Carefully place individual diploid embryos in a depression. For aggregation with tetraploid embryo, place two embryos per depression.

3. Generate the ES cell clumps as described in the Subheading 3.1.

4. Select clumps containing 8–15 loosely connected cells and place them into a drop without depression.

5. Place an individual clump into a depression containing a single diploid embryo or two tetraploid embryos. It is important that the clump is in contact to the embryo. $4n \leftrightarrow ES$ aggregates are usually set up by "sandwiching" the ES cell clump between two tetraploid embryos as illustrated in Figure 3.

6. Carefully place the aggregation dish in the incubator overnight. It is important that the dish is not disturbed, as then the aggregates may become dismantled. If this occurs, the embryo will develop to a blastocyst, but it will not be chimeric.

It is also possible to generate aggregation between two diploid embryos or between one diploid embryo and two tetraploid embryos.

3.5. Transfer of Blastocyst Stage Embryos into Pseudopregnant Surrogate Females

On day 5, the embryos aggregated the day before should have developed to the blastocyst stage and can be transferred into 2.5 dpc pseudopregnant recipient females. Eight to ten embryos are usually transferred into each uterine horn. In cases where the number of embryos outnumbers the numbers of available recipients,

this number can be increased up to 15 if necessary. Alternatively, embryos can be transferred into the oviduct of 1.5 dpc or the uterus of 3.5 dpc pseudopregnant recipient females, though these are not as optimal as 2.5 dpc. A detailed procedure can be found in (30).

3.6. Analysis of Offspring

Depending on the biological question and the resources available, different combinations of aggregation may be set up. The different compositions that can be found upon aggregation are represented in Figure 4. Mutant ES cells can be used to answer various questions related to the embryo proper, as they do not contribute to the TE and PrE derivatives. Analysis will usually require the ability to distinguish the genetic contribution (mutant or wild type) to the various lineages. This may be achieved using various cell autonomous widely expressed reporters, such as *LacZ* gene or various spectrally distinct fluorescent protein-encoding genes (31, 32).

3.6.1. Aggregation 2n↔2n Embryos

Aggregation between wild type and mutant diploid embryos will result in chimeric embryos where mutant cells should contribute to all the lineages, unless they are restricted as a consequence of genetic modification (2, 3). Usually, 8-cell stage embryos from heterozygous mutant intercross are aggregated with wild type embryos. This combination may be used, for example, to determine whether a mutation is cell or noncell autonomous. In addition, the presence of wild type cells in the chimera may delay the onset of the mutant phenotype and thus help uncover a later effect of a mutation (33).
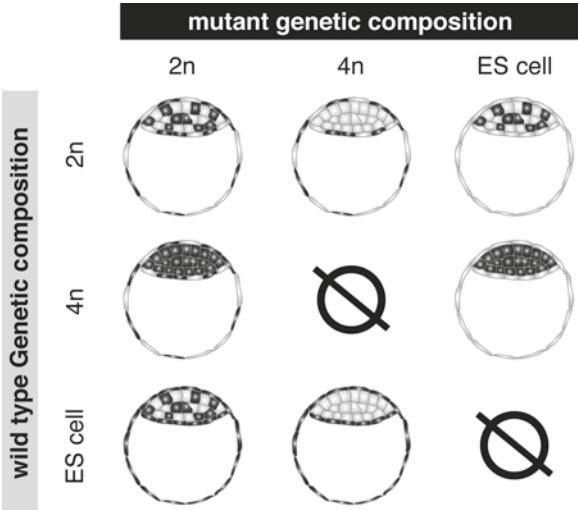

Fig. 4. Developmental potential and contribution to the three lineages of the blastocyst stage embryo. Depending on the combination used for aggregation (2n↔2n, 2n↔4n, 2n↔ES cell or 4n↔ES cell). 2n diploid embryos, 4n tetraploid embryos.

3.6.2. Aggregation 2n↔4n Embryos

In a chimera resulting from aggregation between wild type diploid embryos with tetraploid mutant embryos, mutant cells contribute only to the extraembryonic tissues derived from the TE and PrE. This composition can be used to analyze the extraembryonic phenotype of a mutation. Importantly, it has been observed in $4n↔ES$ cells chimeras that $4n$ cells can be found within the gut of midgestation embryo (34). Support from this observation is the recent demonstration that VE derivatives contribute to the gut endoderm of the fetus (9). Therefore, a special attention should be paid to the analysis of the phenotype of endoderm derivatives.

On the other hand, cells from mutant diploid embryos aggregated with wild type tetraploid embryos will contribute to all the lineages. This can be used to rescue defects in the extraembryonic tissues while the embryo will be derived from mutant cells (*35–39*).

3.6.3. Aggregation 2n↔ES Cells

In the combination generated between wild type ES cells and mutant embryos, the TE and PrE will be composed exclusively of mutant cells while the embryo will be chimeric (40). Mutant ES cells combined with wild type embryos will contribute to the embryo-proper (37, 41).

3.6.4. Aggregation 4n↔ES Cells

In this case, the segregation between wild type and mutant cell contributions will reflect the developmental potential of PrE/TE or epiblast derivatives. Indeed, $4n$ tetraploid cells will be restricted to the PrE and TE derivatives (23), whereas ES cells will contribute solely to epiblast derivatives. This combination is the easiest way to discriminate an embryonic versus extraembryonic phenotype (38, 42, 43). As mentioned in Subheading 3.6.2, $4n$ cells may end up to the gut endoderm derivatives of the embryo and thus attention must be paid to this possibility.

4. Notes

1. New batches of FBS are routinely tested to identify those providing the most undifferentiated ES cell cultures that maintain pluripotency. Briefly, 300 ES cells are plated onto a 60-mm gelatinized tissue culture dish without MEFs (see Note 5). At least two dishes (duplicates) are plated for each condition. ES cells are cultured in ES medium containing 10%, 20%, and 30% FBS, and the medium replaced every 2 days. 7–10 days later, colonies are counterstained with methylene blue or alkaline phosphatase. Serum quality is evaluated by determining the plating efficiency and by screening the morphology of ES cell colonies (a rounded vs. flatter colony shape reveals undifferentiated vs. differentiated cells).

2. Recombinant LIF protein is expensive. We routinely produce and test our own bacterially expressed recombinant protein as originally described in (44). Alternatively, conditioned media from COS cells stably expressing a LIF expressing vector can be used (45).

3. The diameter of the pulled Pasteur pipette should be a little greater than the diameter of the embryo. This gives better control of embryo handling and minimizes the transfer of media during the manipulation of embryos as compared to a pipetter with too wide a diameter, while a pipette with too narrow a diameter will damage the embryos.

4. Depending on the growth rate of the ES cell line being used, a 1/20 dilution may be not sufficient, or may be too great, a dilution. When setting up an aggregation experiment with a new cell line, it is best to titer the cells by preparing several gelatinized plates and plating ES cells at various dilutions ranging from 1/10 to 1/50.

5. Isolation and long-term culture of ES cells usually requires the presence of a monolayer of mitotically inactivated feeder (MEF) cells. Optimally, any given cell line should be maintained on the same type of feeder on it was established. MEF cells are primary cells isolated from mid- to late gestation (12–15 dpc) stage embryos. Mitotic inactivation can be achieved using mitomycin C treatment or γ irradiation. Detailed protocols for MEF isolation and feeder layer preparation are described in (32). Some ES cell lines can be "weaned" off MEFs, and cultured in the presence of LIF on gelatin-coated plates. This is preferable when preparing ES cells for an aggregation experiment as it circumvents the culture contamination with MEFs. However, the morphology of cells will often change and care must be taken to ensure that cells retain their developmental potential when maintained in the presence of LIF.

6. If the ES cell clumps contain more than 15 cells, it is possible to break them up, and therefore reduce their size, by pipetting. If clumps are too small, several clumps can be aggregated.

7. Too high an A/C voltage will cause cell lysis. Blastomeres will lyse either immediately or shortly after pulsing. If this is the case, try lowering the voltage.

8. During the fusion, exposure of the embryos to the mannitol solution must be kept to a minimum period of time. Replace the mannitol solution covering the electrode every 15 min as it tends to dry out, as seen by crystallization at the edges of the drop.

9. It is possible to apply a series of additional pulses to any 2-cell embryos that fail to fuse.

10. Identification and separation of the fused embryos is crucial to ensure that no diploid embryos are used in a tetraploid aggregation experiment; this could lead to a dramatic difference in experimental outcome and misinterpretation of the results.

11. Keep in mind that an extended period of time in Tyrode's solution will compromise the viability of the embryos. Embryos deprived of their zona pellucida are generally sticky and more fragile to manipulation. Avoid touching them directly with the pulled pipette and manipulate them by blowing the medium.

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Chapter 4

Cryopreservation of Mouse Spermatozoa and In Vitro Fertilization

Naomi Nakagata

Abstract

Cryopreservation of mouse spermatozoa has become the foremost technique for preserving large numbers of different strains of mice with induced mutations. Recently, we have established procedures for cryopreservation of mouse spermatozoa and in vitro fertilization using cryopreserved spermatozoa to obtain a relatively high fertilization rate. This chapter attempts to show these procedures in simple terms.

Key words: Cryopreservation, Mouse sperm, In vitro fertilization

1. Introduction

Over the past 20 years, a large number of mice with induced mutations (including transgenes, targeted mutations, and chemically induced mutations) have been produced worldwide (1–5). Moreover, knock-out projects have been started in Europe and USA over the last 2 or 3 years (6). As a result, the number of strains of mice with induced mutations has been rapidly expanding, and maintenance of these strains via standard breeding colonies is becoming a serious problem (7).

Embryo freezing is generally used for this purpose (8). However, it is not particularly economical to freeze embryos for each strain. This is because embryo freezing requires 500 embryos per strain, taken from the oviducts of 25–50 mated females or produced via in vitro fertilization (9). In contrast, 10,000,000–30,000,000 spermatozoa can be frozen immediately after collection from the epididymides of each male. If all frozen-thawed spermatozoa from a single male are used for in vitro fertilization, at least 500 oocytes will be fertilized (10). Therefore, sperm freezing

may provide a much simpler and economical alternative to embryo freezing (11).

The first reports of successful cryopreservation of mouse spermatozoa were published in 1990 (12–14). We were also subsequently successful in the cryopreservation of mouse spermatozoa using an improved method (15, 16).

Since that time, numerous researchers have reported successful mouse sperm cryopreservation using various procedures (17–24). Our group has published numerous papers in which the basic 18% raffinose, 3% skim milk cryopreservation technique has been refined and applied to a variety of different strains and transgenic stocks of mice (10, 25–29). In addition, groups of large-scale ENU mutagenesis screening studies have employed our cryopreservation technique to assess sperm cryopreservation for mouse mutant archiving and the rapid re-establishment of shelf stocks (30, 31).

In general, high fertilization rates are obtained for frozen spermatozoa of the CBA/JN and DBA/2N inbred strain, and some F1 hybid strains (32). On the contrary, the fertilization rate of C57BL/6 frozen spermatozoa remained very low (32), although this could be increased by in vitro fertilization with partial zona pellucida dissected or zona pellucida drilled oocytes (28, 33). C57BL/6 is a major inbred strain and its genetic background is well known. Furthermore, this strain is not only used for the production of transgenic mice, but is also applied as a back-cross for targeted mutant mice (34). Therefore, it was necessary to establish a cryopreservation method for C57BL/6 mouse spermatozoa that could maintain a high fertilizing ability after thawing (35).

Recently, we established a method of in vitro fertilization using preincubation medium containing methyl-beta-cyclodextrin, which yields a relatively high fertilizing ability (36, 37).

In this chapter, I shall restrict myself to a description of the detailed procedure routinely used for mouse sperm freezing and the reproductive technologies concerned with the sperm freezing in my laboratory.

2. Materials

2.1. Cryopreservation and Thawing of Mouse Sperm

2.1.1. Freezing

1. For the cryoprotective agent (CPA) (see Table 1 and Fig. 1): 20-ml disposable conical tube, tip (1,000 μl volume), 1.8-ml microcentrifuge tubes, high speed microcentrifuge, disposable filter unit (pore size 0.45 μm), 1-ml glass ampoules, and twin jet ampoule sealer.

2. For dissection of cauda epididymis: fine scissors, fine forceps, a pair of sharp-pointed forceps, sterile filter paper, 35-mm sterile plastic tissue culture dishes, and mineral oil.

Table 1
Composition of the cryoprotective agent

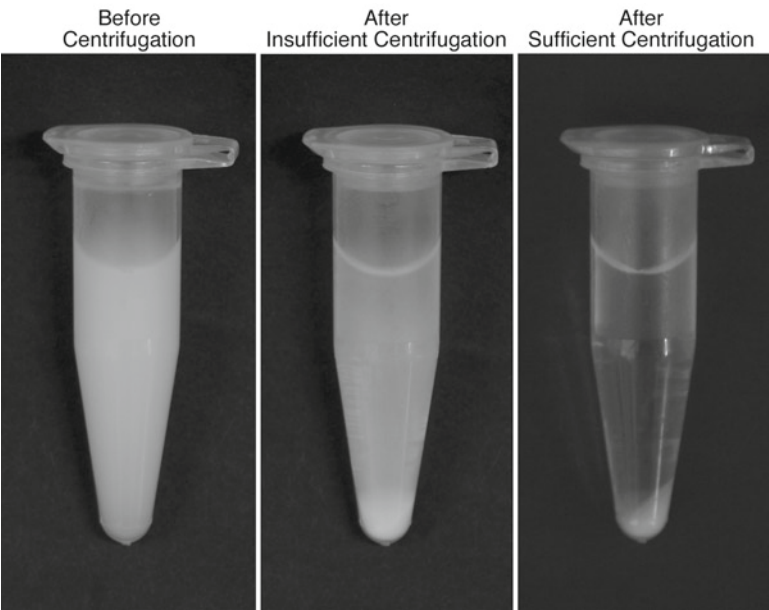| Reagent name	mg/100 ml	Vendor	Catalog number
Raffinose	1,800.0	Sigma	R-7630
Powdered skim milk	300.0	Difco	232100
Glutamine	146.1	Sigma	G-8540

Fig. 1. Appearance of CPA.

3. For sperm quality evaluation: HTF medium (see Table 2) (38), 35-mm sterile plastic tissue culture dishes, mineral oil, incubator (37°C, 5% CO_2), and tip (0.5–10 µl volume).

4. Equipment for sample preparation: micro-spring scissors (5-mm blade), a pair of sharp-pointed forceps, HTF medium, 1-ml syringe, silicone tube, 0.25-ml plastic straw (see Fig. 2), self-adhesive label, and impulse sealer.

5. For sample freezing: freezing canister (see Fig. 3).

6. For sample storage: triangle cassette and liquid nitrogen tank.

2.1.2. Thawing

1. For thawing: water bath (37°C), float for thawing samples (see Fig. 4), fine tissues, fine scissors, straw connector (see Fig. 5), preincubation medium (see Table 3), 35-mm sterile plastic tissue culture dishes, and mineral oil.

Table 2
Composition of HTF medium

Reagent name	mg/100 ml	Vendor	Catalog number
NaCl	593.8	Sigma	S-5886
KCl	35.0	Sigma	P-5405
$MgSO_4 \cdot 7H_2O$	4.9	Sigma	M-7774
KH_2PO_4	5.4	Sigma	P-5655
$CaCl_2$	57.0	Sigma	C-5670
$NaHCO_3$	210.0	Sigma	S-5761
Glucose	50.0	Sigma	G-6152
Na-lactate[a]	0.34 ml	Sigma	L-7900
Na-pyruvate	3.7	Sigma	P-4562
Penicillin G	7.5	Sigma	P-4687
Streptomycin	5.0	Sigma	S-1277
BSA	400	Sigma	A-4378
0.5% Phenol red	0.04 ml	Sigma	P-0290

[a]Assay: 70%
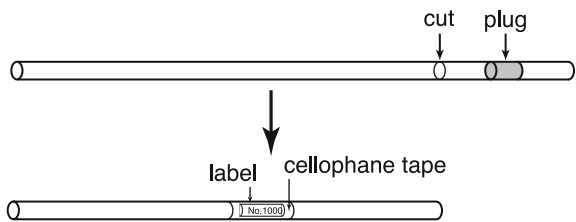
Fig. 2. Preparing the sample container. (1) Cut off the plug portion of the straw. (2) Apply a label with the mouse number printed on it using cellophane tape. (3) Prepare ten straws per mouse in the same manner.

2.2. In Vitro Fertilization

2.2.1. Superovulation

1. PMSG (1,000 IU/ampoule).
2. hCG (3,000 IU/ampoule).
3. Physiologic salt solution (0.9% NaCl).
4. For injection: 1-ml syringe and 27-gauge needle.

2.2.2. Oocytes Collection

1. For dissection: fine scissors, fine forceps, a pair of sharp-pointed forceps, and dissecting needles.
2. For collection: HTF medium, 35-mm sterile plastic tissue culture dishes, and mineral oil.

2.2.3. Fertilization and Embryo Handling

1. HTF medium.
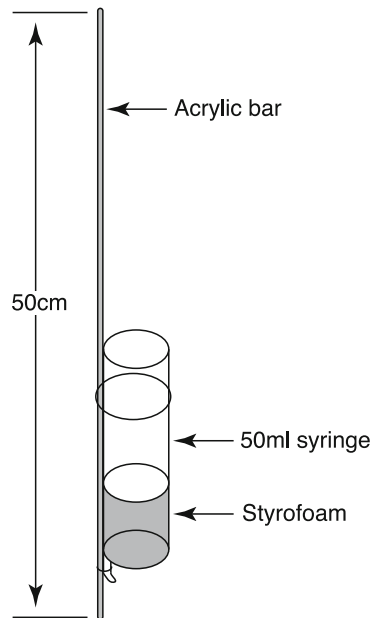2. 35-mm sterile plastic tissue culture dishes.

Fig. 3. Preparing the freezing canister. (1) Insert a piece of styrofoam tightly into the bottom of the syringe. (2) Heat-seal the outlet of the syringe tip. (3) Fix the syringe to the acrylic bar.
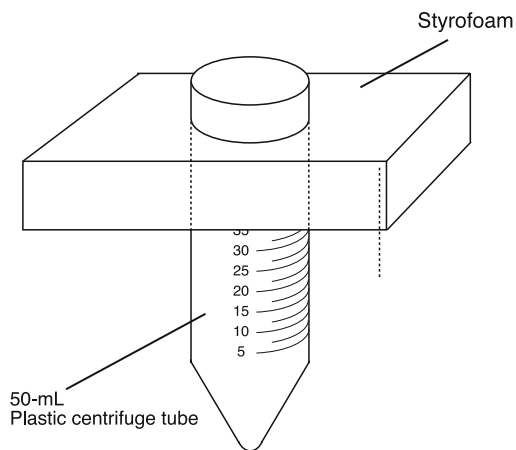

Fig. 4. Preparing the float for thawing samples. Using some styrofoam and a 50-ml plastic centrifuge tube, make the float as shown in the diagram.

3. Mineral oil.

4. Incubator (37°C, 5% CO_2).

5. Wedge-shaped pipette tip (1–200 µl).

2.3. Embryo Transfer

1. mWM medium (see Table 4) (39).

2. For dissection of the wall of the oviduct: a pair of sharp-pointed forceps and micro-spring scissors.
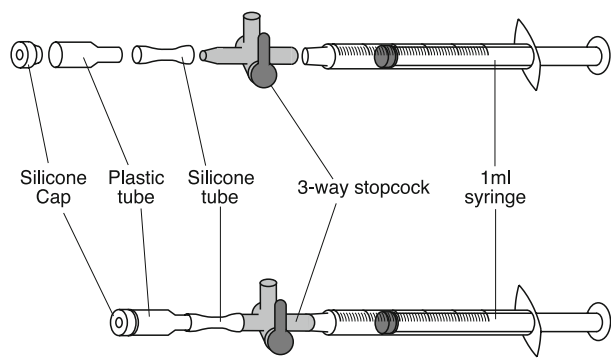
Fig. 5. Preparing the straw connecter. Connect a 1-ml syringe, a 3-way stopcock, a silicon tube, a plastic tube, and a silicone cap in the order presented in the diagram.

Table 3
Composition of the preincubation medium

Reagent name	mg/100 ml	Vendor	Catalog number
NaCl	697.6	Sigma	S-5886
KCl	35.6	Sigma	P-5405
$CaCl_2 \cdot 2H_2O$	25.1	Sigma	C-7902
Glucose (D+)	100.0	Sigma	G-6152
Soduim pyruvate	5.5	Sigma	P-4562
$MgSO_4 \cdot 7H_2O$	29.3	Sigma	M-7774
KH_2PO_4	16.2	Sigma	P-5655
$NaHCO_3$	210.6	Sigma	S-5761
Penicillin G K salt	7.5	Sigma	P-4687
Streptomycin sulfate	5.0	Sigma	S-1277
Methyl-beta-cyclodextrin	98.3	Sigma	C-4555
Polyvinylalcohol	100.0	Sigma	P-8136

3. Methods

3.1. Cryopreservation of Mouse Spermatozoa

3.1.1. Preparation of CPA (see Table 1)

1. Dissolve raffinose, skim milk, and glutamine in 10 ml of distilled water at 60°C in a 20-ml disposable tube.

2. Divide the solution into 1.5-ml aliquots in 1.8-ml microcentrifuge tubes.

3. Centrifuge the sample tubes at $10,000 \times g$ for 30 min at room temperature using a high speed microcentrifuge. (If the

Table 4
Composition of mWM medium

Reagent name	mg/100 ml	Vendor	Catalog number
NaCl	640.0	Sigma	S-5886
KCl	35.6	Sigma	P-5405
KH_2PO_4	16.2	Sigma	P-5655
$MgSO_4 \cdot 7H_2O$	29.4	Sigma	M-7774
$NaHCO_3$	190.0	Sigma	S-5761
Glucose	100.0	Sigma	G-6152
Na-pyruvate	2.5	Sigma	P-4562
Ca-lactate pentahydrate	46.0	Sigma	C-8356
Streptomycin	5.0	Sigma	S-1277
Penicillin	7.5	Sigma	P-4687
0.5% Phenol red	0.2 ml	Sigma	P-0290
20 mM 2-ME	10.0 µl	Sigma	M-7522
100 mM EDTA	50.0 µl	Sigma	E-6635
BSA	300.0	Sigma	A-4278

supernatant is not clear after centrifuging, then centrifuge again until the supernatant becomes clear (see Fig. 1).)

4. Filter the supernatant using a disposable filter unit, and use the filtrate as a CPA.

5. Store the filtered CPA in 0.5-ml aliquots in 1-ml glass ampoules at room temperature. (If the samples are kept in a refrigerator at 4°C for a long time, deposition in the samples separates out in the CPA. Be sure to keep the samples at room temperature.)

3.1.2. Collection of Spermatozoa

1. Kill a male mouse by cervical dislocation and remove the two cauda epididymides aseptically (see Fig. 6).

2. Place the epididymides on sterile filter paper to blot away blood and fluid.

3. Transfer the two cauda epididymides into 120 µl of CPA covered with mineral oil in a 35-mm sterile plastic tissue culture dish (see Fig. 7a).

4. Use a pair of sharp-pointed forceps and micro-spring scissors to make five or six incisions in the epididymides (see Fig. 7b),
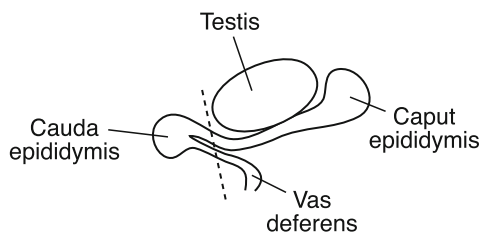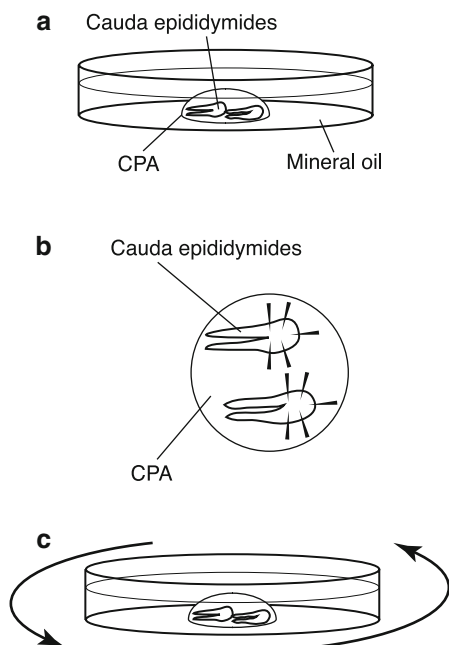
Fig. 6. Removing the cauda epididymidis.

Fig. 7. Preparing the sperm suspension.

and rotate the dish carefully ten times a minute to ensure uniform sperm suspension (see Fig. 7c).

5. After 3 min, remove the cauda epididymides from the well.

3.1.3. Sperm Quality Evaluation

1. Transfer 1 µl of sperm suspension into 100 µl of HTF medium covered with mineral oil in a dish. Place the dish in an incubator for 10 min to disperse the sperm suspension in the HTF.

2. Check the motility level of the collected spermatozoa.

3.1.4. Freezing the Spermatozoa

1. Connect a 1-ml syringe and a 0.25-ml plastic straw using a silicone tube. Carefully aspirate 100 µl of HTF medium, 10 mm of air, and 10 µl of the sperm suspension into the straw successively using a syringe, then add a further 10 mm of air. Seal both ends of the straw using an impulse sealer (see Fig. 8 and Note 1).
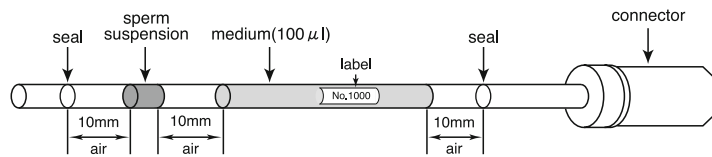
Fig. 8. Filling the sperm suspension into a sample container.
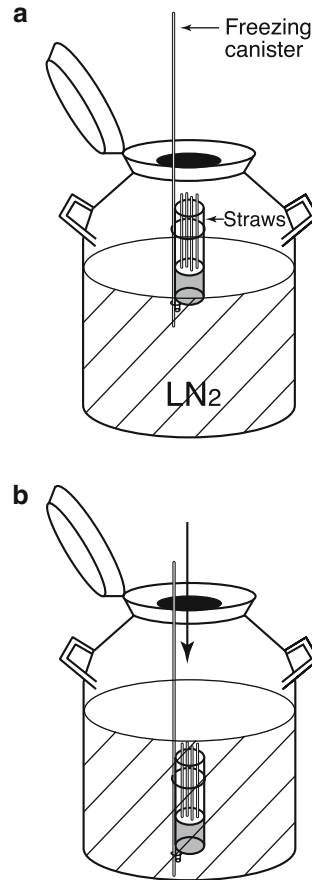

Fig. 9. Freezing the spermatozoa.

2. Create ten samples per mouse in the same manner.

3. Put the samples into a freezing canister and float them on top of liquid nitrogen in a liquid nitrogen tank (see Fig. 9a).

4. After 10 min, briefly immerse the freezing canister in the liquid nitrogen (see Fig. 9b).

5. Transfer the samples into a triangle cassette and store in the liquid nitrogen tank.

3.1.5. Thawing of Spermatozoa

1. Remove a frozen straw from the liquid nitrogen and hold it in the air for 5 s (see Fig. 10a).
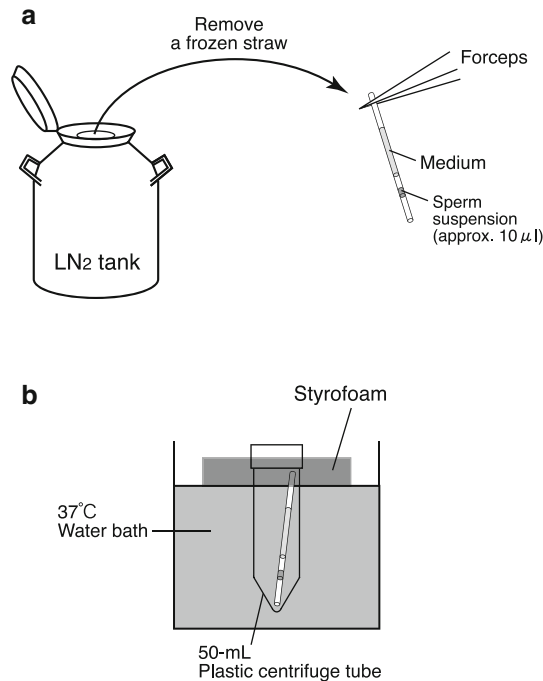
Fig. 10. Thawing the spermatozoa.

2. Immediately immerse the frozen straw in the float for thawing samples (in a water bath maintained at 37°C; see Fig. 10b) for 10 min (see Note 2).

3. After immersion, remove the straw from the float for thawing samples.

4. Use fine tissues to wipe any water from the straw.

5. Cut the sealed end of the straw furthest from the sperm and attach the cut end to the straw connector. Then cut the other sealed end from the straw (see Fig. 11).

6. Transfer only the thawed sperm suspension to a dish containing 90 μl of preincubation medium covered with mineral oil (equilibrated with 5% CO_2 in air for 30 min), and place the dish in an incubator for 30 min (see Fig. 12 and Note 3).

3.2. In Vitro Fertilization Using Cryopreserved Spermatozoa

3.2.1. Superovulation Procedure

1. Prepare the hormones: add 26.7 ml of 0.9% NaCl to PMSG and 80 ml of 0.9% NaCl to hCG and mix together (final concentration: 37.5 IU/ml).

2. Aliquot appropriate amounts of the hormones according to the number of females you shall inject; the aliquoted hormones can be stored for 3 weeks in a refrigerator (4°C).

3. Three days before in vitro fertilization, inject females (8–12 weeks of age) intraperitoneally with 7.5 IU (0.2 ml) of
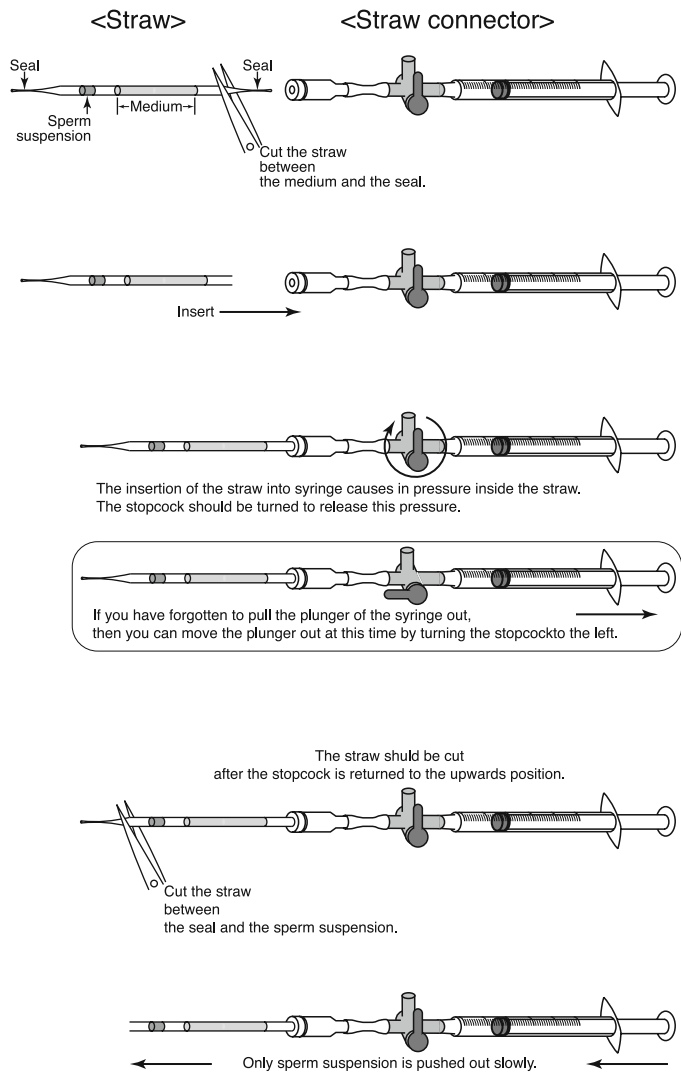
\<Straw\>   \<Straw connector\>

Seal   Seal

Sperm
suspension |←Medium→|

Cut the straw
between
the medium and the seal.

Insert →

The insertion of the straw into syringe causes in pressure inside the straw.
The stopcock should be turned to release this pressure.

If you have forgotten to pull the plunger of the syringe out,
then you can move the plunger out at this time by turning the stopcockto the left.

The straw shuld be cut
after the stopcock is returned to the upwards position.

Cut the straw
between
the seal and the sperm suspension.

Only sperm suspension is pushed out slowly.

Fig. 11. Details of sperm suspension extraction.
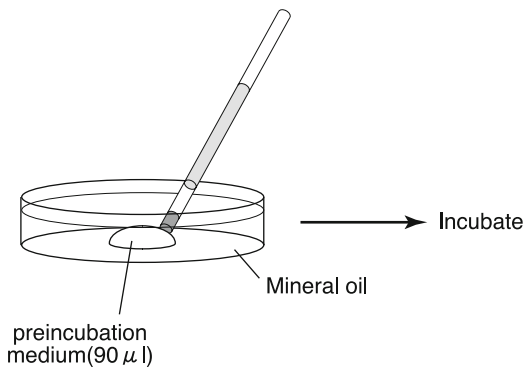
Incubate

Mineral oil

preincubation
medium(90 μl)

Fig. 12. Preincubation of sperm suspension.

PMSG (PMSG is usually administered at the middle of the light cycle, between 14:00 and 17:00 h).

4. One day before in vitro fertilization, inject the females intraperitoneally with 7.5 IU (0.2 ml) of hCG (hCG is to be administered at 17:00 h).

3.2.2. Oocytes Collection Procedure

1. Kill a mature female mouse in which superovulation has been induced approximately 15–17 h after administering hCG.

2. Remove the oviduct, avoiding as much fat, blood, and tissue fluid as possible.

3. Place the tissue on sterile filter paper to blot away blood and fluid.

4. Immerse the removed oviducts in mineral oil held in a fertilization dish.

5. Use forceps to hold the oviduct against the base of the fertilization dish. Use a dissecting needle to tear open the ampoule of the oviduct and release the cumulus-enclosed oocytes from the ampoule. Drag the oocytes into a drop of HTF (4–6 cumulus-enclosed oocytes per drop) (see Fig. 13 and Note 4).

3.2.3. In Vitro Fertilization Procedure

1. Using a wedge-shaped pipette tip, aspirate 10 μl of the preincubated sperm suspension from the edge of the drop (see Fig. 14a, b and Note 5). It is possible to aspirate 10 μl of sperm suspension three to four times per drop.

2. Add 10 μl of sperm to each drop of fertilizing HTF medium containing cumulus-enclosed oocytes.

3. Incubate the oocytes and spermatozoa for 5–6 h in an incubator.

4. After incubation, wash the fertilized oocytes three times in fresh HTF medium (100 μl) and culture overnight.

5. The obtained two-cell stage embryos can now be transferred into recipients.

3.2.4. Embryo Transfer Procedure

In our laboratory, we transfer two-cell embryos through the wall of the oviduct of pseudopregnant recipients (40). This procedure is much easier and simpler than the conventional procedure of embryo transfer (41) and is, therefore, suitable for inexperienced users.
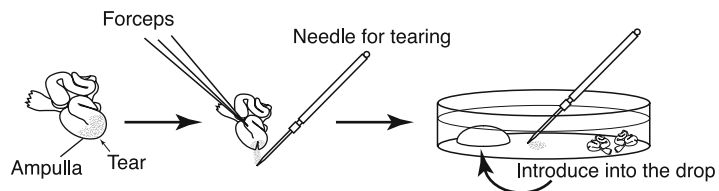
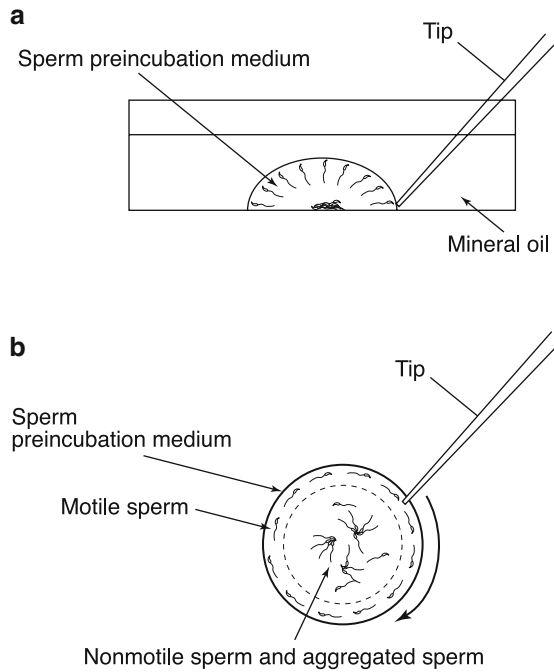

Fig. 13. Collecting the oocytes.

Fig. 14. Collecting the sperm suspension in the preincubation medium.

3.2.4.1. Preparing the Mouse

1. Anesthetize a female mouse.

2. Exteriorize the ovary, oviduct, and part of the uterine horn in accordance with the conventional procedure (41).

3.2.4.2. Preparing the Embryos and Glass Capillary

1. Make a 200-µl drop of mWM in a dish (without mineral oil) and introduce 20 embryos into the drop (see Fig. 15a).

2. Aspirate air and medium at alternative intervals of 2–3 mm into a glass capillary for use in embryo transfer (outer diameter: 200–250 µm). Draw ten embryos into the glass capillary (see Fig. 15b).

3.2.4.3. Embryo Transfer

1. Using a pair of sharp-pointed forceps and micro-spring scissors, dissect the wall of the oviduct between the infundibulum and ampoule.

2. Insert the tip of the capillary containing the embryos into the slit and then push the capillary further into the slit toward the ampoule (see Fig. 16a).

3. Use the forceps to hold the portion of oviduct into which the capillary was inserted.

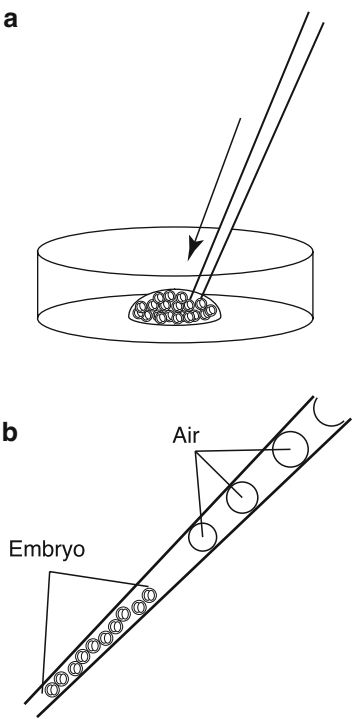4. Expel the embryos and two to three of the air bubbles into the ampoule (see Fig. 16b).

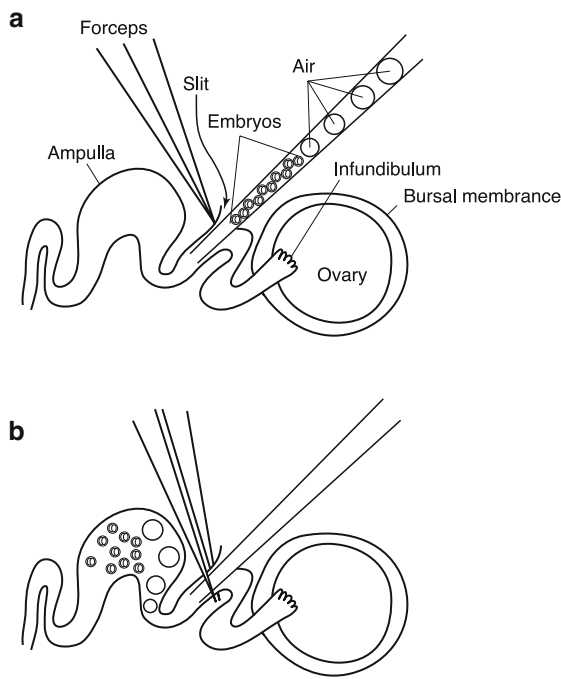Fig. 15. Aspiring the embryos into a glass capillary.

Fig. 16. Transferring the embryos into the oviduct.

Comment: If the embryo transfer is performed successfully, you should be able to see air bubbles through the wall of the ampoule.

5. Withdraw the capillary gently from the slit.

6. Bring the ovary, oviduct, and uterine horn back into the abdomen, and close the skin using wound clips.

7. Keep the mouse warm on a 37°C warming plate until it recovers from anesthesia.

4. Notes

1. Loading 100 µl of HTF medium into the straw prevents the straw from floating on the surface of liquid nitrogen, since the HTF medium acts as a weight that sinks the straw into the liquid nitrogen. Therefore, do not mix sperm suspension and 100 µl of HTF medium.

2. To ensure warming of the frozen sperm, completely immerse the part of the straw containing the sperm in the water bath. Frozen-thawed mouse spermatozoa are sensitive to environmental changes, and if the straw is not kept in the water bath long enough (10 min), the motility of the cryopreserved spermatozoa will be reduced.

3. Do not disturb the dishes containing cryopreserved spermatozoa until the spermatozoa are moving sufficiently within the medium. If the dishes are disturbed before the spermatozoa start to move, then the spermatozoa will not return to their full motility.

4. Be sure to carry out all operations, from sacrificing the female and removing her oviducts to introducing the oocytes masses into a drop of HTF medium, in the shortest time possible (within 30 seconds). Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

5. Spermatozoa with high motility have a tendency to gather near the edge of the drop. So, aspirate the sperm suspension gently from the periphery of the drop, avoiding nonmotile sperm and aggregated sperm.

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Chapter 5

Autopsy and Histological Analysis of the Transgenic Mouse

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Abstract

Over the past decades, transgenic and knock-out mouse models have become common use in research laboratories. Detailed phenotypic characterization of such models is essential for understanding basic mechanisms of normal physiology and disease. Hereto, pathological examination is a very helpful tool. This chapter describes detailed procedures to perform autopsy and immuno-histological analysis of mice.

Key words: Mouse models, Necroscopy, Immunohistochemistry, Pathology

1. Introduction

Often, transgenic and knock-out mice show phenotypes that differ from our expectations. Therefore, it is important to perform a complete pathological examination using both macroscopical and microscopical analyses. It should be noted that apart from (un) expected phenotypes caused directly by the expression of the transgene, the integration of the transgene may also silence genes, which may cause phenotypic changes. For analyzing these phenomena, it is valuable to do routine histological studies of the complete mouse, where all organs are examined in paraffin sections with a regular hematoxylin and eosin staining (see Note 1). If abnormalities are observed in tissues, immunohistochemistry can give extra information regarding the cell types involved.

This chapter describes protocols for autopsy of the mouse; decalcification of the bone; trimming, embedding, sectioning, and staining of the tissues; and a general protocol for immunohistochemistry.

2. Materials

**2.1. Autopsy
of the Mouse**

1. Scalpel.
2. Scissors (12 cm).
3. Forceps.
4. Pliers.
5. Pins.
6. Dissection board.
7. A 10-ml syringe with 25-G needle.
8. Cocher.
9. Spatula.
10. 70% Ethanol.
11. Fixative: 10% buffered formalin: 108 ml of 37% formaldehyde and 892 ml of 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**

Decalcification solution: 40 g NaOH, 827 ml of distilled water, 173 ml of formic acid (caution: dissolving NaOH releases heat).

**2.4. Staining
of the Slides**

1. Mayer's hematoxylin: Dissolve in 1 L of aqua dest. at 80°C, 50 g of potassium alum, 1 g of hematoxylin, 200 mg of sodium iodate, 25 g of chloralhydrate, and 500 mg of citric acid.
2. Eosin: 1% eosin Y (yellowish) in H_2O. Add a crystal of thymol to prevent the growth of molds.

**2.5. Immunohisto-
chemistry**

1. Mouse tissues.
2. 2-Methylbutane.
3. Tissue-Tek (Sakura).
4. Dry ice.
5. Cryomolds.
6. Cryo-microtome.
7. Slide storage box.
8. Silica gel.

9. Phosphate-buffered saline.

10. Dako Pen (DAKO).

11. Avidin/biotin block (Vector).

12. Biotinylated secondary antibody.

13. Fetal calf serum (FCS).

14. ABC Elite kit (Vector).

15. Glycergel (DAKO).

16. Dry acetone, either from a freshly opened bottle or treated (at least overnight) with Molecular Sieve beads (0.5 nm, Merck).

17. Amino-Propyl-triEthoxy-Silane (APES)-coated slides: Clean slides thoroughly (using DECON or other soap). Rinse with tap water and then with distilled water. Dry at 37°C. Dip the slides for 10 s in APES solution in acetone. Dip the slides five 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 2).

Additional information can be found online (1, 2).

1. Kill the mouse by CO_2 asphyxation (see Note 3). In order to avoid autolysis, necropsy must be carried out 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 general condition of the mouse: state of nutrition and development of the skeletal muscular masses, and the presence of skin alterations, fur, and superficial lesions. Spray the fur with 70% ethanol.

3. Perform a median longitudinal cut superiorly to the jaw, taking care to separate the skin from the underlying musculature accurately. Now the superficial cervical (above the salivary glands), axillary (present in the axillary fossa), brachial (in proximity to the angle of the scapula), and the inguinal (close to the bifurcation of the superficial epigastric vein) lymph nodes are visible. They are greyish and small bean shaped. Dissect these first and use a small container for their collection.

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 first page of pathology card. For describing gross findings, on the other side, a table containing the following tissues should be present: 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 gall bladder, 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, and others), Peyer's patches, thymus, skeleton, bone marrow, joints, nose, trachea/larynx, thyroid/parathyroid, brain/meninges, spinal cord, pituitary, epiphysis, ears, and eyes.

4. Take a part of the skin with mammary glands.

5. Remove the salivary glands (ventral cervical region).

6. In the males, isolate the preputial glands, lying above the penis in the connective tissue, which are leaf shaped and of yellowish color.

7. Once the observation of the superficial organs is over, proceed with the examination of the inner cavities of the mouse, starting from the abdomen, then the thorax, and finally the skull.

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-reddish. In order to remove the liver, cut the falciform and coronary ligaments that keep the organ intimately connected with the diaphragm. The gall bladder is visible on the inferior surface of the organ where it appears as a small bag of 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, has a dark-red color, and has a lengthened, oval, slightly curved shape. Take into account volume, consistency, color, margins, and any evident lesions. During the extraction, the pancreas can also be easily removed due to its intimate connection with the spleen. The pancreas is not a compact organ and 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 to the white mesentery.

4. With just one movement, 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 intestines. The small intestine is divided in duodenum, jejunum, and ileum. The duodenum starts from the stomach and is approximately a quarter of the total length, the next part (approximately half of the small intestine) is the jejunum, and the last quart 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 nodule 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, and transfer 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, of opaque pale color 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 adhered to fat pads. Remove the testes together with the epididymis by pulling the fat pats, so that 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, the oviducts, and the ovaries. Remove the whole female genital apparatus by cutting the vagina near the anal opening and lifting it with forceps. From here, proceeding upwards, the mesometrium ligaments, that fix the organs to the posterior wall of the abdomen, are cut 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, parietal pleura and the pericardium are finally separated. 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 the tongue up with forceps and remove all the thoracic organs with scissors until 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, while in the adult, the thymus is atrophic and macroscopically difficult to detect. The lungs are two large organs and 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 approximately 3 ml of formalin in the esophagus using a syringe with 25-G needle until the lungs are nicely blown up and close the esophagus with the cocher. 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. The opening of the cranial cavity

is carried out 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, the skull is removed, and 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 the 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.

All tissues are fixed 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 is: about 3 mm (3).

The following terms are used for the determination of the trimming directions:

*Longitudinal: in the direction of the length axis of the body, an organ or part of an organ.

*Transverse: across the length axis of an organ or part of an organ.

*Vertical: in the direction of the dorsoventral axis of an organ or part of an organ.

*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 stroke in the inguinal region close to the nipples is proposed for the 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 whole organ because of their small size. It is important that a section is taken from the middle area of the lymph node in order 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 glands. Furthermore, the cervical superficial lymph nodes are present immediately above the submandibular salivary glands. The three salivary glands and the mandibular lymphatic center, which consist of two or three lymph nodes, are embedded together. Remove, if necessary, longitudinally a small piece of the salivary gland.

3.2.4. Preputial Gland

Embed the whole preputial gland.

3.2.5. Liver and Gall Bladder

Make a transverse section through the left lateral lobe, the gall bladder, and the right lateral lobe.

3.2.6. Spleen

A transverse section is made through the middle of the organ. This plane of section guarantees the presence of all anatomical structures of this organ.

3.2.7. Pancreas

Embed the whole pancreas.

3.2.8. Small and Large Intestines

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 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 histological evaluation of a relatively large area of tissue that includes both renal poles. This is advantageous for the 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 for 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: While in most of the urinary bladder the urothelium is of ectodermal 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 the 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 the part of the uterus, cervix, and part of the vagina.

3.2.16. Thyroid

One transverse section through both lobes of the thyroid gland, including parathyroid gland with the underlying trachea and esophagus, is required. To consistently include the parathyroid, 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 auricles as well as through 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 optic chiasma including the basal ganglia, septum, cortex, and anterior hypothalamus; the second section at the level of hippocampus containing the cortex and brain stem at the transition of diencephalon to mesencephalon; and the third section containing the cerebellum and pons in transition to medulla oblongata.

3.2.21. Pituitary

Remove the pituitary out of the skull. The pituitary consists of three portions: 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, and
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 upper cervical segment
2. Thoracic cord at mid-thoracic 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, 1½ h.
7. 100% alcohol, 1½ h.
8. 100% alcohol, 1½ h.
9. Toluene, 1 h.
10. Toluene, 1 h.
11. Paraffin, 1 h, 60°C.
12. Paraffin, 60°C, until removing the tissues, but remove the tissues the same day.
13. Embed the tissues in a cassette (see Notes 4 and 5).
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 and blue 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.

On completion of the staining, the section is usually permanently sealed under a thin glass coverslip, the mountant being a natural resin or a synthetic one, almost invariably not mixable with water or ethanol, clear and mount.
Results: Nuclei – blue. Other tissue components – shades of red and pink.

3.6. Immunohisto-chemistry

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 Preparing 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 with the right plane.
3. Work quickly.
4. Put the tissues to be combined in a cryomold appropriate for your particular cryo-microtome.
5. Cover the tissues in the cryomold with Tissue-Tek, making sure not to enclose any air bubbles and that the tissues do not touch each other.
6. Submerge the mold in the pre-cooled 2-methylbutane.
7. 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 cryo-microtome.
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 30 min under a fan 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 or keep at room temperature for a maximum of 2 weeks.

3.6.3. Immunostaining

(All the following steps are performed at room temperature.)

1. Thaw slides (at least for 30 min at room temperature, outside the storage box).
2. Fix the sections in dry acetone for 10 min.
3. Remove from acetone, circle the sections with Dako Pen, and dry for 15 min under a fan.
4. Wash in PBS, 10 min.
5. Incubate the sections with Avidin block (1:5)/4% FCS in PBS, 15 min.
6. Remove the block solution (from step 5) and replace with primary antibody, diluted in 4% FCS/Biotin block (1:5) in PBS, 60 min.
7. Wash in PBS, 3× 1 min.
8. Incubate the sections with biotinylated secondary antibody (see Note 6) in PBS/4% FCS (optionally with 2% normal mouse serum, stand for 10 min before adding to section), (see Note 7), 30 min.
9. Wash in PBS, 3× 1 min.
10. Incubate the sections with ABC solution (made 30 min before use according to the manufacturer's instructions), 30 min.
11. Wash in PBS, 3× 1 min.
12. Develop with AEC according to the manufacturer's instructions.
13. Wash in PBS, followed by distilled water.
14. Counterstain with hematoxylin for 10 s, blue in tap water, and coverslip with Faramount.

4. Notes

1. Useful books for pathological evaluation are: *Ward, J. M. Mahler, J. F., Maronpot, R. R. and Sundberg, J. P. (2000) Pathobiology of the Genetically Engineered Mice. Ames, Iowa, Iowa state university press* and *Maronpot, R. R. (1999) Pathology of the mouse. Cache River Press.*

2. Do not transfer small organs such as lymph nodes together with other organs in 10% formalin but transfer them to a small container.

3. Do not kill the mice by cervical dislocation, since this will cause hemorrhages in the thoracic cavity.

4. Embed the small organs, such as lymph nodes, ovaries, and adrenals together and embed the skin separately.

5. Embed more organs together in one cassette to save time with sectioning.

6. We obtained very good results, with low backgrounds with secondary antibodies raised in donkey.

7. If the primary antibodies are rat monoclonals, some background may occur through cross-reaction of the secondary antibody. To overcome this, pre-incubation of the diluted secondary antibody with 2% normal mouse serum for 10 min may be tried.

References

1. http://www3.niaid.nih.gov/labs/aboutlabs/cmb/InfectiousDiseasePathogenesisSection/mouseNecropsy/. Information on autopsy of the mouse.

2. http://icg.cpmc.columbia.edu/cattoretti/Protocol/MousePathology/mainPageMousePath2.html. Information on autopsy of the mouse.

3. http://reni.item.fraunhofer.de/reni/trimming/index.php. Information on the trimming procedure of the tissues of the mouse.

Chapter 6

Transgene Design

Bart van de Sluis and Jan Willem Voncken

Abstract

Transgenics are powerful mouse models to understand the biological functions of genes. This chapter gives a short overview of the requirements and considerations in designing a transgene. In addition, potential important choices that have to be made in advance for the successful designing and generating a transgenic mouse model are discussed. Methods for DNA purification for microinjection are also provided in this chapter.

Key words: Transgenic, Gene expression, Promoter, DNA construct

1. Introduction

The application of transgenesis has increased exponentially since its introduction in the early 1980s and 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 are both staggering and exciting at the same time. An attempt to give a fully comprehensive overview of all variations in transgene design described in the scientific press would be an illusion and exceed the aim of this chapter. Nevertheless, the strategy to generate 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 biological properties of the transgene (see Subheading 1.1). Secondly, 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 (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; Chapter 9). 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 the purification of large DNA segments, the reader is referred to Chapter 9.

1.1. Origin of the Transgene

In considering transgenic technology, the choice of origin of the transgene, i.e., the species the transgene originates from, is an important one. The origin of a transgene may range from prokaryotes (e.g., reporter genes such as ß-galactosidase) to worms or flies and higher eukaryotes like man. 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 study structure–function relationships in the context of common (mouse) alleles. Second, despite structural divergence, most genes have been well conserved between mouse and man. This may become an important issue when, for instance, the biological 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 homolog, 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 for expressional analysis; such analyses would require quantitation at the DNA and mRNA level, respectively. Structural differences between human and mouse genes make it possible to screen for transgeneity relatively easy. Primary sequence differences, often concentrated in noncoding regions (introns), provide a convenient way to discern 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. Alternatively, discrimination of the transgene-encoded protein can be easily accomplished by adding an epitope to the proteins' amino acid sequence, such as a synthetic Flag-, multiple Histidine (His), protein-derived tags (i.e., Hemagglutinin (HA) , c-myc) or virus-based tag (i.e., V5, PY – polyoma). In addition, follow-up studies such as immunoprecipitation, immunohistochemistry, flow cytometry, chromatin immunoprecipitation (ChIP assay) and protein purification, may significantly benefit from such tags, in case the availability of immunological tools against the transgene product is limiting. Importantly, the effect of tag addition should be evaluated in vitro prior to embarking on in vivo experiments, as the addition of small peptide tags may affect protein function in vivo.

In summary, there is a wide choice in the 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. In addition, an alternative approach to generate transgenic mice is discussed in Chapter 10.

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 need not be preserved

in its entirety though. 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 (see 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 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 spliceosomes 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 regions (MARs) (see Subheading 2.2) which direct transgene expression in a position-independent or cell type-specific fashion (9–17).
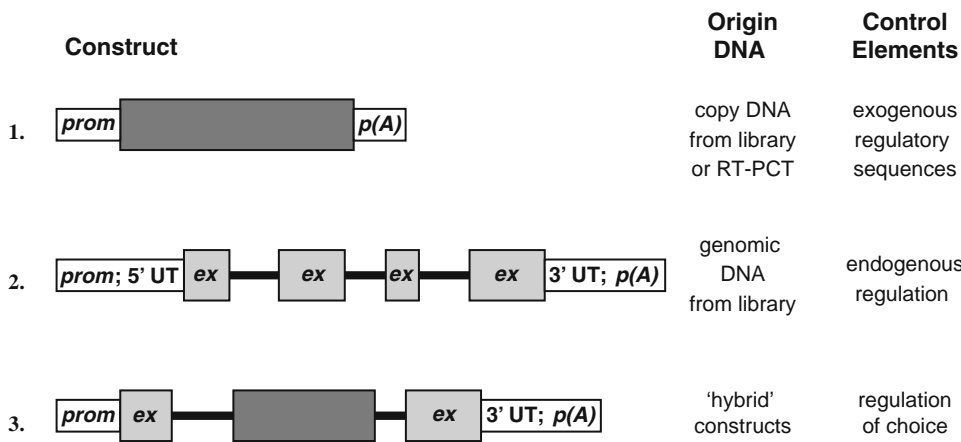
Fig. 1. Schematic overview of basic construct design. The basis of the above scheme is the use of eukaryotic coding sequences. The origin of the coding sequences is indicated in the figure. Since regulatory regions are usually not cloned along with cDNA, these have to be provided "separately" and are 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, the *thin black lines* represent introns (2 and 3).

Regulatory Sequences

1.	_Nature Control Elements:_	Autologous Heterologous
2.	_Expression Profile:_	Ubiquitous Tissue Restricted
3.	_Expression Control:_	Constitutive Inducible

Fig. 2. Regulatory sequences in transgene design. Depending on the nature of the animal model and its specific application, there are numerous choices in as far as the 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.

**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 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 the 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 physiological levels, often with a cell lineage-specific enhancer activity. The application of LCRs in transgenesis is discussed in detail elsewhere (_reviewed in_ (15)). The advantage of including such elements in transgenes is obvious: whereas transgenes with "minimal" promoters may become inactive by the 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 regulatory element within a transgene, there is obvious advantage in 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., which 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 there may be many kb 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, a number of modern cloning techniques have overcome this hurdle: one needs to resort to cloning systems employing P1 artificial chromosomes (PAC), yeast artificial chromosomes (YAC), or bacterial artificial chromosomes (BAC) (see Chapter 9). In addition, other cloning methodologies have been described to generate plasmids for biotechnology purposes, such as recombineering (see Chapter 11). 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 used to generate transgenic mice (see Chapter 9). 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 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, 26–28). 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 bacterial endotoxin (LPS) (29, 30).
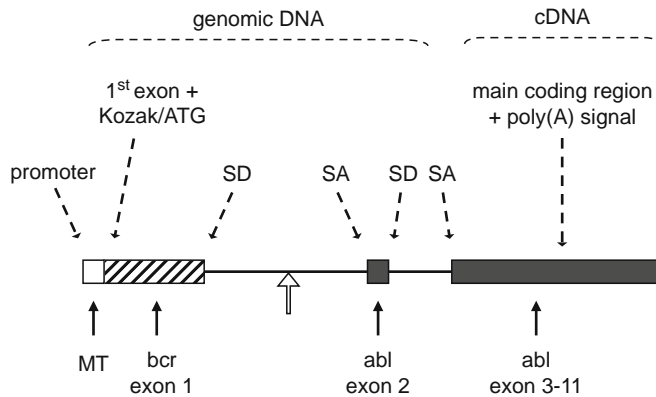
Fig. 3. A textbook example of transgene design. The transgene depicted was constructed to study the development of Philadelphia-positive childhood leukemia in a mouse model. The Philadelphia chromosome, the hallmark of this clinical type of leukemia, results from a reciprocal translocation between chromosomes 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, provide a splice donor (SD) and a splice acceptor (SA) site and preserve 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. Above illustration is adapted after (25).

The use of heterologous promoters and other regulatory elements has become wide spread, and as indicated before, is determined primarily by the aim of the animal model (see Fig. 2). To ensure global and ubiquitous expression of a particular transgene, general-type promoters, such as those derived from *histones, ß-actin,* or housekeeping genes (e.g., *phosphoglycerate kinase* (*PGK*)), 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 proven to function in vivo, or thoroughly test the novel system 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 use in a transgenic animal model.

If tissue-restricted expression patterns are required, specific promoters that confer this selectivity are chosen. In order to determine the minimal requirements for tissue-restricted expression of a particular promoter, it 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 *ß-galactosidase*, *CAT*, *luciferase*, or *GFP*. This approach is standardly used to examine spatio-temporal 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 (31–38). A number of exciting applications of transgenic technology are described in Chapters 9, 12, and 19.

2. Materials

2.1. Transgene Release, DNA Preparation

1. Qiagen Gel Extraction kit.
2. Restriction Endonuclease(s) of choice.
3. Spermidine.
4. Ethidium bromide (10,000× stock = 5 mg/ml in sterile water).
5. Electrophoresis buffer: TAE: 0.04 M Tris-acetate, 1 mM EDTA or TBE: 0.09 M Tris-borate, pH 8.0, 2 mM EDTA.
6. Agarose.
7. Microinjection buffer: 10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA (filter (0.2 μm) sterilized).

2.2. Laboratory Equipment

1. Thermoblock, adjustable temperature control.
2. Microcentrifuge capable of $12,000 \times g$.
3. Electrophoresis equipment.
4. Spectrophotometer.

Optional

5. Elutip-D minicolumns (Schleicher & Schuell).
6. 3 ml and 5 ml polypropylene syringes.
7. Disposable filters (Schleicher & Schuell; Uniflo 0.45 μ).
8. 1 M Tris–HCl pH 7.4.
9. 0.5 M EDTA, pH 8.0.
10. 5 M Sodium chloride, TC (tissue culture) grade.
11. Elutip buff. I: 20 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.2 M NaCl TC-grade.
12. Elutip buff. II: 20 mM Tris–HCl pH 7.4, 1 mM EDTA, 1.0 M NaCl TC-grade.
13. Ice cold 100% EtOH, 70% EtOH.

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 2). Depending on the materials present on location, several methods can be pursued to extract the linearized transgene from agarose gels. Commercially available agarose gel extraction kit, such Qiagen gel extraction kit, may be used. Although optional, some laboratories apply Elutip-D columns for subsequent purification of DNA for microinjection, with very satisfactory and consistent results (see Subheading 3.1.4; Note 8).

3.1.1. Transgene Release

1. For regular-sized transgenes, 15 μg 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 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.

3. Transgene and vector sequences are separated by electrophoresis. Fragments are excised from agarose gels (see Note 7). DNA isolation is carried out according to manufacturer's instructions. Elute DNA with microinjection buffer. Optionally perform an additional DNA purification (see Subheading 3.2).

3.2. DNA Purification (see Note 6)

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$_2$O.

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 (drop wise).

6. Wash both filter and column with 2–3 ml buffer I; remove buffer completely (i.e., flush air through) 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 100% Ethanol; *no extra Sodium acetate is added, no coprecipitants are added* (see Note 9).

10. Precipitate at –20°C, overnight, or 15–20 min on dry ice.

11. Pellet-precipitated DNA at maximum speed for 30 min, 4°C.

12. Wash pellet twice with 70% Ethanol.

13. Remove Ethanol with drawn-out Pasteur pipet and air dry briefly.

14. Dissolve pellet in 50 µl filtered (0.2 µm) *special TE buffer for microinjection*: 10 mM Tris–HCl, pH 7.4, 0.1 mM 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., 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 (39–41). Although the exact mechanisms by which (trans) genes are transcriptionally silenced are not clear on all aspects, 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 biological 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 (42–44), 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 (29).

4. An inserted transgene may affect the expression of (nearby) endogenous genes, which influences the phenotype in an unforeseen manner. Transgene insertion may cause haploinsufficiency, 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 standardly done for ES cell-mediated genetic manipulation in mice (see Chapters 10 and 12).

5. Although without doubt more involving than conventional transgenesis, it is possible to study the behavior of recessive mutations in mice, by overexpression at supra-physiological 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 10, 12, and 15) for the endogenous gene (19).

6. In our experience, the slightest impurities will have a serious impact on the efficiency with which transgenic founders are generated. Often times, residual Ethidium Bromide is a source of trouble. Traces Ethidium Bromide in a DNA preparation is 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.

7. Manipulation of DNA, that is to be used to generate transgenic animals via pronuclear injection, should be done with utmost care: shield DNA as much as possible from UV-light (i.e., day light, 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) are clean when isolating fragments from agarose gels.

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. 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.

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Chapter 7

Inducible Transgenic Mouse Models

Thomas L. Saunders

Abstract

Inducible transgenic mouse models allow for the activation of genes in specific cells and tissues at specific times. Expression levels are dependent on the dose of the agent administered. Effective experimental models are characterized by low background levels of the regulated gene and induction to high levels with sub-physiological levels of inducing agents. The most commonly used methods to control gene expression in mouse models are based on the tet-operon/repressor bi-transgenic system and the estrogen receptor (ER) ligand-binding domain. Less commonly used systems to control gene expression in transgenic mice take advantage of the ligand-binding domain of the progesterone receptor, and the lac and GAL4 inducible systems. The tetracycline-regulated transgenic models are typically designed to activate the expression of the gene of interest in a specific cell type at a specific point in time. The ER is most commonly fused with Cre recombinase, although it can be used with transcription factors, kinases, etc., that are active in the nucleus. Cre-ER transgenes allow for the induction of recombinase activity in specific cells at defined time points. Cre recombinase is most often found in combination with conditional alleles to inactivate gene expression. When used for gene activation, Cre removes stop cassettes from transgenes and thus allows the expression of reporter or other molecules. Thus, the tetracycline-regulated and Cre-ER systems are complementary in mouse models, with utility in the cell-specific activation and inactivation of gene expression.

Key words: Doxycycline, tTA, rtTA, Tamoxifen, Cre-ER, Transgenic mice, Gene expression

1. Introduction

The two principal systems used in transgenic mouse models are the tetracycline (Tc)-regulated transcription factors and the 4-hydroxytamoxifen (4HT)-regulated expression of Cre recombinase. The Tc system is most often used to cause the expression of a gene of interest in a specific cell type. The 4HT system is most often used to cause the inactivation of a conditional allele. Both systems are bi-transgenic, which is to say that they involve the mating of two different genetically modified mice in order to produce

a mouse model in which gene expression can be manipulated. In Tc systems, one transgenic strain carries a Tc-regulated transactivation factor whose expression pattern is controlled by a heterologous promoter/enhancer unit of choice. The second mouse carries a minimal promoter coupled to the gene of interest. When a mouse inherits both transgenes and is treated with a Tc analog such as doxycycline, the gene of interest is expressed. In 4HT systems, one transgenic strain carries a 4HT-regulated Cre recombinase enzyme whose expression pattern is controlled by a heterologous promoter/enhancer unit of choice. The second mouse carries a conditional allele of the gene of interest. When a mouse inherits both transgenes and is treated with a 4HT, the Cre enzyme is translocated to the nucleus where it can inactivate the gene of interest (e.g., by excising a loxP-flanked exon from a gene), or activate gene expression (e.g., by excising a loxP-flanked stop cassette from a gene). Numerous mouse models have been developed with both systems. On occasion, it may be possible to address a biomedical research question by obtaining mice from international repositories or originating laboratories. For example, mice with a 4HT-inducible Cre transgene can be combined with reporter mice to mark cells during embryogenesis or adulthood for cell lineage tracing studies (1–4). The mouse models in these studies are available from animal repositories (see Note 1).

1.1. Tetracycline-Regulated Transgenic Models

The first Tc-regulated gene expression system tested in transgenic mice fused the tet-repressor protein from *Escherichia coli*'s Tn10 transposon to the VP16 transactivation domain from herpes simplex virus (5). When the tet-repressor domain binds to a minimal Tc response element (TRE) DNA sequence consisting of a minimal cytomegalovirus (CMV) promoter followed by seven tandem *tet* operators, gene activation occurs. In the absence of Tc or a Tc analog such as doxycycline (dox), the tTA protein forms a dimer that binds to the TRE and causes the expression of the gene following the TRE. To end transgene expression, dox is administered. Dox molecules bind to the Tc-repressor domain and cause it to dissociate from the TRE, thus ending gene expression. In mouse models where temporal control of gene activation is desirable, the reverse tTA protein (rtTA) is preferred (6). Up-regulation of gene expression is more rapid when a mouse can be treated with an inducer compared to that when waiting for the depletion of an inhibitor. rtTA has four amino acid substitutions in the Tc-repressor domain that invert the response to dox so that rtTA will only bind to the TRE and activate genes after dox molecules bind to rtTA. Subsequent refinement of rtTA protein to increase the sensitivity of the system and to reduce background expression includes further amino acid substitutions in the tet-repressor domain and replacement of the VP16 domain with a trimer of a

12 amino acid minimal activation domain from VP16 (7). The resulting protein is named rtTA2S-M2. Just as the original tTA has evolved, an optimized TRE has been developed (ptet$_{-14}$ or pTRE-Tight) that has lower background expression. The use of these two elements in combination provides the researcher with higher induction levels at lower concentrations of dox.

Additional control over background expression can be gained by adding an inducible transcription repressor. Efforts to develop a repressor are based on the need to control "leaky" expression when the minimal TRE results in low level expression in some cell types. Binding of a repressor to the Tet operon sequences in the TRE blocks background expression. Several repressors have been described. The fusion of tTA or rtTA with the Krüppel-associated box (KRAB) domain of the human ZNF10 (KOX1) or human ZNF354A (KID1) gene produces a hybrid protein that can silence genes within 2–3 kb of the repressor-binding site (8). The human HDAC4 repressor domain can also be used in this way (9). The Tc-repressor protein alone can be used to block expression (10). These systems coordinate the expression of the transactivating rtTA or tTA proteins with repressors to control leaky expression. For example, the use of tTA-repressor with rtTA in the absence of dox establishes a system in which tTA-repressor actively binds to the TRE and prevents any leaky expression from the TRE. When dox is administered, it binds to tTA-repressor and releases it from the TRE while simultaneously activating the rtTA so that it binds to the TRE and transactivates gene expression. The majority of studies with repressors have been performed in cell culture or ex vivo systems. Results on a few transgenic mouse models have produced mixed results, suggesting that the repressors are not ready for routine use in transgenic models (11–13). There is evidence that the ZNF10 repressor bound to the TRE during the first few days of mouse embryonic development permanently silences gene expression (14). Transgenic mouse models with repressors should be designed to prevent repressor binding during pregnancy. In general, this will result in the expression of the inducible gene, which may be undesirable in some research settings.

1.2. Tamoxifen-Regulated Models

In contrast to Tc-regulated transgenic systems, 4HT-regulated expression models are based on a fusion protein that includes the human estrogen receptor (ER) ligand-binding domain fused to a protein that is active in the cell nucleus. Examples include transcription factors (15), mitogen-activated protein kinase kinases (16), and, more commonly, Cre recombinase. Judicious selection of a promoter can be used to direct cell-specific or widespread expression of the fusion protein. The ER domain sequesters the protein in the cytoplasm until 4HT binds to the ER and translocation to the cell nucleus occurs. If a transcription factor is fused to the ER, the downstream targets of the gene will be expressed.

If a kinase is fused to the ER, then it will only translocate to the nucleus and phosphorylate target proteins after 4HT administration. If Cre recombinase is fused to the ER, then the protein will regulate the expression of genes modified with loxP sites (floxed genes).

Depending on experimental design, Cre recombinase can inactivate gene expression by removing an essential exon flanked by loxP sites from a gene (17) or it can activate a gene by removing a floxed stop cassette (4). The addition of the ER to Cre provides temporal control over gene expression and enhances the power of mouse models to address biological questions. The initial Cre-ER fusion protein (18) evolved to Cre-ER^T after introducing one amino acid mutation. Cre-ER^T does not bind endogenous beta-estradiol but is activated by 4HT (19). Cre-ER^{T2} is the most sensitive to 4HT and shows no response to endogenous 17beta-estradiol stimulation. It is characterized by three amino acid mutations in the ER ligand-binding domain (20). Further refinements have been made to improve the activity of the Cre enzyme by use of a codon-optimized iCre recombinase coding sequence (21) joined with two ER^{T2} domains to provide higher 4HT sensitivity and greater recombination activity (22). Judicious selection of transgene promoters in combination with Cre-ER^{T2} or iCre-ER^{T2} can provide both temporal and cell-specific expression of Cre recombinase.

1.3. Transgenic Mice   Genetically engineered mouse models that include Tc-regulated transgenes and Cre-ER^T transgenes have been described in the literature and submitted to repositories (see Note 1). These existing models allow new questions in biomedical science to be addressed in mice that have been previously characterized with respect to background expression and inducibility. For example, by combining B6.Cg-Tg(tetO-DTA)1Gfi/J mice from the Jackson Laboratory with C57BL/6J-Tg(Gabra6-rtTA)1Nak mice from the Riken Bio Resource Center, it should be possible to ablate cerebellar granule cells by the expression of diphtheria toxin A chain (DTA). The extent of cell ablation can be controlled by adjusting the dox dosage. This approach has been used to ablate beta cells in the pancreas (23).

It is important to characterize new inducible transgenic mouse lines for background expression levels, inducibility, and appropriate cell-specific expression patterns. Pronuclear microinjection results in random integration events that can silence transgenes (e.g., when integrated into heterochromatin) or that can activate transgenes (e.g., if integrated into an active genomic promoter/enhancer). When new tTA, rtTA, and Cre-ER transgenic founder mice are developed, the first step in their use as research tools is to evaluate their utility for further experiments. The most straightforward approach to characterize background

expression and inducibility is to mate the mice with a previously described reporter mouse, such as the B6;SJL-Tg(tetop-lacZ)2Mam/J mouse strain (Jackson Laboratory Stock number 002621) (5, 24) for tTA and rtTA transgenic mice. There are a number of mice for use with Cre-ER transgenes; for example, the B6.129(Cg)-Tg(CAG-Bgeo/GFP)21Lbe/J and Tg(CAG-Bgeo,-DsRed*MST)1Nagy/J mice inactivate lacZ expression upon Cre-mediated recombination and then switch on green fluorescent protein or red fluorescent protein, respectively (Jackson Stock numbers 004178 and 005438) (25, 26). After double transgenic mice are generated by breeding, they are treated with dox or 4HT and tested for reporter gene expression. Transgenic founder mice that lack reporter expression in the absence of inducers and produce the appropriate reporter expression pattern after treatment are selected for the establishment of transgenic mouse strains. Methods for analyzing reporter gene expression in transgenic mice can be found elsewhere in this volume.

When new TRE transgenic mice are developed by pronuclear microinjection, they are characterized for the absence of background expression and the ability to respond to Tc-transactivators. In these mice, the goal is to show that the gene of interest cloned behind the minimal promoter in the TRE can be activated by a Tc-regulated transcription factor. A previously described mouse strain with constitutive rtTA transgene expression such as B6. CgGt(ROSA)26Sor[tm1(rtTA*M2)Jae]/J (27) can be used to test TRE transgenic mice for inducibility. Once TRE transgenic founders are sorted out and an animal with the appropriate expression characteristics is identified, it is then used to establish a transgenic mouse strain for further study. The establishment of mouse models with gene targets for inactivation by Cre recombinase involves the development of gene-targeted conditional alleles by homologous recombination in ES cells with gene-targeting vectors. This process is discussed elsewhere in this volume. Suffice it to say that the primary characteristics of a conditional allele are that the targeted gene be expressed at normal levels in the absence of Cre recombinase and that it be transformed into a null allele after excision of DNA sequence by Cre activity.

2. Materials

2.1. Tet-On Plasmids   The pUHrT62-1 plasmid containing rtTA2S-M2 is available from Professors Bujard and Hillen (see Note 2).

2.2. Tet-Responsive Promoter   Tet-responsive promoter, pTRE-Tight (Clontech 631059).

2.2.1. Tet Repressor Plasmids

1. Plasmid pLVCT-tTR-KRAB includes the tet-on tTR-KRAB (Addgene.com, Cambridge, MA, plasmid no. 11643).

2. Plasmid pLVPT-rtTR-KRAB includes the tet-off rtTR-KRAB (Addgene.com, plasmid no. 11777).

2.2.2. ER^{T2} Plasmids

1. Plasmids Cre-ER^{T2} and ER^{T2}-Cre-ER^{T2} (Addgene.com, plasmid numbers 14797 and 13777).

2. Plasmid ER^{T2}-iCre-ER^{T2} is available from Professor Rolf Sprengel (see Note 3).

2.3. Gene Promoters

1. The commonly used pCAGGS plasmid contains a promoter/enhancer unit that is expressed in many cell types. It is a hybrid promoter including CMV, chicken beta-actin, and rabbit globin sequences (28). pCAGGS with additional cloning sites (Addgene.com) (see Note 4).

2. Promoters that drive gene expression in specific cell types of transgenic mice are described in the scientific literature. A comprehensive listing of promoters is beyond the scope of this article.

2.4. Genotyping Primers

1. An essential part of designing a transgene is to ensure that transgenic founder mice can be identified. This is usually accomplished with polymerase chain reaction (PCR) assays (29). Transgene specific primers that will amplify transgene DNA without amplifying mouse genomic DNA are designed to anneal to the rtTA, tTA, Cre, or ER sequences.

2. Oligonucleotide primers (Sigma–Aldrich or other vendor) are resuspended at a final concentration of 10 μM in distilled water prior to use in PCRs.

3. Genotyping PCRs are prepared by adding primers and mouse DNA to a master mix containing Taq polymerase, nucleotides, and buffers such as GoTaq® (Promega M7122, Madison, WI).

4. rtTAM2-S2 forward primer: 5′ CTGGGAGTTGAGCAGCCTAC 3′. rtTAM2-S2 reverse primer: 5′ AGAGCACAGCGGAATGACTT 3′. Primer annealing temperature is 60°C and the PCR product size is 170 bp.

5. tTA forward primer: 5′ ACAGCGCATTAGAGCTGCTT 3′. tTA reverse primer: 5′ CCCCTTCTAAAGGGCAAAAG 3′. Primer annealing temperature is 60°C and the PCR product size is 186 bp.

6. Cre recombinase forward primer: 5′ ACCAGCCAGCTATCAACTCG 3′. Cre recombinase reverse primer: 5′ TTACATTGGTCCAGCCACC 3′. Primer annealing temperature is 60°C and the PCR product size is 198 bp.

7. iCre recombinase forward primer: 5′ GTGCAAGCTGAACA ACAGGA 3′. iCre recombinase reverse primer: 5′ CCAGCAT CCACATTCTCCTT 3′. Primer annealing temperature is 60°C and the PCR product size is 219 bp.

8. ER^T2 forward primer: 5′ AGCACCCTGAAGTCTCTGGA 3′. ER^T2 reverse primer: 5′ GATGTGGGAGAGGATGAGGA 3′. Primer annealing temperature is 60°C and the PCR product size is 153 bp.

9. In addition to performing PCR with transgene specific primers, samples should be subjected to PCR with a primer pair for an endogenous mouse sequence such as the D5Mit425 microsatellite marker that can vary in size between 191 and 211 bp, depending on the genetic background of the mice being tested. D5Mit425 forward primer: 5′ TCGCCTTTC-TTTCCCTCC 3′. D5Mit425 reverse primer: 5′ AAAATTACA-TTTGCATCTGGGG 3′. Primer annealing temperature is 60°C.

10. If desired transgene specific primers can be designed by anchoring one primer in the cell-specific promoter sequence and one in the downstream upstream of tTA, rtTAM2-S2, Cre, iCre, or ER^T2 (see Note 5).

2.5. Doxycycline Preparation

1. Mouse diet with 200 mg/kg doxycycline (Harlan Teklad rodent diet T-7012, Madison, WI).

2. Doxycycline powder (Sigma–Aldrich D9891, St. Louis, MO).

3. Sodium saccharin (Fisher Scientific, S394-500, Pittsburgh, PA).

4. Pellets with slow release of doxycycline designed for 60- or 90-day treatments (Innovative Research of America, Sarasota, FL).

5. Dox stock: dissolve 20 mg of dox in sterile distilled water. Aliquots of stock can be stored frozen at –20°C for up to 6 months.

2.6. Tamoxifen Preparation

1. Tamoxifen (Sigma–Aldrich T5648).

2. 4-Hydroxytamoxifen (Sigma–Aldrich H7904).

3. Ethanol, unadulterated reagent grade or anhydrous ethanol from most chemical houses.

4. Tamoxifen stock: 100 mg/ml of tamoxifen is prepared in ethanol and stored at –20°C.

5. Sunflower seed oil (Sigma–Aldrich S5007).

6. Feeding needles (Roboz Surgical Instrument Co. FN-7920, Gaithersburg, MD).

7. Mouse diet containing 400 mg/kg of tamoxifen (Harlan Teklad).

8. Slow release tamoxifen pellets (Innovative Research of America). Depending on the experimental plan, 5 mg pellets with release times of 60 or 90 days can be used.

2.7. Mouse Tail Tip Genotyping Biopsy Lysis Buffer

1. Lysis buffer: Mix together 1 M NaOH, 500 mM EDTA, pH 8.0, and sterile distilled water to produce 25 mM NaOH, 0.2 mM EDTA, pH 12. If necessary, pH can be adjusted with 1 N NaOH.

2. Neutralization buffer: Prepare 40 mM Tris–HCl, pH 5, to neutralize the lysis buffer before PCR.

3. Methods

3.1. Tc Transgene Plasmid Cloning

1. The essential elements of a plasmid transgene include the promoter/enhancer unit selected for its ability to drive transgene expression in the desired cell, a protein-coding sequence, and a polyadenylation signal. Detailed information on transgene design can be found elsewhere in this volume.

2. Once a cell-specific promoter unit has been identified, it is cloned into the rtTA2S-M2 plasmid, replacing the CMV promoter (nucleotides 1–761). Alternatively, the rtTAS2-M2 coding sequence and SV40 polyA sequence can be cut out of the pUHrT62-1 plasmid as a 1,222 bp EcoRI–HindIII fragment to clone into a plasmid with a promoter. PCR-aided cloning of DNA fragments into vectors is a direct and effective method to build transgenes (30).

3. The cDNA of the protein in question is cloned into the pTRE plasmid polylinker. The plasmid includes the SV40 polyA signal. Inclusion of a Kozak consensus sequence may improve expression.

4. If a repressor is needed to control leaky expression, the tet-on tTR-KRAB repressor, which is active in the absence of dox, is used in conjunction with rtTAM2-S2. The sequence that codes for the tTR-KRAB coding sequence can be removed from pLVCT-tTR-KRAB and placed in a plasmid with a cell-specific promoter and a polyadenylation signal (see Note 6).

3.2. ER^{T2} Plasmid Cloning

1. The Cre-ER^{T2} fragment can be cut out as a 2,004-bp fragment from pCAG-Cre-ER^{T2} by digestion with EcoRI and NotI. The same enzyme digestion of pCAG-ER^{T2}-Cre-ER^{T2} will release the 3,000-bp ER^{T2}-Cre-ER^{T2} fragment. Once isolated, the Cre gene is cloned between a promoter and a polyA signal in a receiving plasmid. This allows the promoter in the

receiving plasmid to control expression of Cre recombinase in specific cell types and provides for temporal control by administration of tamoxifen.

2. The ER^{T2}-iCre-ER^{T2} fragment can be cut out as a 2,670-bp fragment from the pBS-ERT2iCre plasmid by digestion with KpnI and EcoRI. This fragment can then be cloned into a plasmid that contains the desired promoter unit and polyadenylation signal.

3.3. Transgenic Mouse Genotyping by PCR

1. Tail tip biopsies are collected (0.2 mm of the tail tip is cut off with a sterile scalpel) and placed in 0.075 ml of lysis buffer. After heating to 95°C for 10–30 min, add 0.075 ml of neutralization buffer. Add 2 µl of the extracted DNA to a 25-µl PCR containing the desired primer pair and master mix ((31), see Note 7).

2. Amplification conditions for the primers listed above with 60°C melting temperature are 95°C denaturation for 30 s, 56°C annealing for 30 s, 72°C elongation, repeat for 30 cycles, increase the final elongation step to 5 min and then hold at 4°C until the samples are analyzed.

3. Testing DNA samples with the D5Mit425 primer pair will control for false-negative transgene results since all mouse DNA samples should generate a DNA fragment with this primer pair.

3.4. Doxycycline Administration

1. Oral administration of doxycycline by feeding animals a modified diet is effective in regulating transgene expression ((32), see Note 8). The usual mouse diet is replaced with dox rodent diet.

2. When administered in drinking water, dox stock is diluted in distilled water to 0.5–2 mg/ml. Lower doses are recommended for tTA mice so that transgene expression will be activated in a reasonable period of time after dox withdrawal (see Note 9). For the rapid induction of rtTA models, 2 mg/ml of dox-water is used. To overcome the bitter taste of dox-water, saccharin is added (2.5 mg/ml of saccharin). Since saccharin has no caloric value, it will not interfere with the nutrition of the animals, unlike dox-water sweetened with sucrose. Amber water bottles are necessary to protect dox from light-mediated degradation. The bottles are replaced with fresh dox-water once a week (see Note 10).

3. Dox can be administered by intraperitoneal injection of 0.1 ml of 100 µg/ml of dox solution. This provides for a dose of 10 µg. Uninduced control animals are injected with an equal volume of sterile distilled water.

4. Slow release dox pellets are placed subcutaneously in a skin fold on the neck. This can be done using a trochar needle or by a simple surgical procedure (5). Uninduced control animals are treated with a pellet containing no dox.

5. Administration of dox by gavage. A 20-gauge feeding needle is used to deliver 0.1 ml (10 μg) of dox daily. To gavage a mouse, hold the animal gently by the scruff of the neck so that its body hangs down and forms a straight vertical line from the tip of its nose to its stomach. Insert the feeding needle slowly so that the mouse swallows the tube; do not force the tube into the mouse (see Note 10).

3.5. Tamoxifen Administration

1. Oral administration of tamoxifen is effective in inducing prolonged Cre-ER expression ((33), see Note 8). The usual mouse diet is replaced with Harlan Teklad CRD TAM[400] diet (400 mg/kg of tamoxifen).

2. Prior to use, tamoxifen is emulsified in sunflower oil at a 1:10 ratio with a vortex mixer and stored at 4°C for up to 7 days (see Notes 8 and 11). Oil containing ethanol only should be prepared at the same time for administration to uninduced control animals.

3. Administration of tamoxifen by injection. Tamoxifen is re-emulsified just before intraperitoneal injection. Common dosages for widespread Cre-ER activity are 1–9 mg daily for 5 days (19, 34, 35). Single doses of varying concentration may also be given to produce pulses of Cre-ER activity (1, 2). Tuberculin syringes (1 ml with 26-gauge needles) are used for injections. After injecting the oil (0.1 ml for 1-mg dose), pause for a few seconds before removing the needle so that the full dose is delivered. Control animals should receive an equal volume of tamoxifen-free oil.

4. Administration of tamoxifen by gavage. A 20-gauge feeding needle is used to deliver 0.1 ml (1.0 mg) of tamoxifen daily. See note above (Subheading 3.3, item 4) on administration of dox by gavage.

5. Slow release pellets containing tamoxifen are inserted subcutaneously in a loose fold of skin at the animal's neck by trochar or surgical insertion. Uninduced control animals are treated with a tamoxifen-free pellet.

6. Topical application of tamoxifen is accomplished by applying tamoxifen dissolved in ethanol to mouse skin. Areas with fur should be shaved smooth prior to application. Doses of 1 mg per day for 1–5 days are suitable for transgene activation.

4. Notes

1. The international Mouse Strain Resource includes information from multiple repositories (http://www.findmice.org/IMSRSearchForm.jsp). A search for gene or allele names containing "tet" will identify mice with tTA, rtTA, and tet-operon insertions and knock-ins. The search term "Esr1" will identify both Cre-ER mouse models and models that use the ER domain to control gene expression. Prior to importing or working with mice, approval to do so is required from the institutional animal care and use committee.

2. The rtTAs-M2 plasmid can be requested from Professors Hillen and Bujard by submitting the material transfer agreement from this URL: http://www.zmbh.uni-heidelberg.de/bujard/trouble/MTAII.html.

3. Dr. Sprengel can be reached through his research group's URL: http://wmn.mpimf-heidelberg.mpg.de/sprengel.

4. The enhanced version of pCAGGS plasmid is ordered from http://www.addgene.com (plasmid number 11160, pCAGEN).

5. Software for primer design can be found at this URL: http://frodo.wi.mit.edu (36).

6. Care must be taken when this silencer is used to generate transgenic mice since there is evidence that the target sequence of the KRAB repressor will be permanently silenced if dox is not present during embryogenesis (12).

7. Genomic DNA prepared by the hotshot method is not suitable for Southern blot analysis; yields are similar whether skin ear punches or tail tip biopsies are used for extraction, and heating for more than 30 min will not increase DNA yield.

8. Administration of dox and tamoxifen in diet is especially useful in behavioral studies. Treatment of mice by injection or gavage will induce a stress response when the mice are restrained. Activating transgene expression by diet avoids possible confounding effects on behavioral studies because of stress responses induced by restraint of the mice.

9. Dox is absorbed by mouse tissues. Thus, if dox is given to prevent gene expression from tTA, the lowest effective dose should be used so that dox can be efficiently cleared from tissues and tTA-dependent transgene expression activated.

10. Although gavage provides the most accurate means of dosing a mouse, the technique requires some practice before proficiency is attained. For a discussion of the advantages and disadvantages of gavage, see (37).

11. Tamoxifen is metabolized to the active form of 4-hydroxyta-moxifen in the liver. If rapid induction of Cre-ER is required, as in cell lineage studies during embryogenesis, treatment of mice with 4HT in the place of tamoxifen will provide far more precise timing of Cre-ER induction (1).

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Chapter 8

Lentiviral Transgenesis

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Abstract

Conventional DNA injection-based methods are successful in generating transgenic animals and have remained nearly unchanged over the last few decades. Lentiviral vectors are alternative powerful tool for generating transgenic animals, in part because of their ability to incorporate into genomic DNA with high efficiency. This chapter describes lentiviral vectors used to generate transgenic mice and rats. We discuss the protocols and methods in high enough detail such that researchers who are accustomed to creating transgenic animals by pronuclear injection can smoothly transition to using lentiviral transgenesis. We will briefly outline the general principle of the lentiviral expression system and focus specifically on the methods used to generate lentiviral vectors, produce lentiviral particles, inject lentivirus into the fertilized oocytes, and transplant them into the pseudopregnant females. In addition to the surgical aspects of the experiment, we will describe methods to produce high titer lentivirus. Finally, we will discuss the limitations of lentiviral transgenesis and summarize information that will be useful for troubleshooting.

Key words: Lentivirus vector, Transgenic mouse, Transgenic rat, Lentiviral transgenesis, Microinjection, shRNA, Protocol

1. Introduction

At present, the most commonly accepted method to produce transgenic animals is pronuclear injection of DNA into fertilized oocytes. This method has been successful in generating a wide variety of transgenic mice (1) and large transgenic animals (2, 3). However, the DNA injection method is still hampered by a relatively low efficiency, especially in inbred strains of mice and rats as well as larger farm animals (4–6). This problem can easily overcome by using lentiviral vectors, which were initially developed for gene therapy to correct genetic disorders in somatic cells (7). Lentivirus-mediated transgenesis allows the production of a large number of transgenic animals containing a single-copy transgene. The strength of lentiviral trangenesis is the high efficiency in

obtaining transgenic animals from a single experiment. Furthermore, unlike the recombinant Moloney murine leukemia virus vectors, the gene expression from lentiviral vectors does not get silenced during development of the transgenic animal (8). Technically, injecting lentivirus into the oocyte is much easier than microinjecting DNA into the pronucleus, which in several species is difficult to visualize (Fig. 1). The possibility to simply soak embryos in media containing lentivirus suggests that an expensive microinjector system may not even be required in the future to produce transgenic animals (9). Furthermore, by pseudotyping the virus with vesicular stomatitis virus glycoprotein (VSVG), lentivirus can infect virtually any cell types from a variety of species, thus enabling transgenesis in the entire animal kingdom. Lentiviral transgenic birds, pigs, and monkeys have been recently generated (10–12). Despite its enormous potential, lentiviral transgenesis is a specialized technique that needs sufficient experience to make it work.
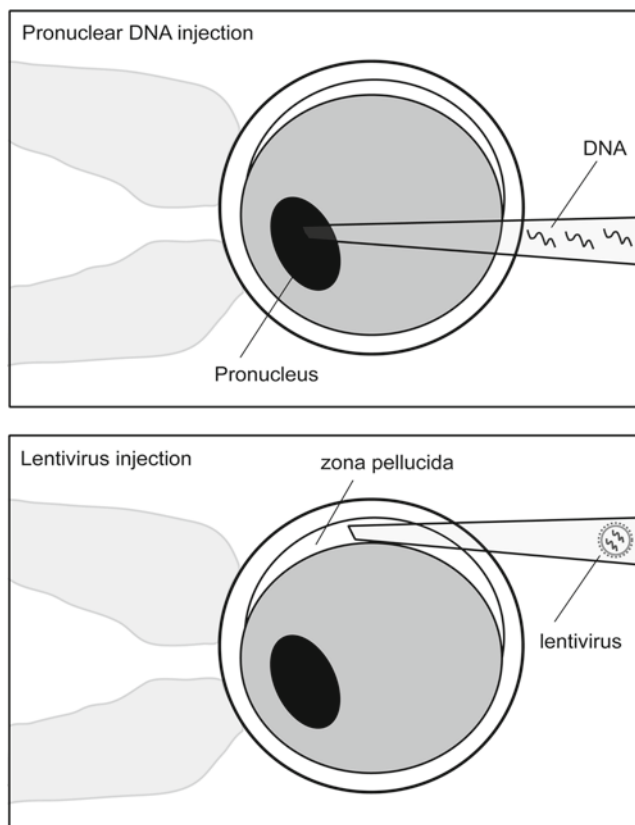
Fig. 1. Schematic overview of DNA microinjection and lentivirus infection. In conventional transgenesis, DNA is microinjected directly into the pronucleus of fertilized embryos (*top*). The pronuclear injection is not very efficient. In mice, the transgene is integrated and expressed in not more than 4% of the injected and transferred embryos. Subzonal lentivirus infection results in higher yields (*bottom*). Viral particles are injected into the subzonal space between the cytoplasmic membrane and the zona pellucida. Subzonal injection has no significant effect on embryonic development.

There are many analogous principles and skills shared among conventional and lentiviral transgenesis. Prior to the start of their study, both authors were experienced with the methods and protocols used for genetic manipulation in mice (13, 14) but were not experts in lentiviral transgenesis. We assume that the reader has some knowledge on embryonic manipulation. If not, we refer to the other chapters in this book related to transgenesis and two textbooks that we consider very helpful in understanding the basics of transgenic technology: "*Manipulating the Mouse Embryo: A Laboratory Manual*" by Andreas Nagy (15) and "*Transgenic Animal Technology: A Laboratory Handbook*" by Carl A. Pinkert (16). The last book is particularly useful for generating transgenic animals in species other than mouse; however, neither of these books deals specifically with lentiviral transgenic technology. In this chapter, we will briefly outline the general principle of lentiviral technology and focus on the methods used to generate lentiviral vectors and produce lentivirus particles. We discuss the important principles of the surgical aspects of lentiviral transgenesis and provide a detailed description of protocols to help researchers who wish to transition from conventional to lentiviral transgenesis. The information provided here is explained in such a way so that readers who are completely new to the field can follow the basic principles. To conduct the lentiviral experiments, we advice new researchers first to seek advice from experts in transgenesis and to establish their basic skills. Moreover, we emphasize all hurdles and trial and errors we experienced ourselves and share all the materials necessary to set up lentiviral transgenesis in your own laboratory.

2. Materials

In addition to the general reagents, equipment, and animals necessary for conventional transgenesis (15), the following materials are needed for lentiviral transgenesis.

2.1. Viral Vectors and Lentiviral Production

2.1.1. Recombinant Lentiviral Vectors

1. Cloning lentiviral vectors requires standard molecular biology materials and facilities.
2. Qiagen Plasmid endofree maxi kit (Qiagen).
3. Luria Broth (LB) medium, ampicillin, and isopropyl alcohol (multiple suppliers).
4. Lentiviral vector DNAs – pVSVG, pCMVΔ8.9, and pFUW plasmids (8, 14).
5. Lentiviral shRNA vector DNA – pLL3.7 (17).
6. High titer TOPP10 competent cells (Invitrogen).

1. Producing lentivirus requires tissue culture facilities under BL2 conditions.

2. Decontamination reagents: 70% ethanol/1% SDS (a squeeze bottle) and bleach (multiple suppliers).

3. 15-cm tissue culture plates (Nunc) and HEKT (obtained from ATCC) or 293FT cells (Invitrogen).

4. 0.2% gelatin coating solution: add 1 g of gelatin (Sigma) to 500 ml of water and autoclave. Keep sterile at room temperature.

5. Culturing media: high-glucose DMEM (Mediatech) supplemented with 10% fetal calf serum (FCS, not heat inactivated; every batch should be tested for efficient lentiviral production) and pen/strep (Invitrogen). Media must be maintained at 37°C before use.

6. Lentiviral vector DNAs. The pVSVG, pCMVΔ8.9, and pFUW plasmids are made with Endofree Qiagen kits. The DNA should be in Endofree TE at a concentration of 0.5 μg/μl.

7. Sterile 2× HBS: 16.4 g NaCl (0.28 M final), 11.9 g HEPES (0.05 M final), and 0.21 g Na_2HPO_4 (1.5 mM final) in 800 ml H2O (use milliQ). Titrate to pH 7.05 with 5 N NaOH. Add H_2O to 1 L. Filter-sterilize through 0.2-μm filter unit (Nalgen). pH must be between 7.05 and 7.12 after filtering.

8. Sterile D-PBS: make from 10× D-PBS. 10× D-PBS is 2.00 g KCl, 2.00 g KH_2PO_4, 80.00 g NaCl, 21.60 g $Na_2HPO_4.7H_2O$, and H_2O to final 1 L (filter-sterilize and store at room temperature). Check pH of 1× D-PBS to ensure that it is of pH 7.1–7.4.

9. Trypsin/EDTA solution from Invitrogen.

10. Concentrating the viral particles was done in a Beckman Ultracentrifuge (Optima L-XP or equivalent), using a SW28 rotor assembly (Beckman), and thickwall polycarbonate tubes (32 ml volume, Beckman).

11. Nalgen filter unit with 0.45-μm pore size filter (Nalgen).

12. Kimwipe and parafilm.

13. Hank's balanced salt solution (HBSS) (Invitrogen).

2.2. Preparation for Surgery

1. Animals: The Sprague–Dawley strain (Charles River Laboratories) was used for rat lentiviral transgenesis. C57BL/6 or FVB mouse strains (Charles River Laboratories) were used for mouse lentiviral transgenesis.

2. Hormones to induce superovulation: human chorionic gonadotropin (hCG) (Sigma); gonadotropin from pregnant mare serum (PMS) (Sigma).

3. All surgical tools (surgical scissors, blades, clamps, needles, threads, drapes, and forceps) were purchased from Roboz. Prior to surgery, all metal tools were sterilized in an autoclave.

4. Sutter P-97 needle puller. Glass tubes: Sutter, borosilicate glass OD 1 mm, ID 0.75 mm (holding pipettes), and micro-injection pipette (Narishige).

5. The transfer pipettes (4 mm OD, 48 in. LG standard wall glass tube PYREX labware).

6. Needle microforge (Narishige).

7. Eppendorf Vario oil microinjector and Eppendorf automated microinjector for optional use to measure pressure of the injection needle.

8. The inverted microscope (such as Nikon) with Leica micromanipulator placed on an air table is identical to those used for routine microinjection for mouse embryos (15).

9. Dissection scope with a boom stand and regular dissection scope (multiple suppliers such as Nikon, Olympus, Leica, and Zeiss).

10. CO_2 incubators (multiple suppliers such as Thermo-Fisher and Levco).

11. Fiber illumination (multiple suppliers).

2.3. Surgery and Microinjection

1. M2 culture media (Sigma), glycerol (Sigma), penicillin–streptomycin (Invitrogen).

2. 35- and 60-mm tissue culture dishes (Nunc).

3. Hyaluronidase (Sigma) for enzymatic removal of follicle cells.

4. Leica micromanipulator and the Eppendorf Cell Tram Vario silicon oil-driven manual injector (Eppendorf).

5. The holding pipette was connected to a Hamilton syringe via a plastic tubing.

6. Table top microcentrifuge (any microfuge that can spin at 14,000 rpm is sufficient).

7. Anesthesia: 60 mg/kg ketamine, 7.5 mg/kg xylasine, and 1 mg/kg midaxolam.

8. Vaseline (antibiotic paste) was applied to the eye to prevent drying.

9. Heat pad (to keep the animal warm postoperation).

10. Flushing holding medium (FHM; Millipore).

11. KSOM (Millipore). For details, see (15).

3. Methods

The rat is the preferred animal model in several areas of research including cardiovascular and neurosciences. Due to the lack of gene targeting technology in rats, mice have been much more successful as experimental animal model in the last two decades (4). Here, we will describe methods and protocols to generate both transgenic mice and rats using lentiviral vectors. We will overview the procedures and specify the expertise necessary to conduct each sub-procedure. The experimental flow diagram is shown in Fig. 2. The methods part of this chapter follows the chronological order of the experimental procedures. First, we will describe how to design and generate the optimal lentiviral vector and produce a high titer lentivirus (Subheading 3.1). It is not exaggerating to say
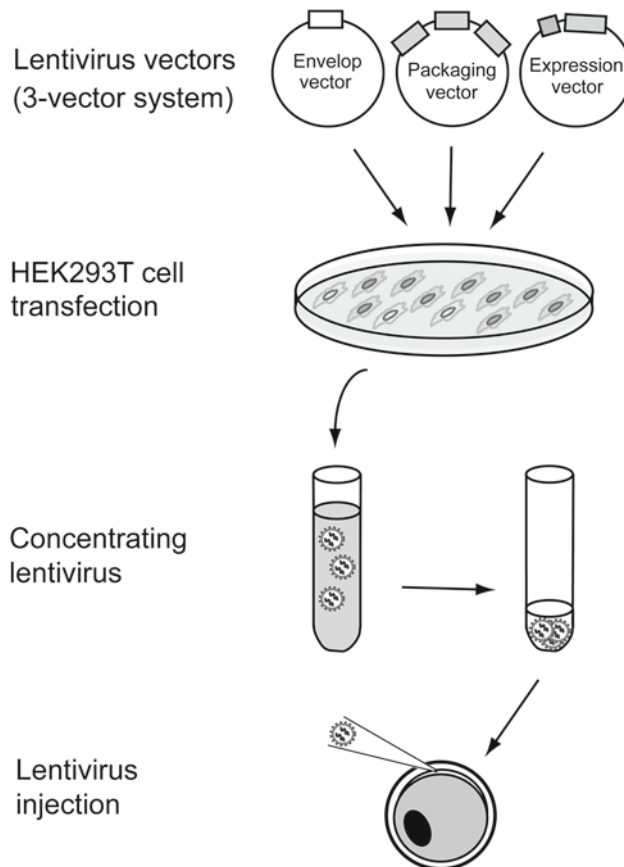
Fig. 2. The experimental flow diagram to generate lentiviral transgenic animals. Design the optimal pFUW expression vector by inserting the transgene of interest and exchanging the promoter sequence. HEKT cells are transfected with three plasmids pVSVG (envelop vector), pCMVΔ8.9 (packaging vector), and pFUW (expression vector) using the calcium phosphate method. High titer viral particles are injected into the subzonal space between the cytoplasmic membrane and the zona pellucida of the developing embryo.

that the most important reagent in the procedure is the high titer lentivirus ($>10^6$ infectious units/μl). Second, we will describe how to schedule the surgery and prepare the animals (Subheading 3.2). Third, we will describe how to do the surgery and microinjection (Subheading 3.3). Fourth, we will describe the post-surgery procedure and how to follow-up on the animals (Subheading 3.4). Finally, we will discuss the limitation and our experience in troubleshooting the hurdles we faced (Subheading 4).

3.1. Viral vectors and Lentiviral Production

The first efficient lentiviral expression system was derived from the human immunodeficiency virus type 1 (HIV-1) and consisted of the following three vectors – a packaging vector, an envelope vector, and an expression vector (18). The biosafety of the system was greatly improved in the second generation vectors, first, by deleting all accessory genes from the packaging vector and second, by developing the self-inactivating (SIN) expression vector, which lacks the transcriptional activation sites from the 3′ long-terminal repeat (LTR) region (Fig. 3). Self-inactivation is a safety feature that turns off transcription of the provirus incorporated in the host genome and reduces the risk of vector recombination with latent retroviral sequences in the host cell genome. Recombinant lentiviral vectors are now routinely used in many areas of biomedical research. At the moment, third generation lentiviral expression systems are available from academic researchers and commercial sources, such as Lenti-X™ Expression Systems (Clontech) and ViraPower™ Lentiviral Expression Systems (Invitrogen). The second generation pCMVΔ8.9/pVSVG/pFUW lentivirus-based gene delivery system we used in our studies was first described by Dr. Inder Verma (18) and modified by Drs. Carlos Lois and David Baltimore to create transgenic mice and rats (8). We did not test other lentiviral expression vectors but expect that other systems should give comparable results.

To increase safety and decrease potential pathogenicity of the virus system, the packaging genes have been split into packaging and envelope vectors. These vectors have deletions in LTR, PSI, and the coding sequences of HIV-1 genome (env, vif, vpu, vpr, and nef) (Fig. 3). The pCMVΔ8.9 packaging vector contains most of the original HIV-1 DNA. The Gag, Pol, and RRE genes from HIV all express proteins required for forming the viral envelope – Gag codes for capsid and matrix proteins, Pol codes for the integrase and reverse transcriptase, and RRE is required for rev action. The pVSVG envelope vector contains the gene for the vesicular stomatitis virus glycoprotein (VSVG). To increase the stability and host range of the mature virus, the lentivirus is produced ("pseudotyped") with VSVG. Recombinant lentiviruses containing VSVG also allow easy concentration to high titers by ultracentrifugation (19). pFUW (expression vector) was derived from pHR′ (7) by making the 3′-LTR (SIN) (18). This vector
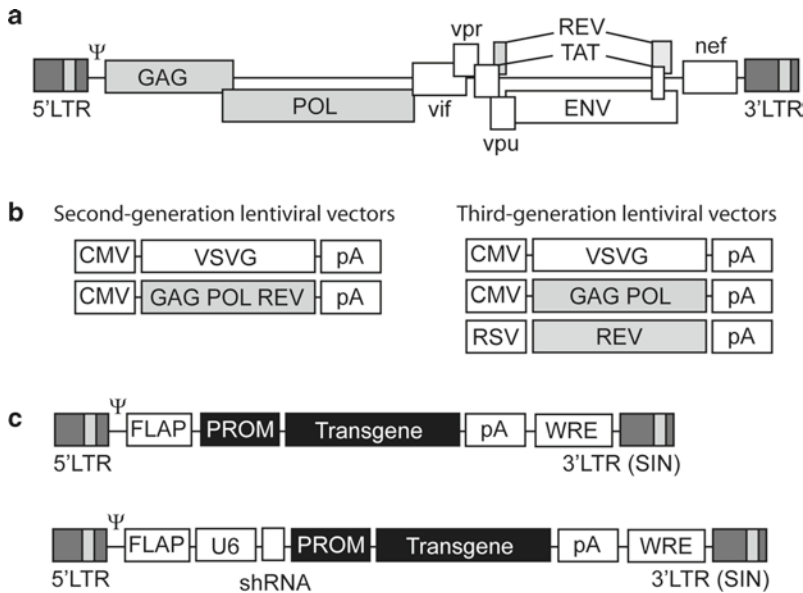
Fig. 3. The lentiviral expression vector system. (**a**) Diagram of the HIV genome. HIV has several major genes coding for structural proteins and several nonstructural ("accessory") genes. The Gag gene codes for the viral capsid, nucleocapsid, and matrix proteins. The Pol gene codes for reverse transcriptase, integrase, and HIV protease. The Env gene codes proteins present in the viral envelope. Rev is responsible for RNA exported from the nucleus before splicing occurs, so that the structural proteins and RNA genome can be produced. The tat, vpr, vif, nef, and vpu code for transactivators and other regulator proteins. (**b**) Schematic representation of the second and third generation lentiviral vector systems. In the second generation system, pCMVΔ8.9 (packaging vector) and pVSVG (envelop vector) contain the trans-acting factors, Gag-Pol-Rev and the envelope protein VSVG. The presence of VSVG in the viral envelope membrane confers the viral particle with the ability to transduce a broad range of cell types. In the third generation system, the Gag-Pol and Rev genes are divided over two plasmids, pMDL g/p RRE and pRSV-REV. (**c**) Lentiviral expression vectors have a carrying capacity of ~8 kb. The pFUW vector contains the ubiquitous ubiquitin promoter followed by a multiple cloning site where the transgene of interest is inserted (*top*). The pLL3.7 vector contains the promoter that drives transgene expression and the RNA polymerase III promoter U6 drives shRNA expression (*bottom*). Prom: any transcription-enhancing promoter can be placed here to modulate spatial, temporal, or quantitative aspects of transgene expression. Transgene: any gene can be inserted in the expression vector. The *gray boxes* represent the self-inactivating deletion in the promoter of the LTR. *HIV* human immunodeficiency virus, *LTR* long-terminal repeat, *GAG* HIV GAG gene, *POL* HIV reverse transcriptase, *ENV* HIV envelope gene, *RRE* rev-responsive element, *CMV* cytomegalovirus, *VSV* vesicular stomatitis virus, *Poly A* polyadenylation signal, Ψ packaging and dimerization signal, *FLAP*: *cPPT* central polypurine tract; *CTS* central termination sequence; *WRE* the woodchuck hepatitis virus posttranscriptional regulatory element.

contains the following virally derived sequences: two HIV-derived LTRs required for integration into the cell genome; the woodchuck hepatitis virus posttranscriptional regulatory element (WRE) to increase transcription (20); the PSI (ψ) gene required for viral RNA transcription and that serves as a packaging signal; the HIV-1 flap element, containing a central polypurine tract (cPPT) and central termination sequence (CTS), to increase the titer (21); and a CMV (cytomegalovirus)-derived enhancer. The pFUW vector does not contain any genes needed for viral replication and cannot replicate itself in mammalian cells. The pFUW vector contains the human ubiquitin promoter for ubiquitous expression of the transgene. The human ubiquitin promoter in

pFUW can be exchanged with any other promoter sequences. We modified pFUW to drive neuron-specific expression by swapping the human ubiquitin promoter with the neuron-specific synapsin I promoter (22).

3.1.1. Recombinant Lentiviral Vectors

1. The pVSVG and pCMVΔ8.9 vectors should not be modified but amplified without any cloning steps. The pFUW vector contains the ubiquitous ubiquitin promoter followed by a multiple cloning site where the transgene of interest is inserted. In our study, we cloned the cDNA encoding for glutamate receptor interacting protein (GRIP) in pFUW and tagged it with HA and FLAG sequences at the carboxyl terminus (22). The tag provides an efficient and convenient way to monitor transgene expression levels and also reveals chimeric expression and/or genetic mosaicism. Green fluorescent protein (GFP) or another fluorescent variant is often used as tag and fused to the transgene to visualize more directly the protein of interest. Investigators have used the expression of GFP in young animals as the primary method for identifying transgenic pups (11, 23). GFP is also useful for checking transfection efficiency during lentivirus production and for quantification of viral titer throughout the procedure.

2. After inserting the transgene, it might also be useful to modify the promoter in order to regulate trangene levels and obtain spatial and temporal expression. The ubiquitin promoter in pFUW can easily be removed and replaced with your favorite promoter. If the optimal promoter sequence is not known, various lentiviral vectors driving the expression of GFP from different promoter sequences should be created and tested in vivo. To determine for neuron-specific expression, the following promoters were tested: a hybrid promoter containing a human CMV enhancer element in front of a chicken β-actin promoter, a mouse Thy1.2 promoter, mouse α-CaMKII promoters in three different sizes, rat Synapsin I promoters in two different sizes, and a hybrid promoter containing two Synapsin I neuron-restrictive silencer elements in front of an SV40 promoter (24). The short (0.5 kb) synapsin I promoter drives high and specific expression of the transgene in vivo. We used this short fragment from the human synapsin I promoter to obtain neuron-specific expression of the transgene in our experiments (22). One of the biggest drawbacks of lentiviral vectors is their limited DNA-packaging capacity. The total genetic material that can be placed into the lentiviral vector is limited to around ~8 kb. Both the promoter and your gene of interest must fit in this relatively short DNA fragment. This imposes a significant restriction to the experiments if the promoters and/or cDNA you intend to insert in the vector are large.

3. The usefulness of transgenic RNA interference (RNAi) to study gene function in mice and rats has now extensively been demonstrated (5, 25). Lentiviral vectors expressing small hairpin RNAs (shRNAs) have been developed and are used to down-regulate specific target genes in transgenic animals (Fig. 3) (26, 27). Recently, tissue-specific and reversible RNAi in transgenic mice and rats has also been reported (28, 29). The pLL3.7 lentiviral shRNA vector works together with the pVSVG and pCMVΔ8.9 vector system and was shown to be successful for in vivo gene knockdown (17). In the pLL3.7 vector, the ubiquitous CMV promoter drives GFP expression and the RNA polymerase III promoter U6 drives the shRNA expression. It is assumed that the RNAi cellular machinery is active in all cell types; however, this is quite difficult to confirm directly. While shRNA expression vectors have been used to knock down target proteins in many cell types successfully, some cell type-dependent variations in the degree of knockdown have been observed (30). Thus, caution should be taken in assessing phenotypes as complete knockdown may not occur in all cells in vivo.

4. To prevent recombination between the bacterial genome and the plasmids, it is important to use the TOPP10 *Escherichia coli* strain to amplify these vectors. Long-term storage of the transformed bacterial stock at –80°C is not recommended. We preserve the vectors in the form of DNA at –20°C and transform a fresh competent cell when DNA needs to be amplified. Because the pFUW vector is nearly 10 kb in size and only a limited number of cloning sites are available, it is important to use high-quality competent cells to do the cloning efficiently. To create high titer TOPP10 competent cells, we used the following protocol (31).

3.1.2. Producing Lentiviral Particles

1. Bio-safety. All tissue culture experiments should be performed in a Biological Safety Cabinet. The biosafety office at your institution must be notified prior to use of the lentivirus system for permission and for further institution-specific instructions. BL2 conditions should be used at all times when handling the virus. All decontamination steps should be performed using 70% ethanol/1% SDS. Gloves should be worn at all times when handling lentiviral preparations, transfected cells, or the combined transfection reagent.

2. Preparing HEKT cells for transfection. Three 15-cm tissue culture plates are needed to produce one lentivirus. Pre-coat the plates with a 0.2% gelatin solution. Add 10 ml of gelatin solution to a 15-cm plate and aspirate to remove the solution. Dry the plate in the tissue culture hood. Gelatin-coated plates can be prepared in advance and stored for later use. Plate 25 ml

of 0.5×10^6 HEKT cells/ml to the gelatin-coated 15-cm plate and let the cells settle for 5 min in the tissue culture hood. Place the plates back into the CO_2 incubator and incubate overnight. It is very important to have evenly dispersed cells across the entire plate to achieve high transfection efficiency. It is essential that the HEKT cells are well-maintained and of relatively low passage number.

3. Calcium phosphate transfection. HEKT cells are co-transfected with the all three lentiviral plasmids. The 15-cm plates with HEKT cells contain 25 ml of media. Remove 5 ml of media from each plate 1 h before transfection. Keep the plates in a CO_2 incubator to stabilize the pH. Two Falcon 2059 tubes are needed to transfect each plate. The first tube contains 260 μl of 2 M $CaCl_2$, 45 μg of DNA (10 μg of pVSVG, 15 μg of pCMVΔ8.9, and 20 μg of pFUW), and sterile H_2O. The total volume is adjusted to 2 ml. The second tube contains 2 ml of 2× HBS. Using a Gilson pipette and a Pasteur pipette with a long tip, bubble the 2× HBS tube and, while bubbling, add the DNA solution (2 ml) drop-wise. After all the drops are in, keep bubbling for another 5 s. Take one plate at a time from the incubator. Tilt the plate and gently rock while adding the transfection mixture (4 ml) into the media. Immediately place the plate back into the incubator and let it incubate for 4–5 h. Aspirate the media and wash the cells with 12 ml of D-PBS twice. Add 20 ml of fresh media.

When transfecting many plates, the transfection mixture (2× HBS and DNA) must be added to the cells immediately after mixing the 2× HBS and DNA solution. A common mistake, for example, is to make ten transfection mixtures first when handling ten plates. Instead, one should make one at a time and add them to the cells immediately after each mixture in prepared. It is convenient to use pFUGW, a vector containing GFP cDNA, as a positive control during virus production. Having a fluorescence microscope close by is convenient to assess the efficiency of transfection immediately.

We have also tested HEK-FT cells from Invitrogen and obtained good quality viruses. The DNA used for transfection must be of high quality, without contaminating particles. Qiagen kits were used to prepare the plasmids. We found that the endofree Qiagen kits gave better results.

4. Concentrating recombinant lentivirus. The culture supernatant is collected into 50-ml centrifuge tubes at 48-h post-transfection and the cell debris is removed by low speed centrifugation ($1,000 \times g$ for 5 min at 4°C). The supernatant is filtered through Nalgen filter unit with 0.45-μm pore size filter. Before filtering the viral media, it is important to

pre-filter 10 ml of media that contains 10% FCS to block nonspecific binding sites on the filter membrane. Next, the filtered viral media is placed into ultracentrifuge tubes and sealed with thin layer of parafilm for biosafety purposes. The tubes will be placed into the buckets of a pre-cooled (4°C) Beckman SW28 rotor and balanced using D-PBS or media, if necessary. The balancing must be done with the rotor bucket and the difference between the paired buckets must be below 0.01 g. Ultracentrifuge the viral media at 25,000 rpm for 90 min at 4°C. Remove the supernatant by inverting the tube, and once inverted, maintain the inverted orientation and place on a few layers of Kimwipes (Kimwipes should be placed on a parafilm to prevent contamination). Let the tube sit for a few minutes. Using a log-tipped Pasteur pipette to aspirate the media along the side walls of the tube, while maintaining the inverted orientation. During this procedure, do not aspirate the bottom of the tube where the viral pellet is formed. After aspirating the residual media from the tube, place the tube in the normal orientation (up-right) and add 50 μl of HBSS to the bottom of the tube, seal the tube with parafilm, and incubate the tube for 1 h at 4°C with gentle shaking. Next, place the tube in a cold room in the dark for 12–18 h without shaking. Finally, pipette the liquid in the tube for about 20 times up and down to resuspend the virus. Caution must be taken to prevent any bubbling, and only the bottom round part of the ultracentrifuge tube should be exposed to the liquid. The viral solution is aliquoted in 5–10 μl, flash frozen in liquid nitrogen, and stored at –80°C. There should be no change in titer with freezing concentrated virus but avoid multiple freeze-thaws.

5. Working practice and lentiviral wastes. All work surfaces including equipment used such as centrifuges and Biological Safety Cabinet should be thoroughly wiped with 70% ethanol/1%SDS. All liquid wastes generated during lentiviral experiments should be immediately decontaminated by mixing with concentrated bleach for at least 30 min. Solid wastes contaminated with lentiviral materials must be autoclaved or incinerated.

3.2. Preparation for Surgery

Fertilized eggs are collected from the oviduct of superovulating young female animals that were housed overnight with a stud male. In this section and the following, we focus on rats since the basic methodology for mice has been extensively described (15). Although embryo collection and mating schemes for rats largely follow the procedures used for mice, some differences occur. One stark contrast between rat embryo and mouse embryo is their viability in vitro. Mouse embryos that have progressed through

the first few cell divisions can still develop when returned to the uterus of a pseudopregnant female. Readers who are familiar with manipulating mouse embryos may recall that the implantation of the injected embryo is done on the next day, allowing the embryo to grow overnight in the CO_2 incubator. In contrast, rat embryos that have divided once are no longer capable of developing. This imposes a time limit for completing embryonic return in each experiment when using rats. An experienced technician who can routinely collect mouse embryo can collect rat embryos without any difficulties.

1. Choice of strain and housing. The Sprague–Dawley strain was used for rat lentiviral transgenesis. Other strains should also work; however, some strains have fewer littermates and will require more work to obtain the same number of transgenic animals. All animals were kept in a light cycle such that the light was on during 6:00 am to 6:00 pm.

2. The four animal groups. Since the cost of each operation including the preparation is significant, we recommend conducting tests to exclude any faulty animals, especially for the stud males and the vasectomized male. The following four animal groups were routinely prepared and stocked.

   (a) Vasectomized males: Vasectomized males (8- to 12-week old) can be obtained from commercial sources (such as Charles River Laboratories). Each vasectomized male should be housed in separate cages. We recommend conducting a "test mating" with a normal reproducing female to show successful vasectomy. If the female rats do not become pregnant, it is good. We kept three to four vasectomized males and housed them in separate cages.

   (b) The embryo acceptors: Pseudopregnant state can only be achieved when the female is in estrus. To match the estrus cycle, the female that has just given birth to new pups was dissociated from the pups and housed together with a vasectomized male. The remaining pups were killed. This procedure must be done on the day before the operation, and thus it is important to prepare pregnant females that will give birth on the day before the operation. Three pregnant animals that were estimated to give birth on days 2, 1, and 0 (day 0 is defined as the day of operation), respectively, were obtained to ensure that we have at least three females giving birth on the day before the operation. One female and one vasectomized male should be kept in each cage. If courtship happens, the embryo acceptors will become pseudopregnant. Only the pseudopregnant females should be used for oviduct transfer operation.

(c) Stud males (the sperm donors): Obtain normal reproducing males. To be safe, we conducted test mating with a normal female to prove that the stud male is competent. Do not use a male that has never been successful in mating. Some young males may not perform as expected. The stud males will mate with the embryo donors during the night before the operation.

(d) The embryo donors: Hormones (PMS and hCG) are injected to induce superovulation. The schedule and the dose of injection are shown in Table 1. The superovulated females will be housed together with the stud males, starting at around 5:45 pm (immediately before the lights turn off) on the day before the operation. To increase the rate of mating, it is important to transfer one female to the cage in which one stud male is housed. The fertilized eggs will be collected at around 12:30 pm on the day of the operation.

3. Timetable and checklist. Creating a timetable and a checklist is critical for the success of the experiments. As described above, we will refer to the day of the microinjection as day 0. A sample timetable and checklist are shown in Table 1. The time of each procedure may be re-adjusted depending on the light cycle of the room in which the animals are housed.

4. Microsurgical tools. Preparing the correct tools for microsurgery is crucial for efficient and successful operation. The tools that are used in mouse cannot simply be re-used for rats, as the size of the organs and embryos are different. In particular, special attention needs to be taken to make the holding needles of the correct size, injection needles with the correct opening, and transfer pipette with different diameters to implant the microinjected embryos into the oviduct of the pseudopregnant female. If a loose opening is present between the inner diameter of the oviduct and the outer diameter of the transfer pipette, the embryo can reverse flow outside the oviduct when pressure is applied through the mouth of the pipette during implantation. We found this to be the frequent cause of error.

(a) The holding pipette. Sutter P-97 needle puller with a box filament was used to pull holding pipettes (Sutter B100-75-10, borosilicate glass OD 1mm, ID 0.75 mm). The condition used were HEAT = 800, PULL = 70, VEL = 8, TIME250, but this may vary depending on the ramp test of the filament. The average size of a single-cell stage rat embryo (100 μm) is slightly larger than that of a mouse embryo (85 μm). To securely hold the rat embryo surrounded with the zona pellucida, one must prepare the holding pipette so that the outer diameter of

Table 1
The preparation and operation schedule for rat lentiviral transgenesis

Calender day	Thursday				Monday	Tuesday	Wednesday	Thursday
Day	−7	−6	−5	−4	−3	−2	−1	−0
Acceptor	Animal arrival 3× E16, 3× E17, 3× E18						Identify the adult females that gave birth on this day. Separate from the pups and place the female into the cage of a vasectomized male.	Check for plug. Use plug positive rats for embryo recipients. We want at least three rats. The plug is often not detectable. The pseudopregnant rat will have higher vascular flow around the ovary and will be easily identified during the surgery.
Embryo donor	Animal arrival 6× 23- to 28-day-old female rats				Inject PMS hormone (15 IU, IP) at 1:00–3:00 pm		Inject hCG hormone (15 IU, IP) at 48 h after PMS injection. Mating (put female with stud male rats, immediately before the dark cycle starts.)	Check for plug.

(continued)

Table 1
(continued)

Calender day	Thursday				Monday	Tuesday	Wednesday	Thursday
Day	**−7**	**−6**	**−5**	**−4**	**−3**	**−2**	**−1**	**−0**
Embryo harvest								From 1 pm harvest embryo from plug-positive embryo donor. Again, the plug is usually not detectable. However, during operation, one can easily identify the sperms in the uterus. The appearance of the fertilized egg is different from that of the unfertilized egg, an observation that was also used to prevent unfertilized egg to be used in microinjection.
Virus injection	The high titer lentivirus should be prepared and stored at −80°C prior to any animal procedures.						Prepare microinjection needles and holding pipettes.	Microinject lentivirus immediately after embryo harvest. Typically, this procedure starts around 2:30 pm.
Embryo implantation						Prepare embryo transfer pipettes.	Autoclave surgical instruments.	Implant embryo back to the recipient mother immediately after injection. At least two operations should be done to ensure success.

the holding pipette is equal to the width of the single-cell stage rat embryo with the zona pellucida. Identical glasswares used in mouse facility were used to create the holding pipette; however, the tip of the pulled pipette was broken under the microforge (Narishige MF-900) at an optimal position to give a larger opening suitable for holding rat embryos. The tip of the holding pipette was heat polished, so that the edge was smooth and the central opening of the pipette was equal to the thickness of the walls. The holding pipette should be Z bent at an appropriate angle to adjust to the micromanipulator and the configuration of the microscope.

(b) The microinjection pipette. The microinjection pipette should be made the day before or in the morning of the operation. P-97 puller was used to pull glass tubes (Narishige GC-1.2). The pulling condition used were HEAT = 720, PULL = 70, VEL = 70, and TIME250, again this may vary depending on the ramp test of the filament. The needles were beveled at an angle of 30° using a needle microgrinder (Narishige EG-400). The optimal opening was determined to be 40–45 psi when the needle air pressure was measured using an Eppendorf electronic microinjector. The size and shape of the tip must be small and sharp enough to penetrate the zona pellucida efficiently. A small tip, however, may clog the viral particles; thus an optimal opening is required. The microinjection pipette should be Z bent in a way similar to the holding pipette.

(c) The transfer pipettes. The transfer pipettes were made by hand-stretching glass tubes (4 mm OD, 48 in. LG standard wall glass tube, 2304040, PYREX labware) that were softened by a burner. After pulling several glass tubes, there should be enough variety from which one can choose the tubes with the specification described below. The thin glass tubes were scorched and cut into 5-cm length pieces. The end that corresponds to the tip was fire polished. The unpolished end was then inserted into a Pasteur pipette whose tip was broken near the root where the narrowing of the diameter starts. The glass tube and Pasteur pipette assembly was secured using melted wax. Caution is needed not to block the airway by applying the melted wax into the pipette tubing. We prepared transfer pipette with different outer diameters (350, 400, 450, and 500 μm). The lattices of the hemocytometer were used to measure the width of the pipettes (note that the smallest increment of the grid is 50 μm). If the fitting between the pipette and the oviduct was too

loose, we switched the transfer pipette during the operation to one with a larger diameter. For biosafety reasons, we placed two filter units in the tubing line to prevent inhaling virus during experiments.

5. Practice sessions using rats. If one only has experience in mouse but no experience in rat embryonic transfer, it is strongly advised to do practice operations. If a single person is conducting the entire procedures of embryo harvest, microinjection, and oviduct transfer, there will be more reasons to practice so that all the procedures complete as scheduled. The microinjected embryo will be viable for only a short period of time and one will like to return as many microinjected embryos as possible within a few hours.

3.3. Surgery and Microinjection

The key to lentiviral transgenesis is to infect single cell embryo with lentiviral particles by microinjecting the high titer viral solution into the space between the plasma membrane of the embryo and the zona pellucida. Once infected into the single cell embryo, the virus will integrate its genetic element into the host genome. Because the transgenic expression is not silenced during development, the expression profile of the transgene among cell types follows the characteristics inherent to the promoter used to drive the transgene. Depending on the number of particles infected, the number of integration will vary. Immediately after microinjecting the lentivirus, one must start the operation to transfer the injected embryos into the oviducts of pseudopregnant females. On average, about 15 embryos are injected into each oviduct of a pseudopregnant female. The operational techniques for rats are similar to that for mice but if the operator has experience only with mouse, a little practice will be required to complete the operation efficiently and with success in rats.

In this section, we describe in detail the procedures on the day of the surgery. The surgery room was reverse pressured compared to the surroundings for biosafety purposes. A beaker with 1:5 Clorox bleach for needle and micropipette disposal was placed. An autoclavable biohazard disposal bag with a container was placed. All surgical metal instruments were autoclaved in bags. A CO_2 incubator, needle puller, microforge, and the microinjection rig were placed in the surgery room. The clock starts ticking at the moment you take out the embryo. The embryo will be of good quality for only 6–8 h in vitro, and thus the embryonic transfer operation must be completed by this deadline.

1. Pre-operation checkup. Make sure all of the instruments are ready. Forge all of the fine glass pipettes according to the specification. It is good to have 2–3 holding pipettes, 5–10 microinjection pipettes, and 12 transfer pipettes (three for each size). Select the animals that you choose to use before

starting the experiments. Especially make sure to use the correct ones and label the cages accordingly to prevent mistakes. We typically prepared three pseudopregnant females and planned to do at least two operations to return the embryos. It is possible to conduct microscopic examination of vaginal smear to confirm that the vasectomized male actually mated with the female; in practice, however, because the "plug check" is not feasible in rats and the examination of vaginal smear requires experience, we omitted this step and used the bloody appearance of the ovary as a criterion to determine pseudopregnancy.

2. Embryo harvest. The pre-operation checkup was done at noon and we started euthanizing the embryo donors at 1:00 pm. The plug check is often difficult in rats. We collected embryos from six young female rats and separated the embryos that were collected from each individual. We used microscopic observation of the embryos to determine if the eggs were fertilized. In this way, we were able to exclude using unfertilized eggs reliably. A procedure that is nearly identical to what is used for mouse was used to collect the embryos. In brief, we created an incision to the abdominal wall and approached the ovary and the oviduct and excised them from the uterus. The oviduct and the ovary were separated by a cut and placed into a 60-mm tissue culture dish filled with 3 ml of M2 media. The single-cell stage rat embryos were dissected from the ampulla of the oviduct in M2 media using procedures identical to those used for collecting single-cell mouse embryos. The cumulus cells (follicle cells) were enzymatically removed by incubating at 37°C for 15 min in M2 media containing 1 mg/ml hyaluronidase. The embryos were kept in microdroplet culture (in CO_2 incubator at 37°C) in M2 media covered with mineral oil until microinjecting the lentiviruses.

3. The configuration of the micromanipulator. The microinjection started immediately after the embryo collection was completed (typically from 2:00 pm). The setup of the inverted microscope (Nikon) and the micromanipulator (Leica) was identical to those used for routine microinjection for mouse embryos. To use the silicon oil-driven manual injector on a Leica micromanipulator, the tip piece of the Leica micromanipulator was changed to the one supplied by the Eppendorf Cell Tram Vario. The holding pipette was connected to a Hamilton syringe via a plastic tubing (care was taken to prevent leakage between each connection). The Hamilton syringe was mineral oil driven and was securely placed at the front edge of the rig outside the vibration isolation table.

4. Setting up the microscope. Turn on the nitrogen tank and microinjector. Place a set of eggs into a drop of oil-covered FHM in the injection dish (~30). Place the injection dish in the center of the microscope stage and focus the microscope at the lowest power on the embryos. Place a holding pipette onto the opposite side of the scope. Lower both pipettes using the course controls into the injection dish and maneuver the needle and the holding pipette so that they are parallel to each other and visible at the lowest magnification. Remove the embryo-containing plate from the stage while loading the needle with the virus.

5. Loading the virus. The virus is taken out of the −80°C freezer immediately before microinjection, spun briefly using a table-top microcentrifuge, and placed on the inner side of the lid of a 35-mm tissue culture dish. We recommend using dry ice to transfer the virus from the freezer to the operation room if the freezer is located in a different location. The virus droplet on the plastic lid was placed on the stage of the microscope and the microinjection needle was loaded with the virus from the tip. During the loading, the virus drop and the needle were visually inspected and the micromanipulator was used to avoid large aggregates of virus that may clog the tip of the needle.

6. Microinjection. After the loading was complete, the plastic dish that contains the embryo in the droplet under the mineral oil was placed on the microscope stage. The tip of the microinjection needle was quickly placed into the culture droplet and constant positive pressure was applied by turning the wheel of the Cell Tram Vario. The holding pipette was then placed into the droplet to stabilize the eggs for injection. The position of contact of the microinjection needle on the zona pellucida should be slightly displaced from the center to prevent the needle from penetrating through the plasma membrane of the embryos. One should not aim the center of the sphere but should try to aim at a position slightly displaced from the center of the sphere. Once the needle penetrates through the zona pellucida, the embryo will be pushed by the injected liquid and therefore, the needle must be removed before the embryo is permanently damaged by the increasing pressure. Inject into the perivitelline space with adequate volume to see an obvious swelling of the perivitelline space. After ~30 eggs have been injected, place eggs into the 35-mm dish with three drops of warmed KSOM.

7. Biohazardous wastes. Unused virus, the injection needles, and the injection dishes should be discarded in biohazardous waste. The needle holders, the injection dish tray, and all Pipetmans used should be placed in the hood under UV light.

8. Post-microinjection procedures. After the microinjection was completed, the injected embryos were inspected under the dissection scope, and undamaged embryos were selected for the following oviduct transfer operation. The oviduct transfer of rat eggs should start the same day around 4:00 pm. The mouse eggs can be incubated overnight. The next morning, count the number of one-cell and two-cell embryos. The ratio should be 80/20, two cell to one cell; an indication that the virus was at an optimal concentration. If the ratio is lower than 80:20, then the virus should be diluted further at the next injection.

9. Oviduct transfer. The rat procedures are analogous to the oviduct transfer for mice, which can be found in (15). Anesthesia was injected (i.p.) based on the following formula: 60 mg/kg ketamine, 7.5 mg/kg xylasine, and 1 mg/kg midaxolam. The surgical area was shaved and cleaned using iodine swab. Vaseline (antibiotic paste) was applied to the eye to prevent drying. Load the embryos into the transfer pipette before opening the mother and pre-select the second set of embryos so that the second loading can be done immediately. The embryos should be positioned between two air bubbles so that the injection process is visible during the surgery. We injected 15 embryos to each oviduct. The ovary and the oviduct were approached from the back of the animal by creating a 2-cm incision to the skin and a similar cut to the abdominal wall. The fat pad surrounding the ovary was grasped with the forceps and the oviduct was pulled out of the body. A clamp was placed on the fat pad to secure the organs. If the animal is pseudopregnant, one should be able to identify the increased bloody appearance of the ovary. It is easy to tell the difference if one has seen a non-pseudopregnant ovary. If the pseudopregnancy cannot be determined with confidence, do not use the animal and move on to the next animal. With a little bit of practice, one can locate the position of the infundibulum, which is often hidden inside the coiled oviduct. A small break will be made in the bursa using a sharp forceps. It is important not to break blood vessels, as the bleeding will obscure the surgical field. Then support the oviduct near the opening with the forceps. The transfer pipette will be placed to the opening of the infundibulum and slid into the oviduct. If the junction between the oviduct and the transfer pipette is loose, one should immediately switch to a transfer pipette with a larger outer diameter. Apply pressure to the mouth of the pipette and visually confirm that the bubble behind the embryos has been injected. Maintain positive pressure while removing the transfer pipette. Place the oviduct back to the abdominal cavity and stitch the abdominal muscles. The skin was sealed using two to three

surgical staples. After the oviduct transfer is completed for one side, move on to the other side. After the surgery is completed, place the animal on a warm pad (~32°C) until the anesthesia fades out.

3.4. Post-Surgery Procedure and Following Up on the Animals

After the embryonic transfer, one will wait until the pups are born. From here on, any scientist who is experienced in handling mice or rats and has skills to genotype transgenic animals can carry on the experiments. We typically obtained 3–12 pups/litter. A small fragment from the tip of the tail is collected and the genomic DNA is extracted for genotyping. Many of the transgenic animals transfer the transgene to the next generation. After several rounds of breeding, it is possible to obtain independent stable transgenic strains from a few transgenic animals (see Note 1–3).

1. Until the P1 pups are born, make sure the mother is healthy and look for complications caused by the surgery (such as abnormal bleeding). Depending on the experiment, the size of the litter may vary. If the litter size is small, the mother may eat the pups. To prevent such happening, one may prepare a foster mother and place the P1 pups among the foster mother's real pups.

2. Determining the copy number of genomic integration is necessary to follow the transgenes during successive rounds of mating. It is, therefore, important to design both PCR-based and genomic Southern-based methods to detect the transgene. For the genomic Southern, it is important to have a unique restriction site in the lentiviral vector. In our study, the genotyping was done using genomic Southern blotting using the DNA that was extracted from a small tail fragment obtained by biopsy (22). For the PCR-based detection, a pair of primer (one inside the open-reading frame of the transgene and another in the promoter of the lentiviral vector) needs to be selected (see Note 4).

3. When we started the project, we took cautions of the biosafety issues and conducted safety test on the born animals. Small volume of blood of each new born P1 pups was collected when the tail biopsy was done for genotyping. The serum from each P1 pup was applied to HEKT cells grown in a 96-well plate and incubated for 2 days. The HEKT cells were fixed and stained to determine using fluorescent microscopy if any infectious virus was present in the serum. A diluted high titer virus that was microinjected should be used as positive control. We never saw any infectious particle in the serum of the P1 pups. Whether this procedure is critically necessary should be discussed with the biosafety office at your institution.

4. Notes

Our most commonly encountered failures were finding no trans-genic animals in the born littermates. In our own experience, the majority of the failures were simply caused by having low titer viruses. The rat embryonic manipulation was highly reproducible and robust once the researcher got used to doing routine operation. In the following, we list some troubleshooting tips.

1. If no pups were born.

    (a) Was the pseudopregnant female really pseudopregnant? Use the most reliable method to determine pseudopregnancy. We found visually inspecting the increased blood flow around the ovary during operation the most reliable method. When we had doubts, we did not implant the embryos and started operating the next available animal.

    (b) Were the eggs fertilized? The appearance of an unfertilized egg and a fertilized egg is different. Use DIC microscope and compare the difference. We often noticed residual sperms in the uterus of the embryo donor. This is another confident way of determining if the donor has mated prior to embryo collection.

    (c) Did you use the transfer pipette with the correct specification? See the section above that describes the specification of the transfer pipettes.

    (d) Were the embryos damaged during microinjection? It is very important to select the best embryos after microinjection of the lentivirus. The number of good embryos will increase with experience and practice.

    (e) Did the transgene interfere with the development? This will require a change in the experimental plan. However, to be confident that the transgene is truly interfering with the development, one may need to demonstrate that by swapping the transgene with GFP, the transgenic rats can be obtained.

2. If pups were born but no transgenic animals were identified.

    (a) Was the viral titer high? Determine if the titer was higher than 10^6 infectious unit/μl. To improve the titer of the lentivirus, one must review the procedure of viral production. Specific points worth mentioning are (1) use healthy HEKT cells, (2) use reagents for calcium phosphate precipitation that give maximum efficiency, (3) use serum that has been proven to produce high titer viruses, and (4) use freshly prepared media.

   (b) Was the vasectomized male truly vasectomized? This can be tested by mating the vasectomized male with a normal reproducing female.

   (c) Did the transgene interfere with the development? If lentivirus transgenesis is routinely performed and the failure is clearly virus dependent despite using high titer virus, it is possible that the transgene has interfered with the development of the animal. In this case, one must change the transgene.

   (d) Was the microinjection done correctly? If enough liquid is not injected into the perivitelline space even if the viral titer is high, the embryo will not get good infection. It is useful to find out how long the needle can stay inside the perivitelline space until the embryo is damaged. We found that it is good to stay longer than shorter, but not to the extent to destroy the embryo.

3. If there is no germ line transmission.

   We do not have enough data to troubleshoot this issue. However, in the majority of the cases, the failure of germ line transmission is dependent on the transgene. The published germ line transmission of transgenic rat that expresses GFP is higher than that of the transgenic rat that expresses GRIP (22). In addition, some alleles are less transmissible than the others.

4. If the expression of the transgene is low.

   The promoter may not be strong enough or the transgene products were not favored by the cells. If the genomic Southern identified the transgene and the genome but the protein product was not detectable from the total cell lysates by Western blotting, try to detect the protein by immunoprecipitation followed by Western blotting. GFP is known to be non-toxic even when it is expressed at relatively high levels in vivo. The best way is to test if the promoter expresses GFP in the transgenic animals.

Acknowledgments

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Transgenesis in Mouse Embryonic Stem Cells

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Abstract

Traditionally, transgenic mice are generated by pronuclear injection of exogenous DNA. This technique has several limitations, including limited control over transgene expression, transgene cytotoxicity, promiscuity and silencing, and founder mouse sterility. Here we describe two protocols to generate transgenic mice from ES cell clones carrying stably integrated exogenous DNA with inducible transgene expression.

Key words: Transgenic mice, ES cells, Inducible expression, Cre recombinase, Doxycycline

1. Introduction

Transgenesis in mouse oocytes is a widely used technique for the creation of human disease models or to study gene function at the level of the whole animal. A drawback of pronuclear injection of exogenous DNA is that there is limited control over transgene expression, making it necessary to generate and characterize many founder lines to obtain a few with optimal transgene expression. Other frequent problems include transgene cytotoxicity resulting in embryonic lethality, transgene promiscuity, transgene silencing, and founder mouse sterility. Many of these problems can be avoided by generating transgenic mice from ES cell clones carrying stably integrated exogenous DNA. Here, we will describe two applications of this approach, both designed to generate transgenic mice with inducible transgene expression. One approach, which was developed in the laboratory of Corinne Lobe, is based on the Z/EG vector system (1, 2). It allows for induction of tissue-specific transgene expression via Cre recombinase-mediated removal of a "floxed" transcriptional "stop" cassette (Fig. 1a). The second approach, which was developed in our laboratory, is
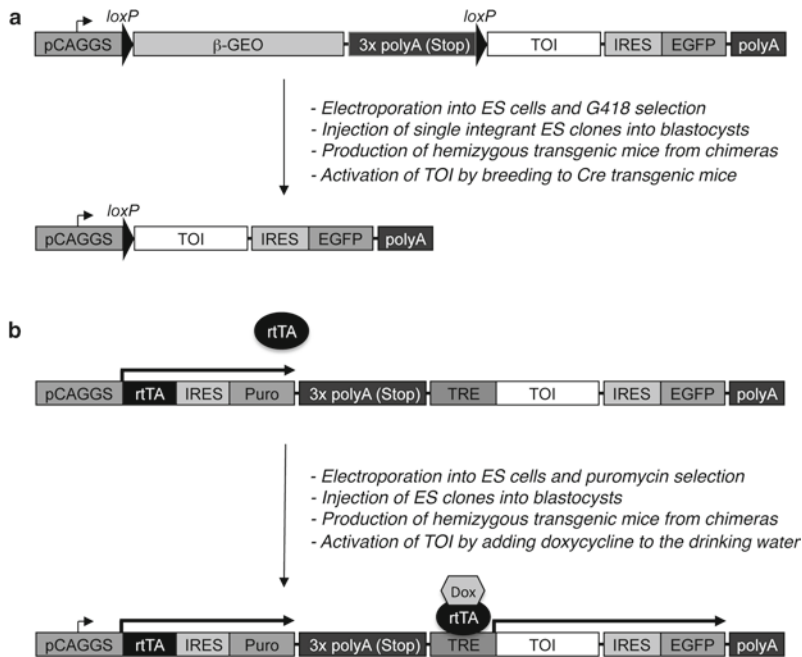
Fig. 1. Schematic representation of the generation of transgenic mice by ES cell-based approaches. (**a**) Generation of Cre recombinase-inducible transgenics using the Z/EG vector. (**b**) Generation of doxycycline-inducible transgenics using the TRIC vector.

based on a so-called TRIC vector system (for tightly regulatable inducible cDNA). It allows for autonomous doxycycline-mediated induction of transgene expression (Fig. 1b). Both approaches include an EGFP marker expressed from an internal ribosomal entry site (IRES) sequence that allows for monitoring of transgene expression. First, we will provide an outline of both methods and then a detailed protocol.

1.1. Cre Recombinase-Inducible Transgenics

Z/EG is a mammalian expression vector designed to generate ES cell-derived transgenic mice (2). It contains the CAGGS promoter, which encompasses the cytomegalo virus (CMV) immediate enhancer and the chicken β-actin promoter. The CAGGS promoter drives a floxed β-geo-stop cassette, consisting of a β-galactosidase and neomycin-resistance fusion gene and three tandemly arranged polyadenylation sites (Fig. 1a). Downstream of this cassette, the coding sequence of the transgene of interest (TOI) should be cloned. It is recommended that the TOI be marked with an epitope tag to facilitate transgene detection. Upon integration, the transgene is inactive and requires Cre recombinase-mediated removal of the β-geo-stop cassette for induction. EGFP is coexpressed from an IRES as a convenient reporter for TOI expression (Fig. 1a). It is important to linearize the transgenic vector to obtain efficient and proper integration into the genome. Unique ScaI and SfiI restriction sites localized within

the backbone of Z/EG are available for this purpose. Linearized DNA is then electroporated into ES cells, and clones resistant for G418 are selected and clonally expanded in 96-well plates (Fig. 2). Each clone is then split into three 96-well plates. Cells in plate 1 are infected with an adenovirus that expresses Cre recombinase, which will remove the β-geo-stop cassette and juxtapose the CAGGS promoter with the coding sequence of the TOI and the EGFP marker. ES cell clones that express EGFP are identified by fluorescence microscopy 2–3 days later. The EGFP intensity of the cells serves as an excellent marker for TOI level of expression and is used to preselect clones with low, moderate, or high TOI protein levels. Clones with the appropriate level of expression are then harvested and subjected to Western blot analysis for the epitope tag to confirm proper TOI expression. Cells in plate 2 are fixed and stained for β-galactosidase activity as an independent measure for CAGGS promoter activity. Once cells in plate 3 reach sub-confluency, they are split again into three 96-well plates. Cells in one plate are frozen when sub-confluent, whereas cells in the other two plates are grown to super-confluency for extraction of genomic DNA. This DNA is used to screen for single-copy transgene integration by Southern blotting. This is important to rule out chromosomal instability resulting from recombination between *loxP* sites at different integration sites. ES clones with suitable transgene expression are reseeded from frozen stocks to
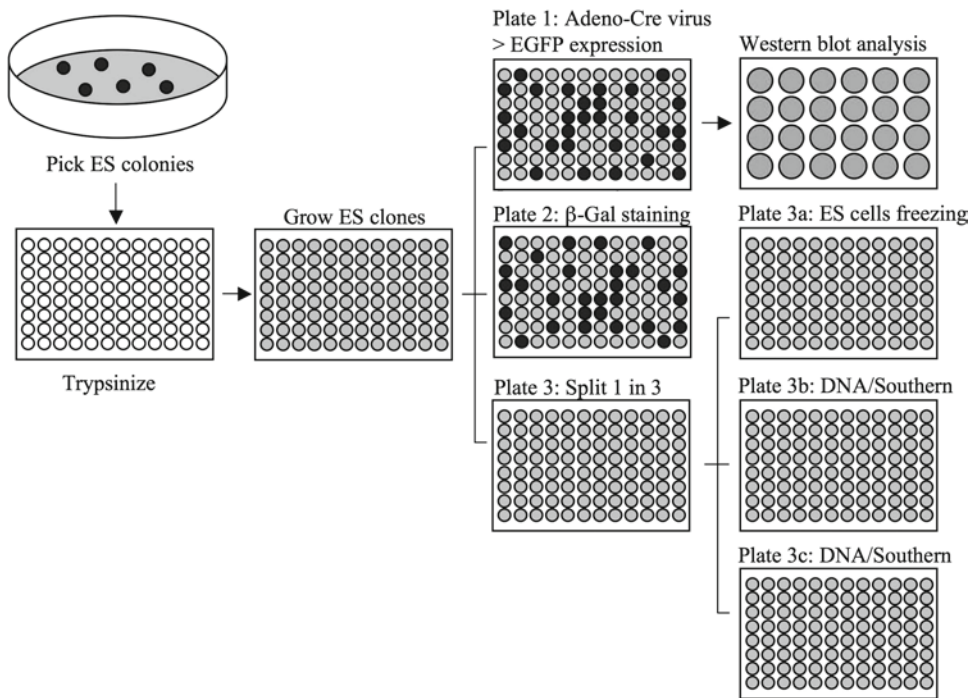
Fig. 2. Schematic overview of the procedure for generating ES cell clones using the Z/EG vector system.

obtain cells for karyotype analysis and production of chimeric mice through blastocyst injection. Male chimeras from independent ES clones are used to generate hemizygous transgenic mice. These mice still contain the floxed β-geo-stop cassette and need to be crossed to Cre transgenic mice to induce the expression of the TOI and EGFP marker (Fig. 1a). A vast collection of well-characterized transgenic mouse strains that express Cre recombinase under the control of different promoters is available to activate transgene expression in a tissue- or cell type-specific fashion. Furthermore, several transgenic mouse strains express Cre recombinase in male or female germ cells, allowing for ubiquitous transgene activation. Thus, there is tremendous flexibility with regard to the spatio–temporal induction of TOI expression once the initial transgenic strains have been established. Another major advantage of this approach is that proper transgene expression can be verified and controlled before the animal is created.

1.2. Doxycycline-Inducible Transgenics

Arguably, the most widely used inducible gene expression system is the Tet-On expression system. One of the disadvantages of this system has been its leakiness, but newly developed reverse tetracycline-controlled transactivator (rtTA) proteins, whose binding to tetracycline-response elements (TREs) is much more dependent on doxycycline, have virtually eliminated this problem. We have designed a novel Tet-On rtTA expression vector, called TRIC, which is tightly regulatable in both cultured cells and mice. In this vector, the CAGGS promoter drives the expression of a tightly regulatable rtTA gene and an IRES-puromycin-resistance gene (Fig. 1b). Immediately downstream of the puromycin-resistance gene are three polyadenylation sites to prevent CAGGS-mediated transcription beyond this gene. The polyadenylation sites are followed by a TRE that binds rtTA exclusively in the presence of doxycycline (Fig. 1b). A short polylinker allows for the insertion of the TOI cDNA behind this element. Downstream of the polylinker is an IRES-EGFP gene that serves as a marker for TOI expression. A major advantage of this vector system is that both the rtTA gene and the TRE-regulated TOI are present in the same vector and will thus co-integrate at the same genomic location, which tremendously simplifies mouse breeding. Again, linearized DNA is electroporated into ES cells, and clones resistant for puromycin are selected and clonally expanded in 96-well plates. Each clone is then split into three 96-well plates (Fig. 3). Cells in plate 1 are grown in the presence of doxycycline, and ES cell clones that express EGFP are identified by fluorescence microscopy 2 days later. Clones with the proper level of expression are harvested for verification of TOI expression by Western blot analysis (using anti-tag antibody). Cells in plate 2 are used to select single-copy transgene integration by Southern blotting, whereas cells in plate 3 are frozen when sub-confluent. ES clones
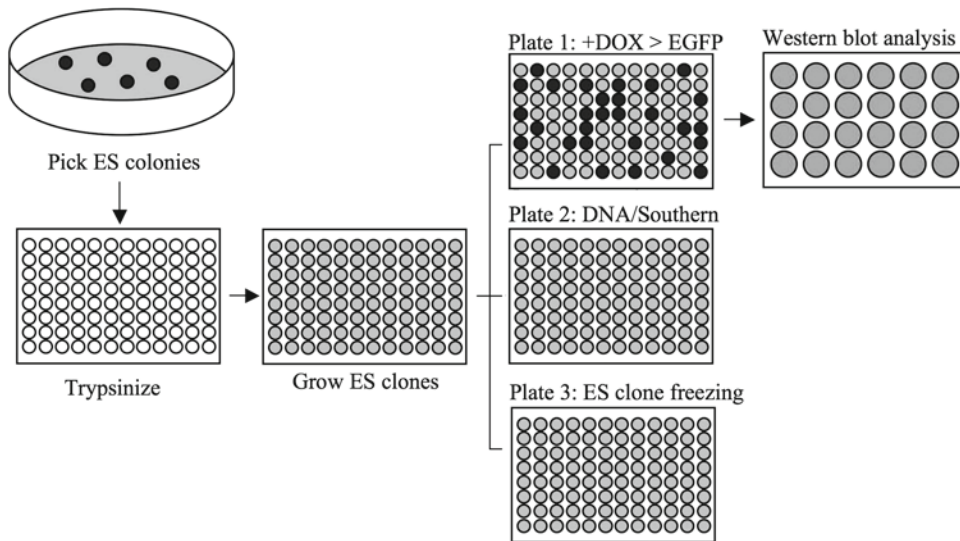
Fig. 3. Schematic overview of the procedure for generating ES cell clones using the TRIC vector system.

with proper transgene expression are regrown and injected into blastocysts to produce male chimeras. Hemizygous transgenic mice derived from these chimeras are provided with drinking water containing doxycycline to induce transgene expression.

A detailed description of the protocol for generating transgenic mice in ES cells is provided below. The protocol is based on the use of the pZ/EG and pTRIC expression systems, but can be easily adapted for use in other expression systems. This protocol is an adaptation of a previously published protocol (3) and is routinely used in the Transgenics and Gene Knockout Core Facility at Mayo Clinic Rochester.

2. Materials

2.1. Equipment for ES cell culture

1. A tissue culture hood with ultraviolet (UV) light, and gas and vacuum (for aspiration).

2. A water-jacketed 37°C incubator with 5% CO_2 and 20% O_2 gas and saturated humidity. Clean it thoroughly before the start of the gene-targeting experiment and use it only for the culture of ES cells and SNLH9 feeders for the duration of the experiment.

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. An inverted fluorescent microscope with 20× objective.

6. A table-top centrifuge with swing-out rotors for 15-mL tubes and 96-well plates.

7. 20- and 200-µl pipettes.

8. Multichannel pipette with a 20- to 200-µl volume range.

2.2. Disposables

1. The following tissue culture-treated flasks, dishes, and plates are used: 25-cm² (T25), 75-cm² (T75), and 150-cm² (T150) culture flasks (use 5, 10, and 20 mL of medium per flask, respectively); 10-cm dishes; 96-well flat- and U-bottom plates; and 24-well plates.

2. Electroporation cuvettes with an electrode gap of 0.4 cm.

3. 1-mL cryogenic vials.

4. 2-, 5-, 10-, and 25-mL disposable pipettes; 15- and 50-mL tubes; and a 50-mL reservoir for multichannel pipettes.

2.3. Tissue Culture Media and Solutions

2.3.1. Feeder Medium

Prepare this medium by adding the following sterile components to 500 mL of Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4,500 mg/L) and low bicarbonate (17 g $NaHCO_3$ mg/mL) (store at 4°C):

1. 50 mL of fetal calf serum (FCS). Long-term serum storage is in 50-mL aliquots at –20°C and short-term storage (for 1–2 months) at 4°C.

2. 5 mL of 200 mM L-glutamine. Store at –20°C.

3. 5 mL of 100 mM sodium pyruvate. Store at 4°C.

4. 5 mL of 10 mM non-essential amino acids. Store at 4°C.

5. 0.5 mL of 10 mg/mL gentamycin. Store at 4°C.

6. 0.5 mL of 5.5×10^{-2} M β-mercaptoethanol. Store at 4°C.

2.3.2. ES Medium

Prepare this medium by adding the following sterile components to 500 mL of DMEM containing high glucose (4,500 mg/L) and low bicarbonate (17 g $NaHCO_3$ mg/mL) (store at 4°C):

1. 90 mL of ES-qualified FCS. Long-term serum storage is at –20°C. Short-term storage (for 1–2 months) is at 4°C (see Note 1).

2. 30 µl of 10.000.000 U/mL ESGRO (LIF). Store at 4°C (see Note 2).

3. 5 mL of 200 mM L-glutamine. Store at –20°C.

4. 5 mL of 100 mM sodium pyruvate. Store at 4°C.

5. 5 mL of 10 mM non-essential amino acids. Store at 4°C.

6. 0.5 mL of 10 mg/mL gentamycin. Store at 4°C.

7. 0.5 mL of 5.5×10^{-2} M β-mercaptoethanol. Store at 4°C.

8. Nalgene filter unit (0.22 µm).

ES medium is filtered using a Nalgene filter unit (0.22 μm). Both feeder and ES media should be stored at 4°C, protected from light, and can be used for up to 1 month if L-glutamine is replaced every other week.

2.3.3. Freezing Medium

1. Freezing Medium (2×): Supplement 30 mL of DMEM with 50 mL of FCS and 20 mL of dimethylsulfoxide (DMSO).
2. Freezing Medium (1×): Supplement 40 mL of DMEM with 50 mL of FCS and 10 mL of DMSO.

Freezing medium is filtered using a Nalgene filter unit (0.22 μm). When stored at 4°C, protected from light, this medium can be used for 1 month.

2.3.4. PBS/0.1% Gelatin

1. Add 0.5 g of porcine skin gelatin to 500 mL of PBS without calcium and magnesium. Dissolve in microwave, filter sterilize using a Nalgene filter unit (0.22 μm), and store at room temperature (RT). Gelatin coating is done by incubating tissue culture surfaces with PBS/0.1% gelatin for 10–15 min (longer incubation time is not a problem).

2.3.5. Others

1. PBS without calcium and magnesium is used to wash cells. Store at RT.
2. TrypLE™Express is used for cell trypsinization. Store at 4°C.
3. β-Galactosidase staining kit.
4. Doxycycline (for TRIC vectors).

3. Methods

3.1. Preparation of Vector DNA for Electroporation into ES Cells

1. Isolate targeting vector DNA from bacteria by alkaline lysis and anion-exchange chromatography.
2. Linearize about 20 μg of transgenic vector DNA with an appropriate restriction enzyme according to the instructions of the manufacturer.
3. Precipitate linearized DNA with ethanol. Add 1/10 vol. of 3 M sodium acetate (pH 5.2) and 5 μl of 20 mg/mL glycogen. Add 2.5× vol. of 100% ethanol. Mix and centrifuge at 18,400 rcf for 10 min at 4°C. Discard the supernatant being careful not to throw out DNA pellet.
4. Rinse with 70% ethanol and centrifuge again for 5 min.
5. Aspirate the 70% ethanol in a tissue culture hood and resuspend the DNA pellet at 0.2 μg/mL in sterile PBS. Analyze 0.5 μg of this DNA solution on a 0.8% agarose gel. Store the linearized vector at –20°C until use.

3.2. Preparation of Irradiated Feeder Layers

ES cells can be grown either on primary or immortalized (STO) mouse embryonic fibroblasts. Our laboratory is using TL1.4 ES cells that have been adapted for growth on STO cells. STO feeders that stably express the neomycin and hygromycin phosphotransferase genes (called SNLH9 feeders) are used to generate transgenics with Z/EG vectors, whereas SNLH9 feeders that also express the puromycin-N-acetyl-transferase gene (SNLH9-Puro feeders) are used to create transgenics with TRIC vectors. Preparation of feeders for use as monolayers is as follows:

1. About 4–5 days before you plan to start growing ES cells, thaw a vial of frozen SNLH9 (or SNLH9-Puro) cells and seed in a T75 flask. Culture at 37°C.

2. When the feeder cells are confluent (usually within 2 days), wash 2× with PBS and add 1.5 mL of TrypLE™Express. After 5 min at 37°C, resuspend the cells in 30 mL of feeder medium and transfer to three T75 flasks.

3. When these three cultures become confluent, trypsinize the cells and suspend them in 20 mL of feeder medium. Count the cells and calculate the number of cells per milliliter. Seed the appropriate amounts of feeder cells in four gelatin-coated 10-cm dishes and one gelatin-coated T75 flask (see Table 1). Reseed the residual cells into the original T75 flasks for use later on (expand this culture if necessary).

4. The four 10-cm dishes and the T75 flask should be nearly confluent the next day. If so, transfer the cultures to a γ-irradiator (e.g., a ^{137}Cesium source) and expose the cells to 3,000 rads.

5. The feeders are now ready for use as ES cell feeders. ES cells can be seeded onto these feeders for up to 5 days post-irradiation.

Table 1
SNLH9 feeder preparation on various tissue culture surfaces

Culture vessel (surface)	Feeder cell number	Amount of 1×10^5 suspension (ml)
T75 flask (75 cm²)	15×10^5	15
10-cm dish (55 cm²)	10×10^5	10
12-well plate (4 cm²/well)	0.6×10^5	0.6
24-well plate (2 cm²/well)	0.3×10^5	0.3
96-well plate (0.5 cm²/well)	0.1×10^5	0.1

3.3. Preparation of ES Cells for Electroporation

It is important to be sure that your frozen stocks of ES cells are truly pluripotent. In other words, they should have a demonstrated ability to produce germline-transmitting male chimeras following their injection into host C57BL/6 blastocysts. If you obtain a new parental ES cell line from another laboratory (or company), it is recommended that you first prepare a large number of frozen stocks (~15–30 vials). Use one of these vials to perform blastocyst injections. A suitable ES cell line should produce a high frequency of germline-transmitting chimeric males in your laboratory.

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 three times, the culture will be 70–80% confluent again within 24–36 h. If diluted six times, this will take about 48 h. If one would inadvertently seed ES cells at too low a density, 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. Thaw a vial of ES cells (~10×10^6) in a 37°C water bath. Transfer the ES cell suspension to a 15-mL tube with 5–10 mL of ES medium. Pellet the ES cells at 180 rcf for 5 min.

2. Aspirate the supernatant, gently resuspend the cells in 10 mL ES medium, and transfer to the T75 flask with irradiated feeders. Incubate at 37°C.

3. When the ES culture is about 70–80% confluent ($20–30 \times 10^6$ ES cells), aspirate the medium, wash the cell culture three times with 5 mL of PBS (gently swirl the dishes to remove dead cells and traces of ES medium), and add 2 mL of TrypLE™Express. Evenly distribute the TrypLE™Express and incubate at 37°C for 5 min.

4. Remove the T75 flask from the incubator and shake it vigorously to generate a single cell suspension. Add 8 mL of ES medium, transfer the suspension to a 15-mL tube, and pellet the ES cells at 180 rcf for 5 min.

5. Aspirate the supernatant, gently resuspend the cells in 5 mL of PBS, and pellet the cells again. Repeat this wash once.

6. After the last wash, resuspend the cells in PBS such that the total volume is 750 μl. Add 50 μl of the 0.2 μg/mL linearized vector solution (10 μg of total DNA for both Z/EG and TRIC vectors). Transfer the 800-μl cell suspension to an electroporation cuvette with a 0.4-cm gap size. Remove potential air bubbles (see Note 3).

7. Place the cuvette in the Bio-Rad Gene Pulser and electroporate at 230 V/500 μF. The time constant should be between 6 and 8 ms. Place the cuvette in the tissue culture hood and let it sit for 5 min (see Note 4).

8. In the meantime, fill a 50-mL tube with 40 mL of ES medium. At 5 min after electroporation, gently transfer the ES cells to the 50-mL tube.

9. Aspirate the medium from the four 10-cm dishes with irradiated feeders and plate 10 mL of aliquots from the ES cell suspension in the 50-mL tube.

10. Exactly 24 h after the electroporation, replace the ES medium on the plates with drug-containing ES medium. The following final drug concentrations are used: G418, 350 μg/mL (for Z/EG vectors) and puromycin, 2 μg/mL (for TRIC vectors). Typically, 8 days after electroporation, ES colonies are ready to be picked. Occasionally, the cells are ready for picking a day earlier or later.

3.4. Picking of Drug-Resistant ES Clones

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 due to three-dimensional growth. The center of an ES colony usually has 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 Figs. 2 and 3 for a schematic overview of the picking procedure.

1. Prepare feeder layers 2–3 days prior to the scheduled picking of the ES cell colonies. For experiments in which Z/EG-based transgenic vectors are used, prepare a total of seven gelatin-coated, 96-well flat-bottom plates with SNLH9 feeders: five plates with 0.1×10^5 feeder cells per well, and two plates with 0.05×10^5 cells per well. For experiments in which TRIC-based transgenic vectors are used, prepare a total of four gelatin-coated, 96-well flat-bottom plates with SNLH9-puro feeders (0.1×10^5 feeder cells per well). For both vector types, prepare two gelatin-coated, 24-well plates with 0.3×10^5 feeders per well (see Note 5).

2. Fill a sterile 50-mL reagent reservoir with TrypLE™Express solution and use a multi-channel pipette to add 25 μl of TrypLE™Express in each of the wells of two round-bottom 96-well plates. Prevent evaporation of the TrypLE™Express solution by placing the plates in the 37°C incubator.

3. Next, load a sterile 50-mL reagent reservoir with ES selection medium and remove the feeder medium from a 96-well flat-bottom plate with 0.1×10^5 irradiated feeders per well.

Add 200 μl of ES medium with 350 μg/mL of G418 or 2 μg/mL of puromycin (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 selection medium in the dish to preserve the normal morphology of the ES clones during the picking (see Note 6).

5. Set an adjustable 20-μl pipette 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 pipette to interrupt the feeder monolayer around the colony (by "drawing" circles around the colony). Then detach the colony and draw it into the pipette tip with 2.5 μl of ES selection medium (see Note 7). Transfer the colony to the first well of the round-bottom 96-well plate with TrypLE™ Express solution (pipette up and down at least five times).

6. Pick up a total of 48 colonies (assuming that the picking time for 48 colonies is less than 25 min). Change pipette 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 irradiated feeder monolayers out of the incubator. Disaggregate the ES cells in row A of the round-bottom 96-well plate using a multichannel pipette (pipette up and down at least 10–20 times). Then, transfer the suspended ES cells to row A of the 96-well plate with feeders. Repeat this procedure for the remaining rows. Incubate the plate in a 37°C incubator.

8. Add 48 additional colonies to this plate by repeating steps 4–7.

9. Usually 2 days after picking, most of the colonies will be 80–90% confluent. The ES cell clones are then ready to be split into three new flat-bottom 96-well plates (0.1×10^5 feeders per well). Separate protocols now apply to Z/EG- and TRIC-based transgenic vectors, which are detailed below.

3.5. Identification of Transgenic ES Clones

3.5.1. Identification of Z/EG-Derived Transgenic ES Clones

1. Replace the feeder medium of two 96-well flat-bottom plates containing 0.1×10^5 SNLH9 feeder cells with 250 μl of ES medium (without G418). Replace the feeder medium of one 96-well flat-bottom plate containing 0.1×10^5 SNLH9 feeder cells with 200 μl of ES medium (also without G418). Return all three plates to the 37°C incubator.

2. Take the 96-well plate with sub-confluent ES cells from the incubator. Aspirate most of the ES medium using vacuum suction. Then remove the remainder of the medium with a multichannel pipette (use clean tips for each row). Wash ES

cells by pipetting 100 µl of PBS gently up and down with a multichannel pipette.

3. After all washes have been completed, add 50 µl of TrypLE™ Express to each well and incubate the plate for 5 min at 37°C.

4. In the meantime, take two 96-well flat-bottom plates with 250 µl of ES medium and one with 200 µl of ES medium from the incubator, and align these three plates in the hood.

5. Place the 96-well plate containing the ES clones in the hood (Fig. 2). Disaggregate the ES cells in row A using the multi-channel pipette by vigorously pipetting the trypsin up and down at least ten times. Then, transfer 50 µl of ES medium from row A of each of the two feeder plates containing 250 µl of ES medium to row A of the plate with the trypsinized ES cells. Dispense 50 µl of ES cell suspension into row A of each of the three 96-well plates with irradiated feeders. When ES cells of all eight rows have been split, incubate all three plates at 37°C. Number the plates 1–3 (Fig. 2).

6. *Plate #1*. Immediately after splitting, add adeno-Cre virus (10 MOI). Refresh the virus at 24 and 48 h. At 54–60 h, remove all but 25 µl of ES medium and measure EGFP expression by fluorescence microscopy. Each clone is scored for EGFP intensity (grade as follows: low, medium, or high) and percentage EGFP-positive ES cells. Select 48 clones with the required level of EGFP expression (low [+], medium [++], and/or high [+++]) and transfer them to a gelatin-coated 24-well plate with 0.3×10^5 irradiated SNLH9 feeder cells (Fig. 2). When ES cultures in these wells become semi-confluent, wash the cells twice with PBS. Add 100 µl of SDS sample (Laemmli) buffer to each well to lyse the cells. Transfer each lysate to an Eppendorf tube and boil for 10 min. These lysates are then used to check for the expression of the TOI by Western blot analysis for the epitope tag.

7. *Plate #2*. ES cells in this plate are stained for β-galactosidase as a measure of CAGGS promoter activity (Fig. 2). This is usually done the day after splitting. We use a β-galactosidase staining kit from Roche. Fixation and staining are carried out according to the manufacturer's protocol. Each clone is scored for both staining intensity (suggested grading: low, medium, or high) and percentage of β-galactosidase-positive ES cells.

8. *Plate #3*. This plate will be used to prepare both frozen ES cell stocks and genomic DNA for Southern blot analysis of transgene copy number (Fig. 2). For this, the ES clones in plate #3 need to be split into three plates. Usually, the ES cells are ready for splitting 2 days after the initial seeding. The procedure entails essentially a repeat of steps 1–5. The only

difference is that one 96-well flat-bottom plate contains 0.1×10^5 SNLH9 cells per well and the other two only 0.05×10^5. The plate with the highest SNLH9 cell density, designated plate #3a, will be frozen at –80°C when the ES cells become 70–80% confluent (usually the next day; the cell freezing step is explained in steps 9 and 10) (Fig. 2). The other two plates, designated plates #3b and #3c, will be used for Southern blot analysis. When the medium turns orange to yellow, refresh the ES medium daily until most ES clones have become super-confluent (usually this takes 4–5 days). To harvest the super-confluent cells, remove the lid, invert the plate, and vigorously tap it into a stack of tissue towels until all residual medium has been removed (do not be afraid to lose ES cells as they will be 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 8). Normally, only one of the two plates will be used for Southern blot analysis. The other plate serves as a backup in case an alternative Southern strategy is needed.

9. To freeze ES clones in plate #3a, aspirate most of the ES medium from the wells using vacuum suction. Remove the remainder of the medium with a multichannel pipette (use clean tips for each row). Wash the ES cells by pipetting 100 μl of PBS up and down in the wells using a multichannel pipette. After washing all wells, add 30 μl of TrypLE™Express to each of the wells and incubate the plate for 5 min at 37°C.

10. Make a 1:1 mixture of ES medium and FCS (ES medium with ~50% FCS) and place it in a multichannel reagent reservoir. Then disaggregate the trypsinized ES cells in row A using a multichannel pipette. Pipette vigorously up and down at least 10–20 times. Add 75 μl of medium from a reagent reservoir to the cells in row A and mix. Repeat the procedure for rows B–G.

11. Finally, mix the contents of each well with 100 μl of ice-cold 2× freezing medium (pipette vigorously up and down at least five times to dilute the freezing medium). Quickly seal the plate with masking tape, wrap it in several layers of tissue towel, and place it in a closed styrofoam box that is kept in a –80°C freezer. ES cells maintain their viability for up to 2 months but should be placed at –145°C if storage beyond this point is necessary.

3.5.2. Identification of TRIC-Derived Transgenic ES Clones

1. Replace the feeder medium of two 96-well flat-bottom plates with irradiated SNLH9-puro feeder cells with 250 μl of ES medium (without puromycin). Replace the feeder medium of one 96-well flat-bottom plate with irradiated SNLH9-puro feeder cells with 200 μl of ES medium (also without puromycin). Return all the three plates to the 37°C incubator.

2. Take the 96-well dish with sub-confluent puro-resistant ES clones from the incubator and divide the cells over the three flat-bottom plates with irradiated SNLH9-puro feeder cells by following steps 2–5 of Subheading 3.5.1. Number the plates 1–3 and process each plate as indicated below (Fig. 3).

3. *Plate #1.* At 24 h after splitting, add doxycycline to a final concentration of 0.1 mg/mL to activate TOI and EGFP expression. At 48 h, remove all but 25 μl of ES medium and estimate EGFP expression by fluorescence microscopy (Fig. 3). Each clone is scored for EGFP intensity (grade as follows: low, medium, or high) and percentage of EGFP-positive ES cells (see Note 9). Select 48 clones with proper EGFP expression (low, medium, and/or high) and transfer them to gelatin-coated 24-well plates with 0.3×10^5 irradiated SNLH9 feeder cells. When ES cells in these wells become semi-confluent, wash the cells twice with PBS. Add 100 μl of SDS sample buffer to lyse the cells. Transfer each lysate to an Eppendorf tube and boil for 10 min. The lysates are used to check for expression of the TOI by Western blot analysis for the epitope tag.

4. *Plate #2.* Use this plate for Southern blot analysis (if necessary, one can create a backup plate for Sourthern blot analysis by splitting the cells into two 96-well plates). Refresh the ES medium daily until most of the ES clones have become super-confluent. To harvest the super-confluent cells, remove the lid from the plate, invert the plate, and vigorously tap the plate onto a stack of tissue towels until all residual medium has been removed. Tape the lid to the plate and freeze it for at least 2 h at –80°C (see Note 8).

5. *Plate #3.* Use this plate to prepare frozen ES cell stocks. The freezing procedure is detailed in steps 9–11 of Subheading 3.5.1 (Fig. 3).

3.5.3. Extraction and Restriction Enzyme Digestion of DNA from ES Cells

Allen Bradley and coworkers (4) have developed the following procedure for extraction and restriction enzyme digestion of genomic DNA.

1. 96-well plates stored in –80°C freezer are incubated for 5 min at RT.

2. Add 50 μl of lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 10 mM 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 pipette (see Note 10).

3. Seal the edges of the 96-well plates with masking tape and incubate them for 3 days at 55°C in a box with water-soaked tissue towels.

4. Pre-cool the plates on ice. In the meantime, prepare a mixture of 10 mL of ice-cold 100% ethanol and 150 µl of 5 M NaCl. Add 100 µl of this mixture to each well using a multichannel pipette. Allow the plates to sit at RT for at least 3 h. Then screen each well 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 to incubate the plate at RT (see Note 11).

5. Spin the plates at 2,200 rcf for 15 min. Discard the supernatant by careful inversion of the plates. The DNA will adhere to the plastic. Gently add 100 µl of 80% ethanol to each well with a multichannel pipette to wash the precipitated DNA. Invert the plates onto paper towels to discard the wash solution thoroughly. Repeat the ethanol wash three times. After the last wash, add 100 µl of 80% ethanol for 30 min. Either store the plates at –20°C or continue with step 6.

6. Invert the plates to remove as much ethanol solution as possible. Leave the plates tilted to air dry for 20 min. Screen the wells for complete evaporation of the ethanol wash solution. If traces of ethanol remain, restriction enzymes will incompletely cut the genomic ES cell DNA.

7. While the plates are drying, prepare a restriction digest cocktail. A typical mixture contains the optimal 1× restriction buffer for the enzyme used, 1 mM spermidine, 100 µg/mL acetylated BSA, 50 µg/mL RNase, and 10–15 units enzyme per sample.

8. Add 35 µl of restriction digest cocktail per well with a multichannel pipette. Tap the plates to ensure that the cocktail completely covers the surface of each well.

9. Seal the plates with masking tape and incubate them overnight in a box with water-soaked tissue towels at the temperature specified by the manufacturer of the enzyme.

10. The next day, suspend each DNA solution by pipetting it up and down using a 200-µl pipette. Add 10–15 units of fresh enzyme to each well and incubate for an additional 24 h.

11. Take 5 µl of sample from 5–10 random wells and run these on a 0.8% agarose gel to check whether the DNA is properly digested. If so, continue as described in Subheading 3.5.4. If digestion appears incomplete, repeat step 10.

3.5.4. Gel Electrophoresis and Southern Blot Analysis

1. Take the plates out of the incubator and add 7 µl of DNA loading buffer to each digest using a multichannel pipette. Place the plates in the refrigerator until electrophoresis (or freeze if longer storage is required).

2. Prepare 0.8–1.0% agarose gels in 1× TAE for electrophoresis of the digests. Use an electrophoresis system that has a capacity for many samples.

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 pipette (punch holes in the gel). Using a scalpel, cut the area of gel to be blotted.

5. To hydrolyze the DNA, soak the gel pieces in 0.25 M HCl for 40 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, and investigator).

7. Carefully pour off the 0.25 N 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 3× SSC for about 30 s. Repeat the rinse one more time.

10. Hybridization is carried out in roller bottles in a hybridization oven (e.g., Stovall Life Science Inc.) at 65°C. Transfer the membranes to hybridization tubes with the DNA side facing the inner tube. Pre-hybridize for 30 min in hybridization buffer at 65°C (hybridization buffer: 30 mL 20 × SSC, 5 mL 100× Denhardt's solution, 60 mL 15% dextran sulfate, 1 mL 10 mg/mL salmon sperm DNA, and 5 mL 10% SDS).

11. While pre-hybridizing the membranes, radioactively label the DNA probe.

12. Replace the hybridization buffer once. Add the denatured probe 30 min later and hybridize overnight at 65°C.

13. First, wash the membrane twice with 2× SSC/0.1% SDS for 10 min at RT (on a Belly Dancer). Then wash with pre-warmed 2× SSC/0.1% SDS for 5 min at 65°C (with agitation). If necessary, continue to wash with the following pre-warmed solutions: 1× SSC/0.1% SDS, 0.3× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS. Wrap properly washed membranes in plastic, and carefully remove excess liquid and expose the films at –80°C.

14. For Z/EG-based transgenesis, it is critical to select transgenic ES clones that have single-copy integrations. Because TRIC-based transgenesis does not involve Cre recombination, it is not essential to select single integrants, although ES clones with single integrations are preferred from a mouse breeding perspective.

3.6. Karyotype Analysis and Long-Term Storage of Transgenic ES Clones

Transgenic ES clones must be thawed and expanded for karyotyping and preparation of frozen stocks. The procedure is the same for both Z/EG- and TRIC-based transgenic ES clones. Selection of transgenic ES clones for reseeding is based on TOI and EGFP expression (as assessed by Western blotting and fluorescence microscopy, respectively) and the transgene copy number (as assessed by Southern blotting).

1. Prepare one gelatin-coated 96-well flat-bottom plate, four 24-well plates, and one 12-well plate with the appropriate amounts of feeder cells (see Table 1) 2–3 days prior to the scheduled reseeding of the transgenic ES cell colonies.

2. Thaw the plate containing the ES cell clones of interest by letting it float in a 37°C water bath. When the freezing medium in the plate has melted, suspend the cells in the freezing medium and transfer them to a 15-mL tube containing 2 mL of ES medium. Spin at 180 rcf for 5 min.

3. Resuspend the pellet in 200 μl of ES medium and transfer the cell suspension to a well in a 96-well plate with irradiated feeders. Incubate at 37°C.

4. Monitor the growth of the ES cells closely and split clones that have reached 70–80% confluency. Aspirate the medium, wash the cell culture two times with 200 μl of PBS, add 35 μl of TrypLE™Express, and incubate at 37°C for 5 min.

5. Remove the plate from the incubator and thoroughly suspend the trypinized cells to generate a single-cell suspension. Add 100 μl of ES medium and transfer the suspension to a 24-well plate with irradiated feeders. The total volume of ES medium per 24-well should be ~1 mL.

6. Once the ES clones reach 70–80% confluency, transfer each clone to three wells of a 24-well plate with irradiated feeders for further expansion. Use two of these wells to make frozen stocks (see steps 7–9) and one well for karyotyping (see steps 10–13).

7. *Freezing*: when the 24 wells become semi-confluent, rinse two of the wells with PBS, add 200 μl of TrypLE™Express, and incubate for 5 min at 37°C.

8. Resuspend the cells and add 1 mL of ES medium. Transfer both cell suspensions to one 15-mL tube and spin for 5 min at 180 rcf.

9. Aspirate the medium from the tube and suspend the cell pellet in 2 mL of 1× freezing medium. Divide the cell suspension over two cryogenic vials. Wrap these vials in tissue towels and place them in a styrofoam box in a −80°C freezer. The next day, transfer the vials to liquid nitrogen for long-term storage.

10. *Karyotyping*: when the 24-well designated for karyotyping becomes semi-confluent, transfer the cells to a 12-well plate with irradiated SNLH9 feeders (see Table 1). When the cells become semi-confluent, add 50 μl of Colcemid per mL of medium (10 μg/mL stock) and incubate at 37°C for 4 h.

11. Harvest the cells by trypsinization, centrifuge at 180 rcf for 5 min, aspirate the supernatant, and suspend the pellet in 5 mL of pre-warmed hypotonic solution (freshly prepared 0.56 g of KCl in 100 mL of MilliQ water) in a 15-mL tube. Incubate at 37°C for exactly 15 min.

12. Add 200 μl of fixative (30 mL of methanol and 10 mL of acetic acid, freshly prepared) and centrifuge at 180 rcf for 5 min. Remove the supernatant. Wash the pellet in 5 mL of fixative, centrifuge at 180 rcf for 5 min, and remove the supernatant. Repeat this wash step once more (if necessary the cells can now be stored at 4°C).

13. Resuspend the pellet in 200 μl of fixative. Pipette about 20 μl of suspension and add two drops on a microscope slide from a height of about 2 ft. Prepare two slides per transgenic ES clone. Place the slides in Giemsa staining solution for 15 min. Wash for 2 min with distilled water and three times for 30 s with MilliQ. Let the slides dry at RT and count chromosomes of at least ten metaphases (use a 100× oil objective). ES clones that have 0–20% aneuploid spreads are selected for microinjection into blastocysts.

3.7. Preparation of Transgenic ES Clones for Blastocyst Injection

1. Prepare a 24-well plate with the irradiated feeder cells as described in Subheading 3.2. Approximately 2–3 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 of ES medium and pellet the ES cells at 180 rcf for 5 min.

2. Aspirate the supernatant, gently resuspend the cells in 1 mL of ES medium, and transfer to a well of the 24-well plate with irradiated SNLH9 feeders. Incubate at 37°C.

3. Once the transgenic clones reach 75–90% confluence (usually within 1–2 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 three times with PBS, add 0.5 mL of TrypLE™ Express, and incubate for 5 min at 37°C.

5. Resuspend the cells in 2 mL of ES medium. Transfer 2 mL of the cell suspension to a 15-mL tube and spin at 180 rcf for 5 min (reseed the remaining 0.5 mL of the cell suspension in a well of a 24-well plate with irradiated feeder cells to allow for re-injection of the ES clone, if necessary).

6. Aspirate the supernatant and resuspend the cells in 250 µl of ES medium. Place the cells on ice. Use them for microinjection within 1–2 h following trypsinization.

7. Typically, 16–24 embryos are injected per transgenic ES clone. Optimally, three to five independent transgenic 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 one can use for selecting the serum with the highest plating efficiency. Test the plating efficiency by seeding 1,000 ES cells onto 10-cm dishes with SNLH9 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 SNLH9 feeders cells should produce sufficient LIF to inhibit the differentiation of ES cells, many investigators using feeders add an extra 1,000 Unit/mL of LIF to the culture medium to assure that the LIF levels remain consistently high.

3. The ES cell/DNA mixture can immediately be shocked. Pre-incubations of the ES cell/targeting vector mixture have no beneficial effect on the ultimate targeting frequency.

4. Substantial cell lysis will occur during electroporation, which will give rise to a somewhat viscous cell suspension. This is a normal phenomenon.

5. Picking 96 ES clones should be sufficient to obtain three to five ES clones that meet all requirements for injection into blastocysts. In instances where more clones need to be obtained, simply pick a total of 192 ES clones.

6. Replacing the medium with PBS will round-up ES cells and will make the selection of ES colonies with optimal morphology more difficult.

7. The 2.5 µl of ES medium does not interfere with the proper trypsinization of the ES colony.

8. Storage at –80°C will crack the cells. This facilitates cell lysis and proteinase K digestion, and increases the DNA yield.

9. For unknown reasons, only a relatively small proportion of the ES cells in each well will be EGFP positive. Also, the level of expression between individual cells is quite variable. It is important to note, however, that such mosaicism is not observed at the level of the whole animal. So, it is not a cause for concern.

10. Lysis buffer lacking proteinase K (incomplete lysis buffer) can be stored at RT. Prepare a 10 mg/mL stock of proteinase K in MilliQ and freeze it in 500 µl of aliquots. Add 500 µl of proteinase K solution to 5 mL of incomplete lysis buffer. The resulting solution is sufficient for one 96-well plate.

11. If the proteinase K digestion is incomplete, it will take longer for the DNA to precipitate.

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Chapter 10

Engineering BAC Reporter Gene Constructs for Mouse Transgenesis

Yu Fu and Peter Maye

Abstract

A culmination of large-scale ideas and efforts has truly allowed for the use of large genomic DNA clones housed in Bacterial Artificial Chromosome (BAC) vectors for biological research. Fundamental advances that have allowed this to happen include (1) the completion of genome sequencing projects and the establishment of highly annotated web-accessible databases allowing for the rapid identity and purchase of BAC clones containing genes of interest. (2) The generation of methodologies to modify BACs genetically, allowing for the rapid creation of gene targeting constructs or transgenic reporter gene constructs using homologous recombination in bacteria.

Recent efforts on our part have capitalized on these advances by using BACs and bacterial recombination methods to generate fluorescent protein reporter transgenic mice to study skeletal biology. The rationale for using BAC genomic DNA clones to engineer reporter gene constructs is based on their much larger size, thus increasing the likelihood that most, if not all, of a gene's respective *cis* regulator elements are present, giving a truer representation of the endogenous gene's expression. In a relatively short amount of time, we have become extremely proficient at generating BAC reporters. Contrary to the widely perceived notion that working with BACs is complex and difficult, we decided to write this chapter to encourage laboratories that are currently using traditional molecular cloning methods to engineer transgenic DNA constructs to strongly consider learning BAC methodologies. As an example, we walk through the steps we took to generate the transgenic reporter mouse line, *Tenascin C* (TNC)-mCherry.

Key words: BAC, Bacterial recombination, GFP, Transgenic, Reporter gene

1. Introduction

The DNA sequencing of genomic DNA libraries and the careful annotation of Bacterial Artificial Chromosome (BAC) clones with open access to the research community has allowed for the rapid identity and procurement of BAC clones, thus transforming the field of molecular genetics. BACs are vectors capable of holding

very large pieces of DNA typically 100–300 kb in size and are commonly used to contain genomic DNA libraries. For the mouse, several BAC genomic DNA libraries exist, with 17 murine genomic DNA BAC libraries derived from a variety of different mouse strains being available from the Children's Hospital Oakland Research Institute (CHORI). However, high-resolution, large-scale genome DNA sequencing has thus far been carried out only on the C57BL/6J strain (1), whose two libraries were constructed by the Roswell Park Cancer Institute (RPCI) and aptly named RPCI-23 and RPCI-24 (2). The mapping of BAC clones against genetic loci can be conveniently viewed by using a web browser at a few different web sites, including Map Viewer from the National Center for Biotechnology Information (3) and the Genome Browser from the University of California, Santa Cruz (4).

In conjunction with the annotation and accessibility of BAC clones, equally important has been the evolution of methodologies to modify large pieces of DNA genetically. Given the large size of genomic DNA inserts, conventional cloning methodologies are not possible. An alternative strategy is to modify the genomic DNA BAC clone genetically while still in the bacteria using homologous recombination. At least two different recombination systems are in wide use, including those based on RecA recombinase and the bacteriophage λ Red recombinase system. We have been successful using both systems, and each system offers different advantages based on the experimental context (5). To create conditional knock-out gene targeting DNA constructs, a number of plasmids and bacterial strains have been generated using the Red recombinase system (6). To create transgenic reporter gene animals, both the one and two vector systems utilizing RecA recombinase work well (7–9).

This chapter will focus on methodologies used to modify BAC clones genetically for the purposes of building reporter gene constructs based on the RecA system. The rationale for using BAC genomic DNA clones to engineer reporter gene constructs is based on their much larger size, thus increasing the likelihood that most, if not all, of a gene's respective *cis* regulator elements are present, giving a more faithful representation of the endogenous gene's expression. This methodology cannot be applied to all genes, given that some genes are very large and extend over multiple BAC clones. Large-scale, BAC-based transgenesis projects such as GENSAT indicate that BAC reporters can be generated from a very high percentage of genes (~85%), with the average transcriptional unit estimated to be ~100 kb in size (10). To provide a comprehensive review of our methodology, we have organized the engineering of a BAC reporter into six consecutive steps (Fig. 1). As an aid to this chapter, we have included some data on the recent construction of a new reporter gene animal model for the gene *Tenascin C*.
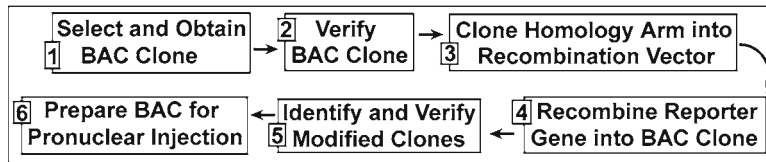
Fig. 1. Diagram organizing the process of generating a BAC reporter in six defined steps. Once all the necessary reagents are obtained, the process typically takes only 2 weeks.

2. Materials

2.1. Growing and Storing BACs

1. LB Media: 10 g peptone from casein, 5 g yeast extract, 10 g NaCl, up to 1 L dH$_2$O, adjust pH to 7.0, and sterilize by autoclaving.

2. Antibiotics: chloramphenicol: 2,000×; 25 mg/ml in ethanol, ampicillin: 1,000×; 50 mg/ml in water, and tetracycline: 1,000×; 10 mg/ml in water.

3. Glycerol stock solution: 65% (v/v) glycerol (sterile), 0.1 M MgSO$_4$, 25 mM Tris-Cl, pH 8.0.

2.2. BAC Purification

2.2.1. BAC Mini-prep

1. P1 Solution: 50 mM Tris, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A.

2. P2 Solution: 200 mM NaOH, 1% SDS.

3. EB Solution: 10 mM Tris-HCl, pH 8.0.

4. 2 M potassium acetate, pH 5.5.

5. Phenol/chloroform.

6. Chloroform.

7. 2-Propanol.

2.2.2. Preparing BAC for Pronuclear Injection

1. Qiagen Maxi kit.

2. 2 M potassium acetate, pH 5.5.

3. Whatman 1 circular filter paper (Fisher Scientific).

4. Phenol/chloroform.

5. Chloroform.

6. 2-Propanol.

7. CL-4B Sepharose (Sigma).

8. Poly-Prep columns (BioRad).

9. Restriction enzyme solutions.

10. Injection buffer: 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 100 mM NaCl.

11. 1,000× polyamine solution: 30 mM spermine (tetrahydrochloride), 70 mM spermidine (trihydrochloride) (Sigma) dissolved in ddH$_2$O and sterile-filtered through a 0.2-μm filter.

2.3. Field Inversion Gel Electrophoresis

1. FIGE mapper (BioRad).
2. Cooling module (BioRad).
3. Variable speed pump (BioRad).
4. Agarose.
5. 1× TBE: 89 mM Tris base, 89 mM boric acid, 1 mM EDTA.
6. Ethidium bromide (10 mg/ml in water).
7. Low-range pulse-field gel marker (New England Biolabs).
8. 6× Loading dye: 12%glycerol, 60 mM Na_2EDTA, pH 8.0, 0.003% bromophenol blue, and 0.003% xylene cyanol.

2.4. Bacterial Recombination

1. pLD53.SC2 and pSV1 vectors (generously provided by Shiaoching Gong).
2. Pir2+ cells (Invitrogen).
3. BAC clone suppliers (BAC-PAC Resource Center, CHORI, Invitrogen).
4. Shaker incubators set at 30°C and 37°C.
5. Incubator set at 42°C.
6. Qiagen Midi Kit.

2.5. Making and Transforming Electro-Competent Bacteria

1. Electroporator.
2. Cuvette with 0.1-cm gap.
3. Sterile dH_2O.
4. Sterile 10% (v/v) glycerol.
5. SOB media: 20 g peptone from casein, 5 g yeast extract, 0.5 g NaCl, up to 1 L with dH_2O, adjust pH to 7.0, sterilize by autoclaving. Just before use, add 2.5 mM KCl, 10 mM Mg_2Cl, and 10 mM Mg_2SO_4.

2.6. Reagents for Cloning Homology Arm and Colony PCR

1. Thermocycler.
2. Taq polymerase.
3. PFX polymerase (Invitrogen).
4. PCR purification kit (Qiagen).
5. 1× TAE (40 mM Tris acetate, 1 mM EDTA).
6. Zymoclean gel DNA recovery kit (Zymo Research).
7. Calf intestinal alkaline phosphatase.
8. T4 DNA ligase.

2.7. Websites and Softwares

1. Genome Bioinformatics: http://genome.ucsc.edu/.
2. NCBI Map Viewer: http://www.ncbi.nlm.nih.gov/projects/mapview/;
3. CHORI: http://bacpac.chori.org/.
4. Vector NTI (Invitrogen).

3. Methods

3.1. Selecting and Obtaining a BAC Clone

The first step of this process is selecting and obtaining a BAC clone containing the gene of interest. Unfortunately, there is no one simple answer to which BAC clone one should choose that will guarantee faithful readout of the reporter gene to the endogenous gene. Gene regulation is complex, with the key *cis* regulatory elements being potentially present far upstream and downstream or within the intron regions of a gene. In our own work, we tend to favor BACs, where the gene is centrally located with perhaps some favoritism toward the upstream region of the gene. As an example, the BAC clone RP24-79K20 we chose for *Tenascin C* (Fig. 2) has 68.5 kb of DNA sequence upstream of the translational start site and 54.8 kb of DNA sequence downstream of the stop codon. BAC clones from RPCI-23 and RPCI-24 are available from the BAC/PAC Resource Center at CHORI. Below we list instructions on how to view BAC clones for a gene of interest and how to download annotated DNA sequence information.

3.2. Viewing BAC Clones Across a Gene of Interest (Contig Map)

1. Go to the *UCSC Genome Bioinformatics* web site (http://genome.ucsc.edu/).

2. Select: *Genome Browser*

3. Make sure the appropriate information is selected, in our case:

   clade: *Mammal*; genome: *Mouse*; assembly: (*use most recent*); position or search term: (*Type in Gene Name Here; i.e. Tenascin*), and select submit.

4. A list will appear, select the appropriate gene. A Contig Map should appear. You should see a list of BAC clone ID's starting with RP23 or RP24 on the left (see Note 1).

5. You can zoom in and out by selecting 1.5×, 3×, or 10× toward the top of the browser page.

6. You can click on the desired Clone ID to find more detailed information about that clone. For example, for TNC, we picked RP24-79K20: Chromosome 4; Start: 63566320; End: 63750190; Length: 183871.

Fig. 2. Schematic of Tenascin C BAC clone RP24-79K20. While the initial selection of BAC clones is gene dependent, we typically favor clones where the exons, shown as *gray vertical bands*, are located in the central region of DNA fragment, leaving plenty of upstream and downstream regulatory sequences. This BAC clone was used to generate a Tenascin C-mCherry reporter mouse shown in Fig. 5.

3.3. Downloading and Viewing an Annotated DNA Sequence for a BAC Clone

1. Open the NCBI Map Viewer: http://www.ncbi.nlm.nih.gov/projects/mapview/.

2. Under Search, select *Mus musculus*, type in your Clone ID (i.e., RP24-79K20), and hit Go.

3. Select the Clone ID again under map element.

4. On the left under *Region Shown*, type in the Start and End coordinates of your Clone ID (For TNC, Start: 63566320, End: 63750190) and hit Go (see Note 2).

5. Toward the top right of the browser, select *Download/View Sequence Evidence*.

6. Change sequence format from FASTA to Genbank and hit Save to Disk.

This downloaded file is a Genbank DNA sequence file with all the annotations, which is extremely useful for visualizing the details of your BAC clone. At this point, we work with the BAC clone in Vector NTI (see Note 3).

3.4. Storing BAC Clones

1. From a bacterial stab, inoculate 2 ml of LB media containing the appropriate antibiotic and grow overnight at 37°C.

2. Place 1 ml of culture into a 1.7-ml screw cap tube. Add 0.5 ml of glycerol stock solution and mix by inverting. Freeze on dry ice and store at –70°C.

3.5. Verifying BAC Clones

Upon receiving the BAC clone, it is important to verify that you received the correct piece of genomic DNA. From the downloaded sequence, identify rare-cutting restriction enzymes that will cut the genomic insert two to four times. Pick three to four restriction enzymes and carry out a diagnostic restriction enzyme digest. Some restriction sites may be present in the BAC vector, so it is worthwhile to obtain annotated DNA sequence information of the BAC vector used for your clone, which is available from CHORI in the form of a downloadable Genbank file or map (http://bacpac.chori.org/vectorsdet.htm). In our experience, most BAC clones cut in a predicted fashion. At the same time, it is not entirely unusual to see some differences from the predicted DNA fragment size. If you are focused on a single gene, I would recommend obtaining two different BAC clones against your gene of interest, allowing you to compare one with the other. Future PCR amplification of homology arm(s) will further verify the identity of your BAC clone as well.

3.6. BAC Mini-prep

1. Inoculate LB media containing the appropriate antibiotic with a frozen glycerol stock, and grow overnight in a shaker incubator at 37°C and ~200 rpm. (Estimate 1 ml of culture per restriction enzyme digestion reaction.)

2. Aliquot 1 ml of culture into 1.5-ml microfuge tubes, centrifuge, and aspirate off LB medium.

3. Carryout alkaline lysis: (see Note 4) thoroughly resuspend bacterial pellet in 300 μl of P1 solution. Then, add 300 μl of P2 buffer, invert two to three times to mix, and incubate for 5 min. Finally, add 300 μl of 2 M potassium acetate and invert two to three times to mix. Chill on ice for 15 min followed by centrifuging at >13,000×G in a microfuge. Transfer the supernatant into a new microfuge tube.

4. Carryout phenol/cholorform extractions: Add 500 μl of a 1:1 mixture of phenol/chloroform to each tube and mix by inverting. Separate into two phases by centrifuging at >13,000×G in a microfuge for 5 min. Transfer the upper aqueous phase into a new tube and add 500 μl of chloroform. Mix again by inverting and centrifuge at >13,000×G in a microfuge for 5 min. Transfer the upper aqueous phase into a new tube.

5. Precipitate BAC by adding an equal volume of 100% 2-propanol. Place on ice for 15 min followed by centrifuging for 30 min at >13,000×G in a microfuge. Wash the pellet with 750 μl of 70% ethanol and centrifuge for 5 min. Remove ethanol and let the pellet air dry for 5 min.

6. Add 10 μl of EB solution to the pellet. Allow 2–5 min for the pellet to go into the solution and store on ice.

3.7. Diagnostic Restriction Enzyme Digestion

Select restriction enzymes that cut two to four times according to the BAC sequence. Set up digestion: 10 μl of BAC DNA from mini-prep, 0.5 μl of enzyme, 2 μl of 10× buffer, and 8 μl of H_2O. Incubate at 37°C for 30 min to 1 h.

3.8. Field Inversion Gel Electrophoresis

1. Prepare a 1% agarose gel in 1× TBE buffer and extra 1× TBE buffer for the electrophoresis chamber. Pre-chill the buffer and gel in the refrigerator for at least 1 h before use.

2. Load the gel with a DNA marker and samples: Cut a thin slice of the Low-Range PFG Marker and slide it into one well of the gel. Immerse the gel into the electrophoresis chamber containing pre-chilled 1× TBE buffer. Add 5 μl of loading dye to each sample and load onto the gel.

3. Run the gel: Turn on the FIGE mapper, select program 4, and hit Start. Initially, run the samples into the gel for 20 min with a cooling module and pump off. After 20 min, turn on the cooling module (set to 18°C) and pump (set to 70). Run overnight for ~16 h (see Note 5).

4. Stain the gel with ethidium bromide: Stop the gel the next morning and stain the gel in 300 ml of 1× TBE buffer containing ~200 μl of ethidium bromide (10 mg/ml).

3.9. Homologous Recombination in Bacteria and Homology Arm Design

For the purposes of modifying large DNA fragments cloned into BAC vectors, homologous recombination systems such as those that utilize the function of the bacterial recombinase A (RecA) have been developed. Both one vector and two vector RecA systems have been developed by Shiaoching Gong and colleagues. The major distinction between these two systems is that with the two vector system, pLD53.SC2 and pSV1, the vector carrying the reporter gene, pLD53.SC2, remains integrated into the BAC clone, while the one vector system, pLD53-SCAEB, allows for the removal of unwanted vector sequences using a resolution step involving the product of the SacB gene (7). While the resolution of unwanted vector sequences is preferable, the one vector system is less high throughput because the process involves cloning two separate homology arms and carrying out two consecutive recombination steps. Moreover, some BAC vectors contain a SacB gene, requiring its removal prior to using the pLD53-SCAEB system (8). In contrast, the two vector system is high throughput involving the cloning of only one homology arm and carrying out one recombination step. The two vector system has been extensively used in the large-scale BAC transgenesis project, GENSAT. Additionally, the vector backbone of pLD53.SC2 does not appear to contribute to or alter the gene expression of its targets. Therefore, for the rapid generation of BAC reporters, we favor the two vector system and will focus on this recombination system.

The two vector RecA recombinase system includes the vectors pLD53.SC2 and pSV1 (Fig. 3a, b). The original pLD53.SC2 contains a multiple cloning site (MCS) upstream of a cassette containing a Kozak sequence, EGFP reporter, and bovine growth hormone polyadenylation signal. This vector confers ampicillin resistance and has an R6kγ replication origin. Plasmids that contain the R6kγ replication origin require the replication protein π, a product of the *pir* gene, to replicate (7, 11, 12). Pir + cells, PIR1 and PIR2, are commercially available from Invitrogen. The pSV1 plasmid carries the RecA recombinase, tetracycline resistance gene, and a temperature-sensitive replication origin allowing it to replicate at 30°C and stop replicating at 42°C (9).

To generate a BAC reporter, a homology arm located a few nucleotides upstream of your gene of interest translational start site is PCR cloned into the MCS of pLD53.SC2. pSV1 is transformed into the bacteria containing your BAC clone of interest, introducing the RecA recombinase into these cells. Then, pLD53.SC2 (containing your cloned homology arm) is transformed into the pSV1/BAC clone bacteria cells. Recombinants are selected for by antibiotic selection and screened first by colony PCR, followed by diagnostic restriction digest. For colony PCR, primers flanking the homology arm should be designed to identify recombinants (Fig. 3c). This protocol generally works with very high efficiency and typically, we only have to pick five to eight bacterial
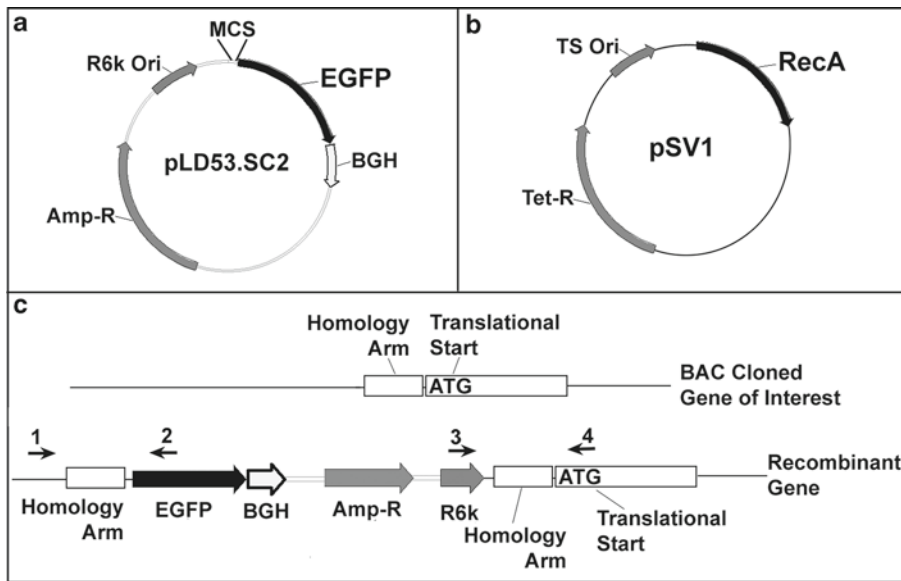
Fig. 3. The two vector recombination system using RecA. (**a**) The pLD53.SC2 vector contains an EGFP reporter gene downstream of a multiple cloning site (MCS), ampicillin resistance, and an R6kγ replication origin. (**b**) The pSV1 vector contains a RecA recombinase, tetracycline resistance, and a temperature-sensitive replication origin (replicates at 30°C, does not replicate at 42°C). pSV1 is transformed into the bacteria containing the BAC clone of interest to introduce RecA. (**c**) A homology arm, typically located a few nucleotides upstream of the translational start site, is cloned into the MCS of pLD53.SC2. Recombination is carried out by transforming pLD53.SC2 (with homology arm) into the bacteria containing your BAC clone and pSV1. Recombinants are initially selected for by colony PCR using primers ((1&2) or (3&4)) that flank the homology arm. Final recombinants are verified by diagnostic restriction enzyme digestion and FIGE (see Fig. 4).

colonies to screen and often identify multiple recombinants in the screening process.

The design of homology arms is context dependent, based largely on the particular gene's DNA sequence. As a general rule, homology arms should be 300–500 bp in size and located a few nucleotides upstream of the translational start site. However, one should not blindly choose this region without first viewing the DNA sequence for repetitive elements or long single nucleotide stretches. Our past experience has shown that homology arms that include repetitive elements do not typically recombine into our region of interest, but perhaps target elsewhere in the BAC insert or within the bacterial genome. Therefore, under difficult circumstances, where repetitive elements or long single nucleotide stretches are present, we favor designing smaller, more selective homology arms. Homology arm size is a flexible variable and we have successfully targeted BACs with homology arms that are only 200 bp in size. Other groups have reported recombining into BACs with as little as 25-bp regions of homology. At the same time, using very small homology arms is typically met with mixed success.

3.10. Making Electro-Competent Bacteria

1. Inoculate 2 ml of LB media containing the appropriate antibiotics with your BAC clone of interest and grow overnight in a shaker incubator at 37°C – 200 rpm. (If bacteria contain pSV1, remember to grow at 30°C.)

2. In the morning, add the 2 ml culture to 50 ml of SOB media (containing the appropriate antibiotics) and grow in the shaker incubator again until $OD_{600} = 0.6$–0.8 (estimate for ~3–4 h at 37°C and longer if grown at 30°C).

3. Transfer the 50 ml culture to a 50-ml conical tube and chill on an ice water bath for 15 min. (It is essential that bacteria stay ice cold for all subsequent steps).

4. Centrifuge bacteria in a pre-chilled centrifuge (4°C) for 10 min at $3,220 \times g$.

5. Pour off supernatant and suspend pellet in 5–10 ml of ice-cold ddH_2O by swirling. After the pellet is resuspended, add more ice-cold ddH_2O up to 40 ml.

6. Centrifuge again as before and repeat step 5.

7. Centrifuge again as before and repeat step 5, except use ice-cold 10% glycerol solution instead of ddH_2O.

8. Centrifuge again as before, decant most of the 10% glycerol solution (try to leave ~200–300 µl). Resuspend pellet by swirling and tapping on the side of the ice bath bucket. Pre-chill 4–6 microfuge tubes on dry ice and pipet 50 µl of competent bacteria into each tube. Store competent bacteria at –70°C.

3.11. Cloning Homology Arm into the pLD53.SC2 Shuttle Vector (see Note 6)

1. Design and order PCR primers to amplify your homology arm containing the appropriate restriction enzyme sites.

2. Carryout a BAC Mini-prep as described above, except resuspend the DNA pellet in 50 µl of EB buffer.

3. Pipet and run a PCR: Use 4 µl of BAC DNA from the Mini-prep as your template in a 100-µl PCR. When possible, use a high fidelity DNA polymerase for this amplification. Our standard reaction mix contains 10 µl of 10× PFX buffer, 2 µl of 50 mM $MgSO_4$, 8 µl of 2.5 mM dNTPs, 2 µl of sense & antisense primers (25 µM), 65 µl of water, 4 µl of BAC DNA, and 2 µl of PFX DNA polymerase. Amplify the homology arm using the appropriate thermocycling conditions. Try to keep the cycle numbers between 21 and 23 to minimize polymerase errors in the amplified product.

4. Verify PCR amplification: Run 4 µl of the PCR product on an agarose gel. Purify the rest of the PCR product using a QIAquick PCR purification kit (elution volume: 40 µl).

5. Restriction enzyme digest: Incubate 20 µl of the purified PCR product and 5 µg of pLD53.SC2 vector separately with the appropriate restriction enzyme(s) at 37°C for 3 h.

6. Gel purify the restriction enzyme-digested PCR product using Zymoclean gel DNA recovery kit and elute with 10 μl of water.

7. Add 2 μl of alkaline phosphatase (CIP) to the restriction enzyme-digested vector and incubate at 37°C for 10 min to prevent self-ligating.

8. Purify vector: Use DNA Clean and Concentrator kit and elute DNA with 10 μl of water.

9. Ligate vector and insert: Set up the following ligation reaction and incubate for 5 min.

   (a) Test: Homology arm: 5 μl, Vector: 5 μl, 2× ligase buffer: 10 μl, Quick T$_4$ Ligase: 1 μl.

   (b) Control: Water: 5 μl, Vector: 5 μl, 2× ligase buffer: 10 μl, Quick T$_4$ Ligase: 1 μl.

10. Electroporate 1 μl of each ligation reaction into 50 μl of Pir + competent *Escherichia coli*.

11. Plate onto LB/ampicillin resistant plates and incubate at 37°C overnight.

3.12. Colony PCR to Confirm Subcloning

1. Pick colonies in the morning and grow in 2 ml of LB/ampicillin (50 μg/ml) medium for ~3–4 h.

2. Take 100 μl of each culture and transfer to microfuge tubes. Centrifuge and decant LB media and resuspend bacteria pellet in 20 μl of H$_2$O.

3. Carryout a PCR with the primers used to amplify the homology arm. Use BAC DNA as positive control. Include a 95°C – 5 min initial step in the thermocycling parameters to break apart the bacteria and liberate the BAC DNA into the reaction. (Reaction recipe: 10× buffer (with MgCl$_2$) 1.25 μl, 2.5 mM dNTPs 1.25 μl, 20uM sense and antisense oligos 0.5 μl each, water 6.9 μl, (5 μ/μl) Taq polymerase 0.1 μl, and 2 μl of resuspended bacteria).

3.13. Homologous Recombination in Bacteria

1. Transform bacterial cells containing your verified BAC clone of interest with 50 ng of pSV1 vector.

2. Grow in a shaker incubator with 1 ml of SOC without antibiotic selection for 1 h at 30°C – 200 rpm.

3. Spread 200 μl of culture onto a chloramphenicol (12.5 μg/ml)–tetracycline (10 μg/ml) containing LB agar plate and incubate at 30°C overnight.

4. Prepare electro-competent bacteria as described in Subheading 3.10.

5. Electroporate 1–2 µl (about 1 µg) of homology arm-pLD53. SC2 DNA into competent BAC clone – pSV$_1$ cells.

6. Grow in a shaker incubator with 1 ml of SOC without antibiotic selection for 1 h at 30°C – 200 rpm.

7. Add 5 ml of LB media containing chloramphenicol (12.5 µg/ml)–ampicillin (50 µg/ml)–tetracycline (10 µg/ml), and grow overnight at 30°C – 200 rpm.

8. Spread 200 µl of culture onto a chloramphenicol (12.5 µg/ml)–ampicillin (50 µg/ml) plate, and incubate at 42°C overnight.

9. Pick colonies and grow in 2 ml of chloramphenicol (12.5 µg/ml)–ampicillin (50 µg/ml) LB media at 37°C for 3 or more hours.

10. Carryout a preliminary screen for recombinants using a colony PCR strategy using primers that flank your homology arm. A standard strategy is to have your sense primer upstream of the homology arm present in the BAC clone and your antisense primer in the reporter gene (Fig. 3).

11. Verify desired recombinants by carrying out a diagnostic restriction digest and FIGE. You need to identify a restriction enzyme that is present in the pLD53.SC2 vector and is also a rare cutter in your BAC clone (we frequently use Pvu1) (Fig. 4).
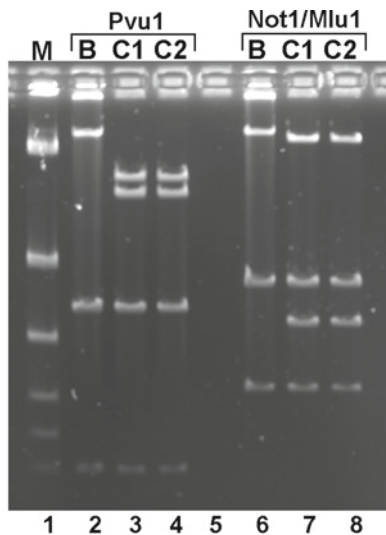
Fig. 4. Confirmation of modified BAC clones. Diagnostic restriction enzyme digestion and FIGE were carried out to confirm candidate TNC-mCherry BAC clones identified by colony PCR. Wild-type BAC (lane B) and two BAC integrate clones (lanes C1 and C2) were digested with Pvu1 or with Not1 + Mlu1. Pvu1 cuts one time on the wild-type BAC, generating two bands of 33 kb and 150 kb. PLD53.sc2 vector brought in another Pvu1 site, splitting the 150 kb band to 69 kb and 80 kb bands. Mlu1 cuts one time on the wild-type BAC, generating 41 kb and 142 kb bands. PLD53.sc2 vector brought in a Not1 site, splitting the 142 kb band to 115 kb and 27 kb bands. The extra lower bands are from the BAC vector backbone.

3.14. Preparing the BAC for Pronuclear Injection (see Note 4)

3.14.1. Day 1: Modified Qiagen Maxi Kit Protocol

1. From a frozen bacterial stock, inoculate two 100 ml cultures (LB media in 500 ml bottle) containing the appropriate antibiotics. Grow overnight at 37°C with shaking at 180–200 rpm.

2. Pour each culture into two separate tubes and spin down cultures at $3,220 \times g$ for 15 min.

3. Discard supernatant and invert the tubes over paper towels to remove extra drops of media.

4. Resuspend bacterial pellets by adding 20 ml of P1 solution to each tube. Gently swirl and view to make sure no clumps of bacteria are present.

5. Prepare fresh P2 solution. Gently swirl the culture and gradually add 20 ml of P2 to each tube. Let the tubes stand for 3–5 min.

6. Gradually add 20 ml of 2 M potassium acetate to each tube and incubate on ice for 20 min.

7. Spin down at $3,220 \times g$ for 10 min to remove most of the heavy particulate.

8. While the samples are spinning down, pre-equilibrate a Qiagen Maxi column with QBT buffer. Place QF buffer in a 65°C waterbath. Also, prepare a filter paper, place it in a funnel, and pre-wet it with sterile, RNAse/DNAse-free water.

9. Set the funnel/filter up over the Maxi column, filter the supernatant from step 8, and let it drip into the Maxi column.

10. Wash the column with QC buffer.

11. Elute the sample with 10 ml of QF buffer and combine them for a total of 20 ml.

12. Carry out phenol/chloroform extractions until the interface looks clean. (Add 10 ml of phenol/chloroform for each extraction, gently mix by inverting the tube, and spin down at $3,220 \times g$ for 10 min. On the centrifuge, remove the brake so the interface does not get disturbed.)

13. Carry out a chloroform extraction: add 10 ml of chloroform, mix by gently inverting the tube, and spin down at $3,220 \times g$ for 10 min.

14. Precipitate BAC by adding an equal volume of 2-propanol and placing the tube on ice for 10 min. (Add 2-propanol slowly with gentle mixing of the contents.)

15. Spin down at $19,750 \times g$ for 30 min. (If possible, use a swinging bucket rotor which will pellet your DNA at the very bottom of the tube, in contrast to a fixed angle which will smear your DNA over a greater surface).

16. Wash with 10 ml of 70% ethanol and spin for 10 min at $19,750 \times g$.

17. Discard ethanol. Remove residual ethanol by inverting the tube over a paper towel, followed by removing the extra drops by aspirating the inside of the tube, avoiding the DNA pellet area.

18. Resuspend the precipitated BAC by adding ~200–250 μl of EB buffer. (Do not pipet up and down to mix). Rotate the tube to move the EB buffer along the bottom surface to solubilize any precipitated BAC. Let the tube stand on ice for 10–15 min.

19. Transfer the BAC to a microfuge tube. First, gently mix by slowly pipetting up and down two to three times followed by transferring it to the microfuge tube.

20. Quantify the BAC yield by measuring on a spectrophotometer. In our hands, we typically get yields of ~30 μg of BAC DNA.

21. Store purified BAC at 4°C (do not freeze).

3.14.2. Day 2: BAC Linearization; Buffer Exchange: Quantification and FIGE Inspection

1. Linearize 10 μg of BAC by cutting with a unique restriction enzyme. If there are no obvious rare-cutting restriction enzyme sites toward the ends of the genomic DNA insert, most BAC vectors contain a rare cutter. For example, pBACe3.6 and pTARBAC1, the BAC vectors for RP23 and RP24 murine libraries, respectively, contain the rare-cutter PI-SceI in their backbone. Carry out a 200–250-μl digest for 2–3 h. Restriction digest reaction: 10 μg of BAC, 20 μl of 10× buffer, 2 μl of restriction enzyme, and make up to 200 μl with water.

2. Prepare 50 ml of pronuclear injection buffer. Mix and run through a 0.2-μm syringe filter.

3. Set up a 2-ml CL-4B sepharose column in a poly-prep chromatography column. Equilibrate the column with ~5–10 column volumes of injection buffer. Column flow rate is ~1 ml/3 min, so it will take 30 min to equilibrate the column. Prepare ten tubes for collection. Collect ~250–300 μl per fraction.

4. After equilibrating the column, allow the injection buffer to run down to the top surface of the sepharose resin and add the contents of your restriction digestion reaction.

5. Immediately start collecting fractions after the sample is applied. After the sample enters resin, gently add the injection buffer to the column ~300 μl at a time. Place the fractions on ice as you move to fill the next tube.

6. Determine the BAC concentration and verify the integrity and quality of the purification by running 25 μl of each fraction on a FIGE (see Notes 7 and 8).
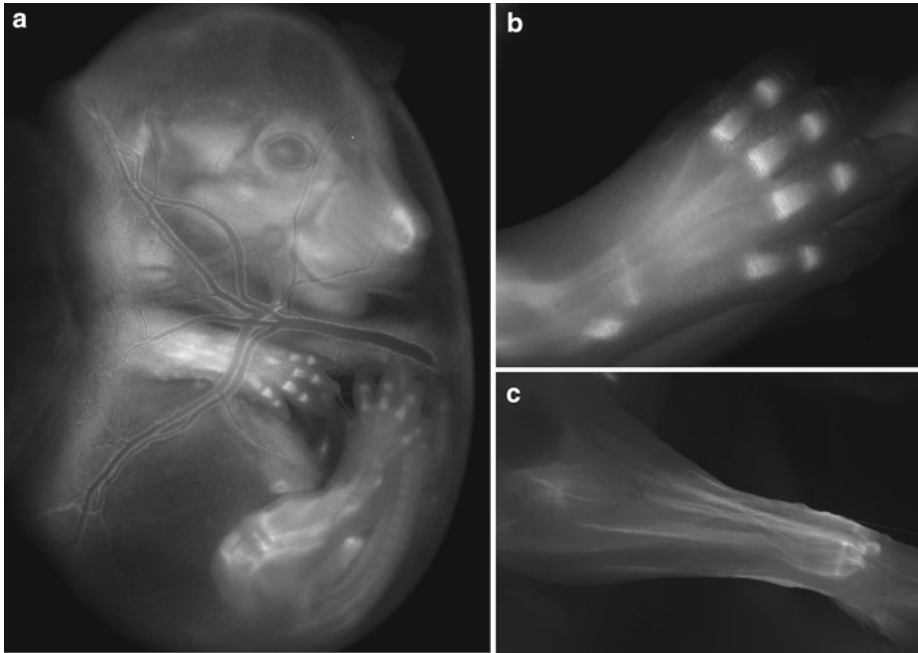
Fig. 5. Transgenic reporter mouse generation. (**a**–**c**) Images from TNC-mCherry reporter mice. EGFP was replaced by mCherry to generate pLD53.SC2-mCherry. Three Tenascin C founder lines were generated. All lines showed comparable expression with some variation in reporter gene intensity. (**a**) Image of TNC-mCherry expression in a E16.5 mouse embryo while still in the yolk sac. Expression is detected in the developing craniofacial structures, developing limbs, and major blood vessels of the yolk sac. (**b**) Image of TNC-mCherry expression in the mouse foot at E17.5 localized within cartilage condensations. (**c**) Image of TNC-mCherry expression in the forearm at 2 weeks of age. TNC-mCherry reporter expression is highly expressed in tendon.

7. Choose your best fraction, dilute it to a concentration of 1.5 ng/μl with injection buffer. Prepare a 3× polyamine solution in injection buffer by adding 3 μl of 1,000× polyamine solution to 997 μl of injection buffer. Then add 20 μl of 3× polyamine solution to 40 μl of your BAC reporter. Your BAC is now ready for pronuclear injection! (see Note 9) (Fig. 5).

4. Notes

1. If you do not see clone ID's in the browser, scroll down and under *Mapping and Sequencing* go to *BAC End Pairs* and select "full" and hit Refresh.

2. If your coordinates are not consistent with the map viewer data, check to make sure you are using the most recent assembly in the USCS genome browser.

3. While we prefer to use Vector NTI, other softwares capable of reading Genbank files are commercially available.

4. To prevent or minimize shearing of BAC DNA and bacterial genomic DNA, do not vortex or vigorously mix at anytime during the purification procedure. With larger BAC purification preps, mix by very gently swirling. From 200 ml of culture, we typically get ~30 μg of BAC measured on a spectrophotometer. If your yields are much greater than this, I would suspect shearing of bacterial genomic DNA in your sample.

5. Our FIGE setup involves an electrophoresis chamber that allows buffer to circulate in and out of the unit. The buffer runs through a cooling unit set at 18°C, which dramatically improves the integrity of the BAC DNA, prevents smearing, and allows for cleaner looking gels.

6. For standard PCR cloning, a variety of kits and strategies can be used. The reagents and kits mentioned in this chapter are what we are currently using, but there are several other options.

7. Purified BAC DNA has a finite life span. Ideally, purification should start about 1 week before pronuclear injection. We typically start our culture on a Sunday night with our transgenic facility injecting on a Friday.

8. For quantifying the concentration of BAC DNA, we typically use the NanoDrop, which is very convenient for measuring low concentration fractions eluted from the CL-4B sepharose column. An alternative approach which we also have used in the past is a Picogreen Assay (Invitrogen).

9. While 1 ng/μl is a standard injection concentration for BACs, you may consider injecting at varying concentrations from 3 ng/μl down to 0.5 ng/μl.

Acknowledgments

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Chapter 11

Targeting Vector Construction Through Recombineering

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Abstract

Gene targeting in mouse embryonic stem cells is an essential, yet still very expensive and highly time-consuming, tool and method to study gene function at the organismal level or to create mouse models of human diseases. Conventional cloning-based methods have been largely used for generating targeting vectors, but are hampered by a number of limiting factors, including the variety and location of restriction enzymes in the gene locus of interest, the specific PCR amplification of repetitive DNA sequences, and cloning of large DNA fragments. Recombineering is a technique that exploits the highly efficient homologous recombination function encoded by λ phage in *Escherichia coli*. Bacteriophage-based recombination can recombine homologous sequences as short as 30–50 bases, allowing manipulations such as insertion, deletion, or mutation of virtually any genomic region. The large availability of mouse genomic bacterial artificial chromosome (BAC) libraries covering most of the genome facilitates the retrieval of genomic DNA sequences from the bacterial chromosomes through recombineering. This chapter describes a successfully applied protocol and aims to be a detailed guide through the steps of generation of targeting vectors through recombineering.

Key words: Recombineering, Gene targeting, Conditional knockout, Mutagenesis

1. Introduction

The first step in gene targeting is the generation of a targeting vector, which requires large DNA fragments manipulation, and, in conventional cloning, multiple and sometimes cumbersome cloning procedures. Homologous recombination, mediated by phage proteins in *Escherichia coli*, was described more than a decade ago (1, 2) and constitutes the foundation for a new molecular biology and genetic technique, namely, recombineering.

*Recombi*nogenic engi*neering* (3), reviewed in (4, 5), or recombination cloning (6), allows for the insertion, deletion, or mutation of DNA sequences, assuaging the need for conveniently located restriction sites. In addition, the desired modification can

be made without leaving any "fingerprints" of foreign DNA. Multiple cloning stages involving intermediate vectors can be bypassed, and therefore, DNA constructs can be accurately assembled in a relatively short time interval.

The recombineering approach described in this chapter is based on a defective λ bacteriophage Red encoded system in *E. coli*, which relies on temperature controlled expression of *exo*, *bet*, and *gam* recombination genes. The expression of all these genes is driven by the λ*P*L promoter, which at 32°C is repressed by the temperature-sensitive repressor *cI*857. Shifting the temperature of the bacterial culture to 42°C inactivates the *cI*857 repressor with subsequent de-repression of λ*p*L promoter, allowing the expression of recombination genes. *Exo* is a 5′-3′ exonuclease that targets double-stranded DNA, digesting 5′ ends and leaving 3′ single-stranded DNA overhangs. The resulting single-stranded DNA overhangs are protected by *bet*, which binds to these overhangs and anneals them to complementary single-stranded DNA. *Bet* cannot promote strand invasion and exchange on its own. *Gam* assists the recombination functions of *exo* and *bet* by inhibiting *E. coli Rec*BCD activity and preventing degradation of linear DNA. In this way, the defective λ bacteriophage Red system can mediate high efficiency recombination between linear DNA (PCR product, linearized plasmid) with homology arms in the 5′ and 3′ ends, and a target DNA already present in the bacteria (plasmid, BAC).

Here we detail a multi-purpose recombineering strategy that can be used to generate concomitantly targeting vectors for classic and conditional knockout, hypomorphic alleles (see Chapter 13), as well as for targeted gene mutagenesis.

The process of generating the targeting vector consists of five steps:

1. Transfer of isogenic BAC into recombinogenic bacterial strains.

2. Introduction of homology arms in the targeting vector backbone (see Fig. 1).

3. Retrieval of genomic region of interest from BAC onto targeting vector, by gap repair (see Fig. 2).

4. Insertion of an orphan *loxP* site. This step is not required if the purpose is to construct a hypomorphic (see Chapter 13) or gene mutagenesis targeting vector (see Fig. 2).

5. Insertion of *frt-neo-frt-loxP* cassette with the same orientation of the orphan *loxP* site if the purpose is to construct a knockout targeting vector (see Fig. 3).

   (a) Insertion of *frt-neo-frt-loxP* or *loxP-neo-loxP* cassette in opposite direction of the targeted gene if the purpose

is to generate a hypomorphic targeting vector (see Chapter 13).

(b) Insertion of *loxP-neo-loxP-mutant exon* if the purpose is to construct a gene mutagenesis targeting vector (see Fig. 4).

2. Materials

2.1. Bacterial Strains

We use bacterial strains EL250 and EL350 (7) and SW105 and SW106 (8). Bacterial strains are available, upon request, from National Cancer Institute – Frederick (9). All strains contain a defective λ prophage, with recombination proteins *exo*, *bet*, and *gam* being controlled by the temperature-sensitive repressor *cI857*.

EL250 and EL350 also contain *Flpe* and *Cre* genes, respectively, under araBAD promoter and AraC regulator. Addition of arabinose in the medium induces the expression of *Cre* or *Flpe* recombinases, mediating recombination between two identical *loxP* or *frt* sites (see Table 1).

Table 1
Bacterial strains used for recombineering

Strain	Genotype	Antibiotic resistance
EL250	DY380[(*cro-bio*A) <> *ara*C-PBAD*flpe*]	None
EL350	DY380 [(*cro-bio*A) <> *ara*C-PBAD*cre*]	None
SW105	SW102[(*cro-bio*A) <> *ara*C-PBAD*flpe*]	None
SW106	SW102 [(*cro-bio*A) <> *ara*C-PBAD*cre*]	None

(see Note 1)

SW105 and SW106 are derived from EL250 and EL350, respectively. These strains contain a fully functional *gal* operon, except for a deletion of *galK*, which allows for efficient BAC modification using *galK*-positive/negative selection (see Note 2).

2.2. Plasmids

We use plasmids PL451 and PL452 (10), which are available, upon request, from National Cancer Institute – Frederick (9). PL451 contains a *neomycin* resistance cassette flanked by two *frt* sites and one *loxP* site. PL452 contains a *neomycin* resistance cassette flanked by two *loxP* sites. The *neo* gene is expressed from both a prokaryotic promoter (*em7*) and a eukaryotic promoter (*Pgk*) (See Table 2).

Table 2
Vectors used for recombineering

Strain	Cassette	Antibiotic resistance
PL451	*Frt-Pgk-em7-Neo-Frt-loxP*	Ampicillin
PL452	*loxP-Pgk-em7-Neo-loxP*	Ampicillin
pDTA.4B	*MC1-DTA*	Ampicillin, tetracycline

For targeting vector backbone, we utilize pDTA.4B, a modified version of pMC1-DTA vector (11), which contains a diphtheria toxin subunit A cassette for negative selection, to allow for enrichment in correctly targeted embryonic stem cell clones (12). A tetracycline cassette flanked by two *Sma*I sites is present in the pDTA.4B vector. Upstream of 5′ *Sma*I site and downstream of 3′ *Sma*I site, there is a set of two unique cutters, *Apa*I/*Sac*II and *Bgl*II/*Xho*I, respectively, which will be used to clone in the homology arms of the genomic region of interest (see Fig. 1).

2.3. Bacterial Artificial Chromosomes

The DNA to be inserted into the targeting vector is retrieved from a BAC. It is important to use BACs with isogenic genomic DNA for the purpose of increasing the efficiency of homologous recombination. BACs derived from C57BL/6 are available from Invitrogen or CHORI. Similarly, BACs derived from 129 can be found in CITB library from Invitrogen. The Ensembl genome browser within a Distributed Annotation Server (DAS) can be used to identify the appropriate clone. We use AB2.2-ES cell-derived BAC clones from Source BioScience/Geneservice (13) (see Note 3).

2.4. Primers

Standard primers of 30–40 bases can be ordered from any preferred oligo provider. For longer primers, 70–100 bases, we recommend using high -quality control vendors. As the length of the oligo increases, the yield of the full -length oligo is reducing, and a 100 mer crude product may contain ~60% full-length oligo. Purification using Polyacrylamide Gel Purification (PAGE) is strongly recommended for oligos longer than 50 mer (see Note 4).

2.5. Media and Selective Plates

1. Low salt lysogeny broth: 5 g yeast extract, 10 g tryptone, 5 g NaCl in 1 L MilliQ. Sterilize by autoclaving (see Note 5).

2. LB broth, Miller (Difco).

3. SOC media: in 900 mL of distilled H_2O, add 20 g of bacto-tryptone, 5 g of yeast extract, 2 mL of 5 M NaCl, 2.5 mL of 1 M KCl, 10 mL of 1 M $MgCl_2$, 10 mL of 1 M $MgSO_4$, and 20 mL of 1 M glucose. Adjust to 1 L with distilled H_2O. Sterilize by autoclaving.

4. Glycerol. Dilute 1:1 in MilliQ (v/v). Sterilize by autoclaving. Store at room temperature.

5. L(+) Arabinose to be dissolved in MilliQ at 10% (w/v) concentration. Sterilize by filtration through 0.22-μm filter. Store at 4°C.

6. Bacto™ Agar.

7. Antibiotics: ampicillin, kanamycin, chloramphenicol, and tetracycline (see Note 6).

2.6. Reagents, Solutions, and Disposables

1. Majority of our restriction enzymes are purchased from New England Biolabs (see Note 7).

2. PCR polymerases: Herculase Hotstart, *Taq*-High Fidelity, and *Taq* Phusion Hot Start High Fidelity (see Note 8).

3. GeneTailor™ Site-Directed Mutagenesis System.

4. pGEM®-T Easy Vector.

5. T4 DNA ligase.

6. ElectroMAX™ DH12S™ cells.

7. Qiagen kits: PCR purification, gel extraction, Miniprep.

8. EtOH 70% in MilliQ (v/v) stored at room temperature. EtOH 100% stored at –20°C.

9. Glycogen. Aliquoted and stored at –20°C.

10. Isopropyl alcohol 100%.

11. NaOAc 3 M, pH 4.5. Store at room temperature.

12. Agarose.

13. Gene Pulser/MicroPulser Cuvettes, 0.1 cm.

14. PCR tubes, 1.7-mL snap-cap microcentrifuge tubes, 15-mL Falcon polypropylene conical tube, and 50-mL skirted centrifuge tubes.

2.7. Laboratory Equipment

1. PCR thermocycler.

2. Shaking water bath with 32–37°C adjustable temperature control.

3. Shaker with 200–300 rpm with 32–37°C adjustable temperature control.

4. Incubators for bacterial cultures with 32–37°C adjustable temperature control.

5. Refrigerated microcentrifuge capable of $25,000 \times g$ (Eppendorf, 5417 R).

6. Refrigerated centrifuge capable of $4,500 \times g$ with swing-bucket rotor for 50-mL tubes (Eppendorf, 5810 R).

7. Electroporator.

8. Water purification system.

9. Electrophoresis equipment.

10. Spectrophotometer.

11. Autoclave.

3. Methods

3.1. Strategy for the Design of Targeting Vector

Strategic planning and a detailed schematic of the individual steps are essential for the successful construction of targeting vector in timely fashion (see Note 9).

1. *Identification of gene locus to be targeted.*

   When choosing the gene locus to be manipulated, it is important to consider a number of factors, enumerated in Chapter 13 and reviewed in (14). Specifically for the method presented here, the size of genomic region to be retrieved, from the BAC into the targeting vector, by gap repair, should be around 8–12 kb, size to allow for efficient homologous recombination in mouse ES cells.

2. *Identification of isogenic BACs spanning the gene locus to be targeted* (see Note 10).

3. *Design of homology arms for gap repair.*

   Choose 400–600-bp regions, namely, HOM1 and HOM2, for the 5′ and 3′ ends of genomic region to be included into the targeting vector. Design PCR primers for amplification of these regions, which should include the specific restriction enzymes necessary for directional cloning into the pDTA.4B retrieval vector, as represented in Fig. 1 (see Note 11). When designing 5′ primer for HOM1, it is very important to insert a unique cutter enzyme, for the final targeting vector, that will be used for linearization (see Note 12).
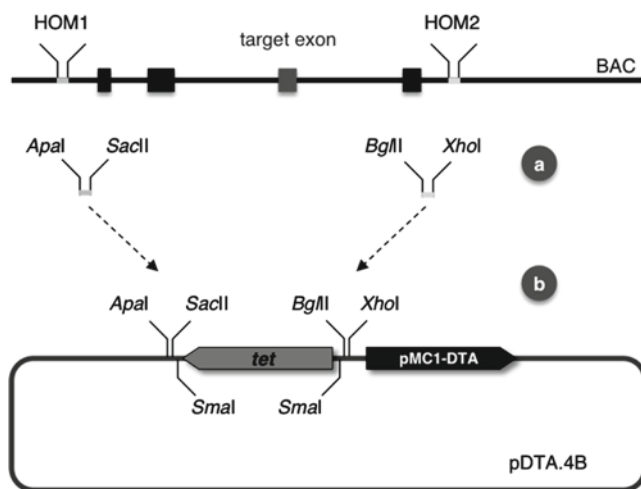
Fig. 1. *Insertion of homology arms into the targeting vector.* (**a**) PCR amplification of homology arms. (**b**) Cloning of homology arms in the targeting vector.

4. *Identification of critical exon and positioning of the orphan loxP site.*

If the purpose is to construct a conditional knockout vector, the first step is the insertion of an orphan *loxP* site. For this, 5′ and 3′ primers that are 70–100 bases in length should be designed to PCR amplify the *loxP-neo-loxP* cassette from PL452 plasmid. Each primer should be composed of 50–75 bases homologous to the region that is going to be targeted, and 20–25 bases homologous to the flanking regions of *loxP-neo-loxP* cassette (see Notes 13 and 14). This will allow the insertion of *loxP-neo-loxP* cassette, with screening in bacteria using kanamycin. To remove the *neo* cassette and leave an orphan *loxP* site, Cre recombinase-mediated, reciprocal recombination of *loxP* sites in bacterial strains EL350 or SW106 should be carried out (see Fig. 2) (see Subheading 3.3.3).
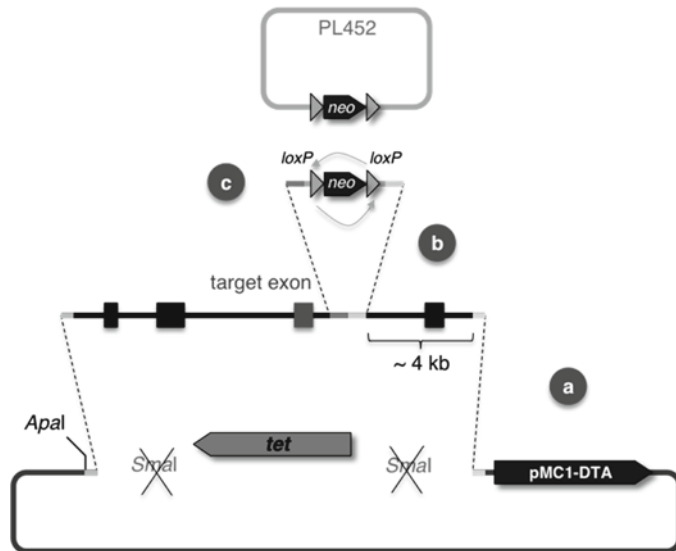
Fig. 2. *Insertion of an orphan loxP site.* (**a**) Gene sequence was retrieved from the BAC by gap repair. (**b**) Recombination between homologous regions will insert the *loxP-neo-loxP* cassette. (**c**) Cre-mediated recombination will generate an orphan *loxP.*

5. *Positioning of frt-neo-frt-loxP cassette.*

If the purpose of gene targeting is to construct a conditional knockout vector, the *loxP* from *frt-neo-frt-loxP* cassette should have the same orientation of the first *loxP* (directional recognition motifs) (see Notes 13 and 14). The design of the 5′ and 3′ primers to PCR amplify the *frt-neo*-frt-*loxP* cassette follows the same principle as that in the previous step. Each primer should be composed of 50–75 bases homologous to the region

that is going to be targeted, and 20–25 bases homologous to the flanking regions of *frt-neo*-frt-*loxP* cassette.

When *neo* resistance cassette is positioned in opposite orientation relative to the modified gene, the normal gene transcription and splicing are affected, resulting in a hypomorphic allele. The detailed mechanism of how the hypomorphic allele is produced and functions is described in Chapter 13 of this book. If the desired insertion in the targeting vector is carried out to generate a hypomorphic allele, then the insertion of *frt-neo-frt-loxP* cassette should be designed to have the opposite orientation relative to the modified gene (see Fig. 3).
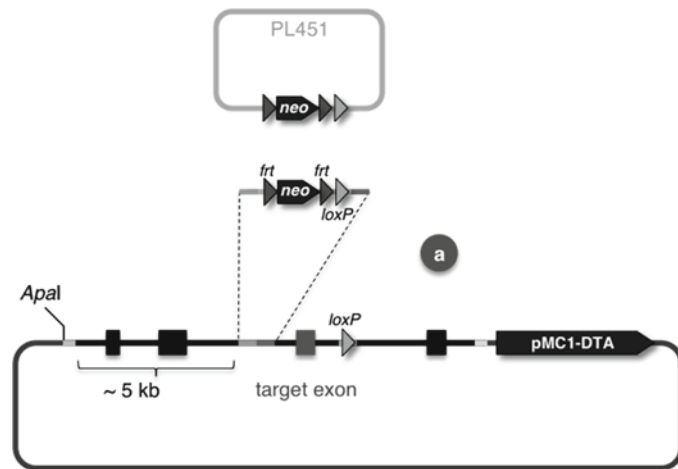
Fig. 3. *Insertion of a frt-neo-frt-loxP cassette.* (**a**) Recombination between homologous regions will insert the *frt-neo-frt-loxP* cassette.

6. *Gene-targeted mutagenesis.*

Insertion, deletion, or single base-pair mutation of a gene can lead to the production of a mutant protein. The encoded protein will have its composition, structure, or function altered, and the effect at the organismal level can be dramatic. Creating such gene alterations is essential to study the in vivo effects of specific protein domain loss of function or to mimic various human genetic pathologies in an animal model. We propose the following strategy for constructing such a targeting vector through recombineering:

After identification of the exon that is going to be mutated, the whole exon or an exon fragment, up to 1 kb in size, will be PCR amplified, from the experimental BAC (see Notes 14 and 15), using primers named as P1 and P2 (see Fig. 4), and then cloned into a commonly used vector for performing desired mutagenesis (see Note 16). The location of the PCR

primers in this step will delineate the beginning of "homolo-gous regions" that will be used in designing the primers for the recombination step, as described below (see Note 17).
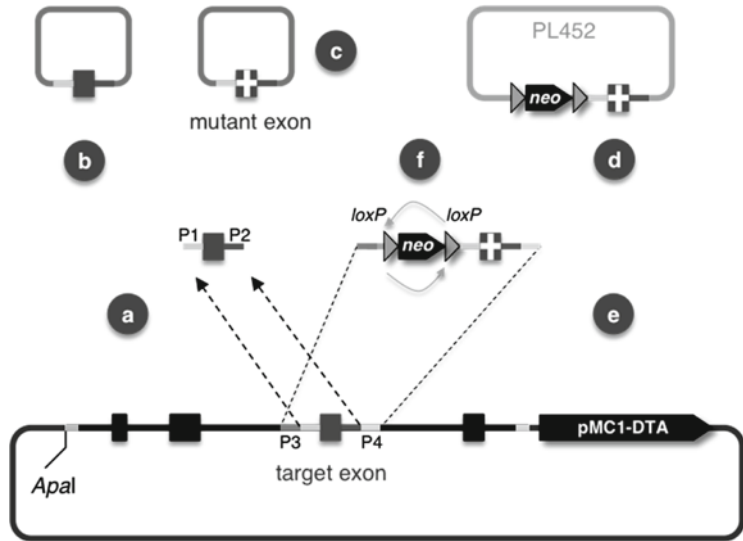
Fig. 4. *Creating a targeting vector for gene mutagenesis.* (**a**) PCR amplification (using primers P1 and P2) of target exon. (**b**) Cloning into mutagenesis plasmid. (**c**) Mutagenesis. (**d**) Cloning into PL452 vector. (**e**) Recombination between homologous regions, P3 and P4, will replace the target exon with mutant exon and introduce a *loxP-neo-loxP* cassette. (**f**) Cre-mediated recombination will generate an orphan *loxP.*

After confirming the mutagenesis, the mutant exon will be cut out and cloned into PL452, downstream of *loxP-neo-loxP* cassette (see Note 18). Next, PCR will be performed of *loxP-neo-loxP-(mut)exon* such that the mutant exon is incorporated into the PCR product. Each primer used for PCR amplification should be composed of 70–75 bases, homologous to the adjacent regions, upstream (P3) and downstream (P4), of primers P1 and P2, respectively, and 20–25 bases homologous to the flanking regions of *loxP-neo-loxP-(mut)exon* (see Note 19). This PCR product will be used in a recombination reaction with the acceptor tar-geting vector harboring the genomic DNA to be targeted (see Note 20). The desired result is the replacement of the intact exon with a mutant exon. Upstream, in the intronic region, the mutant exon will be flanked by the *loxP-neo-loxP* cassette. Subsequent Cre recombinase-mediated, recip-rocal recombination of *loxP* sites will remove the *neo* cassette and will leave behind an orphan *loxP* site. This process will be performed in bacterial strains EL350 or SW106 that express Cre recombinase.

7. *Southern blotting genotyping strategy.*

Screening drug-resistant ES clones for the correct insertion of the gene targeting construct can be performed using Southern blotting. For this, the Southern blotting strategy requires identification of the good genomic cutters that produce easily identifiable changes in the migration pattern. The restriction enzymes of choice can be preserved in the genomic sequence or they can be artificially introduced during various recombination steps. We recommend designing two to three probes, and testing each prior to any recombination steps to ensure that they are specific and hybridize efficiently (see Note 21).

3.2. Retrieval of the Genomic Fragment from a BAC by Gap Repair

3.2.1. BAC Isolation

After receiving the BAC clone, innoculate LB broth containing 12.5 μg/mL of chloramphenicol and 8% glycerol. Incubate the culture at 37°C, for up to 16 h, shaking at 225–300 rpm (see Table 3).

Table 3
Antibiotics concentrations (w/v)

Antibiotic	Plasmids (μg/mL)	BACs (μg/mL)
Ampicillin	100	50
Kanamycin	50	25
Chloramphenicol	25	12.5
Tetracycline	25	12.5

1. Prepare glycerol stock by adding 15% glycerol to the turbid BAC culture. Label and store at –80°C.

2. Isolate BAC DNA using a rapid alkaline lysis protocol. We use the P1, P2, and N3 buffers from QIAGEN® Plasmid Mini kit:

   Transfer 1 mL of BAC culture into 1.7 mL of snap-cap microcentrifuge tube and pellet the cells by centrifugation at $4,500 \times g$ for 20 min at room temperature.

3. Resuspend each bacterial pellet in 250 μL of buffer P1.

4. Add 250 μL of buffer P2 to each tube. Mix thoroughly and gently by inverting the tube six times, and incubate at room temperature for 5 min.

5. Add 350 μL of chilled buffer N3 to each tube. Immediately mix by gently inverting the tube six times, and incubate on ice for 10 min.

6. Centrifuge at $25,000 \times g$ for 10 min at 4°C using a table-top microcentrifuge.

7. Remove the supernatant containing BAC DNA promptly and transfer to a new tube, avoiding the transfer of any white precipitate material.

8. Add isopropanol 1:1 (v/v). Mix gently by inverting 10–20 times.

9. Centrifuge at $25,000 \times g$ for 10 min.

10. Remove supernatant using a flat tip and add 0.5 mL of 70% EtOH. Wash the DNA pellets by inverting the tubes ten times.

11. Centrifuge at $25,000 \times g$ for 10 min at 4°C using a table-top microcentrifuge.

12. Carefully remove the supernatant using a flat tip, as pellets will occasionally become dislodged from the tube.

13. Air dry pellets at room temperature.

14. When the DNA pellets turn from white to translucent in appearance, add 50 μL of MilliQ to each pellet and allow the solution to sit in the tube with occasional tapping of the bottom of the tube (see Note 22).

15. Check by PCR the BAC integrity and presence of desired genomic regions (see Note 23).

3.2.2. Preparation of Electro-Competent Bacterial Strains

1. Inoculate one colony in 5 mL of LB into 50-mL plastic tube and incubate at 32°C, for up to 16 h, shaking at 225–300 rpm (see Notes 24 and 25).

2. Dilute the culture 1:50 (v/v) in a 100-mL Erlenmeyer flask. For each transformation, competent cells resulting from 12.5-mL log-phase culture will be prepared (see Note 26).

3. Incubate at 32°C in a shaker until the culture reaches an OD_{600} of approximate 0.6. This usually takes approximately 3 h.

4. Cool down, to 4°C, a swing-bucket rotor centrifuge and a table-top microcentrifuge. Incubate 50 mL of MilliQ into an ice/water bath slurry.

5. Place the culture for 15 min on ice and then transfer into 50-mL skirted-centrifuge tube (see Note 27).

6. Pellet the cells by centrifugation at $4,500 \times g$ for 5 min at 0°C. Keeping the bacteria ice-cold will increase electro-competency.

7. Carefully pour off the supernatant and resuspend the pellet in 1 mL of ice-cold MilliQ by gently swirling the tubes in the ice/water slurry. This step takes approximate 10 min (see Note 28).

8. Add 9 mL of ice-cold MilliQ and centrifuge the samples at $4,500 \times g$ for 5 min at 0°C.

9. Decant the supernatant and resuspend the pellet in 1 mL of ice-cold MilliQ by gently swirling the tubes in the ice/water

bath slurry. Transfer the bacterial suspension into a 1.7-mL microcentrifuge tube, using a large orifice tip (MBP 1000GGenomic Tip).

10. Centrifuge at $25,000 \times g$ for 10 s, at 0°C, using a table-top microcentrifuge.

11. Remove the supernatant and resuspend the pellet in 1 mL of ice-cold MilliQ by vortexing for a maximum of 5 s at medium speed.

12. Centrifuge at $25,000 \times g$ for 10 s at 0°C, using a table-top microcentrifuge.

13. Repeat steps 9 and 10.

14. Remove the supernatant, resuspend the pellet in 50 μL of ice-cold MilliQ, and store on ice until electroporation (see Note 29).

3.2.3. Inducing the Recombination Function

1. Follow steps 1 and 2 as described in Subheading 3.2.2.

2. When the culture reaches an OD_{600} of approximate 0.6, split the culture into two 100-mL Erlenmeyer flasks.

3. Keep one flask at 32°C (named "un-induced") (see Note 30).

4. Place the other flask (named "induced") in the 42°C water bath. Shake for exactly 15 min at 220 rpm.

5. After 15-min induction, follow steps 4–14, as described in Subheading 3.2.2, for each of the "un-induced" and "induced" samples (see Notes 31 and 32).

3.2.4. Transfer BAC into Recombinogenic Bacterial Strain

1. Pre-cool 0.1-cm electroporation cuvettes.

2. Add 0.1–1 μg of BAC DNA, freshly prepared (see Subheading 3.2.1), to 50 μL of electro-competent cells of each "induced" and "un-induced" cells, freshly prepared as in Subheading 3.2.2.

3. Mix gently by tapping the tube and transfer to a pre-cooled cuvette.

4. Perform electroporation under the following condition: 1.75 kV, 25 μF with the pulse controller set at 200 Ω. The best results correlate with time constants between 4 and 5 ms.

5. Add 1 mL of SOC and transfer to a 15-mL tube.

6. Incubate for 2 h at 32°C, shaking at 225–300 rpm.

7. Plate entire cultures onto two LB plates containing 12.5 μg/mL chloramphenicol and 8% glycerol, and incubate at 32°C.

8. After 24–48 h, emerging colonies can be picked and scaled up in 5 mL of LB containing 12.5 μg/mL of chloramphenicol and 8% glycerol (see Note 33). We recommend checking the

presence and integrity of BAC at this stage by PCR, for five colonies, by inoculating ready-prepared PCR mixes with sterile pipette tips used for colony picking (see Note 23).

9. Make glycerol stock and prepare fresh, induced and un-induced electro-competent cells to be used for gap repair step, described in Subheading 3.2.6.

3.2.5. Insertion of Homology Arms in Targeting Vector

1. Using freshly prepared BAC as template and primers designed as described in Subheading 3.1, PCR amplify the homology arms HOM1 and HOM2 (see Note 34).

2. Confirm single, correct size PCR product by agarose gel electrophoresis (see Note 35).

3. If the desired product is present, proceed with purification by agarose gel electrophoresis for the entire PCR (see Note 36).

4. Isolate the desired band and extract the DNA using gel extraction kit (see Note 37).

5. Although the direct cloning of PCR product into pGEM-T Easy vector is possible, it is recommended to modify the blunt ends of PCR products by A-tailing. Incubate the following mix for 30 min at 72°C: 5–10 μL of PCR product, 2 μL of *Taq* DNA polymerase 10× reaction buffer, 0.8 μL of 50 mM MgCl$_2$, 5 units of *Taq* DNA polymerase, and MilliQ to a final reaction volume of 20 μL (see Note 38).

6. Ligate 1 μL of A-tailing mix into pGEM-T Easy vector (see Note 39).

7. Electroporate the ligation mix into DH12S cells according to standard cloning protocols, scale up ampicillin-resistant colonies and isolate DNA using Miniprep kit.

8. Sequence HOM1 and HOM2 from pGEM-T Easy vector, with the primers used for PCR amplification (see Note 40).

9. Digest pGEM-T Easy-HOM1 with *Apa*I and *Sac*II, and pGEM-T Easy-HOM2 with *Bgl*II and *Xho*I.

10. Using standard cloning protocols, clone HOM1 cut with *Apa*I and *Sac*II into *Apa*I and *Sac*II sites of pDTA.4B, and HOM2 cut with *Bgl*II and *Xho*I into *Bgl*II and *Xho*I sites of pDTA.4B.

11. Confirm the insertion of both HOM1 and HOM2 by enzymatic digestion using the two pairs of enzymes.

3.2.6. Retrieval of Targeted Gene onto Targeting Vector by Gap Repair

1. Digest 5 μg of pDTA-HOM1-HOM2 vector with *Sma*I for 2 h.

2. Perform agarose gel electrophoresis at low voltage, 40–50 V.

3. Isolate the upper size band representing the backbone vector and extract the DNA using gel extraction kit.

4. Proceed with a second digestion with *Sma*I for 1 h (see Note 41).

5. Repeat steps 2 and 3.

6. Measure DNA concentration.

7. Electroporate 50 ng of linear DNA into each of "induced" and "un-induced" samples of recombinogenic and electro-competent (see Subheading 3.2.3) bacteria containing BAC (see Subheading 3.2.4) following steps 1–6 from Subheading 3.2.4.

8. Plate entire cultures of each "induced" and "un-induced" bacteria onto two LB plates, one with ampicillin and the other with tetracycline, and incubate at 32°C (see Table 3).

9. After 16–24 h, pick 30 colonies from the ampicillin plate of "induced" bacteria, in 2 mL of LB media with ampicillin and incubate at 32°C, for up to 16 h, shaking at 225–300 rpm (see Note 42).

10. Isolate DNA using Miniprep kit.

11. Check for successful recombinants by PCR amplification of HOM1 and HOM2, and by several different enzymatic digest using two to three diagnostic enzymes.

12. Choose a correct clone of pDTA.4B-HOM, and transfer it into EL350 or SW106 electro-competent bacterial strains (see Subheading 3.2.2), following steps 1–4 of Subheading 3.2.4 (HOM represents the entire genomic region retrieved from BAC).

13. Transfer the culture to 50 mL of LB with ampicillin, to generate electro-competent cells, and (see Note 43) proceed with the steps from Subheading 3.2.2.

3.3. Insertion of an Orphan loxP Site

3.3.1. Insertion of loxP-Flanked Neo Cassette

1. Using PL452 as template and primers designed as described in Subheading 3.1, PCR amplify the *loxP-neo-loxP* cassette (see Note 44).

2. Confirm single correct sized PCR product by agarose gel electrophoresis (see Note 45).

3. If the desired product is present, add 50 U of *Dpn*I enzyme to the PCR mix and incubate for 2 h at 37°C (see Notes 46 and 47).

4. Proceed with purification by agarose gel electrophoresis at low voltage of 30–40 V.

5. Isolate the desired band and extract the DNA using gel extraction kit.

6. Electroporate 40–50 ng of PCR product into each of "induced" and "un-induced" samples of recombinogenic and electro-competent bacteria containing pDTA.4B-HOM (see Subheading 3.2.6) following steps 1–6 from Subheading 3.2.4.

7. Plate entire cultures of each "induced" and "un-induced" bacteria onto two LB plates, one with ampicillin and the other with kanamycin, and incubate at 32°C.

8. After 16–24 h, pick 30 colonies from kanamycin plate of "induced" bacteria in 2 mL of LB media with kanamycin and incubate at 32°C, for up to 16 h, shaking at 225–300 rpm (see Note 48).

9. Isolate DNA using Miniprep kit.

10. Perform several different enzymatic digest using two to three diagnostic enzymes to identify if recombination occurred (see Note 49).

3.3.2. Recombinant Product Analysis and Purification

After a successful recombination event, excluding gap repair, bacterial cells will contain a mixture of targeted and un-targeted plasmids. To purify out only the recombinated plasmid, we recommend (see Note 50) to proceed as follows:

1. Linearization of DNA obtained in step 9, Subheading 3.3.1, with a unique restriction enzyme (see Note 51) followed by agarose gel electrophoresis and purification using gel extraction kit.

2. Intra-molecular ligation using T4 DNA ligase.

3. Electroporate ligation mix into DH12S cells according to the standard cloning protocols, scale up kanamycin-resistant colonies and isolate DNA using Miniprep kit.

4. Perform several different enzymatic digest using two to three diagnostic enzymes, as in step 10 of Subheading 3.3.1, to check for successful purification (see Note 52).

After purification, the targeted region of the plasmid DNA needs to be sequenced to confirm the insertion of *loxP-neo-loxP* in the desired location.
Finally, choose one clone of pDTA.4B-HOM-*loxP-neo-loxP* and transfer it into EL350 or SW106 electro-competent bacterial strains (see Subheading 3.2.2), following steps 1–4 of Subheading 3.2.4.
Transfer the culture to 30 mL of LB with kanamycin and (see Note 43) proceed with the steps form Subheading 3.3.3.

3.3.3. Cre-Mediated Generation of the Orphan loxP Site

1. Add 100 µL of 10% (w/v) L(+) arabinose to 10 mL of culture from Subheading 3.3.2 and incubate at 32°C, shaking at 225–300 rpm, for 1 h. As control culture, incubate 10 mL of the culture from Subheading 3.3.2, without addition of L(+) arabinose.

2. Plate serial dilution, 1:100, 1:1,000, and 1:10,000 of each "arabinose induced" and "arabinose un-induced" bacterial cultures onto LB plates, with either ampicillin or kanamycin, and incubate at 32°C.

3. After 16–20 h, pick ten colonies, from ampicillin plate of "arabinose induced" bacteria, in 2 mL of LB with ampicillin and incubate at 32°C, for up to 16 h, shaking at 225–300 rpm (see Note 53).

4. Isolate DNA using Miniprep kit.

5. If a restriction enzyme site was artificially inserted during primers design (see Note 13), this enzyme should be the first choice for enzymatic diagnostic digests to check for successful Cre-mediated deletion of *neo* cassette. Additionally, perform two independent enzymatic diagnostic digests.

6. Finally, choose a pure clone of pDTA.4B-HOM-*loxP* and transfer it into EL250 or SW105 electro-competent bacterial strains (see Subheading 3.2.2), following steps 1–4 of Subheading 3.2.4.

7. Transfer the culture to 50 mL of LB with ampicillin and (see Note 43) proceed with the steps from Subheading 3.4.

An alternative approach is to induce Cre expression in EL350 or SW106 cells before making the cells electro-competent.

1. Inoculate an overnight culture of EL350 or SW106 from glycerol stock and incubate at 32°C, for up to 16 h, shaking at 225–300 rpm.

2. Dilute the culture 1:50 (v/v) in a 100-mL Erlenmeyer flask.

3. Add 10% (w/v) L(+) arabinose for a final concentration of 0.1% (v/v) to half of the culture and keep the other half as control.

4. Continue with steps 3–14 from Subheading 3.2.2 to generate electro-competent cells.

5. Choose a clone of pDTA.4B-HOM-*loxP-neo-loxP* and transform into electro-competent cells following steps 1–4 of Subheading 3.2.4.

6. After recovery, transfer the culture to 30 mL of LB with ampicillin.

7. Proceed with steps 2–7 from previous approach.

3.4. Insertion of frt-neo-frt-loxP Cassette

The *neomycin* resistance cassette to be introduced in this step will remain in the targeting vector for positive selection in ES cells. Therefore, this step should be always performed after the insertion of the orphan *loxP* site.

1. Using PL451 as template and primers designed as described in Subheading 3.1, PCR amplify the *frt-neo-frt-loxP* cassette (see Note 44).

2. Proceed as in steps 2–5 of Subheading 3.3.1.

3. Electroporate 40–50 ng of PCR product into each of "induced" and "un-induced" samples of recombinogenic and

electro-competent bacteria (see Subheading 3.2.3) containing pDTA.4B-HOM-*loxP* obtained in step 7, Subheading 3.3.3, following steps 1–6 from Subheading 3.2.4.

4. Follow steps 7–10 from Subheading 3.3.1.

3.4.1. Recombinant Product Analysis and Purification

This section follows the same principle as that of Subheading 3.3.2. After the purification of the targeted plasmid (see Subheading 3.3.2), one clone of the final targeting vector is transferred into EL350 or SW106 and EL250 or SW105 electro-competent bacterial strains (see Subheading 3.2.2), following steps 1–4 of Subheading 3.2.4. We recommend transformation also in DH12S cells, for DNA amplification, and if the test described in Subheading 3.5 is successful, the final vector can be linearized and used for electroporation into ES cells (see Note 12).

Transfer the culture to 50 mL of LB with kanamycin (see Note 43) and proceed as described in Subheading 3.5.

3.5. Analysis of Functional Cre/loxP and Flp/frt Systems

This section follows the same principle from Subheading 3.3.3 using the cells from Subheading 3.4.1.

1. Follow steps 1–4 from Subheading 3.3.3, using both Cre- and Flp-containing bacterial strains.

2. Perform two diagnostic digest tests to check for Flp recombinase-mediated *neo* cassette deletion and for Cre recombinase-mediated removal of targeted exon.

3.6. Gene-Targeted Mutagenesis

3.6.1. Mutagenesis of Targeted Exon

1. Using primers designed as indicated in step 6 of Subheading 3.1, PCR amplify the exon of interest from pDTA.4B-HOM vector (see Notes 15 and 16).

2. Continue with steps 2–7 from Subheading 3.2.5.

3. Perform the desired exon modification, using GeneTailor™ Site-Directed Mutagenesis System or a similar system, according to the manufacturer's protocol.

3.6.2. Cloning of Mutated Exon into loxP-neo-loxP Plasmid

In this step, the mutant exon will be removed from pGEM-T Easy vector and cloned into PL452 vector, immediately downstream of *loxP-neo-loxP* cassette, using standard cloning protocols (see Note 18).

3.6.3. Insertion of loxP-neo-loxP-(mut)exon into Targeting Vector

The *neomycin* resistance cassette to be introduced in this step can remain in the targeting vector for positive selection in ES cells or, if the gene targeting vector construction requires other *neomycin* resistance cassette insertion in a different location, it can be removed by Cre-mediated excision, as described in Subheading 3.3.3.

1. Using primers designed as indicated in step 6 of Subheading 3.1, PCR amplify the *loxP-neo-loxP-(mut)exon* from PL452 host (see Note 19).

2. Proceed as in steps 2–5 of Subheading 3.3.1.

3. Electroporate 40–50 ng of PCR product into each of "induced" and "un-induced" samples of recombinogenic and electro-competent bacteria containing pDTA.4B-HOM (see Subheading 3.2.6) following steps 1–6 from Subheading 3.2.4.

4. Continue with steps 7–10 from Subheading 3.3.1.

3.6.4. Recombinant Product Analysis and Purification

1. Follow steps 1–4 from Subheading 3.3.2.

2. Confirm by sequencing the insertion of *loxP-neo-loxP* in the desired location and confirm the presence of mutation on the targeted exon.

3. Transfer the targeting vector into EL350 or SW106 (see Subheading 3.2.2), following steps 1–4 of Subheading 3.2.4.

4. Analysis of functional Cre/loxP system should be performed as described in Subheading 3.5.

5. After amplification into DH12S cells, the targeting vector can be linearized and used for electroporation into ES cells. (see Note 12).

4. Notes

1. DY380 genotype is F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*Z M15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*) 7649 *gal*U *gal*K *rsp*L *nup*G [λ*cI*857 (*cro-bio*A) <> *tet*], and SW102 genotype is F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*Z M15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*) 7649 *gal*U *rsp*L *nup*G [λ*cI*857 (*cro-bio*A) <> *tet*].

2. Bacterial strains EL250 and EL350 have similar recombinogenic activities and efficiencies with SW105 and SW106, respectively, for the all procedures described in Subheading 3.

3. We recommended ordering two to three different BACs containing the desired genomic region.

4. After the complete design of the strategic plan, we recommend ordering all the primers necessary for all the steps, including sequencing primers. Longer delivery time for longer primers should be considered.

5. When bacteria are grown for electro-competency or induction of recombination function, we recommend growing bacteria in low salt media.

6. For antibiotics concentrations for media and plates, see Table 3.

7. After the complete design of the strategic plan, we recommend ordering all the restriction enzymes necessary for all the steps, including *Dpn*I.

8. We recommend performing PCR amplification using all three different polymerases.

9. We strongly recommend drawing sketches for each individual step.

10. BACs need to be verified by PCR for the presence of DNA sequences that will be used as homology arms. Also, it is essential to sequence these regions together with the genomic regions where the insertion of first *loxP* site and of *frt-neo-frt-loxP* cassette will be performed.

11. We recommend designing two to three distinct homology arms, as some combination might favor the retrieval of targeted DNA from BAC during gap repair. Check whether *Apa*I, *Sac*II, *Bgl*II, *Xho*I, and *Sma*I are non-cutters for these regions.

12. There is a unique *Not*I site located proximal of DTA cassette in pDTA.4B vector. We do not recommend using it for linearization of final targeting vector, as the plasmid backbone will not protect the DTA cassette from degradation in ES cells until homologous recombination takes place. As an alternative, we recommend using *Apa*I, single cutter in pDTA.4B distal of DTA cassette, as its site will be conserved in the targeting vector during all recombineering steps.

13. During primer design, it is recommended to include at least one restriction enzyme that can be used either to analyze for successful recombination events, or if it is a good genomic cutter, for Southern blotting diagnostic genotyping. Also, it is necessary to design primers to be used for sequencing of *loxP* sites integrity and to confirm that the integration of *lox-neo-loxP* cassette occurred at the predicted site.

14. The insertion place of the orphan *loxP* site or *frt-neo-frt-loxP* cassette should be chosen such that splicing will not be affected. For this, a distance of minimum 150–200 bp, upstream of the splice acceptor or downstream of the splice donor, is required. There are several online programs to assist with the splicing prediction (15).

15. It is required to design to have the 5′ primer, P1, at least 200 bases upstream of beginning of targeted exon.

16. Both 5′ and 3′ primers designed in this step, named P1 and P2, should include restriction enzyme for cloning the final mutated PCR product into PL452. Restriction enzymes must be non-cutters for the exonal region and also should not

interfere with removal of mutated product from the muta-genesis vector.

17. Restriction enzyme sites used for cloning will be removed during recombination.

18. PL452 vector has the following unique cutters upstream of *loxP-neo-loxP*: *Bam*HI, *Not*I, and *Sac*II that can be used to clone in the mutated exon.

19. Genomic homology region for 5′ primer (P3) should stop right before primer P1 starts. Genomic homology region for 3′ primer (P4) should start right before primer P2 ends.

20. It is required to sequence the successful recombinants for the presence of the mutation.

21. It is highly efficient to have accurate sequence details of inter-mediary products and of final targeting vector as electronic files, not only in the initial strategic planning step but also during the experiment procedures. We use a freeware plasmid editor (16).

22. Do not use a narrow bore pipette tip to resuspend DNA mechanically. Resuspension may take around 1 h. Store at –20°C.

23. For PCR check, use primers designed to amplify the homol-ogy arms, HOM1 and HOM2.

24. For each bacterial strain, and for each bacterial strain contain-ing BAC or intermediary products of targeting vector, it is required to make 15% glycerol (v/v) stock and store them at –80°C.

25. EL250, EL350, SW105, and SW106 have no antibiotic resis-tance. Bacterial strains must be grown at 32°C. It is required to use only freshly prepared electro-competent cells every time a transformation is needed.

26. The size of the culture depends on the number of samples to be used.

27. We recommend using skirted tubes, which can be centrifuged at $4,500 \times g$.

28. Pipetting should be avoided.

29. For all steps described in this protocol involving electro-competent cells, the preparation of electro-competent cells should follow the steps indicated in Subheading 3.2.2.

30. Every time recombineering is performed, the "un-induced" sample will serve as negative control for recombination efficiency.

31. Bacterial cells that were induced, by heat shock at 42°C, cannot be used for another recombination step.

32. For all steps described in this protocol involving induction of recombineering activity in bacterial cells, it must follow the steps indicated in Subheading 3.2.3.

33. Expect around ten colonies per plate.

34. It is recommended to use various polymerases and a gradient PCR when amplifying BAC sequences, as various polymerases can yield products of various qualities.

35. For each of HOM1 and HOM2, the steps presented in this section must be performed in parallel.

36. If unwanted products are obtained, try to separate the bands by using a gel with appropriate agarose concentration for the desired band size and perform electrophoresis at low voltage, 40–50 V.

37. If ethidium bromide is used, minimizing the exposure of the gel to ultraviolet light is required, because of mutagenic effect of UV radiation.

38. Thermostable DNA polymerase with proofreading activity generates blunt-ended fragments. PCR products obtained using these polymerases need to be modified if they need to be inserted into TA overhang vectors.

39. pGEM-T Easy vector contains *Apa*I and *Sac*II unique sites in the T7 cloning region. HOM1 contains *Apa*I and *Sac*II sites at 5′ and 3′, respectively. In our hands, digestion with *Apa*I and *Sac*II of pGEM-T Easy-HOM1 always resulted in a pure HOM1 product.

40. Alternatively, SP6 or T7 primers can be used for sequencing the inserts from pGEM-T Easy vectors.

41. This step is required to avoid the risk of contamination with the uncut DNA.

42. The control plates are required to assess the efficiency of recombination process. Ampicillin plate of "induced" bacteria should show around 100 colonies. Ampicillin plate of "un-induced" bacteria should have no colonies, as gap repair cannot occur when recombination genes are not active. Tetracycline plates of both "induced" and "un-induced" bacteria should have no colonies, tetracycline cassette being removed from pDTA.4B vector.

43. Make glycerol stock before using the bacterial culture for induction.

44. It is recommended to use various polymerases.

45. A band around 1.9 kb in size is expected depending on the length of genomic homology sequences added to the primers.

46. *Dpn*I treatment is essential for accurate purification of amplified PCR product, to eliminate the circular plasmid DNA contamination and always needs to be followed by gel extraction.

47. Use a maximum of 1 ng of PL452 as template to avoid the risk of contamination.

48. The control plates are required to assess the efficiency of recombination process. Kanamycin plate of "induced" bacteria should show around 30–100 colonies. Kanamycin plate of "un-induced" bacteria should have no colonies, as recombineering of *loxP-neo-loxP* cassette cannot occur when recombination genes are not active. Ampicillin plates of both "induced" and "un-induced" bacteria should have 200–500 colonies, pDTA.4B-HOM providing ampicillin resistance to bacterial cells.

49. PCR amplification can be performed optionally using the same primers as that used in step 1 in Subheading 3.3.1, but it may give false-positive results if minor contamination occurs.

50. Purification can be also obtained by re-transformation in DH12S bacteria of very low amounts, 0.1–0.05 ng, of DNA obtained in step 9, Subheading 3.3.1.

51. We suggest using *Not*I, a unique cutter for pDTA.4B.

52. It is necessary to include the DNA obtained in step 9, Subheading 3.3.1 as control sample.

53. The control plates are required to assess the efficiency of Cre-mediated excision of *neo* cassette and subsequent generation of an orphan *loxP* site. Kanamycin plates of "arabinose un-induced" bacteria should show correlative colony number-dilution, around 50 colonies for 1:10,000 dilution plate. The *neo* gene is expressed from both a prokaryotic promoter (*em7*) and a eukaryotic promoter (*Pgk*); therefore, kanamycin plate of "arabinose induced" bacteria should have no colonies, as kanamycin resistance is lost after *neo* cassette removal. Ampicillin plates of both "arabinose induced" and "arabinose un-induced" bacteria should show correlative colony number-dilution, around 50 colonies at 1:10,000 dilution.

Acknowledgments

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Chapter 12

Generating Conditional Knockout Mice

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Abstract

Gene targeting in ES cells is extensively used to generate designed mouse mutants and to study gene function *in vivo*. Knockout mice that harbor a null allele in their germline provide appropriate genetic models of inherited diseases and often exhibit embryonic or early postnatal lethality. To study gene function in adult mice and in selected cell types, a refined strategy for conditional gene inactivation has been developed that relies on the DNA recombinase Cre and its recognition (loxP) sites. For conditional mutagenesis, a target gene is modified by the insertion of two loxP sites that enable to excise the flanked (floxed) gene segment through Cre-mediated recombination. Conditional mutant mice are obtained by crossing the floxed strain with a Cre transgenic line such that the target gene becomes inactivated *in vivo* within the expression domain of Cre. A large collection of Cre transgenic lines has been generated over time and can be used in a combinatorial manner to achieve gene inactivation in many different cell types. A growing number of CreER^{T2} transgenic mice further allows for inducible inactivation of floxed alleles in adult mice upon administration of tamoxifen. This chapter covers the design and construction of loxP flanked alleles and refers to the vectors, ES cells, and mice generated by the European conditional mouse mutagenesis (EUCOMM) project. We further describe the design and use of Cre and CreER^{T2} transgenic mice and a convenient breeding strategy to raise conditional mutants and controls for phenotype analysis.

Key words: Cre, loxP, Conditional mutant, Mouse mutant, ES cells, Inducible, Tamoxifen

1. Introduction

In the last two decades, gene targeting in ES cells has been extensively used as a powerful tool to generate predesigned mouse mutants and to study gene function *in vivo*. As initially developed, this 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 3,000 knockout (KO) mice have been created following this design. Many of these strains provided valuable information on the biological function of the genes

studied (2). Since these "conventional" KO mice are homozygous for a null allele in the germline, they provide appropriate models of inherited diseases, leading to embryonic or early postnatal lethality in about 30% of cases. Besides this application, germline KO mice do not necessarily represent the optimal tool to study other aspects of gene function *in vivo*, in particular, in adult mice. Therefore, in 1994, a refined knockout strategy termed conditional mutagenesis has been developed that enables to inactivate a target gene only in a selected cell type (3, 4). An overview of the germline knockout and the conditional mutagenesis approach is given in Fig. 1. In a germline knockout strain, the target gene is inactivated in all cells throughout all developmental stages. In conditional mutants, the target gene is modified in the germline but becomes inactivated only in somatic cells. The inactivation of the target gene can be either restricted to a selected cell type without temporal control or be induced at a chosen timepoint in a selected cell type. By these means, gene function in adult mice can be precisely studied in a cell type of choice.

Conditional gene inactivation can be achieved in mice by the cell type-specific expression of the DNA recombinase Cre, while the target gene is modified by the insertion of recombinase recognition (loxP) sites (4). Cre is a site-specific DNA recombinase derived from the P1 phage that recognizes and mediates recombination between 34-bp sequences referred to as loxP sites. As Cre does not require cofactors or accessory proteins to mediate loxP-specific DNA recombination and exhibits optimal activity at 37°C, the Cre/loxP system is the most widely used recombination tool in ES cells and mice (5). Other recombinases such as FLP and PhiC31 Integrase are mainly used for genome engineering
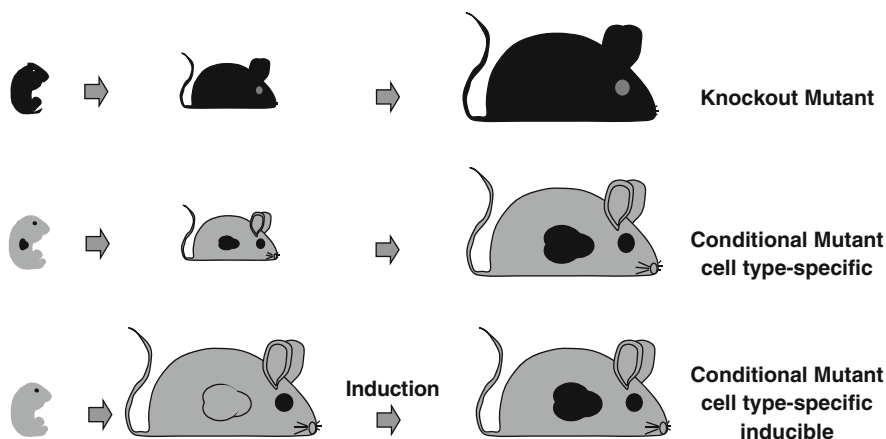
Fig. 1. Gene targeting strategies. *Top*: the germline inactivation of a gene results in knockout mice that exhibit the mutation bodywide throughout ontogeny. *Middle*: gene inactivation in conditional mutants occurs in somatic cells and is restricted to a selected cell type. The timing of gene inactivation cannot be externally induced and may occur at early stages. *Bottom*: in inducible conditional mutants, the onset of gene inactivation in a selected cell type can be regulated, e.g., induced in adult mice.

in ES cells (5). Dre recombinase, an enzyme related to Cre, is the
latest addition to the recombinase toolbox for mammalian cells
(6). Dre has been recently shown to function in mice and may be
suited to complement Cre in future (7). The loxP recognition site
for Cre is composed of two 13-bp inverted repeats separated by
an 8-bp asymmetric spacer that determines the orientation of the
loxP site (8) (Fig. 2a). Cre-mediated recombination 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 obtained. 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. 2b). Although this
reaction is reversible, the equilibrium is strongly biased toward
excision as the circular product is lost by diffusion. This deletion
reaction is mostly used to achieve gene inactivation in conditional
mutants. When two loxP sites are located in opposite orientation
on a DNA molecule, Cre mediates the inversion of the loxP-
flanked sequence (Fig. 2c) (9). As this reaction is also reversible,
an equal ratio of both inversion products is obtained. Furthermore,
mutant loxP sites that recombine with each other but not with
wild-type loxP can be used to exchange gene segments in the ES
cell genome (10).
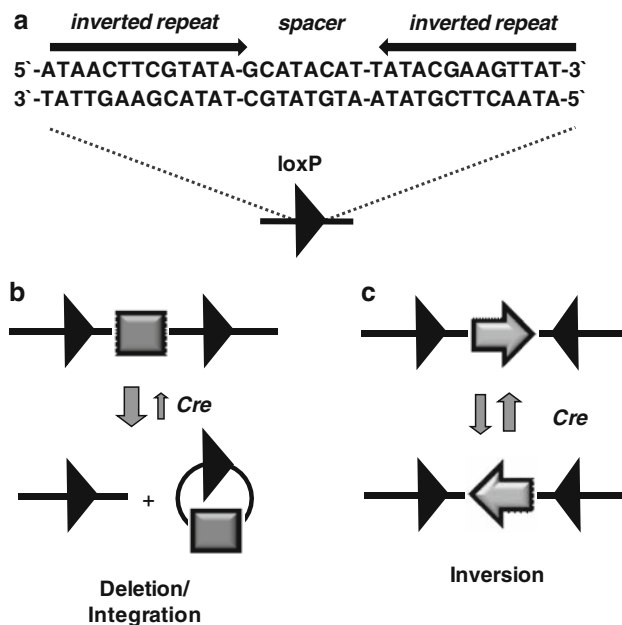
Fig. 2. The Cre/loxP recombination system. (a) Sequence and structure of a loxP site. The 34-bp loxP sequence includes
13-bp inverted repeats flanking an asymmetric 8-bp spacer region that determines the orientation of the loxP site.
(b) Cre-mediated deletion of a DNA segment (*square*) flanked by two loxP sites (*triangles*) of the same orientation.
(c) Cre-mediated inversion of a DNA segment flanked by two loxP sites in opposite orientation.

In conditional mutants, gene inactivation is achieved by the transgenic expression of Cre in a selected cell type, while two loxP sites are inserted into non-coding regions of the target gene. To achieve gene inactivation, the loxP sites are placed in the same orientation into intron regions such that they flank at least one exon of the target gene. Cre-mediated excision of the loxP-flanked region results in the *in vivo* deletion of the coding segment from the genome of recombinase-expressing cells. Upon deletion of the target exon, the mutant gene codes for a nonfunctional protein by loss of an essential domain or as a result of a reading frame shift.

For the generation of conditional mutants, two types of mouse strains are required. The floxed strain harbors the loxP-flanked gene segment, initially introduced into the germline by homologous recombination, while the second strain provides the Cre recombinase gene expressed from a cell type-specific promoter region. Conditional mutant mice are obtained by crossing the floxed with the Cre strain such that the target gene is inactivated within the expression domain of recombinase (Fig. 3). In such standard Cre transgenic mice, recombinase expression and thereby the timing of gene inactivation depend on the developmental activation of the chosen promoter region and cannot be experimentally manipulated. To obtain inducible KO mice, a fusion protein of Cre and a mutant estrogen receptor ligand-binding domain (Cre-ER^{T2}) can be expressed from a cell type-specific promoter (11). The recombinase activity of Cre-ER^{T2} can be induced *in vivo* by the administration of the small molecule inducer tamoxifen. A large collection of standard Cre mice that covers many cell types has been generated over time, while the number of inducible Cre mice is limited but continuously increasing.
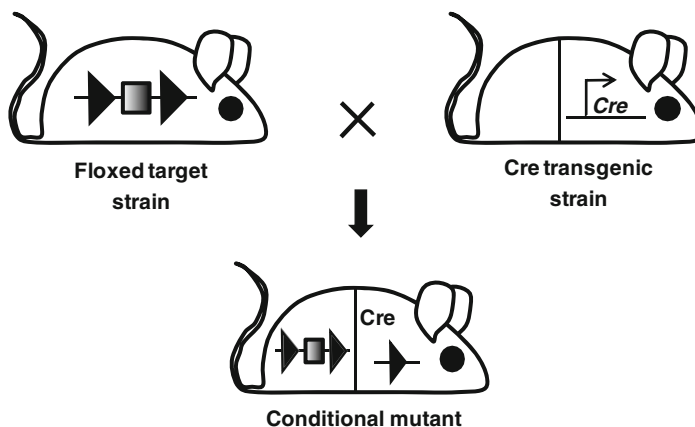
Fig. 3. Generation of conditional mutants. To generate conditional mutants, a strain harboring a loxP-flanked gene segment (floxed allele) (*upper left*) is crossed to mice expressing Cre recombinase in a specific cell type (*upper right*). In double transgenic offspring, gene inactivation occurs through Cre-mediated excision of the floxed gene segment within the Cre expression domain.

There is no general rule to decide a priori whether the germline or the conditional knockout approach is most appropriate for a particular experiment. In many cases, both types of mutants are generated to investigate gene function both during embryonic development and in adult mice. Since a loxP-containing strain can be converted into a classical null allele by crossing it to a "deleter" strain that expresses Cre in the germline, the conditional gene targeting scheme can be universally applied to all knockout projects to obtain both types of mutants.

Referring to conditional KO mice, this chapter first covers the design and use of loxP-flanked alleles. Subheading 3.1 describes a simple PCR-based approach for the generation of vectors for conditional gene targeting applicable in any standard laboratory. Since the number of conditional alleles available from large-scale mutagenesis projects is continuously expanding, self-made vector constructions are not any more mandatory to obtain a conditional mutant. Subheading 3.1.2 covers the design and recombinase-mediated modifications of alleles generated by the European Conditional Mouse Mutagenesis (EUCOMM) project. Next, the generation and use of standard and inducible Cre transgenic mice are described in Subheadings 3.2.1 and 3.2.2. For the use of Cre mice, it is important to characterize the recombinase expression pattern in new strains or to confirm the expression pattern of an established strain. Subheading 3.2.1.1 includes an overview on Cre reporter mice and a protocol for the detection of the β-galactosidase reporter gene in tissue sections. With Subheading 3.3 the chapter closes with a short discussion of the specifics of the phenotype analysis of conditional mouse mutants.

2. Materials

2.1. Vectors for Conditional Gene Targeting

1. Plasmid pEasyflox-DTA (available from the authors).
2. Genomic BAC clone covering the target gene (to be ordered from, e.g., Imagenes, http://www.imagenes-bio.de or Geneservice, http://www.geneservice.co.uk).
3. PCR-qualified low-error DNA polymerase, synthetic oligonucleotides.

2.2. Cre Transgenic Mice

1. Fixative, 4% paraformaldehyde, 5 mM EGTA, 10 mM $MgCl_2$, solved in PBS.
2. X-Gal staining solution, 0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide ($K_4Fe(CN)_6$), 5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 1 mg/mL X-Gal (add fresh before use from 20 mg/mL stock solution in dimethylsulfoxide).

2.3. Inducible Cre Activity

1. Tamoxifen stock solution, 10 mg tamoxifen/mL.

2. For i.p. injection, a 10 mg/mL stock of tamoxifen-free base is prepared.

3. Suspend 1 g of tamoxifen-free base (Sigma #T5648) in 10 mL of 100% ethanol. Add 90 mL of sunflower seed oil (Sigma #S5007) and stir the suspension for several hours until the tamoxifen is completely dissolved. Store the stock solution in aliquots at –20°C for up to 4 weeks.

3. Methods

3.1. Vectors for Conditional Gene Targeting

The design of conditional KO alleles follows two main objectives: (1) a pair of loxP sites must be inserted into intron regions of the target gene such that the encoded protein is inactivated through Cre-mediated excision of the loxP-flanked exon(s) and (2) the target gene should not be disrupted by the presence of the loxP sites prior to Cre-mediated recombination. This section presents two approaches to obtain conditional alleles in ES cells. The first approach involves self-designed conditional alleles based on PCR amplification of the gene targeting vector homology arms, a method applicable in any laboratory that is experienced in molecular biology. The second approach relies on predesigned conditional alleles that are available from the EUCOMM project as ready-made targeting vectors, targeted ES cell clones, and mouse lines. The EUCOMM vectors and alleles are accessible under http://www.eucomm.org. A complete overview of all alleles generated by the international gene targeting programs is available at http://www.knockoutmouse.org. Previously published mouse mutants as valuable resource can be searched using the genes query of the mouse genome informatics pages of the Jackson laboratory (http://www.informatics.jax.org). Before considering the construction of self-made targeting vectors, we recommend a careful analysis of the databases of the growing public resources.

3.1.1. Self-Designed Conditional Vectors for Conditional Gene Targeting

3.1.1.1. Vector Design

A conditional construct can be assembled from three DNA segments of the target gene that can be amplified by PCR from a genomic template (Fig. 4). The construct's segments A and C serve as homology regions that mediate recombination with the chromosomal target gene. Segment B comprises the loxP-flanked region of a conditional allele that is selected for Cre-mediated excision. The combined length of the homology arms A and C usually adds up to 5–10 kb. The 5′ homology arm A should have a size of at least 1 kb and include a unique restriction site at its 5′ end for linearization of the vector before electroporation of ES cells.

The loxP-flanked segment B usually has a size of 0.5–2 kb and includes one or more exons of the target gene to disrupt the target protein by Cre-mediated excision. We recommend to place loxP sites at least ~250 bp apart from the exon boundaries to reduce the risk of interference with splicing signals. Within the size limit of ~2 kb, often only a single exon can be flanked by loxP sites. Therefore, the target gene must be carefully analyzed for its genomic structure using, e.g., the Ensembl genome database (http://www.ensembl.org) to define a "critical" exon, the deletion of which will disrupt gene function. In general, the critical exon is the first exon downstream of the gene's exon 1 that exhibits different reading frames upon splicing to its preceeding and its following exon. Thereby, the genomic deletion of the critical exon leads to reading frame shift within the mRNA of the recombined allele and to a premature stop codon. This will result either in the production of a truncated, nonfunctional protein or in the degradation of the mutant mRNA by the nonsense-mediated decay mechanism. Alternatively, if a functional domain of the target protein is known, a specific exon, e.g., coding for a protein kinase domain, can be chosen as a critical exon. The third genomic segment C represents the 3′ homology arm of a conditional gene targeting vector with a size of 2–5 kb. The size of segment C should be at least equal to the length of segment B to provide sufficient sequence space for homologous recombination downstream of the second loxP site. For the positive selection of stably transfected ES cell clones, a neomycin resistance (neo) gene, flanked by FRT recognition sites for FLP recombinase, must be included into the vector next to one of the loxP sites (Fig. 4). Most vectors further include a negative selectable marker such as the diphtheria toxin A chain (DTA) expression cassette to reduce the frequency of ES cell clones containing random vector integrations.

To define the boundaries of segments A and C, the respective genomic sequences should be further analyzed for the location and number of DNA repeats. We recommend to minimize the number of repeat elements within the vector's homology arms. Upon completion of the vector design, a strategy for the screening of transfected ES cell colonies must be defined. We usually screen genomic DNA prepared from ES cell clones in 96-well plates by Southern blot analysis. For this purpose, repeat-free DNA segments of 600–700 bp, located next to the 5′ and 3′ homology arms, can be amplified by PCR from the genome and used as external probes for Southern blot hybridization. To design a Southern blot screening strategy, the sequence of the targeted locus should be assembled using appropriate software (e.g., Vector NTI, Invitrogen, Carlsbad, USA) Invitrogen is the supplier of the vector NTI software and analyzed for restriction site differences in comparison to the wild-type locus. The neo gene expression cassette, as a unique part of the targeted locus, includes
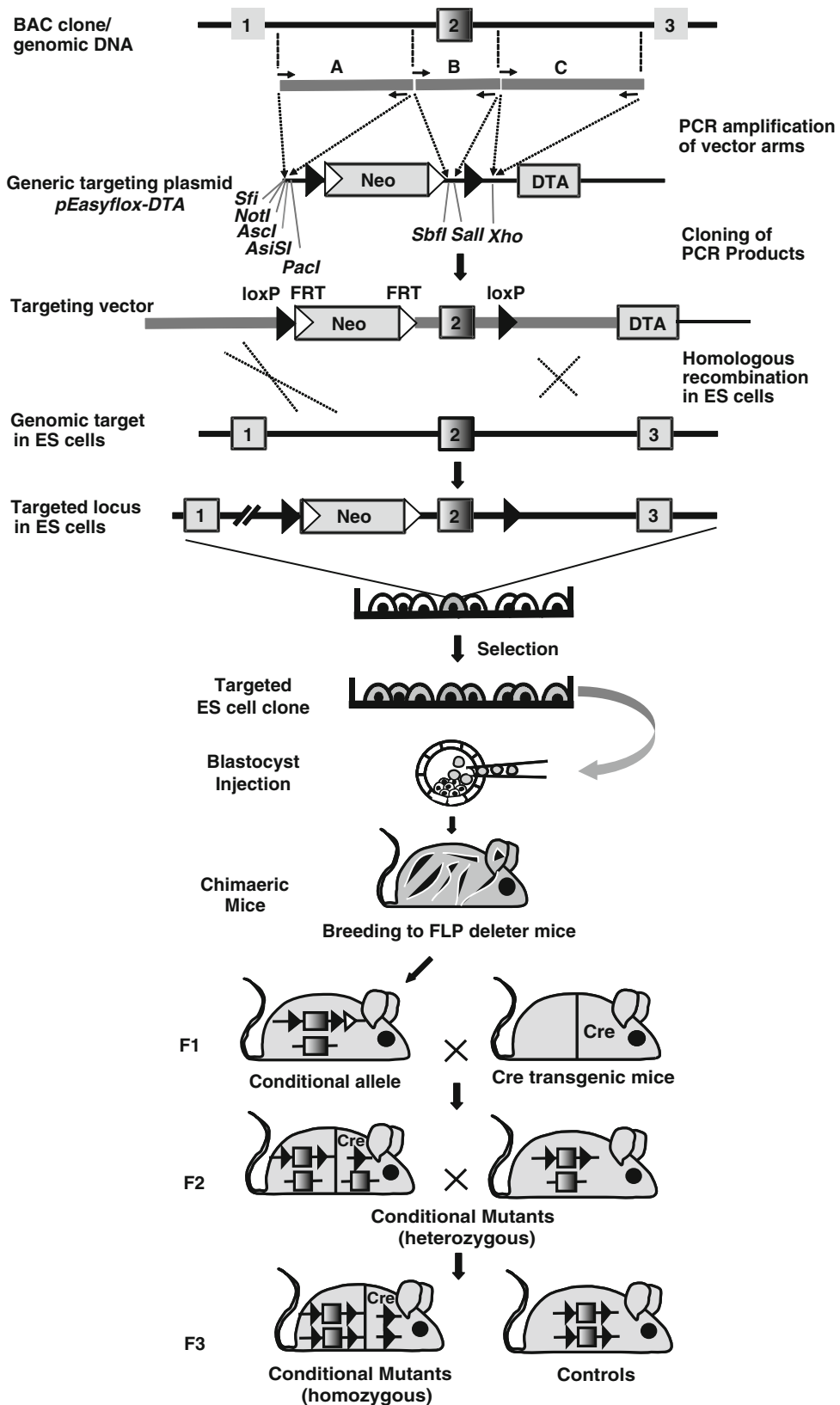
Fig. 4. Conditional gene targeting vectors and conditional mutants. Scheme for the PCR-based assembly of a vector for conditional gene targeting, starting from a hypothetical target gene that contains three exons (*numbered rectangles*). The targeting vector is assembled by PCR amplification of the genomic segments **a**–**c**, which represent its 5′ homology region

restriction sites for, e.g., EcoRI, EcoRV, PstI, and XbaI (see below). By comparison of the restriction maps of the wild-type and targeted locus, enzymes can be identified that allow the clear identification of recombinant ES cell clones.

3.1.1.2. Vector Construction

For the construction of vectors for conditional gene targeting, we use the generic plasmid backbone pEasyflox-DTA, which contains two loxP sites, a FRT-flanked neo expression cassette, and a DTA gene (Fig. 4). This vector includes NotI, AscI, AsiSI, and PacI sites for insertion of the 5′ homology arm (segment A), a SbfI and SalI site for insertion of the loxP-flanked segment B, and a XhoI site for cloning of the 3′ homology arm (segment C). The three segments should be PCR amplified using a proofreading DNA polymerase such as Phusion (New England Biolabs) or Herculase (Stratagene), and fully sequenced upon cloning of the PCR product. The primers for PCR amplification must include the restriction sites chosen for cloning of the respective genomic fragment into pEasyflox-DTA. As part of the vector design, the absence of these sites within the amplified segments must be confirmed.

The frequency of homologous recombination between a targeting vector and its genomic target sequence is strongly reduced if the homologous sequences are not identical (12). To ensure proper recombination, the segments A–C for vector construction should be amplified from the genome of the same mouse strain as used to establish the ES cell line chosen for the targeting experiment. As template for PCR, we use BAC clones of the RPCI-23 genomic library, derived from the mouse strain C57BL/6 J. BAC clones that cover the genomic region of interest can be found via the Ensembl mouse genome database (http://www.ensembl.org) and ordered from, e.g., Imagenes (http://www.imagenes-bio.de, Berlin, Germany) (see Note 1). Such C57BL/6-based gene targeting vectors can be transfected into the C57BL/6-derived ES cell line Bruce4 (13) or JM8 (14), or into F1 ES cell lines such as IDG3.2 (15) or V6.5 (16), derived

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Fig. 4. (continued) (**a**), the 3′ homology region (**c**), and the loxP-flanked region (**b**). *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. The PCR primers must include restriction sites that are compatible to sites within the generic targeting plasmid pEasyflox-DTA. This plasmid includes a NotI, AscI, AsiSI, and PacI site for cloning of the 5′ homology region **a**; a SbfI and SalI site for insertion of the loxP-flanked region **b**; and a XhoI site for cloning of the 3′ homology region **c**. Finally, the gene targeting vector contains a FRT-flanked neomycin resistance gene (neo) and the second, "critical" exon is flanked by loxP sites. The diphtheria toxin expression cassette (DTA) allows negative selection of ES cell clones carrying random integrations of the vector. Upon electroporation of the targeting vector into ES cells, neo resistant colonies are selected. Targeted ES cell clones that result from homologous recombination of the vector with its genomic counterpart can be identified by the analysis of genomic DNA. Through microinjection of targeted ES cells into blastocysts, germline chimeric mice are generated. By crossing chimeras to a FLP deleter strain, the FRT-flanked neo selection marker is excised and the conditional, loxP-flanked allele (neo-free) for conditional gene targeting is generated. Mice harboring the conditional allele are crossed with Cre transgenic mice (F1 generation). In the next mating cycle (F2), offspring that are heterozygous for the conditional allele plus the Cre transgene are crossed to heterozygous conditional mice. In the resulting F3 generation, offspring are obtained that are homozygous for the conditional allele and heterozygous for the Cre transgene representing the conditional mutants. Littermates that are homozygous for the conditional allele but do not contain the Cre transgene can be used as controls.

from (C57BL/6 × 129) hybrid blastocysts. Upon completion of vector cloning, the homology regions of the targeting construct should be sequenced to avoid the transfer of undesired mutations into the target gene. Before the transfection of ES cells, the gene targeting vector must be linearized at its 5′ end. For this purpose, pEasyflox-DTA contains a SfiI and a NotI site (Fig. 4).

The electroporation of ES cells and the selection and screening of neomycin-resistant colonies are a multi-step procedure that exceeds the scope of this chapter and are described elsewhere (17). Identified recombinant ES cell clones are injected into blastocysts for the production of germline chimeric mice to transmit the conditional allele to their offspring. In its initial configuration, a targeted allele includes the neomycin resistance expression cassette and that must be later excised from the genome to ensure the normal function of the conditional allele. The neo gene cassette can be conveniently removed from the targeted locus by crossing conditional (neo positive) offspring with FLP recombinase transgenic (deleter) mice (18) (see Note 2). Double transgenic offspring derived from this cross have lost the neo gene by recombination between the FRT sites flanking the selection marker. These offspring can then be further crossed to a Cre transgenic strain of choice, resulting in the first mice that harbor one copy of the conditional allele (neo negative) and the Cre transgene (Fig. 4). Half of these offspring will also contain the FLP transgene and should be excluded from breeding. Next, the intercross of conditional males and females will deliver the first homozygous conditional mice. The further breeding strategy may be adapted to the specific Cre line in use since some of these strains exhibit (undesired) recombinase expression in the male or female germline (see Note 3). In case of a Cre line with deleter activity in the male germline, it is required to cross homozygous conditional males (without Cre) with homozygous conditional females that also contain the Cre transgene. The offspring of such matings can be classified by genotyping into the experimental group of homozygous conditional mutants (with Cre) and the control group of homozygous conditional littermates (without Cre) that should be equal to a wild-type control (Fig. 4) (see Note 4). To confirm the proper function of the conditional allele, the expression levels of its mRNA or protein in homozygous conditional mice should be compared to that of wild-type mice.

3.1.2. Conditional Alleles Generated by Large-Scale Mutagenesis Programs

Several large-scale research programs have been launched in recent years to generate public libraries of mutated mouse ES cells. The main programs are the Knockout Mouse Project (KOMP; USA), the EUCOMM (Europe), the North American Conditional Mouse Mutagenesis Project (NorCOMM; Canada), and the Texas A&M Institute for Genomic Medicine (TIGM; USA). It is the common goal of these programs to generate

mutations for all known mouse genes. The alleles generated by these consortia are mostly of conditional nature. These efforts are coordinated by the International Knockout Mouse Consortium (IKMC), which will publish on its website a searchable list of all available vectors, ES cell clones, and mice (http://www.knock-outmouse.org). All reagents of these programs are distributed to scientists via specialized distribution centers (http://www.komp.org, http://www.eummcr.org). By ordering ES cell clones from these ES cell libraries, the generation of conditional KO mice can be greatly simplified and accelerated.

The key feature of the basic EUCOMM/KOMP conditional allele design is the "critical exon", which will be the target for Cre excision (Fig. 5a). The critical exon is determined by four criteria. (1) Its deletion will cause a frame shift in the remaining mRNA, leading to a disrupted mutant protein, or even complete absence of protein by the mechanism of nonsense-mediated mRNA decay. (2) The exon is present in all transcript isoforms (splice variants) of the targeted gene. (3) The size of the exon is below 1 kb, to assure that the distance of the loxP sites is short enough for efficient Cre recombination. (4) The size of the flanking introns is at least 0.5 kb, so that loxP sites can be placed in non-conserved regions that are not required for endogenous splicing. In some instances, to fulfill the design criteria, several small exons are grouped together as one group of critical exons.

Beside the floxed critical exon, an EUCOMM/KOMP targeting vector contains 5′ and 3′ homology arms that mediate homologous recombination, and a central targeting cassette that disrupts gene function and reports gene expression by a lacZ reporter. Two versions of targeting cassettes are in use: promoter-less and promoter-driven (Fig. 5a).

The promoterless targeting vectors are used for the targeting of genes that are expressed in ES cells at sufficient levels. This strategy utilizes the promoter of the targeted gene to drive the expression of the neo resistance, which results in high targeting frequencies of the vector. Most EUCOMM/KOMP targeting vectors contain a targeting cassette with a neo resistance that is driven by a β-actin promoter. This allows the targeting of all genes, irrespective of their expression status in mouse ES cells. The β-actin:neo cassette is removable by treatment with Cre or Flp recombinases (see below).

The alleles generated after targeting with a EUCOMM/KOMP conditional vector are "knock-out first" alleles (19). That is, in the first version of the allele, gene function is inactivated by splicing of upstream exons to a splice acceptor in the targeting cassette (Targeted mutation 1a, Tm1a, Fig. 5b). To render this allele into a conditional version, the targeting cassette has to be removed by Flp recombinase, which leaves loxP sites flanking a "critical" exon behind (Tm1c, "conditional", Fig. 5b). The Flp excision is typically performed by breeding the Tm1a mice with a
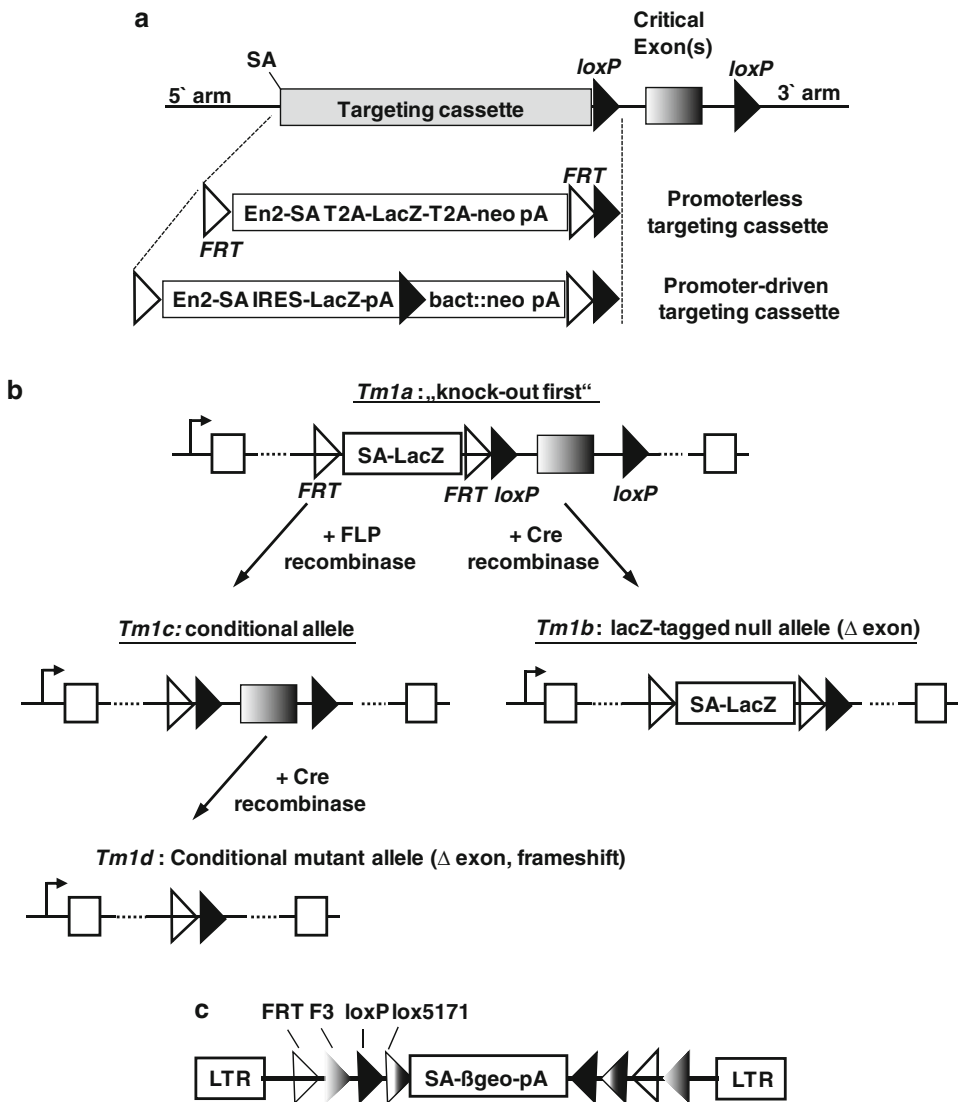
Fig. 5. EUCOMM conditional alleles. (**a**) Basic design of the EUCOMM/KOMP conditional targeting vector, and the two main targeting cassettes used in the program. (*SA* splice acceptor, *En2-SA* Engrailed2 splice acceptor, *T2A*, *T2A* self-cleavage peptide sequence, *pA* polyadenylation signal). (**b**) Possible modifications of the EUCOMM/KOMP allele by successive recombinations with Flp or Cre recombinases. (**c**) The EUCOMM conditional gene trap vector. Heterotypic recombination sites for Flp and Cre recombinases allow inversion and conditional re-inversion of the cassette.

Flp germline deleter mouse. This conditional allele can subsequently be mutated by breeding to a mouse line that expresses Cre recombinase in a time- and tissue-specific manner (Tm1d, "deletion", Fig. 5b). Alternatively, the original allele can be treated with Cre recombinase to remove the critical exon, which results in a lacZ-tagged, germline null allele (Tm1b, "beta-Gal reporter", Fig. 5b).

The vectors that are generated by the EUCOMM/KOMP programs can also be used by researchers to assemble vectors with other targeting cassettes. For this purpose, the intermediate versions of the targeting vector can be obtained, and a targeting cassette of choice (e.g., a GFP or Cre cassette) can be inserted by Gateway cloning.

In addition to targeted ES clones, the EUCOMM program has also generated a library of conditionally mutated ES cells by gene trapping. The gene trap vector used in EUCOMM contains the FlipROSAβGeo cassette, which utilizes the Flex principle (20) for conditional mutagenesis. The FlipROSAβGeo trap cassette consists of a splice acceptor, a β-galactosidase reporter, a neomycin resistance gene, and a polyadenylation signal. Two pairs of heterotypic recombination sites for Cre and FLP recombinases, respectively, are positioned in inverted orientation around the gene trap cassette (Fig. 5c). This allows inverting the cassette two times. The first inversion, e.g., by the Flp recombinase of a FLP deleter mouse strain, silences the mutation and creates a conditional allele. The second inversion, e.g., by tissue-specific Cre recombinase, reinstates the mutation.

3.2. Cre Transgenic Mice

3.2.1. Constitutive Cre Activity

Cre transgenic mice can be produced by random integration of Cre expression vectors through pronuclear injection or by the targeted insertion of Cre in frame with the initiation codon of an endogenous gene (5) (Fig. 6). The first approach is more straightforward considering vector construction and its introduction into the germline. However, an appropriate promoter region tested for transgenic expression is required. Since the level and pattern of transgene expression often varies greatly depending on its copy number and integration site, multiple transgenic independent founder lines must be raised and analyzed to identify a useful strain. In Cre transgenic mice, the expression properties of the selected promoter region determines the onset and cell type-specificity of gene inactivation while the level of recombinase expression determines its efficiency.

Transgene constructs for the expression of Cre recombinase are designed in a similar manner to other cDNA expression constructs for transgenic mice (Fig. 6a). A DNA fragment containing the promoter region of choice is combined with the coding sequence of Cre. The Cre gene should be preceded by an intron providing a splice donor and acceptor site to increase mRNA expression *in vivo*, e.g., by the hybrid intron described in (21). This unit must be followed by a polyadenylation signal, e.g., the bpA element derived from the bovine growth hormone gene (22). Useful vectors containing the P1 bacteriophage-derived Cre coding region (Genbank accession No. X03453), such as pgk-Cre-bpA, can be found in the plasmid collection of Addgene (http://www.addgene.org). In most cases, a Cre coding region that includes an SV40 T antigen nuclear localization sequence (like in pgk-Cre-bpA) is
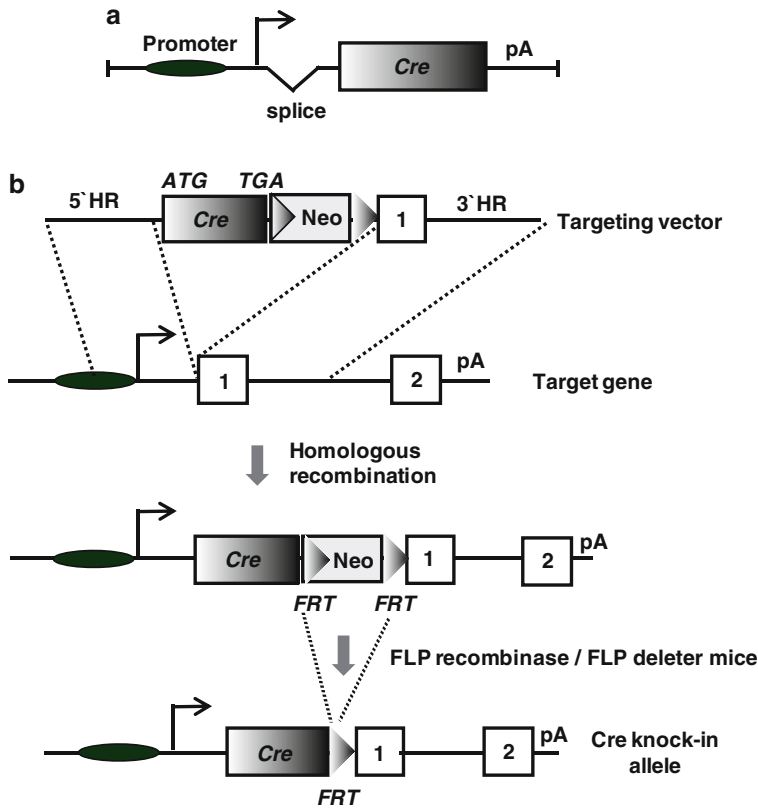
Fig. 6. Generation of Cre transgenic mice. (**a**) Schematic Cre expression vector for the production of transgenic mice by pronuclear injection. A promoter region characterized for gene expression in transgenic mice drives the transcription of the Cre coding region. An intron, providing splice donor and acceptor sites for efficient transgene expression, is placed upstream of Cre, followed by a polyadenylation signal sequence (pA). (**b**) Insertion of Cre into an endogenous gene by gene targeting. The targeting vector contains 5′ and 3′ homology regions (HR) of the target gene and the Cre coding region (including start (ATG) and stop (TGA) codons), followed by an FRT-flanked neomycin resistance expression cassette (Neo). The start codon within the first exon of the target gene is replaced by the ATG of Cre such that the position of the initiation codon is unchanged. The FRT-flanked neo gene is deleted from the targeted locus by transient expression of FLP recombinase in ES cells or cross of the Cre/Neo mouse strain to a FLP deleter line.

used, although Cre also carries endogenous nuclear targeting determinants (23). Alternatively, a codon-optimized Cre gene (iCre) can be used that leads to increased levels of recombinase in mammalian cells (24).

To reduce the effects of the integration position on transgene expression, Cre lines are increasingly derived as BAC vector transgenic mice. BAC vectors that carry genomic DNA segments of 150–250 kb are thought to include most regulatory elements of a chromosomal gene and are less influenced by the genomic environment. Due to their large size, BAC transgenes cannot be assembled by standard cloning procedures but require protocols such as ET cloning or recombineering for homologous recombination in *Escherichia coli*. A protocol for the construction of BAC transgenic Cre mice can be found in (25).

As an alternative to the transgenic approach, the coding sequence of Cre can be inserted downstream of the promoter region of an endogenous gene by homologous recombination in ES cells (Fig. 6b). By this knock-in strategy, Cre expression is regulated optimally since all control elements of the targeted gene are present at their natural chromosomal position. Furthermore, the targeting approach can be applied to any gene without the need for previously characterized promoter regions. A replacement type vector for the targeted insertion of Cre should be constructed such that the initiation codon of the targeted gene is replaced by the coding region of Cre (Fig. 6b). We suggest to add a polyadenylation signal at the end of the Cre coding region to avoid the transcription of exons located downstream of the Cre stop codon and mRNA destabilization by nonsense-mediated decay (26). To enable the selection and identification of targeted ES clones, a selection marker gene must be included in a targeting vector. The selection marker must be later removed from the genome to avoid a potential disturbance of the targeted conditional allele (27). We recommend to use an FRT-flanked neo expression cassette that can be recovered from, e.g., pEasyflox-DTA (see Subheading 3.1.1).

Gene targeting is, however, more laborious compared to conventional transgenesis. In addition, a nonfunctional allele of the gene utilized for Cre expression is created and this may be of disadvantage for phenotype analysis and its interpretation. This complication has been addressed through the expression of Cre via an internal ribosomal entry site to avoid the disruption of the endogenous gene (28, 29). Alternatively, the virus-derived self-cleaving T2A peptide (30) may provide in future another option for Cre expression by fusion of a T2A-Cre sequence to the C-terminus of a target protein.

More than 150 Cre transgenic and knock-in mouse strains have been described that enable conditional mutagenesis in a variety of cell types and tissues. An overview of the published Cre lines and their respective recombinase activity profiles is given in the Cre-X database (http://nagy.mshri.on.ca/cre) (31). A listing of all published Cre lines can also be obtained through a gene search with the term "Cre" at the Jackson Mouse Genome Informatics website (http://www.informatics.jax.org).

To characterize the recombinase expression profile of new and established Cre mouse strains, multiple Cre reporter alleles are available. Such indicator strains contain a loxP-flanked DNA segment that initially prevents the expression of a β-galactosidase, GFP, or other reporter gene from a coupled promoter region. Reporter strains based on randomly integrated transgenes may not exhibit reporter activity in all tissues but can be useful to map Cre activity in tissues of confirmed reporter activity. The most widely used indicator strains were constructed as knock-in alleles into the widely

expressed Rosa26 locus and express β-galactosidase or fluorescent proteins (Table 1). The expression of the β-galactosidase reporter can be monitored in tissue sections by using the histochemical X-Gal stain or by immunohistochemistry using specific antibodies. The latter method allows the detailed documentation of reporter expression by co-staining with cell type-specific markers. In this chapter, we provide a protocol for the histochemical detection of β-galactosidase activity.

3.2.1.1. Protocol: X-Gal Staining of Tissue Sections

The appropriate staining method depends on the tissue of interest. To get a first impression of the activity pattern of Cre recombinase, one should perform whole mount staining of all major mouse tissues. If the staining pattern is already known and one or more tissues are to be analyzed in more detail, histological sections of these tissues should be analyzed as they give detailed information about the regional and cell type-specific distribution of recombinase activity.

1. *Tissue preparation*

   For sections of large organs (e.g., brain), perfuse the mouse intracardially with fixative (see Subheading 2.2) following standard procedures before preparing the organs. After perfusion, tissues should be post-fixed in the same solution for 1–1.5 h at 4°C, but not longer as this would impair the activity of β-galactosidase. For whole mount stainings of smaller organs and of embryos, perfusion is not necessary.

2a. *Whole mount staining of organs or embryos*

   (a) After preparation, the tissues are incubated in fixative at 4°C for 1–5 h, depending on tissue size. Do not fix for longer than 5 h.

   (b) The tissues are washed three times for 20 min in phosphate-buffered saline (PBS) and then placed in the X-Gal staining solution (see Subheading 2.2) overnight at 37°C.

   (c) Afterwards, the tissues are washed in PBS and stored in PBS (plus Na-Azide) until documentation.

2b. *Staining of tissue sections*

   (a) After perfusion of the mice and dissection, the organs are embedded in 4% agarose dissolved in PBS.

   (b) From the organs, 100-mm thick sections are cut with a vibratome and directly mounted on Superfrost Plus slides.

   (c) The sections are air-dried for 1 h at RT and then the slides are incubated in X-Gal staining solution (see Subheading 2.2). In case of strong signals, the intensity of the staining should be monitored every hour, whereas samples with weak signals can be incubated overnight at 37°C.

Table 1
Cre reporter mice

Reporter name	Reporter prior recombination	Reporter upon recombination	Promoter region	Insertion type	References
Rosa-lacZ	none	β-galactosidase	Rosa26	Rosa26 knock-in	(51, 52)
Rosa-GFP	none	eGFP	Rosa26	Rosa26 knock-in	(53)
Rosa-YFP	none	eYFP/eCFP	Rosa26	Rosa26 knock-in	(54)
Rosa-RFP	none	tdRFP	Rosa26	Rosa26 knock-in	(55)
mT/mG	RFP (tdTomato)	GFP	pCAGGS	Rosa26 knock-in	(56)
Z/AP	β-galactosidase	alkaline phosphatase	pCAGGS	Transgene	(57)
Z/EG	β-galactosidase	eGFP	pCAGGS	Transgene	(58)

(d) The slides are washed in PBS, fixed in 4% paraformaldehyde (in PBS), and finally either counterstained (e.g., with Nuclear-Fast Red) or directly covered with a suitable aqueous mounting medium.

3.2.2. Inducible Cre Activity

Inducible gene inactivation was first shown in transgenic mice expressing Cre from a promoter that is inducible by interferon (32). In addition, the tetracycline-regulated gene expression system was used to control Cre in specific mouse lines (33, 34). As another possibility for the local induction of gene inactivation, viral Cre expression vectors can be delivered by intravenous or stereotaxic injection into mice (35–37). Presently, the most frequently used technique to achieve inducible and cell type-specific Cre activation is provided by fusion proteins of Cre with the ER^{T2} mutant ligand-binding domain of human estrogen receptor (amino acids 282–595, Fig. 7a) (38, 39). The CreER^{T2} fusion protein can be constitutively expressed in mice using a cell type-specific promoter region, but does not exhibit recombinase activity unless a specific activating ligand is supplied. In the absence of this ligand, the ER^{T2} domain is bound by heat shock proteins that presumably block recombinase activity by sterical hindrance.
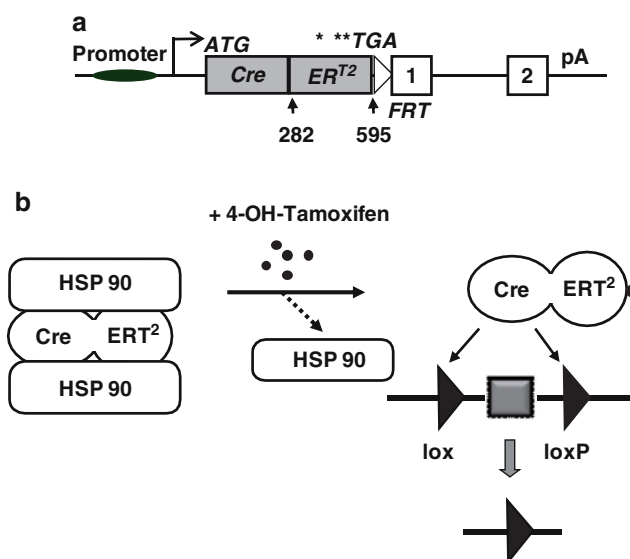
Fig. 7. The tamoxifen-inducible Cre-ER^{T2} system. (**a**) Structure of a BAC transgene or knock-in allele for the cell type-specific expression of Cre-ER^{T2} fusion protein. In Cre-ER^{T2}, the coding region of Cre (including the ATG start codon) is fused to the ligand-binding domain of the human estrogen receptor (ER^{T2}, residues 282-595) that provides a stop codon (TGA). The ER^{T2} ligand-binding domain contains three point mutations (***) that render ER^{T2} unresponsive to the natural ligand estradiol. As shown in Fig. 6, the start codon of Cre-ER^{T2} replaces the initiation codon of the BAC transgene or endogenous target gene. (**b**) Principle of the tamoxifen-inducible Cre-ER^{T2} protein. The Cre-ER^{T2} protein is constitutively expressed in a selected cell type but remains inactive by binding to heat shock proteins (HSP90) that interfere with Cre activity. Cre-ER^{T2} can be activated by the ER^{T2} ligand 4-OH-tamoxifen that leads to the dissociation of HSP90 proteins. Upon HSP90 dissociation, the fusion protein is released from the inactive complex and mediates the excision of a loxP-flanked DNA segment via the active Cre domain.

The ER^{T2} domain is unresponsive to the natural ligand estradiol due to the change of three amino acids. However, recombinase activity of the fusion protein can be induced by the synthetic ligand 4-hydroxy-tamoxifen, which releases the heat shock proteins from the ER^{T2} domain (Fig. 7b).

Mouse strains expressing CreER^{T2} in a selected cell type can be generated like Cre transgenic lines by random integration of a CreER^{T2} expression construct or by knock-in of the CreER^{T2} coding region into an endogenous gene. The CreER^{T2} coding region can be recovered, e.g., from the plasmid pCAG-CreER^{T2} available from Addgene (http://www.addgene.org).

A variety of transgenic and knock-in mouse strains expressing CreER^{T2} in specific cell types were described in the last years, demonstrating the feasibility of this technique in all major organs including the brain. Since the information on these strains is scattered in the literature, Table 2 provides a compilation of the more than 30 published lines. Although the CreER^{T2} technique is working efficiently, it must be noted that the treatment of mice with tamoxifen which acts as an antagonist of the wild-type estrogen receptor, can cause side effects and experimental artifacts. It has been further described that the treatment of CreER^{T2} transgenic mice with tamoxifen, but not the treatment of Cre negative controls, induces the death of lymphoma cells (40). Acute tamoxifen treatment also causes behavioral alterations – 4 weeks after the treatment, most but not all of these effects are normalized (41). It is, therefore, essential to include control groups to test for the potential side effects of tamoxifen and the activation of Cre in a specific experimental setting. The treatment of littermates containing a CreER^{T2} transgene and the conditional allele of interest with the tamoxifen vehicle alone does not represent a sufficient control. In addition, CreER^{T2} transgenic littermates and groups of mice that harbor only the conditional allele should be treated with tamoxifen and compared to the experimental group containing both genetic modifications.

3.2.2.1. Protocol: Tamoxifen Induction

The current standard method to induce CreER^{T2} activity is the intraperitoneal (i.p.) injection of the free base of tamoxifen dissolved in oil. The free base of tamoxifen is inactive on CreER^{T2} but is metabolized in the liver to the active ligand 4-OH-tamoxifen. Alternatively 4-OH-tamoxifen can be directly injected, but this compound is more costly than the free base of tamoxifen. Both compounds were shown to induce recombination in all mouse tissues including the brain. For complete induction of CreER^{T2} in adult mice, the 10 mg/mL tamoxifen stock solution (see Subheading 2.2) is injected i.p. daily for 5 consecutive days at a dosage of 40 mg/kg body weight (this equals to 4 µL stock solution per 1 g body weight). However, for complete induction in the brain, it was shown that two injections per day for 5 consecutive days are required (42).

Table 2
CreER^T2 mouse lines

Specificity	Promoter	Name (-CreER^T2)	Insertion type	References
Widespread	Rosa26	Rosa26	Knock-in	(59, 60)
	CMV enhancer/chicken β-actin	CMV-actin	Transgene	(61)
	CMV enhancer/chicken β-actin	CAGG	Transgene	(62)
	Ubiquitin C	Ubc	Transgene	(63)
Keratinocytes	Kreatin 5	K5	Transgene	(11)
	Kreatin 14	K14	Transgene	(64)
Melanocytes	Tyrosinase	Tyr	Transgene	(65, 66)
Hepatocytes	α1-antitrypsin promoter	αAT	Transgene	(67)
	Serum albumin	SA	Transgene	(68)
Smooth muscle	SM22α	SM	Knock-in	(69)
Skeletal muscle	Skeletal muscle α-actin	HAS	Transgene (PAC)	(70)
Cardiac muscle	α-myosin heavy chain	α-MHC	Transgene	(71)
Osteoblasts and odontoblasts	Chollagen 1 α1 chain	Col1a1	Transgene	(72)
Chondrocytes	Chollagen 2 α1 chain	Col2a1	Transgene	(73)
Adipocytes	Adipocyte fatty acid binding protein	aP2	Transgene	(74)
Astrocytes	Glial fibrillary acidic protein	GFAP	Transgene	(75, 76)
	Astrocyte-specific glutamate transporter	GLAST	Knock-in	(77)
Neuronal stem cells	Nestin	Nes	Transgene	(78)
Schwann cells	Proteolipid protein	PLP	Transgene	(79, 80)
	P0 fused to connexin 32	P0Cx	Transgene	(79)
	Calcium/calmodulin-dependent protein kinase 2 α	CamKIIα	Transgene (BAC)	(42)
Neurons, forebrain	Frizzled10	Frizzled10	Transgene	(81)
Cortical neurons	$Na_v1.8$	$Na_v1.8$	Transgene	(82)
Nociceptive Neurons in DRG	Villin	Vil	Transgene	(83)
Epithelium, intestinal	Kidney-specific cadherin	KspCad	Transgene	(84)

(continued)

Table 2 (continued)

Specificity	Promoter	Name (-CreER[T2])	Insertion type	References
Epithelium, renal	Keratin 18	K18	Transgene	(85)
Internal epithelial organs	Cited1	Cited1	Transgene (BAC)	(86)
Epithelium, nephronic	Tie2 receptor tyrosine kinase	Tie2	Transgene	(87)
Endothelium	Vascular endothelial cadherin	VECad	Transgene	(88)
	Pdgfp	Pdgfb	Transgene (PAC)	(89)
Endothelium, vascular	Pitx3	Pitx3	Transgene (BAC)	(90)

A more convenient and less stressful alternative to the intraperitoneal injection of tamoxifen is the oral administration of tamoxifen citrate with the food. For this purpose, tamoxifen citrate is mixed with the normal chow to a concentration of 360 mg/kg chow. The duration of the treatment depends on the desired tissue, but previous studies have shown that for most tissues, 1–2 weeks are sufficient (43).

3.3. Concluding Remarks

Conditional mutagenesis has been used to address various biological questions in adult mice that could not be resolved with germline mutants, often because a null allele results in embryonic or neonatal lethality. However, researchers who consider to work with conditional mutants should be aware that different classes of mutants are obtained in combination with noninducible or inducible Cre mice, and that these mutants address questions on gene function in a different way, since the phenotypes that are observed in cell type-specific conditional mutants represent the combined consequence of gene inactivation in the developing and adult organism.

In case the experimental question concerns the role of a gene in different cell types, noninducible cell type-specific Cre strains are presently the most pragmatic choice since a large collection of tissue-specific strains is available. The vast majority of the published conditional mutagenesis experiments belongs to this noninducible class. A good example for this approach is the comparison of the cell type-specific inactivation of the insulin receptor in skeletal muscle, pancreas, and liver. Cell type-specific gene targeting revealed a prime role of insulin receptor for glucose homeostasis in the liver but not the skeletal muscle, and also showed the role of insulin signaling in pancreatic insulin secretion (44–46). In order to select a Cre line for conditional mutagenesis, one should be

aware of the onset of recombinase expression during development. Any promoter region used for the cell type-specific expression of Cre becomes activated in the course of pre- or postnatal development. Conditional mutants generated with noninducible cell type-specific Cre mice are distinct from germline knockouts by their regional pattern of gene inactivation, but like in classical knockouts, gene function is lost during development. Therefore, phenotypes observed in cell type-specific conditional mutants represent the combined consequence of gene inactivation in the developing and the adult organism.

In case the experimental question concerns the role of a gene specifically in the adult body, inducible Cre mice are the best choice to generate inducible conditional mutants. Inducible gene targeting allows the analysis of gene function in adults since these animals can undergo normal embryonic and postnatal development until the time of gene inactivation. In the first inducible knockout experiment, the B lymphocyte antigen receptor was deleted in mature B cells. Since this receptor is also essential for the development of B cells, its function in mature B cells could only be addressed by inducible gene inactivation. In many other cases, target genes can be deleted either early in development or later in adults. To address the difference between these approaches, we recently compared the phenotypes of noninducible and induced conditional mutants for the *Braf* gene in forebrain neurons. We found common phenotypes in both types of mutants but noninducible and induced mutants also exhibited distinct phenotypes (unpublished results). Therefore, a complete description of gene function may require both types of conditional mutants, each type reporting on gene function at a particular phase of ontogeny.

In conclusion, conditional mutagenesis is a field in progress since the resources of conditional alleles and inducible Cre mice are growing rapidly. The number of conditional mouse lines generated by individual researchers can be estimated to be in the range of some hundred lines (see also (47)). These resources are considerably expanded by the EUCOMM program that produced until mid 2009 more than 2,100 conditional alleles in ES cells and 266 mouse lines, and the program is continuously expanding its resources. In the coming years, these resources will strongly promote the application of conditional mutagenesis in mice.

4. Notes

1. We prefer BAC clones of C57BL/6 J origin since PCR can be initiated from a template of low complexity, compared to the use of genomic DNA. In addition, PCR-associated mutations can be easily identified by comparison of the PCR products to

the C57BL/6 genome sequence in the available web resources (http://www.ensembl.org, http://www.genome.ucsc.edu).

2. The first germline Cre deleter mouse strain was found among transgenic lines produced with the minimal promoter of the tetracycline-regulated gene expression system (48). The use of this strain is inconvenient since this transgene is X-chromosome linked and exhibits mosaic recombination. We presently use an unpublished Cre knock-in strain into the Rosa26 locus on the C57BL/6 background from TaconicArtemis (*Gt(ROSA)26Sortm16(cre)Arte*, http://www.taconic.com). This transgene, however, can be maintained only in the heterozygous state, presumably due to Cre toxicity in homozygous Rosa26-Cre mice. The same company offers a FLP germline deleter strain on the C57BL/6 background.

3. The germline recombination activity of lines expressing Cre from a cell type-specific promoter is anecdotic and hard to retrieve from the literature. Reference (40) gives information on strains used in Immunology. Concerning brain-specific lines, we observed deleter activity in the male but not the female germline of CamKII-Cre (49) and Nestin-Cre mice (50). For CamKII-Cre mice, the frequency of germline recombination greatly varies among different conditional alleles from ~2% to almost 100%.

4. Phenotypic changes observed in homozygous conditional mutants most likely result from the loss of gene function by Cre-mediated inactivation of the conditional allele. However, Cre mice may also exhibit distinct phenotypes that should be controlled before the use of a particular strain for a conditional experiment. Whether such phenotypes result from Cre recombinase activity or an effect of transgene insertion remains unclarified. In case of strong recombinase expression, Cre toxicity has been observed in cultured fibroblasts and male germ cells (40), which likely result from the recombination of cryptic loxP sites present in the mouse genome.

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Chapter 13

Hypomorphic Mice

Darren J. Baker

Abstract

The use of genetically engineered mice has become a standard approach in order to study the physiological contribution of genes in a variety of life-science disciplines. Classical and conditional gene-targeting methods aimed at generating knock-out mice that lack gene products have been useful, but may be limited in their scope. If the gene of interest is essential for cell viability, little insight can be gained into the in vivo function of these genes. A hypomorphic approach, utilizing many of the same methods employed for traditional gene targeting, allows one to disrupt the function of genes to a lesser degree and bypass the lethality caused by many gene mutations. The purpose of this chapter is to introduce the concepts behind how hypomorphic alleles impede normal genetic function and provide information necessary to construct a targeting vector successfully for use in ES cells to generate ultimately mice with lower than normal amounts of an endogenous protein of interest.

Key words: Hypomorphic alleles, Gene targeting, Animal models

1. Introduction

Classical and conditional gene knock-out strategies have been widely used to create animal models for human disease or to study gene function at the level of the whole animal (1, 2). Animal models using classical gene targeting have been essential for dissecting many cellular processes, including tumor suppression (3). Tumor suppressors are not essential genes by definition, and, therefore, complete knock-outs of these genes usually develop in adult animals. For genes other than tumor suppressors, sometimes a heterozygous knock-out animal has a sufficient reduction in total protein to develop an overt phenotype that can be further analyzed for the involvement of the particular gene in a condition. Many single heterozygous mice fit these criteria. However, some genes may be essential during embryogenesis, but then may

be dispensable later in life. In these cases, a conditional knock-out approach using the standardized cre-lox or frt-flp transgenic models to disrupt genes in the entire adult animal or only in certain tissues can be employed to determine if late life ablation of this gene has any cellular consequences (4, 5). These models, therefore, have been able to extend the findings of classical knock-outs. However, both classical and conditional gene knock-outs are limiting if the protein of interest is essential for cell viability. If the complete removal of a protein is incompatible with cellular survival and heterozygous knock-out mice are not sufficient to promote a phenotype, little knowledge is likely to be gained into the physiological function of these genes. Under this scenario, how can one further dissect the potential functions of this gene at a physiological level? A further reduction of the biological activity of a protein would be required that still is sufficient for cell survival. Implementing a gene-targeting technique utilizing hypomorphic gene-targeting alleles makes expression of more protein than that expressed by knock-out alleles possible, but significantly less than that of corresponding wild-type alleles (5–7). Animal models utilizing this approach have become standard and appear in over 500 manuscripts as of the beginning of 2009. When used in combination with knock-out alleles, hypomorphic alleles can enable the production of a series of mice with various expression levels (8), allowing for one to decipher the degree of protein reduction necessary to promote a phenotype. These techniques vary significantly from transgenic RNA interference (RNAi)-induced gene silencing-mutant mice (9), although both approaches are designed to reduce the total amount of endogenous protein. Transgenic RNAi mouse models come with a caveat that accompanies most transgenic models: the insertion site of the transgene is random and may disrupt essential genes. Moreover, the degree of protein reduction can vary greatly from founder to founder in the first generation of the transgenic offspring based on the insertion site and the number of insertions. In addition, shRNAs may nonspecifically target other mRNAs. These off-target effects may vary temporally and in different tissues with varied mRNA expression patterns depending on the cell type. These unfortunate off-target effects are nearly impossible to assess firstly in cell culture systems. The transgenic RNAi founders need to be assayed to determine what the reduction of the gene of interest is and the overall protein reduction required to mediate a physiological phenotype. Therefore, hypomorphic gene targeting, where the insertion sites are known, provides for determination of physiological consequences of genes without the potential effects of transgene insertion. The purpose of this chapter is to introduce the two distinct strategies for generating hypomorphic alleles for gene targeting. Furthermore, important considerations in order to ensure success when designing hypomorphic alleles will be addressed.

2. Materials

Once the targeting construct is obtained, the process of gene targeting, including all reagents and supplies, is as described (1). Therefore, the method of gene targeting will not be further described here. Basic targeting vector construction utilizes technology and materials that are used for molecular biology subcloning. For those without the capacity to do routine cloning, there are many companies that will assist in the construction of the targeting vector for a fee (e.g., inGenious and Ozgene).

2.1. Generation of Hypomorphic Targeting Vectors

1. pNTKV1901 (Stratagene).
2. pHTKV1901 (neo replaced by hygro cassette, available from Dr. Jan van Deursen upon request).
3. High-fidelity polymerase (i.e., Herculase enhanced DNA polymerase, Stratagene).
4. Mouse embryonic stem (ES) cell DNA or BAC DNA of region intended for targeting.
5. pGEM-Easy (Promega).
6. Phenol:chloroform:isoamyl alcohol (25:24:1).
7. 3 M NaOAC (pH 4.8).
8. Glycogen (20 mg/ml).
9. 100% and 75% ethanol.
10. Taq DNA polymerase.
11. QiaQuick PCR purification kit (Qiagen).
12. QiaQuick gel extraction kit (Qiagen).

2.2. Western Blotting

1. Dry ice and liquid nitrogen.
2. 2¾ in. porcelain CoorsTek mortar and pestle (Fisher).
3. Standard laboratory scale, accurate to 0.1 mg.
4. Dulbecco's phosphate-buffered saline (DPBS).
5. Laemmli sample buffer (Bio-Rad).
6. Steamer.
7. Western running and transfer device (Bio-Rad).
8. Immobilon PVDF membrane (Millipore).
9. Washing buffer: TBST (TBS containing 0.1% Tween-20).
10. Blocking buffer: 5% (w/v) nonfat dry milk in TBST.
11. Secondary antibodies: anti-goat and anti-rabbit IgG conjugated to horseradish peroxidase (Southern Biotechnology).
12. SuperSignal West Dura Extended Duration substrate (Thermo Scientific) and BlueLite film (ISC Bioexpress).

3. Methods

3.1. Hypomorphic Allele Production by Cryptic Splicing

The most commonly used method for generating hypomorphic alleles in ES cells utilizes the neomycin phosphotransferase II gene (neo). This gene contains cryptic splice acceptor and donor sites in both its sense and anti-sense orientation, and has been widely used for the generation of knock-out animals by replacing exons of the gene of interest (3). However, simply targeting the neo gene for insertion into an intron of an endogenous mouse gene creates a mutant allele that generates pre-mRNA molecules containing the cryptic exon in both orientations (Fig. 1, see Notes 1 and 2). For hypomorphic alleles, however, the orientation of the neo gene needs to be opposite of the direction of the endogenous gene, otherwise the polyadenylation signal of the neo gene will be used and a functionally null gene will be created (see Note 3). During splicing, the cryptic exon may either be included in the final mRNA product or be spliced out. In mice, it seems that splicing into the cryptic exon occurs preferentially (6). The net result is that protein translated from this transcript will be prematurely truncated, because the neo introduces stop codons in all three reading frames. Mature transcripts that splice out the cryptic exons will result in normal mRNA and, therefore, normal wild-type protein. The overall effect of the neo insertion is a
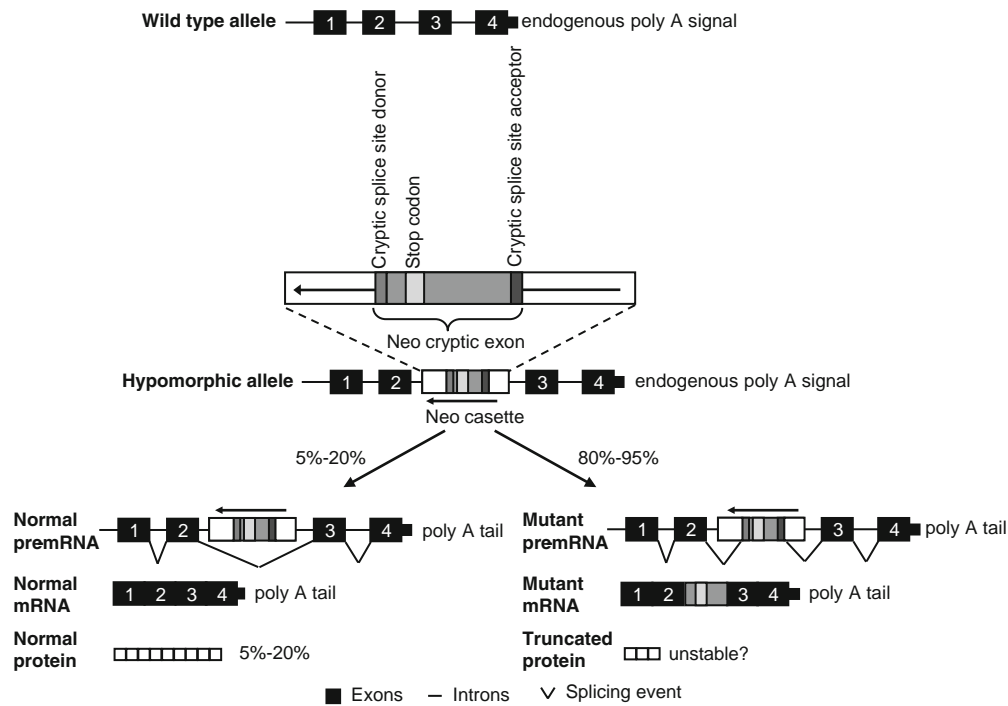
Fig. 1. Mechanism of protein reduction by cryptic splicing using a neo cassette.

substantial protein reduction. This decrease may vary from gene to gene but appears to be consistent between independently targeted ES clones, typically resulting in the production of 5–25% of normal protein (5, 6, 10–13). The variability between these models could result from differences in regulation of protein at the post-translation level. In these scenarios, the small amounts of normal wild-type mRNA can yield significantly higher amounts of steady state protein than proteins that may be turned over more rapidly.

By definition, neo-based hypomorphic mouse models have targeting vectors designed that confer drug resistance to G418 (3). Successful hypomorphic gene targetings have used the pMCI-neo (Stratagene) cassette introduced into early introns of some essential genes (5, 6, 10), although recent strategies using pNTKV1901 have also been successful (Dr. Jan van Deursen, personal communication). By amplifying 5′ and 3′ homology arms by PCR and insertion into the multiple cloning sites (MCS) of pNTKV1901, a usable hypomorphic construct is created (see Notes 4 and 5). It appears as though the neo cassette, as long as it contains the cryptic exon and the stop codons in all three reading frames, is all that is relevant. The promoter driving neo resistance seems to also have no influence on the expression levels of protein in the animal. In order to avoid the generation of pre-mRNA molecules that contain most of the wild-type mRNA sequence, the neo cassette should be inserted in an early intron.

3.1.1. Neo Hypomorphic Targeting Vector Construction

1. The first step is to determine the homology arms to insert 5′ and 3′ of the neo cassette in pNTKV1901. The length of each arm should be between 4 and 5 kb for an overall homology of the targeting construct of at least 8.5 kb. With less homology, the rate of homologous recombination decreases dramatically, whereas it is difficult to clone a total homology of over 10 kb.

2. Primers that include restriction sites at the ends to facilitate cloning into the MCS of pNTKV1901 to amplify these homology arms should then be ordered. Primers can be ordered from a variety of commercial sources (e.g., Invitrogen or IDT Labs). It is advisable to create primers that can be cloned into sticky ends of the vector to enrich for the successful ligation of these fragments. Although it does not matter which homology arm is inserted first, verify that the restriction enzymes used in the second digest will not cut the fragment inserted in the first step. The forward primer used for the 3′ arm should include the endogenous gene sequence immediately after the sequence used in the 5′ arm reverse primer to minimize the amount of non-homologous DNA sequences.

3. PCR amplify the homology arms using high-fidelity polymerases (such as Herculase enhanced DNA polymerase) from either bacterial artificial chromosome (BAC) DNA or ES cell DNA (see Notes 6 and 7).

4. To facilitate cloning, it is necessary to polish the ends of this PCR product to ligate into pGEM-Easy. To polish, add H_2O to the PCR sample until the final volume is 100 µl. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Vortex for 2 min at maximum speed ($20,000 \times g$).

5. Centrifuge for 5 min at room temperature at 14,000 rpm ($20,000 \times g$). Transfer the upper aqueous phase to a new tube and note the volume.

6. To this tube, add 1/8 vol. of 3 M NaOAC (pH 4.8), 5 µl of glycogen (20 mg/ml), and 2.5 vol. of ice-cold 100% ethanol.

7. Mix by inversion and centrifuge for 15 min at 4°C at a speed of 14,000 rpm ($20,000 \times g$).

8. Aspirate the supernatant, add 250 µl of 75% ethanol, and vortex for 1 min. Then centrifuge for 1 min at 14,000 rpm ($20,000 \times g$).

9. Remove all alcohol and add 20 µl of the following mixture:

   (a) 2 µl 10× Taq polymerase buffer.

   (b) 0.8 µl 50 mM $MgCl_2$.

   (c) 0.5 µl dNTPs (25mM per dNTP).

   (d) 0.2 µl Taq polymerase (Invitrogen).

   (e) 16.5 µl H_2O.

10. Incubate for 30 min at 72°C.

11. Add 100 µl of PB buffer from Qiagen PCR purification kit following the manufacturer's protocol. Elute in 30 µl of H_2O and ligate into pGEM-Easy as suggested by the manufacturer.

12. Screen colonies for insertion of homology arm, digest out fragment, and gel purify according to standard procedures (Qiagen gel extraction kit). Ligate these purified fragments into pNTKV1901 digested plasmid which was alkaline phosphatase treated and purified from gel. Transform bacteria and screen for the presence of the properly ligated construct. Repeat these procedures for the second homology arm insertion.

3.2. Premature Polyadenylation-Mediated Hypomorphic Alleles

An alternative approach to generating hypomorphic alleles in ES cells utilizes an expression cassette consisting of the polyoma EPy441 enhancer, the HSV-tk promoter, the coding region of the hygromycin phosphotransferase gene (hygro), and the HSV-tk polyadenylation signal (Fig. 2). When inserted into an intron in the same transcriptional direction as the endogenous gene, the hygro gene HSV-tk polyadenylation signal will cause early transcription termination from the endogenous promoter (7, 14).
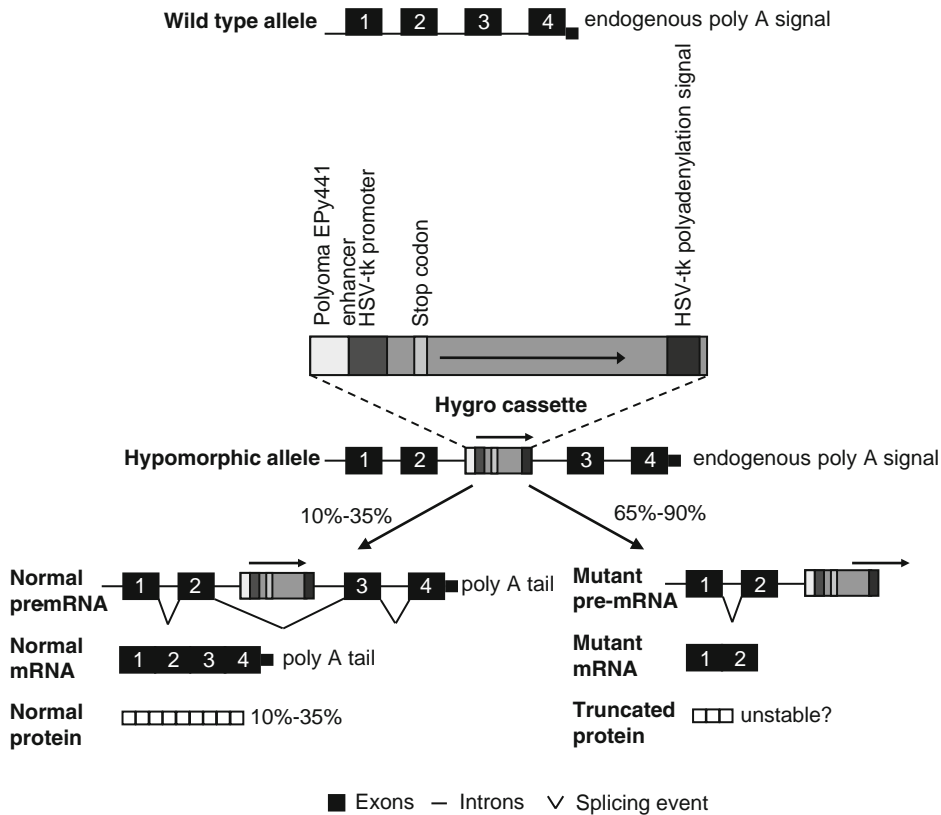
Fig. 2. Premature polyadenylation mechanism for protein reduction using hygro casette.

The resulting truncated pre-mRNA will subsequently generate a truncated mRNA, resulting in a non-functional protein. The termination of transcription due to the HSV-tk polyadenylation signal does not occur in all cases, however, as this region is not robustly regulated. In these cases, transcriptional termination occurs at the endogenous polyadenylation site, generating a full-length pre-mRNA that also harbors the hygro cassette. During processing, the hygro cassette will be spliced out, as it does not contain cryptic splice acceptor or donor sequences. This will yield wild-type mRNA that can then be translated into fully functional protein. The hypomorphic alleles generated by premature polyadenylation produce about 20–35% of normal protein, suggesting that the frequency of polyadenylation at the HSV-tk poly(A) site is higher than that of slippage (7, 15). Other polyadenylation sites could alternatively be used; however, the strength of the polyadenylation site is a key factor to consider. If the signal is too strong, the hygro gene insertion would recapitulate the scenario of a complete knock-out allele, and no wild-type mRNA or protein will be created.

The generation of targeting constructs for hygro hypomorphs is similar to that for neo hypomorphs, just that the hygro cassette is inserted instead of the neo cassette. Therefore, it is quite simple to generate targeting constructs for both hygro and neo hypomorphs in side-by-side cloning procedures to expedite the generation of mutant mice, especially in scenarios where one is unsure what level of reduction of the gene is able to still confer survival. For hygro hypomorphs, simply replace the vector of pNTKV1901 with pHTKV1901 and clone as described in Subheading 3.1.1. Hypomorphic mice utilizing pHTKV1901 appear to produce slightly more protein than pNTK1901 hypomorphs (Dr. Jan van Deursen, personal communication).

3.3. Generation of a Series of Mutant Mice with Progressive Protein Reductions

Using various combinations of wild-type, hypomorphic and knock-out alleles, one can generate a series of mice with a graded reduction in protein levels (Fig. 3). Mice with one wild-type and one hypomorphic allele, either neo or hygro targeted, but collectively termed +/H, produce less protein than wild-type (+/+) mice but more than heterozygous knock-out (+/−) mice. Mice homozygous for the hypomorphic allele (H/H) typically produce less protein than heterozygous null mice, but more than mice with one knock-out and one hypomorphic allele (−/H mice). Homozygous null (−/−) mice are incapable of producing any functional protein (see Note 8). In order to visualize the actual protein reduction across this series, standard Western blotting of mouse embryonic fibroblasts (MEFs) or various mouse tissues is most commonly used and further described below (see Subheading 3.3.1). Alternatively, an indirect approach, such as quantitative real-time PCR (qRT-PCR) using primers specific to wild-type mRNA, can be utilized (16). The basics of this technique are well documented. However, for the use in hypomorphic
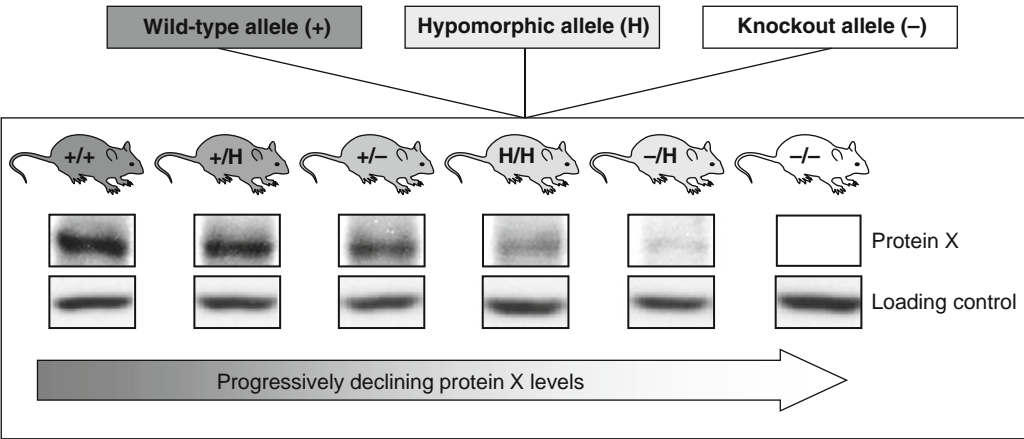
Fig. 3. Overview and representative protein reduction of a series of mutant mice than can be generated from wild-type, hypomorphic, and knock-out alleles. In this example, hygro-based hypomorphic alleles were utilized.

mice, the design of primers used to detect mRNA is crucial and needs further explanation. For the hypomorphic gene-targeting schemes depicted in either Fig. 1 or Fig. 2, one would need to design an exonic forward primer that spans the end of exon 2 and the beginning of exon 3, to allow for the determination of the relative amount of normal mRNA using standard qRT-PCR techniques in any genotype. These values could then be normalized to a housekeeping gene, and the relative expression for the hypomorphic allele can be ascertained (17). The forward primer would not hybridize to mutant mRNA and there would be no contribution in the total expression in this analysis.

3.3.1. Western Blotting for Tissues of Mutant Mice

1. Collect mice at a defined age (i.e., 2 months old) and kill using carbon dioxide inhalation.

2. Snap-freeze the tissue/tissues of interest by placing in labeled 1.7-ml tubes and dropping into liquid nitrogen.

3. Transfer these samples to –80°C for long-term storage. Alternatively, proceed directly to step 4.

4. Chill mortar and pestle on dry ice. Using large forceps, dip the mortar into liquid nitrogen to fill. Return to dry ice.

5. Place the tissue to be prepared for lysate in the liquid nitrogen and immediately begin to pulverize the tissue.

6. Continue to pulverize until all liquid nitrogen has evaporated. Transfer tissue powder to new 1.7-ml microcentrifuge tube that has been cooling on the ice. Weigh immediately and record the weight of tissue added. Do not add more than 50 mg into this tube and place on dry ice. Any remaining powder can be returned to the original tube and stored at –80°C indefinitely.

7. Once all samples to generate lysates have been placed on dry ice, preheat lysate boiler to 100°C. For each sample, first add 10 μl of DPBS per milligram of tissue powder to resuspend, and place on ice. Then add 10 μl of 2× Laemmli to the tube, vortex at maximum speed ($20,000 \times g$) for 10–15 s, and pierce a small hole in the top of the centrifuge tube. Place in lysate boiler at 100°C for 15 min. After boiling, vortex for 10–15 s, centrifuge briefly, and load 10 μl into a well of a precast Bio-Rad Tris–HCl gel. The lysates can be stored at –20°C for future use.

8. Run and transfer proteins as per standard Western blotting techniques.

3.4. Transgenic RNA Interference in Mice

Using conventional transgenic techniques, shRNA-expressing constructs can be introduced into one-cell mouse embryos, resulting in reduced expression of the targeted genes (18). The transgene is transmissible to future generations through the germline. This approach comes with the typical caveat associated with transgenic

mice, which is that certain lines may have significantly higher impairments in protein production due to integration site effects. Therefore, many independently derived lines need to be monitored in order to determine which specific line is of the most interest. However, by making the RNAi-based vector inducible, by using the Tet-On system for instance, the amount of protein in the adult animal can be modulated, allowing one to temporally regulate the amount of protein. Moreover, in this case, the uninduced cohort can be used to control for integration site effects. This approach can, therefore, yield significant insight into how certain proteins behave during the tumorigenic process and may be useful to assess oncogene and nononcogene addiction. Using an inducible shRNA approach then addresses critical questions that hypomorphic mice based on classical gene targeting cannot, because inducible modulation of protein expression in hypomorphs is impossible.

4. Notes

1. Hypomorphic alleles, unlike knock-out alleles, leave the coding sequence of the gene intact. Therefore, targeting is done exclusively in intronic regions. To avoid interfering with splicing endogenous exons, one should choose to target an intron that is at least 500 bp in size.

2. Early introns should be targeted, as insertion in late introns of genes may result in partially active or dominant-negative truncated proteins.

3. The orientation of the neo insertion must be in the reverse orientation to avoid premature polyadenylation, while hygro gene insertions need to be in the same orientation as the gene of interest in order to achieve premature polyadenylation.

4. The use of a multi-purpose cloning vector, such as pNTKV1901-frt/loxp or pHTKV1901-frt/loxp, can create hypomorphic alleles as well as conditional knock-out alleles via a single gene targeting (19, 20). These vectors may prove useful for those interested in creating a series of mice with graded reductions in protein levels without the added cost of additional gene targetings.

5. Do not use the *Not*I site of the targeting vector for cloning, as this site is reserved for linearization of the targeting construct.

6. Recombineering, a technique further described in another chapter of this book, can be used to generate neo and hygro hypomorphic gene-targeting alleles for creating mutant mice.

7. If using PCR-based methods for generation of the hypomorphic targeting alleles, amplification should be performed with high-fidelity DNA polymerases, such as Herculase enhanced DNA polymerase (Stratagene), on genomic DNA extracted from the ES cell line that will be targeted (21) or from BAC clones from the same genetic background as the targeted cells. Many BAC clones of the SV129 genetic background, a standard ES cell line used for targeting, can be acquired from GeneService, UK (http://www.geneservice.co.uk).

8. It is strongly advised to have an antibody for the protein that is to be made hypomorphic that is directed against the N-terminus of the protein. This allows one to determine if stable truncated proteins are being created by the use of standard Western blotting. If no truncated proteins are detected, one can be assured that the phenotypic consequences in the animal are due to insufficiencies of the protein, rather than dominant-negative effects of these abnormal proteins.

Acknowledgments

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Chapter 14

MICER Targeting Vectors for Manipulating the Mouse Genome

Chunhong Liu, Paul F. Szurek, and Y. Eugene Yu

Abstract

The mouse has become an important model for understanding human development, physiology and disease because of its genetic and biological similarity to humans. Desired mouse mutants with precise genetic alterations can now be generated through gene targeting in mouse embryonic stem cells. The rate-limiting factor in a gene-targeting experiment is the time needed for cloning to construct targeting vectors. The establishment of the Mutagenic Insertion and Chromosome Engineering Resource has made available targeting vectors for the insertional mutagenesis of a large number of mouse genes as well as for chromosome engineering throughout the mouse genome. This unique resource has enriched the repertoire of the genetic reagents for targeted manipulation of the mouse genome.

Key words: MICER, Gene targeting, Targeting vectors, Insertional mutagenesis, Chromosome engineering

1. Introduction

As a premier model organism, the mouse has played a critical role in understanding human biology and disease. An unparalleled advantage of using mouse models over other mammals is our ability to manipulate its genome. Using embryonic stem (ES) cell techniques, a wide spectrum of genomic alterations can now be engineered in mice, including single-gene knock-outs, single-base-nucleotide alterations, conditional mutations, and megabase rearrangements. To facilitate these efforts, various genetic reagents have been established and, among them, the Mutagenic Insertion and Chromosome Engineering Resource (MICER) offers its specific advantages for gene- or genomic region-targeted manipulations of this species (http://www.sanger.ac.uk/PostGenomics/mousegenomics) (1).

In this chapter, we will discuss the methodological details germane to the utilization of MICER vectors for manipulating the mouse genome, particularly for generating a mutant allele of a gene or a larger genomic rearrangement in ES cells.

MICER was established by indexing more than 150,000 clones from the *5′HPRT* and *3′HPRT* insertional targeting vector libraries (1, 2). Each clone contains a drug resistance gene as a positive selection marker, 5′ or 3′ portion of an *HPRT* minigene, a *loxP* site, and a coat color marker (Fig. 1). An individual *5′HPRT* MICER targeting vector is established by sequencing the end
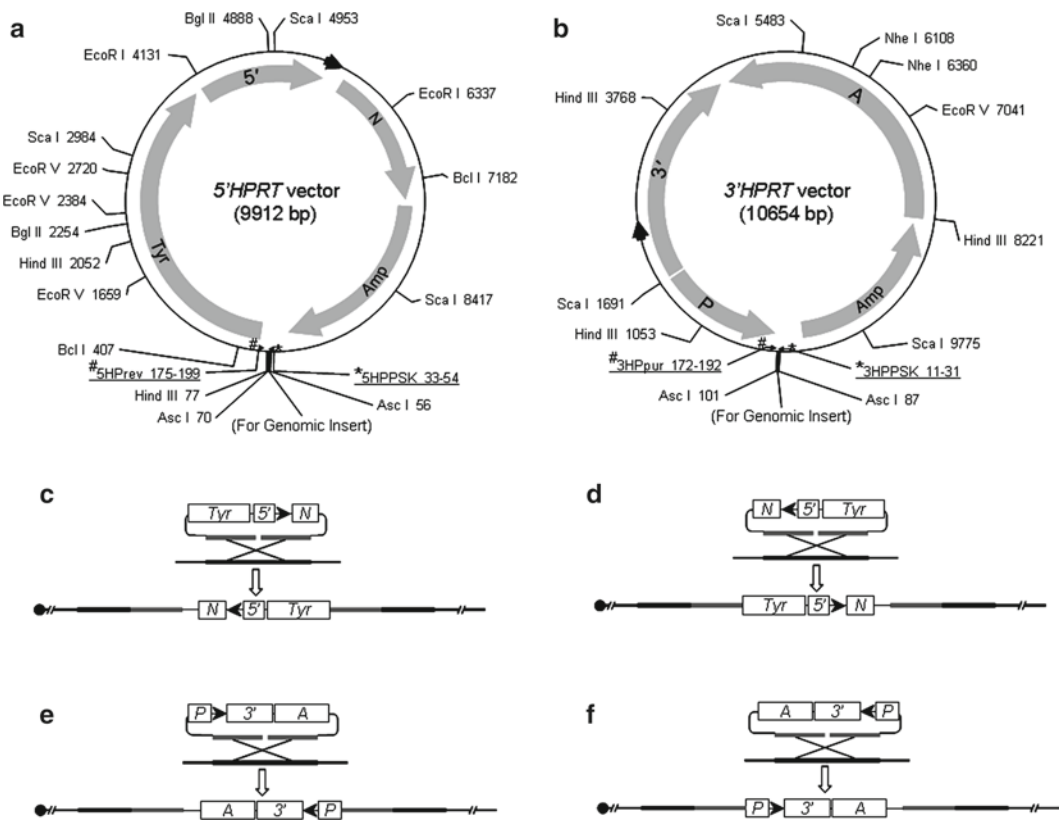
Fig. 1. Plasmid backbones of MICER vectors and gene targeting using MICER vectors in ES cells. (**a** and **b**) The plasmid backbones of a *5′HPRT* and *3′HPRT* MICER vectors, respectively, with selected restriction enzyme sites. (**c**–**f**) Gene targeting in ES cells. MICER vectors can be used to insert *loxP* sites, positive selection markers, the *HPRT* fragments and coat color markers to predetermined loci in the ES cell genome. (**c** and **d**) Presence of the neomycin resistance gene and (**e** and **f**) the puromycin resistance gene enables different targeting events to be identified. The complementary, but nonfunctional, *5′HPRT* and *3′HPRT* fragments are derived from an *HPRT* gene. *Dark and gray bars* represent regions of homology between the MICER vector and the genomic locus. The vector is linearized in the homology region before being electroporated into ES cells. X represents recombination between the vector and the genome. An orientation of the targeted *loxP* site on the chromosome is determined by the orientation of *loxP* in a MICER vector (compare the orientations of *loxP* in **c**, **d** and in **e**, **f**). *Filled triangle loxP* site, *5′* nonfunctional 5′-*HPRT* cassette, *3′* 3′-*HPRT* cassette, *N* neomycin-resistance gene, *P* puromycin-resistance gene, *Tyr* tyrosinase transgene, *A* agouti transgene, *Amp* ampicillin-resistance gene.

sequences of the mouse genomic insert using the sequencing primers located external to the cloning site, 5HPrev (5′-TGAAGAAAGTTGAGGAGAGTTTTC-3′) and 5HPPSK (5′-TTGGCCGATTCATTAATGCAG-3′) (Fig. 1). For a *3′ HPRT* MICER targeting vector, the sequencing primers are 3HPpur (5′-AGACAATAGCAGGCATGCTG-3′) and 3HPPSK (5′-CGTC CCATTCGCCATTCAGG-3′) (Fig. 1). All MICER vectors are displayed in the Ensembl Mouse Genome Browser (http://www. ensembl.org/Mus_musculus/Info/Index). Individual MICER vectors are available from Geneservice (http://www.geneservice. co.uk), and their identities can be confirmed by using the aforementioned sequencing primers.

The data from mouse mutants generated using *5′ HPRT* MICER vectors showed that the tyrosinase transgene (*Tyr*) in a MICER vector leads to a change of eye color from pink to dark ruby and a change of coat color from albino to cream (Fig. 2a). This mutant phenotype exhibits incomplete penetrance and variable expressivity: in some targeted loci, *Tyr* led to a change of eye color but not coat color (Fig. 2a). The presence of two copies of *Tyr* led to a tan coat color, which is apparently caused by a gene
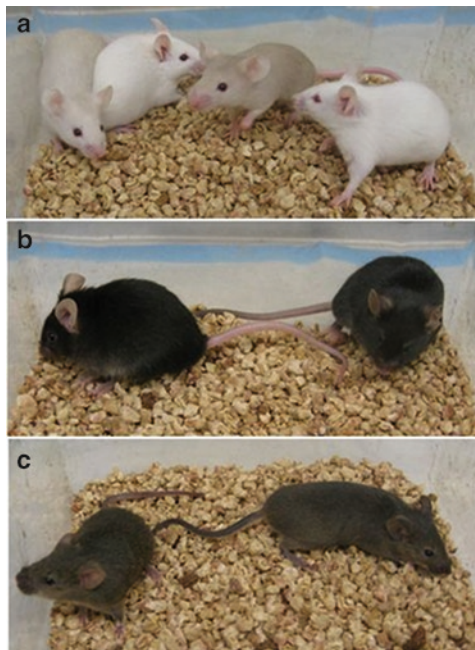
Fig. 2. The phenotype of coat color markers in MICER vectors. (**a**) The tyrosinase transgene in a *3′ HPRT* MICER vector led to a dark ruby eye color (*left*, *second to the left*) and cream coat color (*left*) in the albino coat color background. The presence of two copies of the tyrosinase transgene led to a tan coat color due to the gene dosage effect (*second to the right*). *Right*, a wild-type mouse. (**b** and **c**) Compared with wild-type mice (**b**, *right*; **c**, *right*), mutant mice carrying a 5′ *HPRT* MICER vector exhibited a light tail color in the black (**b**, *left*) or agouti (**c**, *left*) coat color background due to the presence of the agouti transgene.

dosage effect (Fig. 2a). The phenotype of *Tyr* is discernible only if the background coat color is albino. The data from mouse mutants generated using *3′ HPRT* MICER vectors showed that the agouti transgene (*A*) in a MICER vector led to a light tail color in all the loci targeted so far in a black or agouti coat color background, which is most evident around three weeks of age (Fig. 2b, c). However, a gene dosage effect was not apparent for the agouti transgene. During breeding, these coat color markers can be used for tracking the mutant alleles, eliminating the need for PCR- or Southern blot-based genotyping.

2. Materials

2.1. MICER Vectors for Generating a Mutant Allele of a Gene

1. MICER vectors (Geneservice, Source BioScience plc, http://www.geneservice.co.uk).
2. MICER vector DNA can be purified by using Qiagen plasmid purification kits (Qiagen, Valencia, CA).
3. ES cells: W4129S6 cell line (3) (Taconic Farms, Inc., Hudson, NY) or AB2.2 cell line and related feeder cells (SNL76/7 and SNL76/7-4) (4) (Allan Bradley, Wellcome Trust Sanger Institute).
4. Restriction enzymes for digesting MICER vectors (New England BioLabs Inc., Ipswich, MA).
5. For the reagents needed for Southern blot analysis, see ref. 5.

2.2. Chromosome Engineering Using MICER Vectors

The same materials as described in Subheading 2.1 are required with exception that only ES cells carrying an inactivated endogenous *Hprt* locus can be used for chromosome engineering, such as AB2.2 ES cells.

3. Methods

3.1. Mutagenic Insertion of a Gene Using a MICER Vector

MICER vectors are insertional targeting vectors. Based on extensive comparisons of mutants generated using replacement vectors and those generated using the appropriate MICER vectors, the mutant phenotypes were indistinguishable (6–8) (Yu and Bradley, unpublished data). Therefore, appropriate MICER vectors could be used for generating mutant alleles of genes in ES cells. The feasibility of using a specific MICER vector for generating a gene mutation in ES cells will depend upon the selected vector, the strain background of ES cells, the homology region in the vector and the genotyping strategy used to identify the targeted allele.

3.1.1. MICER Vectors for Generating a Mutant Allele of a Gene

Gene targeting using a MICER vector will result in duplication of the homology region in the vector and insertion of the entire plasmid backbone of the vector in the targeted locus in the ES cell genome (Figs. 1, 3 and 4). The consequence of the recombination between a MICER vector and the ES cell genome will depend on the relative position of the homology region of the vector in a gene. If the homology region in a MICER vector contains the first or last exon, targeting will result in the duplication of the 5′ or 3′ end of the gene, respectively. It is possible, under some circumstances, that a MICER vector containing the first exon may result in disruption of the regulatory element of the gene and thereby generate a null allele. However, it is also possible that such a targeting event may not lead to a null allele because the critical region of the regulatory element may remain intact after targeting. The probability of generating a null allele is even lower if the MICER vector contains the last exon of the gene because regulatory elements usually are not located at the 3′ end of a gene. Therefore, to generate a null allele, it is advisable to select a MICE vector in which the homology region contains an encoding exon(s) but is devoid of both the first and last exons of the gene. Using this type of MICER vectors may lead to a frameshift mutation.
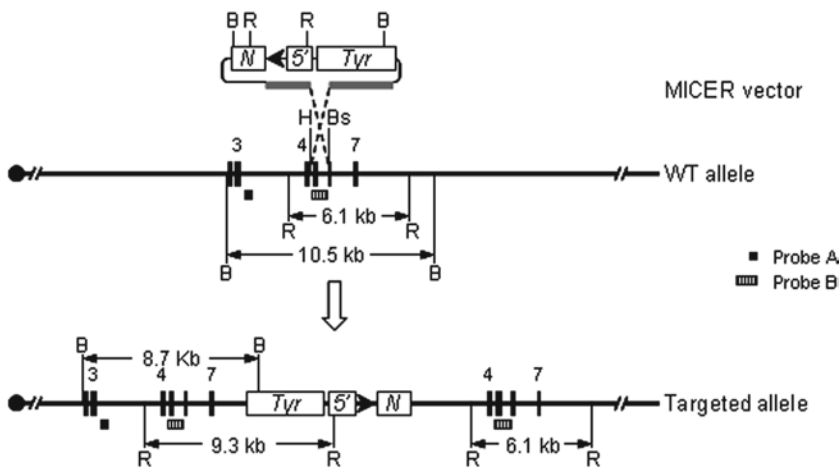
Fig. 3. The strategy to generate a mutant allele of a gene using a MICER vector. The example shown is for the targeting of the *Inpp5k* gene (11). To generate a gap probe (Probe B), the MICER vector was digested with the restriction enzymes HpaI and BstBI. The larger digested fragment was ligated back after filling in the sticky end and to re-generate a unique BstBI site. Before electroporation, the modified MICER vector was linearized by digesting with BstBI. Homologous recombination between the modified MICER vector and the genomic locus led to the repair of the gap (10), the duplication of the genomic homology region in the MICER vector and the insertion of the entire plasmid backbone. The presence of the poly(A) signals for termination of transcription after the *Tyr* gene in the plasmid backbone led to the truncation of the transcript. The probes and diagnostic restriction fragments of Southern blot analysis are indicated. The heterozygous mutant can be identified by digesting ES cell or mouse DNA with *Eco*RI and the gap probe (Probe B). The heterozygous and homozygous mutants can be identified by digesting mouse DNA with BclI and the gap probe or the external probe (Probe A). *B* BclI, *Bs* BstBI, *H* HpaI, *R*, *Eco*RI. *Filled triangle loxP* site, *5′* nonfunctional 5′-*HPRT* cassette, *N* neomycin-resistance gene cassette, *Tyr* tyrosinase transgene.
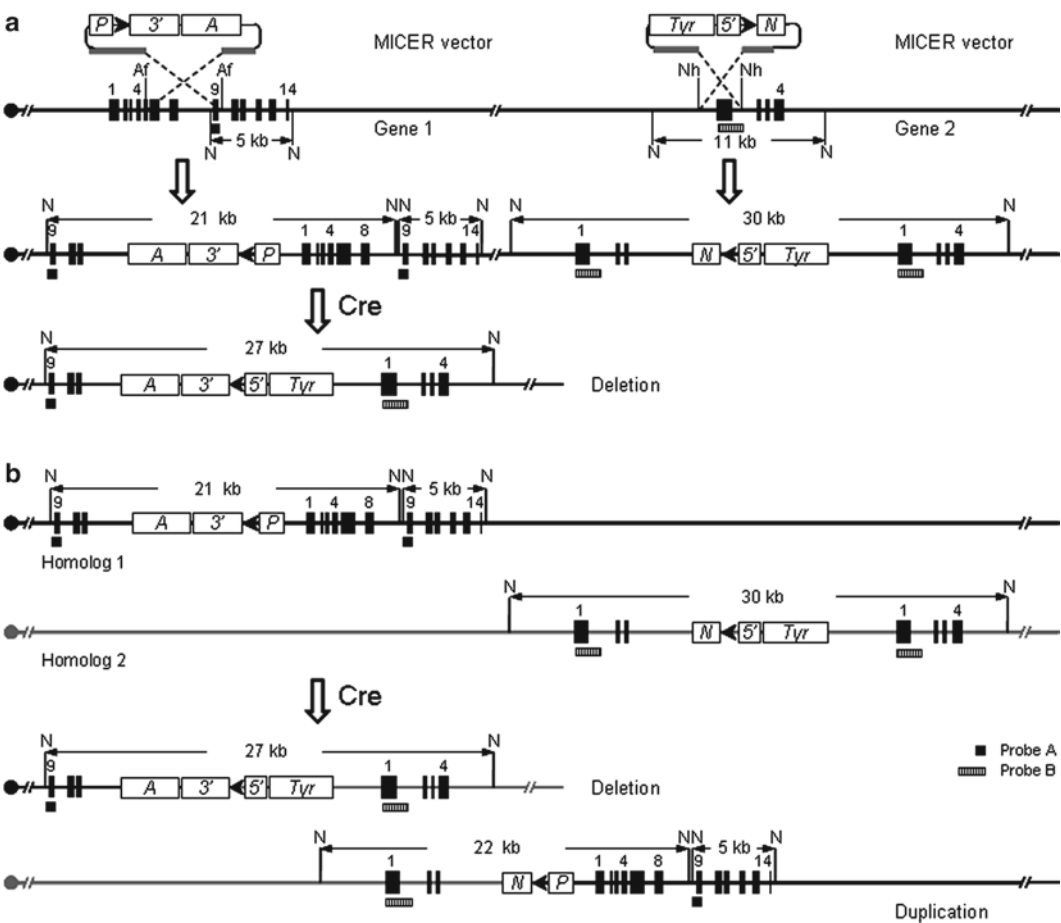
Fig. 4. The strategy to engineer chromosomal rearrangements using MICER vectors. The example shown is for engineering a deletion and a duplication between *Mpo* (Gene 1) and *Chad* (Gene 2) (18) (Yu and Bradley, unpublished data). (**a**) *3′ HPRT* and *5′ HPRT* MICER vectors were used to target the *loxP* sites to *Mpo* and *Chad*, respectively, *in cis*: To generate the gap probes (Probes A and B), the MICER vectors were digested with the restriction enzymes to generate the modified MICER vectors. The digested fragments carrying the plasmid backbones were ligated back. Before electroporation, the modified MICER vectors were linearized by digesting with the appropriate restriction enzymes. The targeted locus was identified by a Southern blot analysis. After the introduction of a *Cre*-expression vector into the double-targeted ES cells, a deletion was generated from the *cis* recombination, which was confirmed by a Southern blot analysis. (**b**) Targeting two *loxP* sites *in trans* using the same targeting vectors in (**a**). After the introduction of a *Cre*-expression vector into the double-targeted ES cells, a deletion and the reciprocal duplication were generated from the *trans* recombination, which were confirmed by a Southern blot analysis. For the strategy to identify ES cell clones carrying double-targeted alleles *in cis* or *in trans*, see ref. 14–16. *Af* AflII, *N* NdeI, *Nh* NheI. *Filled triangle loxP* site, *5′* nonfunctional 5′-*HPRT* cassette, *3′* nonfunctional 3′-*HPRT* cassette, *N* neomycin-resistance gene, *P* puromycin-resistance gene, *Tyr* tyrosinase transgene, *A* agouti transgene.

The presence of the poly(A) signals for termination of transcription in the plasmid backbones may also lead to mutant transcripts caused by abnormal terminations of the transcripts. To shorten a truncated transcript, it is desirable to select a MICER vector in which the genomic homology region is mapped towards the 5′ end of the gene. An example of MICER vector-based targeting

strategy for generating a mutant allele of a gene is presented in Fig. 3.

If a homozygous knockout of a gene in ES cells is desired, two MICER vectors with different plasmid backbones could be used by sequential targeting on both homologs of the same chromosome. The appropriate MICER vectors with different positive selection markers for such a purpose are available for 382 genes (1). Furthermore, since MICER vectors with a single selection marker can be used directly to inactivate 1,803 genes, the complementary vectors used for inactivate the second allele of a gene on the second chromosomal homolog can be easily generated by switching the plasmid backbone of these vectors. This can be achieved by using the AscI restriction enzyme sites that flank the mouse genomic insert (Fig. 1a, b) (1).

3.1.2. ES Cells for Targeting with a MICER Vector

DNA used for the mouse genomic inserts in MICER targeting vectors was isolated from AB2.2 ES cells that were derived from a 129SvEv blastocyst (1, 4). To obtain the targeted clones with a sufficient frequency, it is desirable that the ES cells used in a gene targeting experiment are isogenic to the genomic inserts in a MICER targeting vector (9).

3.1.3. Genomic Homology Region in a MICER Vector

Compared with replacement vectors, MICER vectors as insertional targeting vectors generally have higher targeting frequencies (10). However, the size of the homology regions is still an important factor affecting whether the targeted ES cell clones can be isolated. If there are multiple MICER vectors available for targeted disruption of a specific gene, the size of the mouse genomic inserts in a vector should serve as one of the selection criteria. Four and one-half to nine kilobases should be a desirable size for the homology region in a MICER vector (10). Although successful targeting has been obtained using a MICER vector with less than 4.5 kb of the homology region, shorter homology will undoubtedly reduce the targeting frequency and significantly increase the probability of failing to isolate the targeted ES cell clones after a routine screening.

Before a MICER vector is electroporated into the ES cells, it will need to be linearized using a restriction enzyme. The site of the linearization should occur in the genomic homology region but not in the plasmid vector backbone (Fig. 3). Each side of the homology region separated by the linearization site should be about half the size of the total genomic insert. However, since insertional targeting vectors have a higher targeting efficiency, a size as short as 1.5 kb may also be sufficient for a shorter homology region (10). If no desirable unique restriction enzyme site is available, a restriction enzyme site that occurs more than one time in the homology region may also be used for linearization as long as the final combined homology region left in the linearized targeting vector is larger than 4.5 kb.

In the example of the targeting of *Inpp5k* (Fig. 3) (11), the original total length of the genomic homology region in the MICER vector is 6.5 kb. To generate a gap probe (see below), the MICER vector was digested with HpaI and BstBI to delete a 1.1-kb HpaI-BstBI fragment from the homology region. The modified MICER targeting vector was linearized by BstBI digestion before electroporation to generate the genomic homology regions of 2.2 kb and 3.2 kb.

3.1.4. Genotyping Strategy for Identifying a Targeted Allele

Southern blot analysis is routinely used for screening ES cell clones in gene targeting experiments, including those using MICER vectors. A successful genotyping to identify a targeted clone requires a desirable diagnostic restriction enzyme site and a DNA probe. The selected restriction enzyme sites shown in the plasmid backbone in Fig. 1a, b may be used in a diagnostic restriction enzyme digestion in a Southern blot analysis for genotyping of ES cells and mice since we have found the restriction enzymes for these sites except AscI work well with DNA isolated from ES cells and mouse tissues. The presence of repetitive DNA sequences in a targeted region may sometimes complicate the design of a Southern blot strategy to differentiate between the wild-type allele and the targeted allele. Therefore, before the start of a gene targeting experiment in ES cells, it is necessary to establish a genotyping strategy and a probe(s) for Southern blot analysis. When multiple MICER vectors are available for targeting a specific gene, this effort will facilitate selection of a desirable targeting vector. A probe for Southern blot analysis can be established based on the genomic sequence external to the homology region, such as Probe A in Fig. 3, and generated by a PCR reaction using ES cell genomic DNA or DNA from a BAC clone as a template. One of the selected restriction enzyme sites could be located in the homology region, such as the *Eco*RI sites in Fig. 3. However, it will be difficult to differentiate heterozygous mutant mice from homozygous mutant mice using this strategy. To identify a homozygous mutant, the selection of restriction sites located outside the homology region is preferred, such as BclI in Fig. 3 and NdeI for Gene 2 in Fig. 4. The use of a DNA fragment from the plasmid backbone or the genomic insert as the probe for Southern blot analysis should be avoided since all ES cell clones selected based on a neomycin- or puromycin-resistance marker will yield a positive band(s). However, a probe in the gap area of the homology region in the vector may be considered (Probe B in Fig. 3; Probes A and B in Fig. 4). To do that, a gap region needs to be removed from a MICER vector, which will be repaired in ES cells during the targeting event (10).

3.2. Chromosome Engineering Using MICER Vectors

Cre/*loxP*-mediated chromosomal engineering can be used to generate large genomic rearrangements with predetermined endpoints, such as deletions, duplications, inversions and translocations (12–16).

One of the rate-limiting factors in a chromosome engineering experiment is the time needed to construct targeting vectors. To maximize efficiency, MICER vectors were developed to contain all the genetic elements required for chromosome engineering. These vectors cover the entire mouse genome, and there is at least one MICER vector available for an average of 39 Mb of the mouse genome for chromosomal manipulation (1).

MICER-based chromosome engineering in ES cells includes three elaborate steps: (1) using a MICER vector to target *loxP*, one of the two complementary but non-functional fragments of an *HPRT* gene (*5′ HPRT* or *3′ HPRT*) to the first endpoint in the ES cell genome with a positive selection marker; (2) using another MICER vector to target a second *loxP*, the complementary *HPRT* fragment to the second endpoint with an alternative positive selection marker; (3) introducing Cre to the double-targeted ES cells to induce recombination (Fig. 4). Recombination between the targeted *loxP* sites re-generates a functional *HPRT* gene. Culturing these ES cells in a medium containing hypoxanthine, aminopterin and thymidine (HAT) selects for clones that carry the functional *HPRT* gene and therefore the rearranged chromosomes (13, 15, 16). Because the engineered ES cell clones carrying the desired chromosomal rearrangements are isolated using the *HPRT*-based selection, only parental ES-cell lines with an inactivated endogenous *Hprt* gene, such as AB2.2 line, can be used in these experiments (4, 17).

The gene targeting procedure to insert *loxP* to two endpoints in MICER-based chromosome engineering is similar to that used for generating a mutant allele of a gene. Depending upon the type of chromosomal rearrangement, the relative orientations of the two targeted *loxP* sites need to be defined. For generating a deletion and a duplication, the two targeted *loxP* sites need to be orientated in the same direction in relationship to the centromere or telomere. The correct orientation of *loxP* on a chromosome can be achieved by choosing the desired orientation of the *loxP* in a MICER vector based on the genomic sequence. If the orientation of the *loxP* in a MICER vector is not desirable, it can be corrected by digesting the vector with AscI and inverting the orientation of the plasmid backbone. If the orientation of the genomic sequence at an endpoint is unknown, vectors with two different orientations need to be tested.

The genotyping strategy of the targeting event is also similar to that used for generating a mutant allele of a gene (Figs. 3 and 4). However, an additional genotyping strategy is required to identify ES cell clones carrying the chromosomal rearrangement. Since a Cre/*loxP*-induced chromosomal rearrangement will alter the affected genomic regions including the associated restriction enzyme sites, sometimes the restriction enzyme sites used for genotyping the earlier targeting events may be different from

those used for genotyping the chromosomal rearrangement. To verify the presence of the desired chromosomal rearrangement, fluorescence in situ hybridization (FISH) using BAC probes and array-based comparative genomic hybridization (aCGH) are often required (18, 19). However, Southern blot analysis is preferred for the first screening to identify the ES cell clones that carry the desired chromosomal rearrangement because it can analyze a large number of ES cell clones in a single experiment.

An example illustrating the Cre/loxP-mediated recommendation strategy for generating and analyzing a chromosomal deletion and the reciprocal duplication is shown in Fig. 4. Two loxP sites were sequentially inserted into the two endpoints located at the Mpo and Chad genes on mouse chromosome 11 and oriented in the same direction in relationship to the centromere. The 3′HPRT MICER vector for targeting the first loxP site to Mpo was digested with AflII to delete 4.0-kb from the genomic homology region where the gap probe (Probe A) was isolated. Before electroporation, the modified MICER targeting vector was linearized by digesting with AflII. The total genomic homology region in the modified MICER targeting vector is 6.0 kb with 3.5- and 2.5-kb homology regions after AflII digestion. The targeted locus was identified by digesting ES cell DNA with NdeI and Probe A in a Southern blot analysis. The 5′HPRT MICER vector for targeting the second loxP site to Chad was digested with NheI to delete a 2.8-kb fragment from the genomic homology region. Before electroporation, the modified MICER targeting vector was linearized by digesting with NheI. The total genomic homology region in the modified MICER targeting vector is 4.5 kb with 2.7- and 1.8-kb homology regions after NheI digestion. The targeted locus was identified by digesting ES cell DNA with NdeI and the gap probe (Probe B) in a Southern blot analysis. Afterwards, the Cre-expression vector was electroporated into the double-targeted ES cells to induce the desired rearrangements through the cis (Fig. 4a) or trans recombination (Fig. 4b), which were confirmed by digesting the ES cells with NdeI and Probe A or B in a Southern blot analysis. A similar strategy can also be used to generate and analyze an inversion in ES cells. The main difference in engineering an inversion is that the two-targeted loxP sites need to be orientated in the opposite directions (13, 15, 16).

If a gene is larger than 20 kb, using a replacement vector to generate a deletion of the entire gene will not be very efficient. Therefore, chromosome engineering could be a useful alternative to deleting a large gene. In a mammalian genome, including the mouse genome, there are many gene clusters in which a number of genes in a family are located adjacent to each other. Since genes in a cluster may have a redundant function, generating and analyzing a mutant carrying a null allele of a single gene may not be feasible for the genetic analysis of these genes in a cluster.

To understand the function of the entire gene cluster, it is desirable to delete the entire region using chromosome engineering (20).

There is a lot of interest in using MICER vectors for generating conditional alleles. However, such an application may only be feasible under special circumstances. Since targeting using MICER vectors causes duplication of the homology region and insertion of the plasmid backbone, a conditional allele could be engineered only if the targeting of MICER vectors would not affect normal transcription of the floxed gene as well as the neighboring gene(s). To establish a desirable conditional allele, two targeted MICER vectors need to be located on the same homolog of a chromosome. ES cell clones carrying the desired targeted alleles on the same chromosomal homolog could be identified by FISH analysis using MICER vectors as the probes (Yu et al., unpublished data).

Acknowledgments

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Chapter 15

Knock-In Approaches

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Abstract

Molecular genetic strategies to study gene function in mice or to generate a mouse model for a human disease are continuously under development. The application and importance of knock-in approaches are increasing. This chapter elaborates on novel developments for the generation of knock-in mice. Special emphasis is given to recombinase-mediated cassette exchange, a new emerging knock-in strategy that enables easy generation of a series of different knock-in mutations within one gene.

Key words: Knock-in, Mutation, Site-specific recombinase, LoxP, Cre, FRT, Flp, att, ΦC31, RMCE, FLEX switch

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1. Introduction

Molecular genetic strategies to study gene function in mice or to generate a mouse model for a human disease are until today mostly based upon either conventional transgenesis via pronuclear injection of fertilized oocytes or (conditional) targeted gene ablation via knock-out ES cell approaches, as discussed in detail in the preceding chapters. The first method is predominantly used to add genetic information in order to achieve overexpression of wild-type (or mutant) protein in particular tissues, and the latter predominantly to (conditionally) inactivate genetic information. However, conventional transgenesis with antisense or siRNA encoding constructs is currently also used to ablate expression of genes, whereas random or precisely targeted introduction of additional gene constructs in ES cells can be applied to overexpress any gene of interest. Furthermore, precisely targeted manipulation of ES cell can also be used to introduce tailor-made modifications of a particular gene, allowing more subtle analysis of the function

of the gene. By such a knock-in approach, the protein-coding region of a gene can be replaced, e.g., by sequences encoding a reporter protein or a homologous protein of a different species. Also, smaller modifications such as introduction of small tags and point mutations can be introduced into the protein-coding region of a gene.

This chapter provides a brief overview of applications and examples of different knock-in approaches. Successful application of a knock-in approach is likely to unravel new insights into the function of a particular gene, which might lead to novel scientific questions. To answer these questions, it is often necessary to introduce additional modifications in the same gene. A knock-in strategy using recombinase-mediated cassette exchange (RMCE) might then be a suitable, time-saving alternative for repeated application of conventional knock-in strategies. Several different RMCE systems, which were developed during the last years, will be considered. For application of one of these RMCE methods, the method based upon Flp recombinase in combination with heterotypic FRT sequences, a detailed protocol is described in Chapter 16.

2. Knock-In Technologies and Strategies

2.1. Simple Knock-Out/Reporter Knock-In

The procedure to generate knock-in mice via homologous recombination (HR) in ES cells is in principle identical to the procedure to generate standard knock-out mice. Thus all the general guidelines, protocols to target ES cells, etc., to generate knockout mice, which are discussed elsewhere in this volume, apply also for knock-in mice. Even the basic design of a linear construct to target the ES cell is quite similar. Such a construct should contain somewhat centrally in the construct a selectable marker gene, enabling positive selection, whereas both ends should be made up of flanking sequences homologous to the gene to be targeted by homologous recombination. Presence of an additional selection marker at either end of the targeting construct for negative selection against random integration is not considered here, but could be included. In contrast to a standard knock-out targeting construct, the knock-in construct contains additional sequences that have to be knocked in immediately upstream of the selection marker gene. Among the first knock-ins generated are reporter knock-in mice in which an incomplete exogenous reporter gene is inserted into a gene in such a way that it acquires the transcriptional regulation of the targeted gene. The incomplete reporter gene itself lacks promoter and enhancer control elements, thus expression of the reporter relies on control elements provided by the targeted allele. As depicted in Fig. 1, such a reporter knock-in
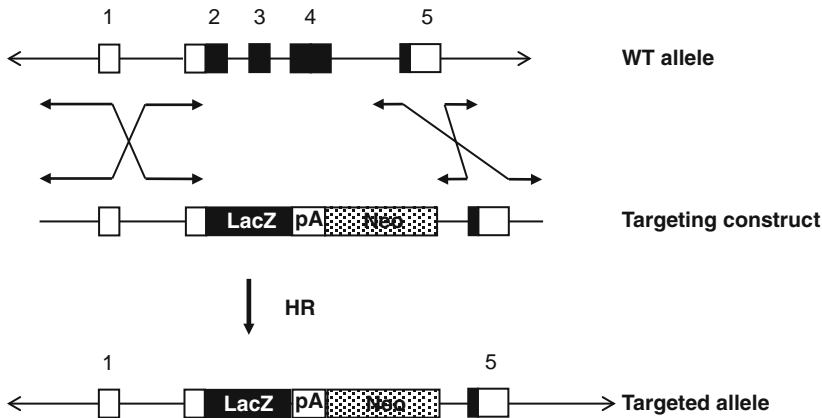
Fig. 1. Simple knock-out/reporter knock-in. Schematic representation of a strategy to target a gene, existing out of five exons, with a knock-out/reporter knock-in construct. At the 5′-side, the flanking sequence for HR is fused at the initiation codon in exon 2 to a LacZ-coding region followed in 3′-direction by a sequence containing a polyadenylation signal (pA) to terminate transcription, a complete selection marker gene (Neo) including its own promoter and polyadenylation signal (*not indicated*), and finally the 3′-side flanking sequences for HR, which start somewhere in the intron preceding exon 5 of the gene. After HR within the flanking sequences (*indicated by crossed double arrows*), expression of LacZ acquires the transcriptional regulation of the targeted gene. Furthermore, the expression of the original protein is concomitantly knocked out due to the removal of the protein-coding sequences. The remaining exon 5 is no longer part of a transcript. For the wild-type (WT) allele, open boxes represent 5′-end and 3′-end untranslated sequences in exons, whereas the black boxes represent translated sequences in exons.

construct could be designed to replace protein-coding sequences with the open reading frame for β-galactosidase (LacZ), a polyadenylation signal (pA), and a complete Neo gene with its own promoter and polyadenylation signal. Since the exogenous reporter gene carries its own polyadenylation signal, the more downstream located polyadenylation signal of the endogenous gene has lost its function. Furthermore, the presence of a selection marker gene downstream of the functional polyadenylation signal in the reporter gene is believed to not interfere with the expression of the knocked in reporter gene. However, even more precisely tailor-made knock-out/reporter knock-in targeting constructs, which make use of the genuine endogenous polyadenylation signal and allow removal of the selection marker gene, could be designed as explained in the next paragraph discussing sophisticated functional knock-ins. Anyhow, because the endogenous protein-coding sequences are lost due to their replacement by reporter gene sequences, the resulting targeted allele is at the same time a genuine knock-out allele. Except in case of haplotype insufficiency, which could compromise normal development and expression, heterozygous mice are ideal research tools to study the expression of the gene during development and adult life via monitoring the expression of β-galactosidase using biochemical reactions or antibodies. Homozygotes can be studied as a standard germline knock-out, in which anomalies occurring via the

read-out of the reporter gene can be more easily studied by comparison with phenotypically normal heterozygotes and wild types. Examples of such knock-out/reporter knock-in approaches can be found in studies by Allen et al. (1) and Makita et al. (2) in which *Sstr2*, encoding the somatostatin receptor 2, and *Taz*, encoding a transcription factor, were inactivated, respectively.

2.2. Sophisticated Functional Knock-Ins

2.2.1. Application and Design of Sophisticated Functional Knock-Ins

In principle, the knock-out/reporter knock-in, as described in the previous paragraph, can be considered as an initial but relatively simple kind of a functional knock-in. This strategy allows monitoring of the expression of the targeted gene, while being a knock-out at the same time. However, the ongoing development to manipulate genes in a tailor-made fashion led to the development of many different and more sophisticated functional knock-in strategies. Such an approach often, but not necessarily, ablates also the expression of the wild-type version of the original endogenous gene by complete or partial replacement of the protein-coding sequence. It should be noted that a modification by introduction of a stop codon or a partial deletion, resulting merely only in a truncation of the protein or removal of one or more functional protein domains, is also considered here as functional knock-in. In comparison to conventional transgenesis using pronuclear injection to insert a modified version of a gene randomly in the genome, the targeted functional knock-in has several advantages: (1) the analysis is often greatly simplified, because a wild-type deficient background is created simultaneously; (2) the spatial and temporal patterns of expression are likely identical or close to that of the replaced wild-type endogenous gene; and (3) there is a reduced risk for undesired insertional mutagenesis, a problem linked to random integration.

The key element in the design of a successful knock-in strategy is positioning the expression of the modified endogenous gene under transcriptional control of all known *cis*-acting elements of the endogenous gene, even if the protein-coding region is replaced completely by an exogenous protein-coding sequence. Thus, knock-in targeting vectors should be constructed in such a way that eventually the knock-in genes acquire the transcription patterns of the endogenous gene. Two of such targeting knock-in vectors are shown in Fig. 2a, b. The first vector is designed to introduce a point mutation into the protein-coding region of a particular exon, whereas the second one is intended to fuse a green fluorescent protein (GFP) reporter sequence to the carboxy terminus of the endogenous protein.

Of course, the targeting constructs should contain centrally a selectable marker gene for positive selection. Since the presence of such a marker gene within a gene could interfere with the expression of the modified gene, the selection marker gene is positioned between two LoxP or FRT recombination target
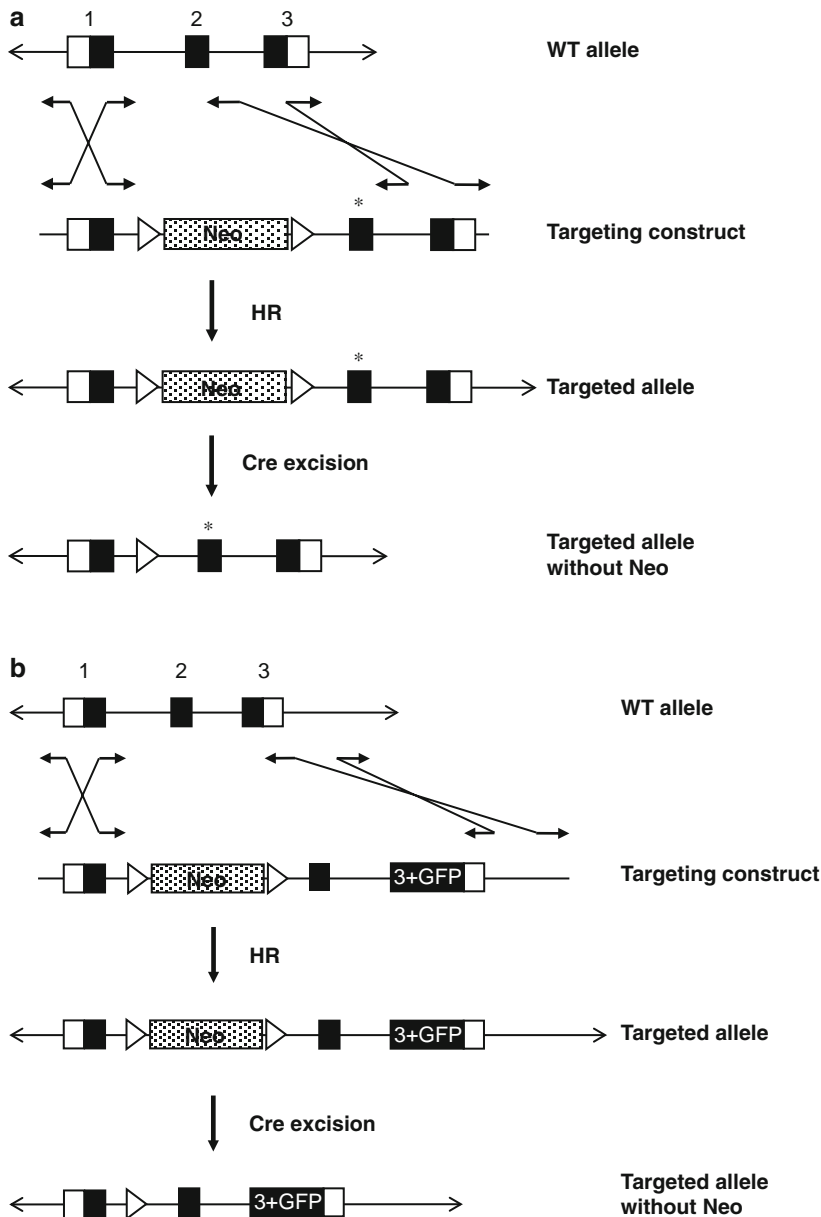
Fig. 2. Sophisticated functional knock-ins. (**a**) Schematic representation of a strategy to target a gene, existing out of three exons, with a modified exon 2 (e.g., insertion, deletion, or modification of an amino acid residue or a protein domain indicated by *asterisk*) and a Neo gene flanked by LoxP or FRT sequences (*indicated by open triangles*) present within intron 1 sequences. After HR in the flanking sequences (*indicated by crossed double arrows*), the targeted gene contains both the Neo gene and the modified exon 2. After removal of the Neo gene by Cre or Flp in vitro in ES cells or in vivo in the mouse, the resulting targeted gene encodes the desired modification, whereas only a small LoxP or FRT sequence is left behind upon excision of the selection marker gene, which otherwise could interfere with the expression of the targeted gene. (**b**) Schematic representation of a strategy to target the same gene as in (**a**) with a modified exon 3, in which coding sequences for the GFP reporter protein are fused in frame to the 3′-end of the protein-coding sequence of the wild-type gene, together with a Neo gene flanked by LoxP or FRT sequences present within intron 1 sequences. After removal of the Neo gene, the targeted gene encodes a GFP fusion protein.

sequences in order to remove it either already in vitro in the ES cells themselves, or later on in vivo by the activity of Cre or FLP recombinases. Excision of the marker gene leaves behind only a small insertion of a single LoxP or FRT sequence, usually in an intron at a position where it is expected not to interfere with the expression of the targeted gene – please note the similarity of this aspect with conditional knock-outs. However, this excisable selection marker gene could also be positioned into 5′- or 3′-untranslated region sequences, or upstream or downstream of the gene, depending on the size of the gene, the position of the modification in the gene, etc.

Depending on the ultimate goal of the knock-in approach, many different targeting knock-in vectors can be designed in order to (1) insert a stop codon to truncate the encoded protein; (2) insert, change, or delete amino acid residues or protein domains within coding sequences of an exon; (3) insert or delete exons, resulting in an *in frame* insertion or deletion of protein domains; (4) replace the complete endogenous protein-coding sequences by the coding sequences of an exogenous gene; (5) insert a cDNA encoding an additional open reading frame into the 3′-untranslated region preceded by an internal ribosome entry site (IRES), resulting in concomitant expression of an additional gene (e.g. ,Cre) and the endogenous protein of the targeted gene, etc. It should be noted that such knock-in sequences in the targeting construct can consist of genomic or cDNA sequences, or a combination of both. Precise fusion of reading frames, introduction of correctly positioned splice donor and/or acceptor sites, introduction of an IRES sequence, etc., in the targeting construct can be presently easily achieved tailor-made using a combination of sequential cloning and several specific PCR strategies to build the targeting construct.

The potential knock-in strategies described thus far are all aimed at modifying the protein-coding sequences of the targeted gene. However, using a similar approach, *cis*-acting elements that control transcription and translation of the modified gene, which still expresses the wild-type protein, can be changed deliberately in order to study their relevance and function or just to modify expression of the targeted gene in a desired fashion. Enhancers, promoters, and elements controlling translation and stability of the transcribed mRNA could be inserted, modified, or even deleted. Of course, such modifications, even the ones with deletions, can also be considered as knock-ins.

2.2.2. Examples of Sophisticated Functional Knock-Ins

As explained above, the knock-in technologies to generate functional knock-ins can serve many different objectives. A limited number of examples of successfully generated knock-ins are given below.

Knocking in a point mutation in the endogenous glucocorticoid receptor, which ablates its ability to bind DNA and to activate transcription but does not interfere with its transcriptional repression activity, facilitated the understanding of the mechanism of steroid action in multiple different pathways through DNA-binding-dependent and -independent mechanisms (3). Presence of a proline motif in the co-stimulatory receptor CD28 was shown to be essential for the regulation of T-cell function (4).

Knocking in the cell cycle gene *Cdk2* into the locus of its paralog *Cdk1* resulted in embryonic lethality and loss of meiotic function of Cdk2, revealing divergence of functions (5). Knock-in in the human receptor CXCR2 in the locus of its mouse ortholog revealed that the human gene can functionally replace the mouse gene. In addition, this knock-in mouse is an attractive model to test novel pharmaceuticals designed to antagonize human CXCR2 in vivo (6).

Functional knock-in 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 its mouse ortholog. These sorts of models can be used to better understand the molecular mechanisms that cause the pathophysiological states, and also to test different therapies that might ameliorate the severity of disease. Recent examples of such experiments include the creation of a knock-in of multiple CAG repeats within the huntingtin gene, which recapitulates juvenile Huntington's disease (7). Recently, research in this model revealed that the drug ampakine reduced the severity of the disease by up-regulating BDNF (8). Similarly, a knock-in approach in the *Ott* gene was used to create a OTT-MAL fusion oncogene, which mimicks a t(1, 22) (p13;q13) translocation specifically associated with a subtype of pediatric acute megakaryoblastic leukemia (AMLK) in humans. The fusion oncogene induced AMLK in the knock-in mice (9).

The significance of differential splicing of the lipoprotein receptor *Apoer2* gene for modulation of synaptic plasticity and memory by Reelin was revealed by a knock-in approach which resulted in the expression only of a transcript lacking the alternatively spliced exon 19 encoding a part of the intracellular domain of this receptor (10).

Zhou and colleagues designed a knock-in strategy to introduce a tandem affinity purification (TAP) tag into any gene of the mouse in order to enable large-scale tagging of mouse genes and systematic identification of protein complexes during development (11).

Also, Cre recombinase protein-coding sequences are often knocked in into a specific gene in order to generate a Cre mouse having Cre recombinase expression, according to the expression pattern of the targeted gene. A very special approach was followed by Bäckman and colleagues who introduced Cre recombinase

protein-coding sequences preceded by an IRES in the 3′-end UTR of the dopamine transporter *Dat* gene. This knock-in gene synchronizes expression of Cre recombinase with expression of the dopamine transporter in dopaminergic neurons without effecting the expression level of the transporter itself (12).

2.3. Series of Knock-In Alleles

The development until today of several different sophisticated knock-in strategies to study diverse aspects of gene function might invite researchers to introduce several modifications as series of independent single modifications and combinations thereof into a gene of the mouse. Such extended functional knock-in approach requires repetition of the laborious, time-consuming, and costly procedure of targeting a construct in ES cells and indentifying clones resulting from proper homologous recombination. In case multiple different modifications need to be knocked in, application of a RMCE method might be a good time- and money-saving alternative to generate such a series of different knock-in alleles. In case of RMCE, a first cassette is initially introduced into the gene of interest by homologous recombination. Subsequently, this parental ES cell line can be used repeatedly to introduce alternative cassettes with different modifications into the gene very efficiently. Several existing systems for such an efficient exchange of cassettes by different site-specific recombinases in ES cells will be discussed.

2.3.1. Site-Specific Recombinases

The bacteriophage P1-derived Cre in combination with its specific recombination target sequence LoxP (34 bp) and the *Saccharomyces cerevisiae*-derived Flp in combination with its specific recombination target sequence FRT (48 bp) are commonly used for the removal of a positive selection marker gene flanked by LoxP or FRT sequences from a targeted allele and/or for conditional inactivation of a targeted allele, as explained in Fig. 2 and elsewhere in this volume. The recombination activity of these site-specific recombinases (reviewed by (13–15)) in the mouse genome is bidirectional (insertion/excision) and is schematically depicted in Fig. 3a. However, thermodynamically, the excision reaction is preferred. Furthermore, the non-replicating extrachromosomal circular excised DNA will be diluted upon consecutive cell divisions. Thus the equilibrium will finally be driven toward the excised situation with only a single recognition sequence remaining in the genome. Therefore, despite the bidirectional activity of Cre and Flp, single LoxP and FRT sequences targeted into the genome of the mouse are not really ideal for targeting the integration of circular plasmid DNA carrying sequences to be knocked in. A third recombinase system that could be used to target integration is the bacteriophage ΦC31-derived integrase, which recombines the heterotypic recombination target sequences attB (34 bp) and attP (39 bp) (reviewed by (13–15)). As depicted in Fig. 3b, recombination between attB and
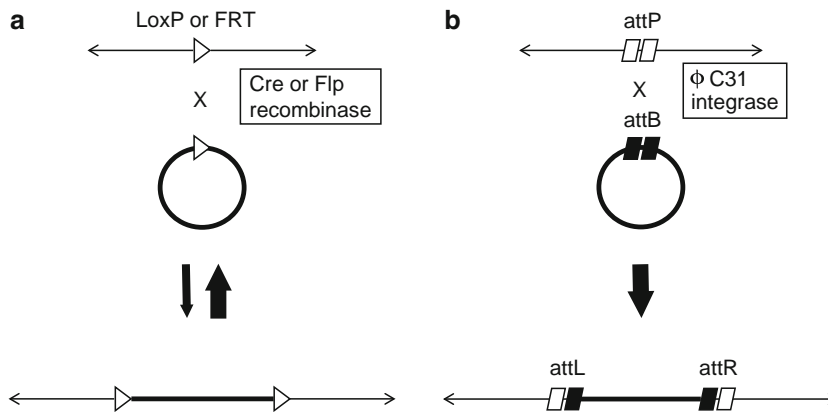
Fig. 3. Site-specific recombinases. (**a**) The Cre/LoxP and Flp/FRT recombination systems. Recombination between two identical recombination target sequences (LoxP or FRT) (*open triangle*) results in a bidirectional insertion/excision of a circular DNA molecule in/from a genomic sequence. Thermodynamically, the excision reaction is preferred. (**b**) The ΦC31 integrase system. Recombination between heterotypic attP (*open double parallelograms*) and attB (*black double parallelograms*) recombination target sequences results in an unidirectional insertion of a circular DNA molecule in the genomic sequence, since the resulting chimeric attL and attR sequences are not substrates for the integrase.

attP results in insertion of the circular DNA molecule in the genome flanked by two hybrid attL and attR sequences. These hybrid attL and attR sequences are not substrates for the integrase. Therefore, the recombination by ΦC31 is unidirectional and could easily be used to target integration of circular plasmid DNA. A disadvantage of use of the ΦC31 recombinase system, however, is the presence of multiple pseudo-attP sequences with partial sequence identity to attP sequences in the human and mouse genomes, which appear to be capable of recombining with incoming attB-carrying plasmid sequences (reviewed by (13, 15)). Finally, it should be noted that integration of circular plasmid DNA by ΦC31, and also by the Cre/LoxP or Flp/FRT systems, results anyhow in a usually non-preferred co-integration of prokaryotic sequences present in a plasmid.

2.3.2. Recombinase-Mediated Cassette Exchange

Elegant systems to use the above-discussed recombinases to knock-in sequences at preferred targeted loci, avoiding undesired co-integration of prokaryotic plasmid backbone sequences, emerged when methods for RMCE were designed (reviewed by (13–15)). These RMCE methods are based on the replacement of cassettes flanked by two non-interacting recombination target sites. For a system based on ΦC31, two naturally occurring heterotypic recombination target sequences attB and attP – attB can only recombine with attP and not with attB itself, and the same applies for attP – were already available. For Cre and Flp, several mutant recombination target sequences have been developed by introducing nucleotide substitutions in one of the inverted repeats (13 bp) or the spacer sequence (8 bp) in between, which make up

these recombination target sequences. It should be noted that unlike the 34-bp sequence of LoxP, the 48-bp sequence of FRT contains two copies of a 13-bp (inverted) repeat, intervened by an extra nucleotide at one side (16–18). Because these mutant heterotypic LoxP and FRT recombination target sequences can only recombine with an identical target sequence, recombination between two different heterotypic recombination target sequences does not occur. The principle of RMCE using heterotypic LoxP or FRT sequences is depicted in Fig. 4a. A double recombination between identical heterotypic recombination target sequences results in exchange of cassettes, one present in the genome and the other on a circular plasmid. Alternatively, a combination of single LoxP and FRT sequences can be used. It should be noted that the exchange is bidirectional and is expected not to have a preferred direction, unless maybe in case large differences in sizes
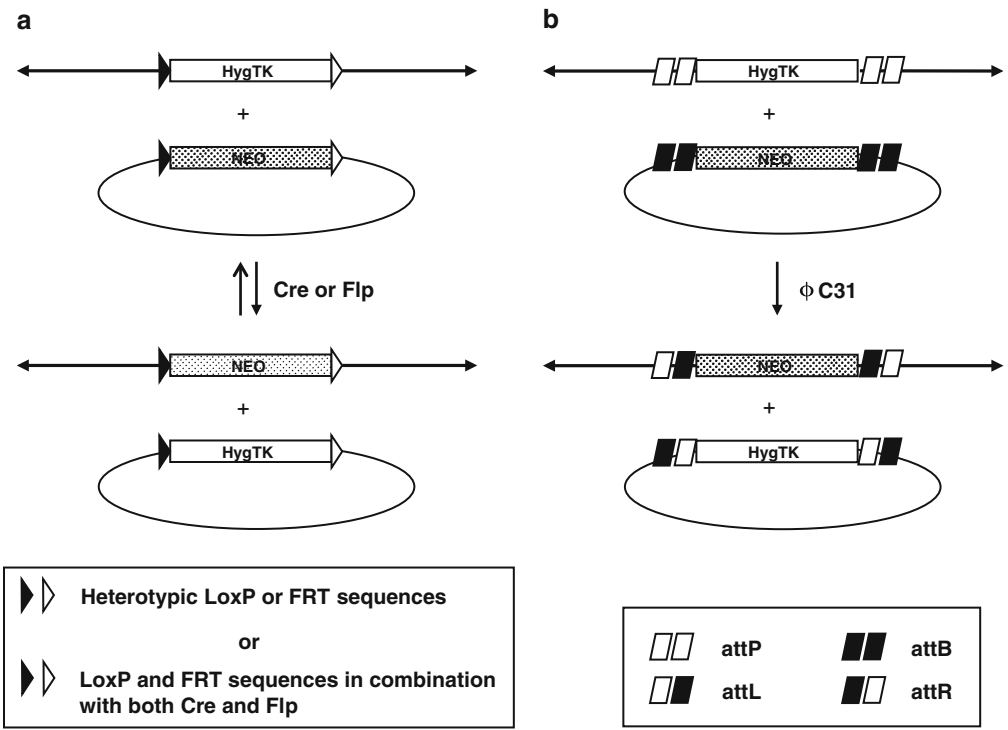
Fig. 4. Principle of recombinase-mediated cassette exchange (RMCE). (**a**) By the activity of transiently expressed Cre or Flp recombinases, a HygTK gene flanked by heterotypic LoxP or FRT sequences present in a genomic sequence is exchanged in a double recombination event for a Neo gene flanked by the same heterotypic recombination target sequences present in a circular plasmid. The exchange is bidirectional. By application of the appropriate negative (ganciclovir against the presence of HygTK) and/or positive (G418 for the presence of Neo) selection, only cells resulting from the intended exchange will survive. Alternatively, single LoxP and FRT sequences can be used together, instead of two heterotypic sequences of either one of the systems. (**b**) By the activity of transiently expressed ΦC31 integrase, a HygTK gene flanked by attP sequences present in a genomic sequence is exchanged in a double recombination event for a Neo gene flanked by attB sequences present in a circular plasmid. The exchange is unidirectional. Selection is to be used to eliminate cells that did not recombine.

of the cassettes are involved. However, because such an exchange is achieved in vitro in, e.g., ES cells by transient expression of the recombinase, selection can be applied to obtain the desired outcome in a dividing cell culture, which results in dilution and final disappearance of the circular DNA molecules together with the transiently expressed recombinase. Figure 4b shows RMCE using ΦC31 and attB/attP sequences. In contrast to RMCE using the Cre or Flp system, RMCE using ΦC31 and attB/attP is unidirectional, which is advantageous.

2.3.3. Potential Applications of RMCE for Generation of Knock-Ins

In the examples used here to illustrate some potential applications of RMCE (Fig. 5), the use of Flp and heterotypic FRT sequences is assumed; however, other systems could be used likewise. The starting point for the application of RMCE for the generation of knock-in mice is the introduction of an exchangeable cassette in ES cells by homologous recombination, replacing parts of the gene to be targeted. The exchangeable cassette contains a HygTK selection marker gene allowing positive selection by hygromycin B and negative selection by ganciclovir. After targeting of the cassette flanked by heterotypic FRT sequences, such a parental ES cell line could subsequently be used repeatedly to generate different knock-ins in the same gene by an exchange of the HygTK cassette for different sequences. Preferably, the 5′-end of the HygTK cassette flanked with the heterotypic FRT sequences is introduced in an intron, whereas its 3′-end should be positioned in a more downstream intron or even downstream of the last exon, replacing an internal or a 3′-end part of the gene, respectively. The FRT sites remain present in the locus after the exchange and should not be present in the protein-coding region. Furthermore, the exchange plasmid to be used for the actual RMCE in the next step must contain the appropriate knock-in sequences and preferably an excisable selection maker gene flanked by LoxP sequences. To achieve the exchange, the parental ES cell line is electroporated with a mixture of a plasmid for transient expression of Flp and the exchange plasmid. It should be noted that in case only negative selection with ganciclovir against the HygTK cassette would be applied, the selection marker gene could be omitted from the exchange plasmid. A theoretical example for the application of RMCE is depicted in Fig. 5 showing a gene with five exons. Exon 2 (encoding the initiation codon) to the last exon (encoding the stop codon and polyadenylation site) are replaced via homologous recombination by the HygTK cassette flanked by the heterotypic FRT sequences (Fig. 5a). Through subsequent RMCE, this cassette can be replaced by knock-in sequences encoding a modification (Fig. 5b). Alternatively, sequences encoding a specific splice variant ablating a particular exon, sequences encoding a GFP fusion protein, sequences encoding a completely different protein-coding

a **Generation of parental ES cell line with targeted HygTK cassette**
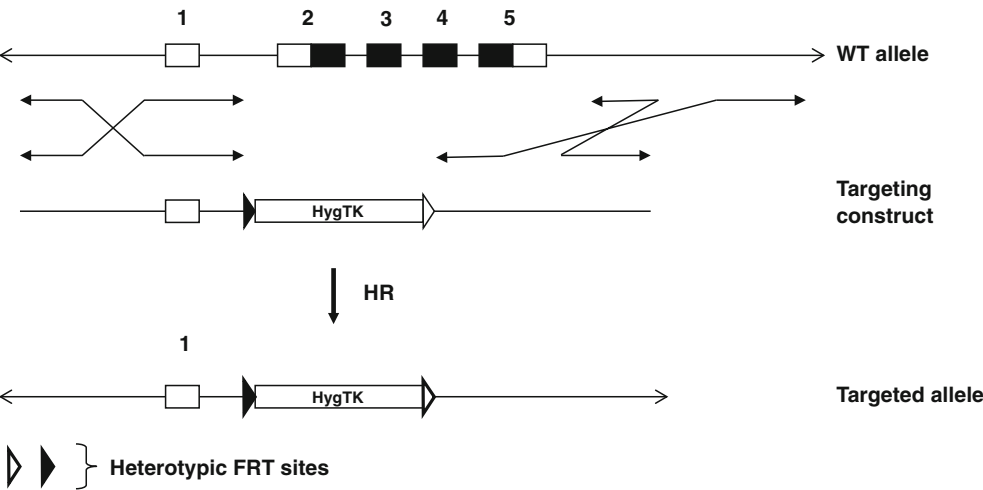
b **RMCE restoring the gene modified**
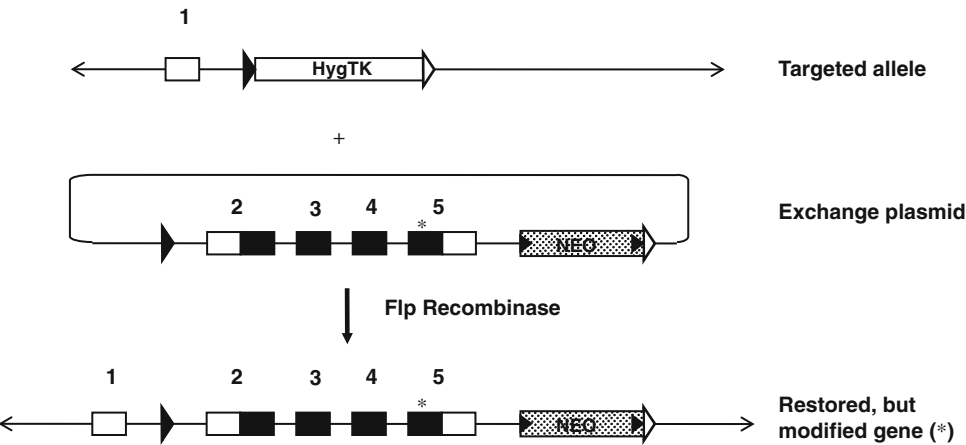
c **Some alternative exchange plasmids**
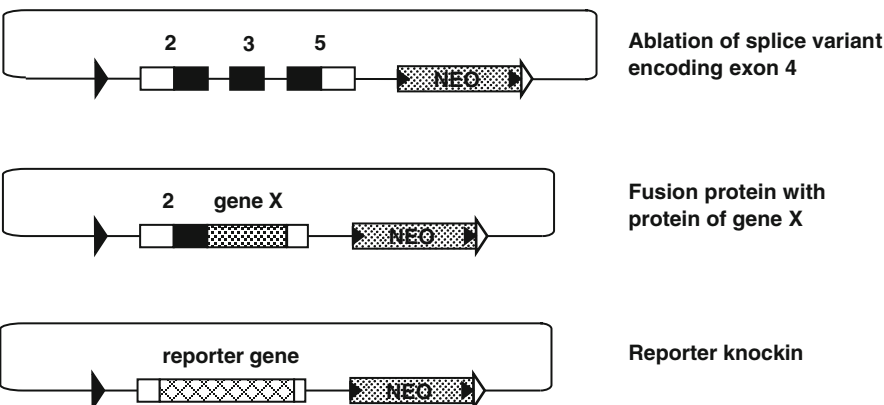
sequence (e.g., reporter gene), etc., could be knocked in by RMCE (Fig. 5c). Unless the excisable selection marker gene is positioned downstream of the gene, it will very likely be necessary to remove it in order to avoid interference with the targeted gene. In principle, the targeted gene could also be restored by wild-type sequences with extra LoxP sequences, enabling 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 could be removed and eventually restored with the modified versions. In case of larger genes, it might be necessary to replace only a part of the gene and to focus on modifications of that particular part of the gene (e.g., particular protein domains such as signaling motifs, a catalytic domain, etc.). 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 contrary, a parental ES cell line will very often be suitable for the generation of a classical germline knock-out, leaving the possibility for future modifications open.

In the given examples for potential application of RMCE, the focus is on the modification of the protein-coding region. As already mentioned, however, a RMCE-based knock-in approach can also be used to introduce multiple modifications in regulatory elements such as enhancers, promoters, etc.

2.3.4. Examples of Application of RMCE to Generate Knock-Ins

Actual application of RMCE to generate knock-in mice emerged from preceding research on the application of RMCE in mammalian cells in vitro, including mouse ES cells (reviewed by (13–15)). Bode and colleagues developed the RMCE method involving Flp and heterotypic FRT sequences in established cell lines and mouse ES cells (16–18). It should, however, be noted that the loci carrying the initial exchangeable cassette were introduced randomly at unknown loci in the genome of the cells. This proof-of-principle experiment for the application of RMCE using Flp/FRT in ES

Fig. 5. Different potential applications of RMCE. Application of RMCE using Flp and heterotypic FRT sequences for the generation of knock-ins is schematically depicted here for a theoretical gene existing out of five exons (*black boxes represent the protein-coding sequences in exons* 2–5). (**a**) The generation of a parental ES cell line suitable for RMCE is shown. A large part of the gene is replaced due to HR by the HygTK cassette flanked by heterotypic FRT sequences. (**b**) RMCE is used to restore the gene by reintroduction of the replaced sequences encoding now a modification in exon 5 (e.g., insertion, deletion, or modification of an amino acid residue or a protein domain indicated by *asterisk*). The 3′-end FRT sequence in the exchange plasmid is preceded by a Neo selection marker gene (flanked by LoxP sequences for removal later on) to allow positive selection for cells with the intended exchange. (**c**) A limited number of alternative exchange plasmids are shown as illustration for the usefulness of the parental ES cell line for the generation of different modifications. Adapted from Roebroek et al. (2003), Transgenic mouse: Methods and Protocols, Chapter 10, pag. 197, Fig. 3, with permission from Humana Press.

cells triggered, however, the use of RMCE in combination with a preceding homologous recombination targeting step in order to make knock-in mice.

First, Cesari and colleagues targeted the *Elk-1* locus, encoding a family member of Ets transcription factors, on chromosome X in ES cells. They used a HygTK gene flanked by heterotypic FRT sequences to allow for the subsequent generation of alternative alleles of interest by RMCE (19). Knock-out mice generated from this null allele, in which the HygTK cassette replaced essential exon sequences, are viable and do not reveal strong phenotypes, apart from male sterility. This male sterility was not due to *Elk-1* deficiency, but rather due to the presence of HygTK expression, which has previously been related to this defect. Replacement of HygTK in the *Elk-1* knock-out allele by Neo via application of RMCE in ES cells resulted in a Neo-containing null allele, which finally gave fertile *Elk-1* knock-out male mice. However, no additionally generated *Elk-1* knock-in mice were reported thus far.

Second, the authors of this chapter applied RMCE using Flp/ FRT in ES cells in order to generate *Lrp1* knock-in mice (20). The objective was to generate knock-in mice with inactivating mutations in protein domains of the multifunctional lipoprotein receptor LRP1. Due to the large size of this gene (about 100 kbp, 89 exons), the knock-in strategy involving RMCE was designed to target only the 3′end exons (exon 76–89), as schematically depicted in Fig. 6. To reduce the complexity of plasmid construction, a combination of genomic and cDNA sequences was used in the exchange plasmids. Re-introduction of wild-type *Lrp1* sequences resulted in a phenotypically normal mouse, whereas inactivation of a proximal NPXY motif (NPTY→AATA, encoded by exon 88) in the intracellular domain of LRP1 resulted in late fetal liver destruction, causing perinatal death. On the contrary, inactivation of a distal NPXYXXL motif (NPVYATL→AAVAATL, encoded by exon 89) revealed initially no obvious phenotype. Finally, inactivation of a furin cleavage site in LRP1, essential for a maturation step (RHRR→AHAA, encoded by exon 76), showed only a mild liver phenotype. Recent comparative analyses of the proximal NPXY and distal NPXYXXL double mutant mouse showed an earlier lethal phenotype compared to the proximal NPXY mutant. Analyses of the derived MEFs demonstrated that inactivation of the proximal NPXY motif impairs early steps in the biosynthesis of mature LRP1 (21). Further characterization of the distal NPXYXXL mutant in the background of an LDLR-deficient mouse model for atherosclerosis revealed enhanced postprandial dyslipidemia and atherogenesis, pointing toward an atheroprotective role of this motif (22).

Also, RMCE based on Cre and heterotypic LoxP sequences was recently used to generate knock-in mice. Sato and colleagues (23) used the application of RMCE to study endothelin1/endothelin
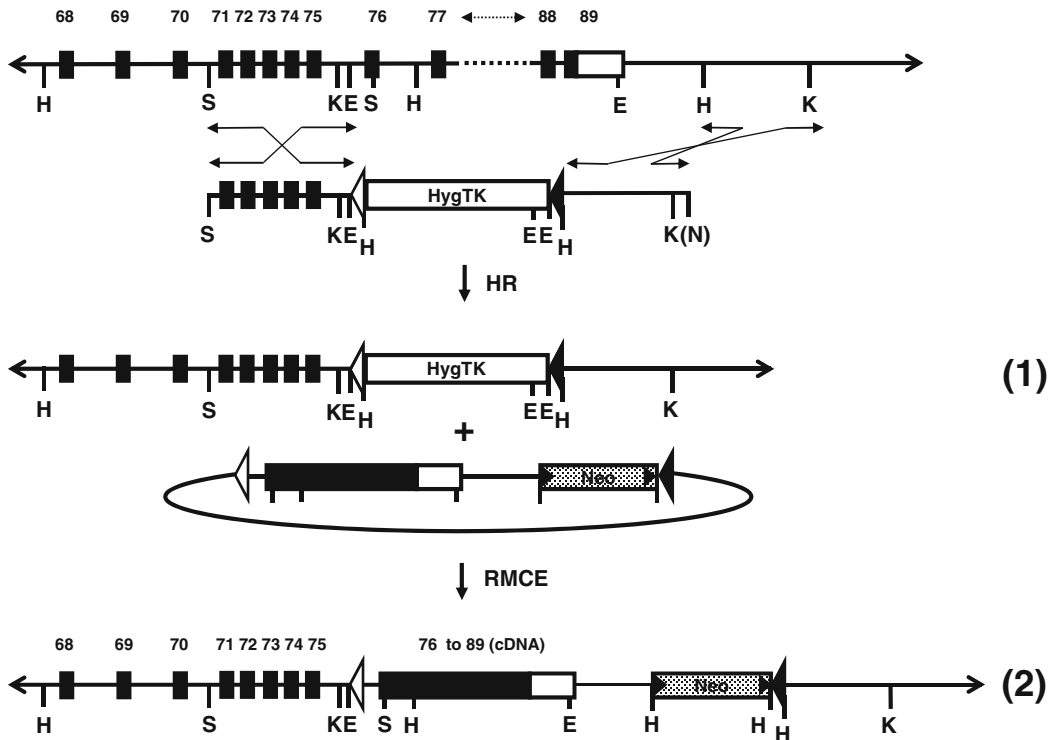
Fig. 6. Application of RMCE in the *Lrp1* gene. By HR, the 3′-end part of the *Lrp1* gene (3′-part intron 75 to sequences downstream of exon 89) is replaced by an exchangeable cassette encoding a HygTK gene and flanked by heterotypic FRT sequences (*large open and filled triangles*) (1), which can subsequently be replaced via RMCE with initially removed *Lrp1*-coding sequences (genomic and cDNA sequences), restoring the *Lrp1* gene, either wild type or mutant (2). For selection purposes, a Neo expression cassette flanked by LoxP sequences (*small filled triangles*) was included in the exchange plasmid, downstream of the *Lrp1* sequences. Restriction enzyme sites: *E Eco*RI, *H Hin*dIII, *K Kpn*I, *N Not*I, *S Sac*I. Adapted from Roebroek et al. (2006) (20), Molecular and Cellular Biology 26, 605–616, Fig. 1b, with permission from the American Society for Microbiology.

type A receptor signaling in pharyngeal arch development. Knock-in of LacZ-coding sequences was used to generate an *Ednra* knock-out/reporter knock-in mouse. Homozygous mice demonstrated perinatal lethality and craniofacial abnormalities, which were almost identical to the phenotype observed in mice lacking *Edn1* or *Ednra*. Knock-in of *Ednra* cDNA rescued the *Ednra*-null phenotype, whereas knock-in of *Ednrb* cDNA, encoding sequences of a paralogous gene, resulted only in a partial rescue. Additionally, the generation of a modified *p53* allele with an exchangeable cassette in ES cells was reported, which should enable the generation of multiple P53 knock-in mice by application of RMCE (24). Although no knock-in mice resulting from RMCE are described yet, the *p53* allele with the exchangeable cassette could be used successfully for RMCE not only in the ES cell themselves, but also in mouse embryonic fibroblasts (MEFs) derived from a generated, heterozygous mouse carrying this

modified *p53* allele. Furthermore, Shmerling and colleagues reported the application of RMCE in the β-actin locus to generate heterozygous mice having strong and ubiquitous expression, driven by the β-actin promoter, of promoterless transgenes (e.g., different reporter genes) targeted into this locus (25). RMCE was not only applied successfully in ES cells, but also in fertilized oocytes by pronuclear co-injection of an exchange plasmid and Cre protein into the fertilized oocytes, which were derived from mice carrying the modified β-actin locus.

Finally, application of RMCE using ΦC31 and attB/attP sequences to generate knock-in mice is reported by Hitz et al. (26). Into the *Rosa26* locus in ES cells, an exchangeable cassette was targeted by HR, allowing subsequent application of RMCE, which resulted in the introduction of an RNAi construct encoding shRNAs for three genes of the MAPK signaling pathway, of which the expression is driven by an U6 promoter. Due to the combination with a stop cassette and loxP sequences interrupting the shRNA sequences, which have to be removed first, the inactivation of the signaling genes by shRNAs could be triggered in a time- and tissue-dependent manner via controlling expression of the Cre gene crossed into the mice. It should be noted that for the expression of the RNAi constructs in the knock-ins, *cis*-acting elements of the *Rosa26* locus itself are not used at all. In this sense, this RMCE strategy in the *Rosa26* locus is an alternative use of RMCE as discussed so far. The *Rosa26* locus, as well as the *Hprt* locus, is often used as a suitable predetermined recipient locus for a single copy of a transgene carrying all of its own *cis*-acting elements using ES cell technologies as an alternative for pronuclear injection of fertilized oocytes, resulting in random integration of mostly multiple copies of the transgene. Of course, this *Rosa26* or *Hprt* approach can be used in combination with RMCE. In fact, the *Hprt* locus is also already used for application of RMCE based on Cre and heterotypic LoxP sequences: human sequences derived from two different bacterial artificial chromosomes, over 100 kbp in size and encoding the *Myo7a* gene, were introduced in this locus by RMCE and were shown to complement the shaker-1 mutation as expected (27).

2.4. Additional Applications of Heterotypic Recombination Target Sequences to Generate Knock-Ins

Besides for RMCE, the developed heterotypic recombination target sequences can be used for many other specific applications in manipulation of the mouse genome (reviewed in (13–15)). The only alternative discussed here is the flip-excision (FLEX) switch method designed by Schnütgen et al. (28) enabling a Cre-dependent switch to turn the expression of a gene off, while the expression of another is turned on. Schnütgen et al. used this approach to conditionally knock-out the retinoic acid receptor γ gene (*Rarg*), converting it at the same time into a LacZ reporter gene. This FLEX switch is based on the reversible inversion of a sequence between recombination target sequences such as LoxP

and FRT in case these sequences have an opposite orientation compared to a reversible excision in case these sequences have the same orientation (as explained in detail in a preceding chapter). In the situation where two sets of heterotypic target sequences are used, a 100% irreversible switch toward the inversed configuration can be obtained. In combination with a knock-in strategy, this method could be used to switch in a spatiotemporal fashion from the expression of a wild-type gene to the expression of a modified gene. A theoretical example for such FLEX switch approach is given in Fig. 7 with further detailed explanation in the legend. Such a FLEX switch could be used together with a
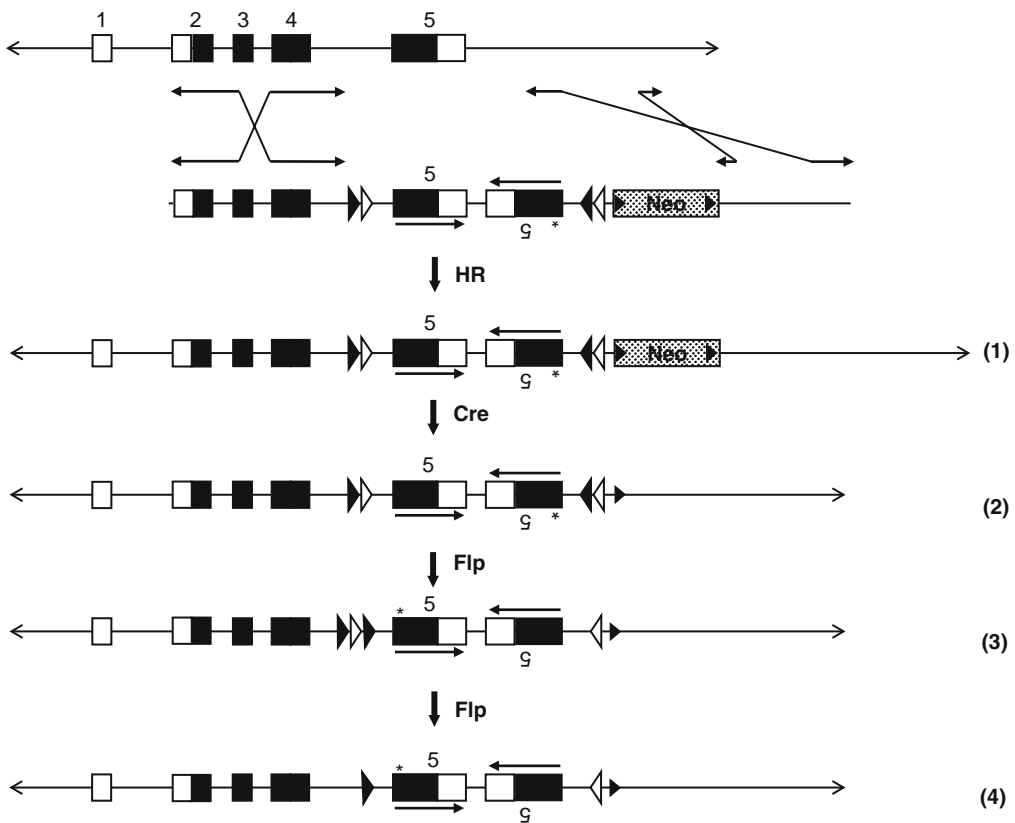
Fig. 7. Schematic representation of a FLEX switch knock-in strategy. The 3′-end of a theoretical five exon gene is replaced via HR by sequences containing from 5′ to 3′ a set of heterotypic FRT sequences, the last exon, exon 5, with some flanking sequences in sense orientation followed by a modified last exon (e.g., insertion, deletion, or modification of an amino acid residue or a protein domain indicated by *asterisk*) with flanking sequences in antisense orientation, a second set of heterotypic FRT sequences in opposite orientation, and a Neo selection marker gene flanked by LoxP sequences (1). After removal of the Neo gene by Cre (2) in vitro in ES cells or in vivo in the mouse, Flp expression in vivo in the mouse results in inversion of the exon 5 sequences by recombination between either pair of identical FRT sequences (3) (only inversion due to recombination between the *open triangles* is shown, the alternative possibility will give the same final result). As a consequence of the inversion, the other pair of identical FRT sequences (here the *filled triangles*) obtains a similar orientation, enabling an excision by Flp of a small circular DNA molecule with two heterotypic FRT sequences. So finally, the inversed sense and antisense exon 5 sequences remain flanked only by single heterotypic FRT sites, which cannot recombine any further (4). Due to the irreversible inversion, which occurs only in tissues that express Flp, only the modified gene can be expressed in these tissues instead of the wild-type gene.

RMCE approach by combination of the different recombinase systems: RMCE involving ΦC31 and attP and attB sequences, LoxP sequences to flank an excisable selection marker gene, and finally, heterotypic FRT sequences to enable the FLEX switch as discussed in Fig. 7.

3. Concluding Remarks

The different knock-in approaches discussed in the previous paragraphs show that site-specific recombinases are instrumental for the generation of sophisticated functional knock-ins and a series of knock-in alleles generated by RMCE. Combination of two or even three recombinase systems allows the generation of even conditionally expressed knock-ins. The number of potential applications of these site-specific recombinases will surely grow further in the near future as they enable the design of tailor-made modifications, allowing fine tuning of our efforts to elucidate the function of a gene in detail.

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Chapter 16

Generation of a Series of Knock-In Alleles Using RMCE in ES Cells

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Abstract

Recombinase-mediated cassette exchange (RMCE) is a powerful tool to generate a series of knock-in mutations into a particular gene of the mouse. It uses standard ES cell technologies to introduce an exchangeable cassette into the gene of interest by homologous recombination. Because the introduced exchangeable cassette is flanked by heterotypic specific recombination target sequences, site-specific recombinases can be used in a subsequent RMCE step to exchange the cassette by other sequences. Here, an experimental procedure for the application of RMCE in E14 ES cells using heterotypic FRT recombination target sequences and the site-specific recombinase Flp is elaborated.

Key words: Knock-in, Mutation, Site-specific recombinase, FRT, Flp, RMCE

1. Introduction

The principles of recombinase-mediated cassette exchange (RMCE) and its potential applications are discussed in detail in Chapter 15. The starting point for the application of RMCE for the generation of knock-in mice is the introduction by homologous recombination (HR) in ES cells of an exchangeable cassette flanked by heterotypic recombination target sequences, replacing parts of the gene to be targeted. After introduction of such a cassette, the resulting parental ES cell line can subsequently be used repeatedly to generate a series of different knock-ins, e.g., different mutations in the encoded amino acid sequence, in the targeted gene by an exchange of the cassette for modified sequences flanked by the same heterotypic target sequences, present in a replacement plasmid. Transient expression of a site-specific recombinase is used to achieve this exchange, which, e.g., results in a functionally restored but modified gene.

The protocol presented here refers especially to the RMCE step in the procedure as was applied for the generation of several different *Lrp1* knock-in mice (1, 2), which is also explained in detail in Figure 6 of Chapter 15. For application of RMCE in ES cells, most of the general guidelines and protocols to target ES cells via HR also apply for this special application. Of course, composition of media, culture conditions, use of feeder cells, etc., depend on the ES cells to be used. For RMCE in *Lrp1*, the ES cell line E14 was used (3).

2. Materials

2.1. Plasmids

1. The plasmid for targeting the *Lrp1* gene in ES cells by HR and the exchange plasmids used in the RMCE procedure are described in detail in (1) and schematically depicted in Figure 6 of Chapter 15. The HygTK cassette, which was incorporated into the plasmid for targeting *Lrp1*, was derived from a plasmid described in (4). This cassette allowed positive selection by hygromycin B and negative selection by ganciclovir. The exchange plasmid contained a Neo resistance gene for positive selection by G418.

2. For transient expression of the Flp recombinase, the Flp expression plasmid pCAGGS-FLPeIRESpuro was used (5).

2.2. ES Cells and Cell Culture

1. For RMCE in *Lrp1*, the ES cell line E14 was used (3).

2. The E14 ES cells were grown on mitomycin C-treated feeder cells. The ES cell culture medium, Glasgow MEM supplemented with non-essential amino acids, sodium pyruvate, glutamine, and 2-mercaptoethanol, finally contained 20% fetal calf serum (FCS) and 30% Buffalo rat liver (BRL) cell-conditioned medium to supply for leukocyte inhibitor factor (LIF).

3. Methods

The first step to target the exchangeable cassette into the *Lrp1* gene by HR is not worked out in detail here, because general guidelines and protocols to target and analyze ES cells are discussed elsewhere in this volume. It should be noted, however, that of 300 isolated hygromycine B-resistant clones, only one resulted from proper HR. The next RMCE step was quite effective, since efficiencies of this exchange step varied from 38% to 61% for the different exchange plasmids upon G418 selection (1).

This difference in efficiency illustrates the power of application of RMCE in order to generate a series of knock-in mutants compared to that of knock-in approaches starting all over again from the start with targeting for HR. Please see also Note 1 regarding the potential use of a combination of negative and positive selection.

The experimental procedure for RMCE in the *Lrp1* gene in E14 ES cells is given below in detail. It should be noted that this RMCE procedure worked equally efficiently for another gene in these E14 ES cells (Roebroek et al., unpublished data) and can also be used for the application of RMCE in mouse embryonic fibroblasts (MEFs) (see Note 2).

1. Before electroporation, 133 μg of pCAGGS-FLPeIRESpuro, the Flp expression vector, is mixed together with 40 μg of exchange plasmid DNA (both supercoiled circular DNA) in a total volume of 40 μl of sterile TE.

2. DMEM (450 μl) containing 10% FCS and a suspension of 5×10^6 ES cells, heterozygous for the exchangeable cassette introduced previously by HR (# ES cells = # total cells – # feeder cells), is mixed with 30 μl of the DNA sample (100 μg of Flp expression vector plus 30 μg of exchange plasmid).

3. The mixture is transferred to an electroporation cuvette and pulsed with an electroporator set to 200 V and 960 μF (Gene Pulser® electroporation system, Bio-Rad). These conditions are identical to the conditions used for HR to introduce the exchangeable cassette in the ES cells.

4. The entire electroporated cell suspension is seeded in a 75-cm² culture flask on a feeder layer (3×10^6 mitomycin C-treated feeder cells) in ES cell culture medium.

5. The cells are cultured for about 36–48 h (depending on the density and growth of the ES cells) without selection pressure to let the recombination events happen.

6. Due to the transient expression of the Flp recombinase, possibly mixed clones are formed. To reduce the chance on selecting such mixed clones, the ES cells are trypsinized and replated at a very low density of 2.5×10^5 ES cells per 25-cm² culture flask.

7. After a 24-h incubation period, the ES cell culture medium is replaced by the same medium, but containing 400 μg/ml of G418.

8. G418-resistant clones (about 5–25 surviving clones per flask) are picked after 8–10 days for further growth and for genetic analysis, as described elsewhere in this volume.

4. Notes

1. In a pilot experiment, a combination of G418-positive selection (to select for the Neo gene present in exchange plasmid) and ganciclovir-negative selection (to select against the HygTK gene) was used, resulting in 100% efficiency of the RMCE procedure, but the resulting E14 ES cell clones grew poorly under the conditions tested. G418 selection alone yielded an efficiency of about 50%, without a negative impact on growth of the ES cells. To avoid a potential negative impact on the capacity of the manipulated ES cell lines to go germline, this double selection was no longer applied, especially because the high efficiency of G418 selection alone proved to be sufficient. Several different experiments, in which RMCE was applied, resulted in an overall efficiency between 38 and 61% of correctly exchanged, selection-surviving ES cell clones, which were subsequently used to generate knock-in mice.

2. With equal efficiency, RMCE could also be applied in immortalized MEFs derived from homozygous mice, which themselves resulted from RMCE in ES cells. The above-described protocol was applied unchanged except that MEFs instead of ES cells, normal DMEM/F12 medium containing 10% FCS, and no feeders were used. The objective of this application of RMCE in MEFs was to obtain additional different *Lrp1* knock-in MEFs by double replacement of a particular knock-in mutation by another one using two exchange plasmid constructs encoding an identical knock-in mutation, but two different selection marker genes. Although two subsequent rounds of RMCE could be performed successfully with high efficiency, the final outcome was not satisfying due to the apparent tetraploidy of the used immortalized MEFs, which implies the need of four subsequent rounds of RMCE to obtain the desired new "homozygous" knock-in mutant MEFs (Reekmans et al., unpublished data).

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Chapter 17

Selection of Targeted Mutants from a Library of Randomly Mutagenized ES Cells

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Abstract

A method for random relatively unbiased mutagenesis of ES cells with a mutagenic retroviral vector is described. An orderly assembly of mutant ES cells in multi-well plates is generated. 3D pooling of the wells of the assembly allows quick PCR search for insertions in genes of interest. Mutant ES cell clones are then isolated from the positive wells and used to produce mutant animals using conventional techniques.

Key words: Retroviral insertion, Random mutagenesis, ES cell, Knock-out mice, 3D pooling, Gene targeting

1. Introduction

The mouse is an invaluable model organism for the understanding of human diseases and development. The mouse and human genomes share highly conserved genes, most of which are not well characterized for their function.

Knock-out mice provide useful information for functional annotation of genes. Two types of techniques, gene targeting (1) and gene trapping (2), are widely used for the production of mutant mice. Gene targeting involves construction of a targeting vector for each individual gene and relies on homologous recombination of the vector DNA with chromosomal DNA of ES cells. Gene trapping employs random insertion of mutagenic vectors into the genome, which is followed by drug selection for vector insertions within genes. Because of the high demand of knock-out mice in scientific community, international programs are initiated which aim to generate a public resource of ES cell lines

containing mutation in every genes (3). Both gene targeting and gene trapping are utilized toward this goal.

On the contrary, a series of recent studies indicate that a significant proportion of the mammalian genome is transcribed (4). The proposed transcribed regions are by far larger than the conventionally defined gene region, even reaching to more than 90% of the entire genome according to some reports. It is impractical to knock-out all these transcripts by gene targeting method. Many of the newly identified transcripts appear to be non-coding and weakly expressed, which may be difficult to mutate by gene trap approach. Although the significance of this transcription is still under debate, this observation raises anticipation for a novel genetic approach that allows for mutating ES cell genome with the complexity high enough to cover entire transcripts.

Here, we describe a method for saturating mutagenesis in mouse ES cells with retroviral vector insertion (5). It involves the following steps.

1. Random mutagenesis of ES cells with a highly mutagenic retroviral vector. We simply select for all vector insertions, and do not employ drug selection into gene regions as conducted in convectional gene trap approach. This is critical to achieve saturating mutagenesis of any unpredicted transcripts.

2. Generation of a large-scale, orderly assembly of mutant ES cells in multi-well plate format, followed by cell freezing and genomic DNA extraction.

3. PCR screening for the cell pools containing mutant clones with vector insertions in desired genomic locations.

4. Isolation of mutant ES cell clones.

Because of the simplicity of the procedure, it is possible to automate several labor-intensive steps, allowing for the construction of large-scale mutant ES cell library. Our mutant library contains ten million independent mutant clones, and 90% of the genes screened so far are mutated. It will also be possible to apply our approach to other pluripotent stem cell lines, such as recently reported rat ES cells.

In addition to the retroviral vector, new types of mutagenic vectors have been developed in recent years, such as human immunodeficiency virus-based vectors and transposon vectors derived from Sleeping Beauty (6), piggyBac (7), and Tol2 (8). Each vector appears to have different specificity for genomic integration sites (9). The principle of our method can be readily applied to these vectors and will help to further increase the complexity of the mutations.

2. Materials

2.1. Media

ES cells were grown on Dulbecco's Modified Eagle's Medium (DMEM, high glucose, with L-glutamine, Invitrogen), supplemented with 15% fetal bovine serum (FBS, Hyclone, batch preselected for optimal ES cell growth), non-essential amino acids (NEAA, Invitrogen), penicillin–streptomycin (Pen-Strep, Invitrogen), 100 μM 2-mercaptoethanol (Sigma), and 1,000 U/ml of LIF (ESGRO, Invitrogen).

Mouse embryonic fibroblasts (MEFs) were grown on DMEM with 10% FBS, NEAA, and Pen-Strep.

2.2. Cells

Primary MEF feeder cells were produced from C57BL/6J-Tg(pPGKneobpA)3Ems/J mice (The Jackson Laboratories) and γ-irradiated, essentially as described in Ref. (10).

Before library construction, 129S1/SvImJ ES cells were re-derived to obtain cells with high probability of germline transmission. It is known that pluripotency of ES cells gradually deteriorates with culturing due to accumulated mutations and epigenetic changes. Therefore, we first picked up a number of individual ES cell colonies, karyotyped them, and used euploid clones for blastocyst injections. Clones yielding more than 90% chimeras were chosen for further testing. A few of those contributed to the germline, with probabilities in excess of 90%.

2.3. The Vector and the Virus-Producing Cell Line

The retroviral vector we use has been described previously (5) and has the following important elements: (a) splice acceptor, stop codons in all three reading frames, polyadenylation (polyA) signals, and transcription terminator – all to assure target gene inactivation; (b) a phosphoglycerate kinase (PGK) promoter-driven neo marker to select clones with insertions and *LoxP* sites to remove the marker, if necessary; (c) an encephalomyocarditis virus internal ribosome entry site (IRES) and tetracycline-controlled transactivator, which are not needed for gene inactivation, but serve as a plug-in to an inducible gene-inactivation system described elsewhere by Zeng et al. (11). Upon insertion, only one orientation of the vector relative to the direction of transcription of the target gene is inactivated. Our data confirm the high mutagenicity of the vector.

The vector is produced by a virus-producing cell line, made by transfection of the retroviral vector into NIH-3T3-based packaging cell line GP + E-86 having gag-pol and env genes of Moloney murine leukemia virus integrated in two different locations (12). High-titer virus-producing clones were identified and used to infect mouse ES cells through co-cultivation.

3. Methods

3.1. Summary of the Technique

Co-cultivation with a virus-producing cell line is used to infect mouse ES cells with the highly mutagenic retroviral vector. Cells with independent vector integrations are arranged into ordered arrays in multi-well plates. The arrays are duplicated to produce two identical copies, one for storage and the other for 3D pooling of wells for subsequent DNA isolation. Pooled DNA is screened by PCR using vector-specific primers and primers from a gene of interest to find whether this gene has a retroviral insertion. Positive PCR results showing the same size fragment in three different dimensions of the ordered array of wells are used to identify the wells containing ES clones with the insertions in a gene of interest. ES cells from the well are expanded and the clone of interest is isolated through sequential subcloning procedure detailed below. The isolated ES cell clone is used for standard blastocyst injection, chimera production, and mating to achieve germline transmission of the mutation.

3.2. Mutant ES Cell Library Construction

The library is constructed in units consisting of ten 96-well plates with ~500 different mutant ES cell clones per well. A unit contains ~0.5×10^6 independent mutant ES cell clones. Figure 1 depicts the main steps of the library unit construction. The detailed protocol is as follows (see Note 1).

Throughout this protocol, neomycin-resistant primary MEFs are used as feeder cells. MEFs could be either Mitomycin C treated, or γ-irradiated. Other feeder cells could be used; however, MEFs are the feeders of choice.

Day (−2). Thaw ES cells on MEF feeder cells.
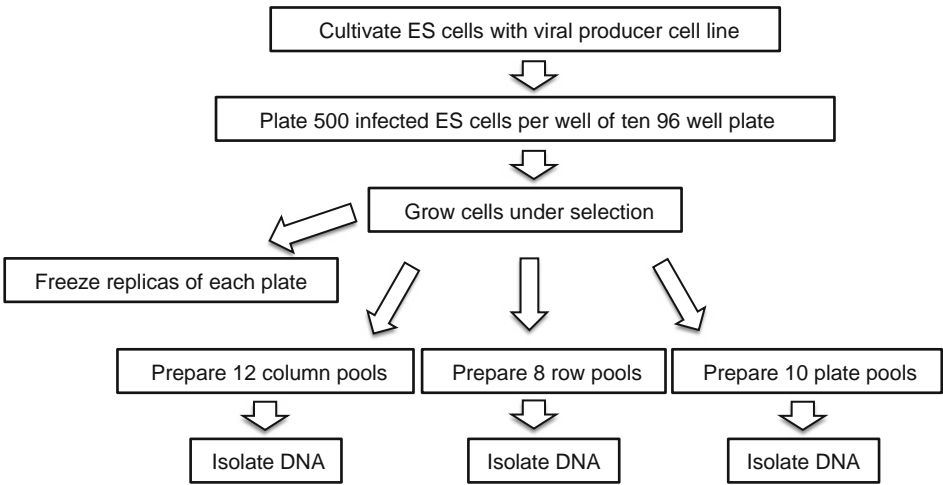
Fig. 1. Library construction. Chart for construction of a library unit of randomly mutagenized ES cells. The unit consists of ten 96-well plates with ~500 different ES clones per well, or a total of ~0.5 million mutant clones.

Day (−1). Make 11×10 cm dishes of virus producers (5×10^6 of virus producer cells per 10-cm dish)

Day 1. Treat virus-producer cells with mitomycin C: make 90 ml of mitomycin C medium (0.9 ml of 100× mitomycin C stock + 89 ml of regular medium; mitomycin C stock is prepared by dissolving 2 mg of mitomycin C in 2 ml of PBS and filtering through a 0.22-μm syringe filter) and add 7 ml/10 cm dish. Incubate for 2.5–3 h at 37°C. Wash three times with 10 ml of PBS (with Ca^{2+} and Mg^{2+}). Add 7 ml of ES medium + 4 μl of 10 mg/ml polybrene/dish. Trypsinize ES cells and plate 1.5×10^6 in 3 ml of the medium per plate on ten dishes with virus producers, and keep the eleventh dish w/o ES cells.

Day 2. Replace medium in co-cultivation dishes. Prepare ten 96-well plates and two control 10-cm dishes with MEFs.

Day 3. Transfer infected ES cells into 96-well plates: use cells from one 10-cm co-cultivation dish to plate 4,000 ES cells/well of one 96-well plate. Use virus producer cell line plate dish without ES cells to correct the cell numbers. Start G418 (250 μg/ml) selection. Name the plates P1 to P10. To determine plating and infection efficiencies, plate 1,000 ES cells per 10-cm dish without G418, and 3,000 ES cells per 10-cm dish with G418. Our particular ES and virus producer cell lines usually displayed ~40% plating efficiency and ~30% efficiency of infection, which means that 4,000 ES cells per well eventually result in ~500 individual clones of infected ES cells per well. On subsequent days, replace G418-containing ES medium every day.

Day 5. Make twenty 96-well plates with MEFs for use on day 7.

Day 6. Make sixty 15-cm dishes with feeder cells for use on days 9 and 12.

Day 7. Split each of the 96-well plates to two identical 96-well plates. This step is done to provide ES cells with a room for growth.

Day 9. Dispense 45 μl of 2× freezing medium per well of ten 96-deep-well plates (we used 96 tube clusters of Matrix storage tubes, Thermo Scientific Cat. #4147. These clusters allow easy retrieval of individual positive tubes after their identification for further processing). Replace regular medium with ES cell medium in thirty 15-cm feeder dishes. Label the dishes *N*P1–*N*P10, *N*C1–*N*C12, and *N*RA–*N*RH, where *N* is the number of the library unit, and P, C, and R stand for pools corresponding to plates, columns, and rows, respectively. Trypsinize and combine cells from the two sister 96-well plates to one 96-well plate, mix by pipetting. Transfer 45 μl from each well to 96-deep-well plates with 2× freezing medium. Pipette to mix. Seal the deep-well plates with the sealing foil and place in a styrofoam box with a layer of dry ice covered with two layers of Utility Wipes. Transfer to a −80°C freezer after at least 30 min. Transfer to a vapor-phase

liquid nitrogen freezer next day. Transfer 45 μl to *NP1–NP10*: all wells of each plate are combined into the plate pools. Transfer 45 μl to *NC1–NC12*: the wells from identical columns from all ten plates of the unit are combined into column pools. Transfer 45 μl to *NRA–NRH*: the wells from identical rows from all ten plates of the unit are combined into row pools. Figure 2 shows the principle of the 3D pooling. Each well in the library unit is defined by a unique combination of plate, column, and row pools to which it belongs.

Day 12. Replace regular medium with ES medium in thirty 15-cm feeder dishes (made on day 6). Trypsinize and collect pooled cells from 15-cm dishes plated on day 9. *Plate pools are treated differently from column and row pools.* Label freezing vials *NC1–NC10* and *NRA–NRH*, 3 vials of each number, a total of 60 vials. For column and row pools, trypsinize each pool, resuspend in the medium, transfer half of the content to a 15-ml screw-cap tube, spin down for 10 min at $130 \times g$, discard supernatant, suspend pelleted cells in 3 ml of freezing medium, and dispense into three freezing vials. Freeze the vials at –80°C in a styrofoam tube rack for subsequent transfer to liquid nitrogen. For each column and row pool, transfer the other half of the cell suspension to a 50-ml tube (a total of 20 tubes), spin for 10 min at $130 \times g$, discard the supernatant, resuspend the pellet in 10 ml of PBS (without Ca^{2+} and Mg^{2+}), re-precipitate the cells, and store them at –20°C or –80°C till DNA isolation. For plate pools, label 15-cm feeder dishes *NP1–NP10*, three dishes of each number, a total of 30 dishes. Trypsinize each pool, resuspend in the medium, use one half of the suspension to plate on three 15-cm feeder dishes, transfer the other half of the cell suspension to a 50-ml tube (a total of ten tubes), spin for 10 min at $130 \times g$, discard the supernatant, resuspend the pellet in 10 ml of PBS (without Ca^{2+} and Mg^{2+}), re-precipitate the cells, and store them at –20°C or –80°C till DNA isolation.

Day 14. Label 30 freezing vials *NP1–NP10*, three for each number. Similarly, label fifty 50-ml tubes, five for each number.
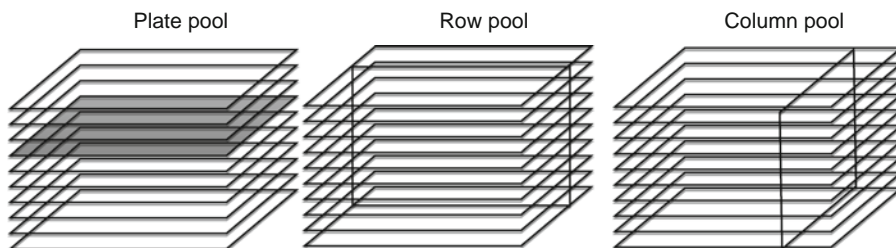
Fig. 2. Pooling scheme. Pooling strategy for the wells of a library unit. In addition to freezing a replica of each plate, cell suspensions from each well were pooled in three different ways: plate pools combined all wells of a given plate, row pools combined identical rows from all plates, and column pools combined identical columns from all plates. Every well of the library unit is defined by its 3D address – a unique combination of the plate, column, and row pools.

Trypsinize each pool, resuspend in the medium, combine, and thoroughly mix cell suspension from each three identical plate pool dishes plated on day 12. Use one-sixth of the suspension to spin cells down, resuspend in freezing medium, and dispense in three freezing vials per plate pool. Freeze the vials at –80°C in a styrofoam tube rack for subsequent transfer to liquid nitrogen. Equally divide the rest of the ES cell pool into five 50-ml tubes, spin for 10 min at $130 \times g$, discard the supernatant, resuspend the pellet in 10 ml of PBS (without Ca^{2+} and Mg^{2+}), re-precipitate the cells, and store them at –20°C or –80°C till DNA isolation.

DNA isolation. DNA was isolated from cell pellets of plate, row, and column pools using QIAGEN Genomic Maxi kit. The resulting amount of DNA (~0.5 mg) allows quite significant, but finite number of screening PCRs. When DNA runs out, a new portion could be isolated from frozen cell pellets in the case of plate pools, or (for plate, column, and row pools) the pools could be regrown from frozen stocks and DNA re-isolated.

A single library unit constructed as described in this section contains $\sim 0.5 \times 10^6$ individual clones of mutant ES cells. A bigger library could be assembled by building multiple units. We found out that with 20 units ($\sim 10^7$ ES clones), we were able to find insertions in more that 90% of genes we have screened (see Note 2).

There is no need to finish making a library unit before starting the next one. The units could be staggered by few days in such a way that several units are constructed simultaneously (one has to avoid overlapping labor-intensive days, for example, day 9 from one unit and day 12 for another).

3.3. Mutant ES Cell Library Screening

The library is screened using nested PCR with gene-specific and vector-specific primers, as shown in Fig. 3. The vector-specific primers were common to all genes screened and were carefully selected in order to avoid amplification of false-positive fragments derived from endogenous retroviral sequences. Gene-specific primers were designed for each gene and were usually targeted to the 5′ end of the gene. The annealing temperature of each primer pair was set to 65°C and the parameters for intra- and inter-molecular interactions between the primers are set to the highest

Fig. 3. Screening for gene-specific insertions. Screening library for insertions in the gene of interest. *Hollow arrows* show positions of PCR primers. First PCR reaction is run with the primers marked "1" and the nested reaction with the primers marked "2." This design is necessary due to the high complexity of the pools being analyzed, each containing thousands of independent vector insertions.

stringency allowed by the program. Specificity of the primers used was evaluated by BLAST of the mouse genome. QIAGEN HotStarTaq DNA polymerase was used for all PCRs. The first designed screening primer pair worked for ~75% of genes. For the rest, primers were redesigned.

We first screened genomic DNA from plate pools and subsequently determined the 3D address of the mutation of interest by screening genomic DNA from column and row pools. This order of screening implies that DNA from plate pools is more heavily used. This is the reason why plate pools are treated differently on day 12 of library construction – more DNA is needed. We reliably detected insertions within 2–3 kb of the gene-specific primer. On average, we ran ~100 PCRs per gene in order to identify mutations in a target gene and an additional ~100 PCRs per gene in order to determine the 3D address of the mutation of interest in our library. When the 3D address of the mutation is established, it makes sense to determine the exact site of the insertion by sequencing. It could be done by purifying and directly sequencing a PCR fragment produced during screening of any of the pools using vector-specific sequencing primer facing outwards.

DNA pools being screened have an average complexity of ~5×10^4 individual ES cell clones (~100 wells times ~500 clones/well). A minimum of ~2 µg of a pool DNA was used in each PCR (5 µg of DNA gives more reliable results). Assuming the molecular weight of diploid mouse genome to be ~4×10^{12}, 2 µg of genomic DNA contains ~3×10^5 copies of diploid genome. Therefore, 2 µg of pooled genomic DNA includes on average ~6 copies of genome from each individual ES cell clone, which is close to the limit of PCR detection. Total amount of genomic DNA isolated from a plate pool in our library construction protocol is ~2,500 µg, which is enough to screen 1,250 genes using 2 µg per gene, or 500 genes using 5 µg per gene. After this, frozen copies of cell pools have to be regrown to replenish the source of genomic DNA. In practice, it is convenient to screen 8 units (one-third of a library) of a library of 24 units (~12×10^6 individual clones) at a time. In our experience, insertions in ~50% of genes could be found by screening only one-third of a library, another third is required for ~30% of genes, and the remaining 20% have to be screened over the entire library (genes that have no insertions also fall into this category). The ability to screen many genes over partial library increases the total number of genes that could be screened before it is necessary to regrow the pools. Use of long-PCR may further expand the limit of insertion detection and may help identify desired mutants in a smaller scale library.

Due to differential growth rates of different individual clones, complexities of the pools will be reduced with the repeated rounds of regrowth, resulting in inability to use one constructed library indefinitely. In our experience, however, at least two rounds of

the regrowth are possible without a significant loss in pools'
complexity. This will bring an estimate for a total number of genes
that could be screened using a single library to several thousands,
a significant part of the entire genome.

3.4. Isolation of ES Cell
Clones

Figure 4 shows a workflow of isolation of the ES clone of interest
after it was localized in the library through PCR screening. The
following protocol is organized by days from the start of the pro-
cedure. Note that the day numbers serve for reference only.
Exercise common sense and good judgment to move to the next
step depending on how the cells grow and look (see Note 1).

    Day 0. Prepare a 24-well feeder cell plate.

    Day 1. Thaw positive wells from mutant ES cell library, use of
24-well plate allows processing of several clones in parallel.
Prepare four 96-well feeder plates per one positive library well.
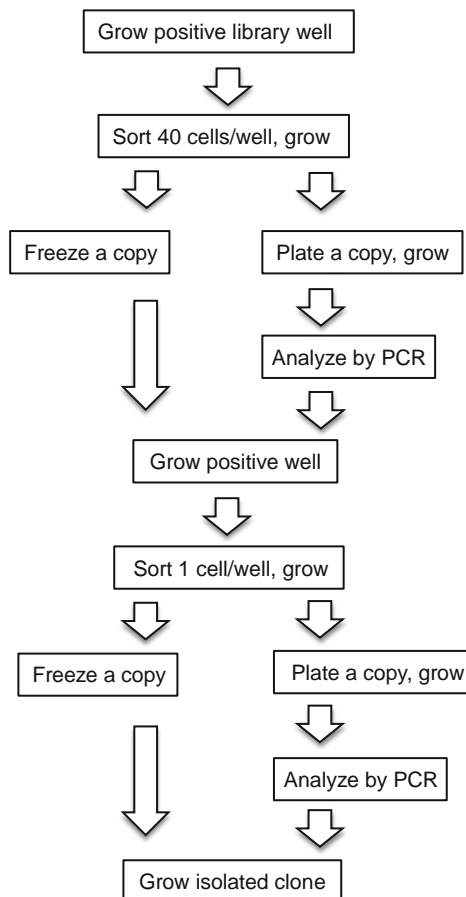
Fig. 4. Isolation of individual clones. Chart for isolating the clone with mutation in the
gene of interest from a library well identified by its 3D address in PCR screening. Each
well contains ~500 different mutant clones. Most clones could be isolated in two rounds,
as shown. Some clones require an additional round, as described in the protocol.

Day 3. Trypsinize and resuspend ES cell pool. Distribute ~100 cells per well of four 96-well plates. This step could be done either with a cell sorter, or by counting cells and diluting them to an appropriate density. A plating efficiency of ~40% results in ~40 distinct clones per well (plating efficiency is lower when cell counting and dilution are used instead of a cell sorter, likely because the sorter allows to recognize and disregard feeder cells that have different light scattering properties). Part of the remaining cells is frozen in liquid nitrogen, and another part washed with PBS and used for DNA isolation.

Day 4. Isolate DNA from the frozen cells of the positive cell pool and analyze by PCR to confirm that the desired clone is present in the pool.

Day 6. Prepare four 96-well feeder plates per clone being isolated.

Day 7. Trypsinize 96-well ES cell plates made on day 3 and thoroughly resuspend the cells. Transfer half of the cells to a plate containing 2× freezing medium, seal with a foil, and place in a styrofoam box with a layer of dry ice covered with two layers of Utility Wipes. Transfer to –80°C freezer after at least 30 min. Store at –80°C until the positive wells are identified. Transfer the other half of the cells to a fresh 96-well plate with feeders, and place the plate back in a CO_2 incubator.

Day 9. Wash the cells twice with 200 μl per well of PBS (without Ca^{2+} and Mg^{2+}). Add 50 μl of H_2O per well and incubate at 95°C for 1 h in a Saran Wrap-covered, glass baking dish with wet tissue at the bottom. Remove from the oven and spin the plates briefly to shake the condensate from the lids. Add 10 μl/well of 2.5 mg/ml of proteinase K solution and pipette to mix. Incubate at 56°C for 2 h in a Saran Wrap-covered, glass baking dish with wet tissue at the bottom. Transfer the baking dish with the plates to 95°C for 1 h to inactivate proteinase K. Remove from the oven, spin the plates briefly to shake the condensate from the lids. Analyze 5 μl from each well by nested PCR similar to ES library screening (start at least first PCR on the day of DNA isolation). Make a 24-well feeder cell plate.

Day 10. Finish PCR and run gels. Transfer the cells from positive well(s) of 96-well plate(s) to the 24-well plate.

Day 11. Prepare six 96-well feeder plates per one positive well.

Day 12. Trypsinize and resuspend ES cell pool. Distribute one cell per well of four 96-well plates and ten cells per well of additional 2 plates. Again, this step could be done either with a cell sorter, or by counting the cells and diluting them to an appropriate density. Note that no matter what method is used, less than half of the wells of the "one per well" plates will actually contain ES cell clones. The remaining cells are frozen in liquid nitrogen.

Day 14. Prepare six 96-well feeder plates per one positive well.

Day 15. Trypsinize 96 well plates with single clones and transfer as much as possible to a new 96-well plate with feeders. This step is important for speeding up ES cell growth.

Day 17. Trypsinize 96-well ES cell plates made on day 15 and thoroughly resuspend the cells. Transfer half of the cells to a plate containing 2× freezing medium, seal with a foil, and place in a styrofoam box with a layer of dry ice covered with two layers of Utility Wipes. Transfer to –80°C freezer after at least 30 min. Store at –80°C until the positive wells are identified. Transfer the other half of the cells to a fresh 96-well plate with feeders and place the plate back in the CO_2 incubator.

Day 19. Extract DNA from plates made on day 17 the same way as done on day 9. Prepare a 24-well feeder plate. Start PCR.

Day 20. Finish PCR and run gels. The process of ES clone isolation is concluded when a positive well is found on one of the "one cell per well" plates. Occasionally, no positive wells are found; in such a case two additional "ten cells per well" plates are analyzed by PCR and the positive well is subject to another round of cloning (the above procedure is repeated starting at day 12). Identified positive wells are thawed, trypsinized, and transferred to a 24-well feeder plate. The resulting clone should be frozen for permanent storage (several vials) and propagated for injection (see Notes 3 and 4).

4. Notes

1. Durations of individual steps of this protocol (Subheadings 3.2 and 3.4) were optimized for the growth rate of the particular ES cell clone that we used. It is likely that the other ES cells with slightly different growth rates will require readjustments of the duration of at least some steps.

2. With the library in place, its screening and isolation of clones are straightforward and relatively simple. Library construction, on the contrary, involves significant initial effort, which is not justified if the final goal is limited to obtaining knock-outs in few genes of interest. The described approach is optimal for large groups of genes (hundreds to thousands) to be mutated.

3. The rate-limiting step of the technique is the isolation of ES cell clones from a positive library well. Usually, a single mutant clone is isolated. Therefore, the probability of this clone to contribute to germline is of crucial importance, is essential for the efficiency of the technique, and requires very high quality of the ES cells used for the library construction. We used a subclone of our original ES cells that were devoid of

aneuploids and able to provide very high rate of germline contribution. As the result of these precautions, the chances for a single clone purified as described to contribute to germline transmission of the mutation were about 70–75% in our hands. If a gene of interest has more than one vector insertion present in the library and the respective clones are isolated, the likelihood of germline transmission of either of the mutation is more than 90%.

4. All the described procedures could be performed in a manual mode; however, the efficiency of processing multiple multi-well plates is much higher using liquid-handling robotic equipment for all stages of the procedure including setting up PCR plates, and cell sorter during ES clone isolation.

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Chapter 18

Generation of Genetically Modified Rodents Using Random ENU Mutagenesis

Ruben van Boxtel and Edwin Cuppen

Abstract

The generation of genetically modified animals using *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis is a fast and highly effective method. The technique is based on treating male animals with the supermutagen ENU, which randomly introduces mutations in the spermatogonial stem cells. By breeding these animals with untreated females, an F1 population is generated in which each individual carries unique random ENU-induced mutations, which can be retrieved using either genotype-driven or phenotype-driven approaches. No complicated cell culturing techniques are required and since no foreign DNA is introduced, the mutant animals that are generated are not transgenic. Here, we describe the detailed protocols for ENU mutagenesis and for mutant retrieval.

Key words: ENU mutagenesis, Mutation discovery, Phenotype-driven screens, Genotype-driven screens, Mutant rodent models

1. Introduction

The availability of genetically modified model organisms has greatly benefited the research of human physiology and disease. Traditionally, one of the most efficient methods to generate mutant alleles of genes is the use of chemical mutagenesis. In rodents, *N*-ethyl-*N*-nitrosourea (ENU) was shown to be the most potent germ line mutator (1). The technique is based on treating male animals with the ENU, which very efficiently generates point mutations in the DNA of spermatogonial stem cells. The mutagenicity of ENU results from the ability to transfer its ethyl group to nucleotides in the DNA, which can cause mispairing and subsequently point mutations after several rounds of replication (2). Due to the randomness of ENU, every spermatogonial stem cell

will contain unique mutations throughout the genome, which will give rise to a heterogeneous population of sperm cells. Subsequently, the mutagenized males are crossed with untreated females to generate an F1 population, in which every individual contains these unique heterozygous ENU-induced mutations (Fig. 1).

After an F1 population has been established, the mutations that affect protein function have to be retrieved. Depending on the research question, two different approaches can be used. Firstly, phenotype-driven screens are excellent tools for gene discovery and to dissect developmental and biochemical pathways that underline a given phenotype. The challenge in these forward screens lies in assessing the phenotype of interest in a consistent and systematic manner, i.e., the phenotype should be clearly distinguishable in affected compared to unaffected animals. Multiple phenotypes can be scored in rapid but broad primary screens (3), followed by more detailed, phenotype-specific follow-up screens. Mutations underlying the phenotype of interest are mapped by crossing the mutagenized genetic background into a mapping strain. Subsequently, known single nucleotide polymorphisms between the two strains are genotyped in order to locate the genomic locus containing the mutation.

Secondly, gene-driven screens are extremely useful for determining the function of specific genomic elements. Especially, the availability of fully sequenced genomes has increased the popularity of the latter approach. DNA is taken from F1 animals derived from chemically mutagenized founders, and screened in a targeted way for heterozygous mutations in a set of predetermined genes of interest. Subsequently, interesting mutations, like for example mutations that result in the introduction of a premature translational stopcodon (nonsense) or mutations that change a functionally important residue (missense), are bred to homozygosity by crossing heterozygous animals. Different methods have been used to screen the DNA of F1 animals, like a yeast-based assay that specifically identifies mutations interfering with the translation of the protein (4) and CEL1-based nuclease cleavage (5) and Mu transposase-based detection (6), both depending on heteroduplex DNA detection, which arises after denaturing and reannealing DNA fragments containing a heterozygous mutation. However, mutation discovery by dideoxy resequencing (7) is still considered to be the golden standard, and although this method is relatively expensive, it is well suited for scaling and automation. In addition, all the different mutation types can be picked up at similar sensitivity.

The efficiency of ENU mutagenesis-based reverse genetics approaches is essentially dependent on the induced mutation frequency, which was found to be strain and dose dependent (7, 8), and the effectiveness of the method of choice for retrieving the mutations.
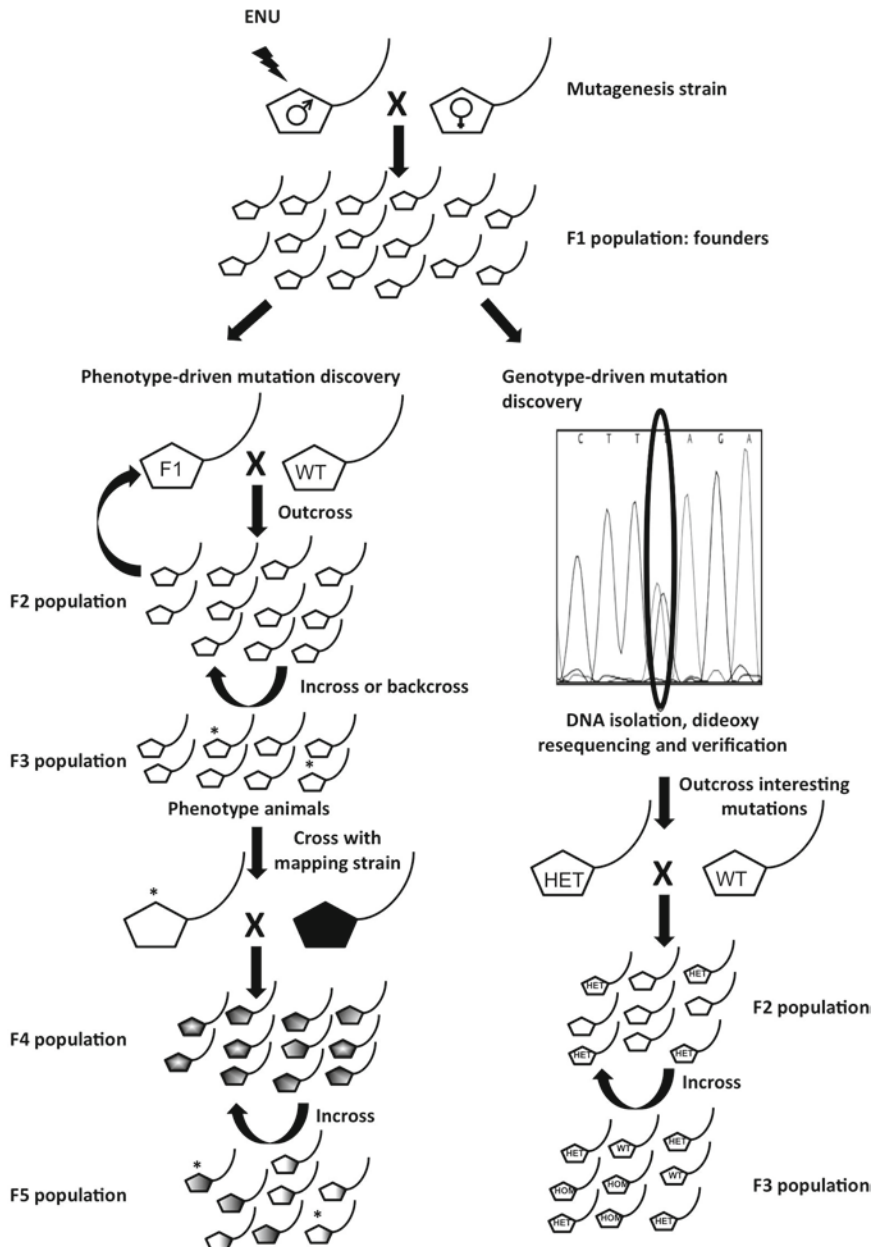
Fig. 1. Schematic overview of the ENU mutagenesis approach. By crossing mutagenized males with untreated females an F1 population, in which all individuals carry unique random ENU-induced mutations, is generated. Depending on the research interest, both genotype-driven and phenotype-driven approaches can be applied for mutation discovery. For a phenotype-driven approach, the F1 animals are crossed out with untreated animals of the same strain (or alternatively of a mapping strain). The animals of the F2 generation are subsequently backcrossed (for high retrieval of homozygous mutations) with the F1 founder or crossed with brother–sister mating. The animals of the F3 generation will carry random. ENU-induced homozygous mutations and can be assessed for the phenotype of choice. For mapping, an animal that displays the phenotype of choice is crossed with an animal of a mapping strain. Subsequently, the progeny is crossed with brother–sister mating in order to generate unaffected (wild type and heterozygous mutant) and affected (homozygous mutant) animals, which can be used for mapping using linkage analysis. For a genotype-driven approach, DNA is taken from the F1 animals, which is screened for heterozygous ENU-induced mutations in genes of interest. After verifying mutations of interest in an independent PCR and sequencing reactions, the F1 animals carrying the mutations are crossed with untreated animals of the same strain. Homozygous mutant animals are obtained by crossing heterozygous mutant animals.

In both mice and rats, the highest observed ENU-induced mutation rate is approximately one mutation every 1.2–1.5 Mb (7, 9–13). However, by taking advantage of a DNA mismatch repair (MMR)-deficient genetic background (14), a system that was shown to be involved in the repair of ENU-induced damage (15), the mutation frequency could be increased by more than twofold in the rat (16). In addition, the mutation spectrum in this MMR-deficient background changed in such a way that the chance of introducing nonsense mutation was increased by 20% (16), resulting in an overall increase in efficiency of about 2.5-fold. This shows that a considerable part of the ENU-induced damage is cleared by DNA repair mechanisms and, importantly, that these repair mechanisms at least in part bias the mutation spectrum of ENU.

Advantages of the ENU mutagenesis technique are that (a) the animals are not transgenic since no foreign DNA was introduced, (b) ENU mainly introduces point mutations, which resembles the most common form of human genetic variation, (c) ENU mutagenesis does not depend on complicated cell culturing techniques or oocyte manipulation, and (d) the technique allows for the retrieval of allelic series, including knockout alleles, but also many missense mutations in genes of interest. These alleles could have hypo- or hypermorphic effects influencing effective gene dosage and/or, they could affect specific residues that are essential for protein–protein interactions or catalytic activity, providing a powerful tool for dissecting gene function.

Here, we describe detailed protocols for performing ENU mutagenesis in rats and mice and for retrieving mutations of interest using either a genotype-driven or phenotype-driven approach.

2. Materials

2.1. Reagents and Equipment

1. Safety wear for protection during ENU solution preparation and the treatment of animals: lab coat, gloves, mouth mask, and goggles.

2. One-way absorption paper and 0.1 M sodium hydroxide (NaOH) solution.

3. Syringes of 10 and 50 ml, needles of 21G, filters (∅ 0.2 μm) disposable cuvettes and parafilm.

4. Ethanol (100%).

5. Phosphate–citrate buffer: 0.1 M NaH_2PO_4, 0.05 M citric acid, and set pH at 5.0 with phosphoric acid. The buffer is filter sterilized.

6. Weigh scale.

7. Spectrophotometer.

2.2. ENU Stock Solution Preparation

1. Roughly 1 h prior to the planned injections the ENU bottle (IsoPac) is unpacked and the metal lid is removed (see Note 1).

2. Using a syringe 5 ml of 100% ethanol is injected and the solution is shaken vigorously (see Note 2).

3. The ENU is dissolved in 95 ml of the phosphate citrate buffer by slowly adding the solution using a syringe and allowing for depressurization, followed by shaking vigorously for approximately 5 min.

4. The ENU solution is filter sterilized ($\varnothing$ 0.2 μm).

5. The concentration of the ENU is determined by measuring the optical density (OD) using a spectrophotometer at 395 nm wavelength of a 1:10 dilution in the phosphate–citrate buffer of the ENU stock solution. Subsequently, the concentration is calculated by assuming that 1 OD unit equals a concentration of approximately 1 mg/ml. (see Note 3).

6. ENU solutions should always be transported in a closed vial. A 0.1 M NaOH solution should always be within reach to quickly neutralize any spillages (see Note 4).

7. Dissolved ENU should be used within 1 h after preparation. Do not store dissolved ENU (see Note 5).

2.3. Waste Removal

All disposals should be soaked in 0.1 M NaOH for a few minutes before discarding. All waste should be designated as hazardous and removed by a central waste facility. The working areas that were used for preparing the ENU stock solution and for injections should be cleaned with 0.1 M NaOH.

2.4. DNA Isolation and Mutation Discovery by Dideoxy Resequencing

1. Instruments and tubes/deep-well plates for tissue collection.

2. Tissue lysis buffer: 100 mM Tris–HCl (pH 8.5), 200 mM NaCl, 0.2% SDS (w/v), 5 mM EDTA, and 100 μg/ml of freshly added Proteinase K.

3. Chemicals: phenol:chloroform (1:1), isopropanol, 70% ethanol, and sterile 10 mM Tris–HCl (pH 8.0).

4. Primers designed for a nested PCR amplifying exons of genes of interest.

5. 5× PCR buffer: 25 mM of tricine, 7.0% glycerol (w/v), 1.6% DMSO (w/v), 2 mM $MgCl_2$ and 85 mM NH_4Ac, and pH 8.7 with 25% ammonia (w/v).

6. Taq polymerase for PCR amplification.

7. dNTPs for PCR amplification.

8. PCR machine with 96- and/or 384-well blocks.

9. Standard gel electrophoresis unit, agarose and ethidium bromide.

10. Sequencing chemicals: BigDye (v3.1; Applied Biosystems) and Sanger BigDye Dilution Buffer version 2 (SBDDv2; Applied Biosystems). BigDye mix should be kept at –20°C and in the dark until use.

11. Ice-cold 80% ethanol and precipitation mix: 80% ethanol (w/v) supplemented with 40 mM NaAc.

3. Methods

3.1. ENU Mutagenesis

3.1.1. Optimal Dosage and Strain Differences

The efficiency of the ENU-driven forward and reverse genetic procedures is essentially determined by the germ line mutation frequency. Theoretically, a higher dose of ENU would induce a higher mutation frequency; however, this also negatively influences fertility and viability. To determine the optimal dose one should consider (1) a treatment that results in sufficient fertile males, which is considered to be at least 25% of the treated animals after a full cycle of spermatogenesis; (2) the mortality rate before and after the treatment as animals should be viable for generating an F1 population; (3) the mutation frequency in the F1 population. Importantly, it was shown that weekly administration of two or three low doses of ENU increased the mutagenicity compared to a single high dose (8).

In addition, the efficiency of ENU is highly strain-dependent, which should be considered when selecting a strain for an ENU mutagenesis experiment. In both mice (8) as rats (7), it was demonstrated that while some strains are well suited for ENU mutagenesis, others are either intolerant for the toxic effects or show suboptimal breeding properties.

The experimental steps for ENU mutagenesis in mice and rats are virtually the same, although the ENU dose per kg of body weight is much lower in the rat. As it is well documented that the optimal dose is dependent on the strain as well, it is recommended to use the documented optimal dose per species and strain (7, 8) or when no data is available for the strain of choice, to first determine the optimal dose.

3.1.2. ENU Treatment

Male mice or rats of 10–12 or 12–14 weeks old, respectively, are used for ENU treatment, which is administrated via an intraperitonial (IP) injection. For safety reasons, two persons should perform the injections, and they should wear gloves, lab coat, goggles, and a mask during the procedure. The volume of ENU solution injected should be calculated for each animal using the equation (optimal strain-dependent split dose of ENU × kg bodyweight)/ (10 × measured OD). If necessary, the ENU solution can be diluted with phosphate–citrate buffer.

1. Prepare a room for the ENU treatment by covering the floor with the one-way absorption paper to absorb any spilled ENU.

2. Weigh the males, calculate the volume of ENU, and administrate it by IP injection.

3. Repeat the treatment weekly until the optimal dose is reached.

4. Monitor the animals' health at least weekly by determining body weight and general status. Generally, a persisting loss of bodyweight (up to 10%) during and/or directly after injections is a reason to take an animal out of the study.

5. Three weeks after the last injection mutagenized males are crossed with untreated females to determine the fertility rate. It is known that the fertility of ENU-treated males drops immediately after the treatment, which is probably due to the toxic effect of the mutagen that results in a depletion of both mature sperm and spermatogonial stem cells and can be indicative for an efficient mutagenesis. Whereas completely sterile mice can regain fertility after the ENU treatment (8), other animals like rats rarely regain fertility (17). The F1 animals that are born during this first breeding are counted and discarded. These animals are not suited for screening to reduce the chance of producing chimeras (see Note 6).

3.2. Generation of the F1 Library

After a full cycle of spermatogenesis (~60–70 days after first ENU injection) the ENU-treated males are crossed with untreated females to produce the F1 progeny, which can be screened for mutations using phenotype-driven or genotype-driven approaches. In case of a genotype-driven approach, a large living repository, which can subsequently be screened for mutations in genes of interest, can be generated. However, if space is limited, breeding using a rolling-cycle model can be applied. In this model, the F1 animals are screened before weaning for a panel of genes of interest and only animals that carry interesting mutations are retained. Notably, male F1 animals that will be discarded can be archived by freezing sperm and tissue samples. These permanent frozen libraries can be screened for mutations in genes of interest indefinitely and have been created for both mice (18) and rats (6).

3.3. Genotype-Driven Mutation Discovery

Depending on the research question different approaches can be chosen for mutation discovery. For the genotype-driven approach, prior knowledge about the gene sequences is required in order to amplify the target regions. Different methods can be applied for targeted mutation discovery in genes of interest. Here, we describe a highly efficient protocol for retrieving mutation using nested PCR amplification followed by dideoxy resequencing. This procedure is cost-effective and can be applied for small-scale experiments as well as easily be automated using standard liquid handling robotic systems for systematic and large-scale screenings.

3.3.1. DNA Isolation

1. A tissue sample, like ear, toe, or tail material, is collected from each F1 animal for DNA extraction.

2. The sample is incubated overnight at 55°C in 400 μl of tissue lysis buffer, preferably under shaking or rotating conditions.

3. If tissue debris is present (which is likely from a tail clip), the samples should be centrifuged at maximal speed for 1 min and the supernatant transferred to a fresh tube. The pellet can be discarded.

4. 400 μl of phenol/chloroform (1:1, v/v) is added, and the mixture is vortexed vigorously for 2 min followed by centrifugation for 3 min at maximal speed. Subsequently, the aqueous layer is transferred to a new tube, and this step is repeated. After the second centrifugation, 300 μl of the aqueous layer is transferred to a new tube.

5. The gDNA is precipitated by adding 300 μl of isopropanol and by inverting the tube ten times. The sample is centrifuged at $16,000 \times g$, at 4°C for 20 min. The supernatant is removed and the pellet is washed with 100 μl of 70% ethanol. The sample is again centrifuged at $16,000 \times g$, at 4°C for 5 min and the supernatant is discarded. The sample is spun an additional minute, and all remaining ethanol is carefully removed using a pipette.

6. The DNA is dissolved by adding 500 μl 10 mM Tris–HCl (pH 8.0) and incubating at 55°C for 10 min with occasional vortexing and can be stored at –20°C.

3.3.2. High-Throughput Resequencing

This protocol assumes the use of standard 384-well plates and a GeneAmp® PCR system 9700 (Applied Biosystems), although other brands are expected to perform equally.

1. The first PCR reaction contains 5–10 ng of template DNA, 2 μl of 5× PCR buffer, 0.1 mM of each dNTP, 0.2 μM of each primer (see Note 7), and 0.2 units of Taq polymerase in a total volume of 10 μl and is carried out using a touchdown thermocycling program (94°C for 60 s; 15 cycles of 92°C for 30 s; 65°C for 30 s with a decrement of 0.2°C per cycle; and 72°C for 60 s followed by 30 cycles of 92°C for 30 s; 58°C for 30 s; 72°C for 60 s; and 72°C for 180 s).

2. After thermocycling, the first PCR reaction is diluted with 20 μl of Milli-Q water, and 1 μl is hatched into the second PCR mix (by pipetting or using 96 or 384 needle pin-replicators), which contains 1 μl of 5× PCR buffer, 0.1 mM of each dNTP, 0.2 μM of each primer, and 0.1 units of Taq polymerase in a total volume of 4 μl. The second PCR is carried out using a standard thermocycling program (94°C for 60 s; 35 cycles of 92°C for 20 s; 58°C for 30 s: 72°C for 60 s; and 72°C for 180 s).

3. It is highly recommended to test several samples of the second PCR on a 1% agarose gel containing ethidium bromide for the presence of the correct amplification product before sequencing.

4. If the correct amplification product is present, the second PCR mix is diluted with 20 μl of Milli-Q, and 1 μl is hatched into the sequencing mix, which contains 1.9 μl of sequencing buffer, 0.1 μl of BigDye v3.1, and 0.4 μM of sequencing primer in a total volume of 4 μl. The sequencing reaction is carried out using a specially designed thermocycling program (40 cycles of 92°C for 10 s, 50°C for 5 s, and 60°C for 120 s).

5. Before proceeding to capillary sequencing, the sequence fragments have to be purified using ethanol precipitation. To each well 30 μl of precipitation mix is added and the mixtures are vortexed vigorously and spun at maximal speed, at 4°C for 40 min in a cooled plate centrifuge. The supernatant is discarded, and the pellet is washed with 30 μl of ice-cold 80% ethanol. The samples are spun an additional 10 min, the supernatant is discarded, and the samples are air-dried for approximately 15 min preferably protected from light. Finally, the precipitate is dissolved in 10 μl of Milli-Q.

6. The plates are analyzed on the 96-capillary 3730XL DNA analyzer (Applied Biosystems) using the standard RapidSeq protocol on a 36 cm array.

7. The sequencing reads that belong to one amplicon are aligned, which facilitates the discovery of heterozygous point mutations (see Note 8).

8. All candidate mutations have to be verified in an independent PCR and sequencing reaction in order to exclude false positives.

3.4. Phenotype-Driven Mutation Discovery

Phenotype-driven screens are excellent tools for gene discovery underlying a specific phenotype interested in a specific phenotype. The F1 population carrying heterozygous mutations can directly be tested to screen for dominant phenotypes or the animals can be outcrossed followed by either a backcross to the F1 founder or brother–sister incrosses (Fig. 1), resulting in F3 animals with random homozygous ENU-induced mutations, which can be screened for recessive phenotypes (19). In addition, mutations underlying a phenotype of choice can be screened for in a genome-wide fashion (20) or by applying a region-specific approach, like using a balancer chromosome (21). Here, we limit ourselves to explaining phenotype-driven screens for recessive ENU-induced mutations.

3.4.1. Breeding Scheme

Recessive mutation discovery depends on crossing animals with heterozygous ENU-induced mutations and scoring the phenotype

of interest in their progeny, which will have random homozygous mutations. After assessing these animals for the phenotype of choice, the causative mutation has to be mapped by crossing affected animals to a mapping strain, followed by brother–sister incrosses of their progeny. These animals are then assessed for the phenotype, and the causative mutation can be mapped using linkage analysis (Fig. 1). Alternatively, F1 animals can be directly outcrossed with the mapping strain. In this way, the F3 progeny that is assessed for the phenotype of choice can be directly used for mapping. The mapping strain is usually a relatively far diverged strain, and sufficient genomic information should be available for both strains in order to perform linkage analysis.

1. Cross the F1 animals with untreated animals of the same or, alternatively, a mapping strain of choice (see Note 9) to generate the F2 generation.

2. An F3 generation, carrying random homozygous ENU-induced mutations in their genomes, is subsequently generated by incrossing the F2 animals or by backcrossing to the F1 founder in order to increase the amount of random homozygous mutations.

3. The F3 animals are screened for the phenotype of choice.

4. Affected F3 animals are crossed with the mapping strain to produce an F4 population in which all individuals are heterozygous for the causative mutation (see Note 10).

5. DNA is isolated of both affected and unaffected animals using the protocol described above.

3.4.2. Mapping   The genomic loci containing the mutations that are underlying the phenotypes of interest can be mapped using linkage analysis, which is currently most efficiently performed using panels of single nucleotide polymorphism (SNPs) for which the different strains have been genotyped (22, 23), as previously described (24, 25).

3.5. Outcrossing   After identifying interesting mutation or an interesting phenotype, it is important to maintain the line by outcrossing the heterozygous carriers with untreated animals of the same strain. In addition, these crosses are important to eliminate unwanted ENU-induced background mutations. Generally, six to ten backcrosses to the parental strain are considered to eliminate most of the background mutations. However, background mutations that are close to the mutation of interest are hard to evade and could be a potential problem. Nevertheless, the chance that a linked mutation landed in a genetic feature that attenuates the phenotype under study is small. Indeed, estimates concerning the impact of the background mutations indicate that the potential problem should to be exaggerated (26). When phenotyping animals, there should always be a 1:1 relationship between phenotype and genotype, and

littermates should always be included as controls. The ultimate control would be the generation of a second allele of the same gene, which would not carry the confounding background mutation and should verify the phenotype.

1. Animals of the F1 population that harbor mutations of interest are crossed with untreated animals of the parental strain.

2. If the causative mutation is known, the progeny are genotyped to determine if they inherited the induced mutation and the heterozygous animals are retained. If the causative mutation is not known, the carriers in the progeny are discriminated by brother–sister incrosses assessing the phenotype of interest in their offspring.

3. Further outcrossing of the mutation to the parental strain genetic background is performed by backcrossing heterozygous animals of each subsequent generation to the parental strain. In every outcrossing stage, homozygous animals can be generated by intercrossing two heterozygotes. This procedure also yields nonhomozygous littermates that could serve as controls in experiments.

4. Notes

1. People who work with ENU should be aware of the genotoxic effects and should take some precautions beforehand. When preparing the ENU stock solution wear gloves, lab coat, goggles, and a mask. ENU solution should be prepared in a high-flow chemical hood. Prepare a 0.1 M NaOH solution to neutralize spillage (see Note 4).

2. It is recommended to thaw the ENU isopac before dissolving it because it greatly facilitates dissolving the ENU.

3. The final concentration of the ENU stock differs between batch numbers and should always be determined by measuring the OD_{395nm}. Typically, this concentration varies between 6 and 8.5 mg/ml. In ENU mutagenesis experiments in rodents, the concentration of ENU is generally measured at a wavelength of 395 nm; however, in other ENU experiments, a wavelength of 238 nm might be used (e.g., zebrafish ENU mutagenesis). For consistency purposes, it is recommended to use OD_{395nm}.

4. Since the half-life of ENU decreases by increasing pH and is less than a minute at high pH alkaline solutions (0.1–1 M NaOH) should always be in close proximity when handling ENU. Any ENU spillage should be poured with 0.1 M NaOH and left stand for few minutes before cleaning.

5. The half-life of ENU in the phosphate–citrate buffer, which is at pH 5.0 is approximately 80 h. At pH 7.0, this decreases to 34 min, which would approximate the half-life of the chemical in the animal body. Therefore, we advise using the ENU solution for injection within 1 h after preparation because of its high instability when dissolved.

6. There is a high chance of retrieving chimaeric animals when animals are used that were born before 10 weeks after the last injection. This is probably due to ethyl adducts that originates from mutagenized sperm in the fertilized oocyte, which can result in heterogeneous mutation fixation in different lineages.

7. The nested PCR set-up described here assumes the use of primers that were designed with an optimal annealing temperature of 58°C. We make use of publicly available Web-based information system called LIMSTILL, LIMS for Induced Mutations by Sequencing and TILLing (http://limstill.niob.knaw.nl), which allows for automated primer design.

8. For aligning and analyzing the sequence reads PolyPhred (27) software can be used, which automatically detects the presence of heterozygous single nucleotide substitutions. The LIMSTILL software (see Note 7) also allows for automated annotation in order to determine the effect of the retrieved mutation.

9. Depending on the phenotype of interest and mutation frequency, another round of outcrossing with an untreated animal of the same genetic background of the mutagenized strain can be assessed in order to dilute the ENU-induced mutations.

10. If affected F3 animals are unable to breed, an F2 animal that was used to generate affected animals can be used to cross with the mapping strain. However, since this F2 animal is a heterozygous carrier of the causative mutation, by chance only 50% of the progeny will be heterozygous carrier of the mutation. Brother–sister incrosses have to be used to determine which individuals are the carriers.

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Chapter 19

Bone Marrow Transplantations to Study Gene Function in Hematopoietic Cells

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Abstract

Immune cells are derived from hematopoietic stem cells in the bone marrow. Experimental replacement of bone marrow offers the unique possibility to replace immune cells, to study gene function in mouse models of disease. Over the past decades, this technique has been used extensively to study, for instance, macrophage function in atherosclerosis. In this chapter, we describe the methods for both full bone marrow transplantations and T cell-specific transfers.

Key words: Immune cells, Macrophages, Bone marrow transplantation, T cells, Mouse, Atherosclerosis

1. Introduction

1.1. General

All cellular components of the blood originate from hematopoietic stem cells in the bone marrow. Pluripotent hematopoietic stem cells give rise to two lineages of specialized white blood cells (Fig. 1). The lymphoid lineage produces B cells, T cells, Natural Killer (NK) cells; the myeloid lineage gives rise to granulocytes, monocytes/macrophages, and dendritic cells (DC). In addition, erythroblasts (the precursors of erythrocytes) and megakaryocytes (which produce platelets) are produced from the myeloid lineage (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 complete new permanently functioning hematopoietic system after transplantation with donor bone marrow. The transplantation results in a gradual replacement of the recipient cells by cells from a donor of
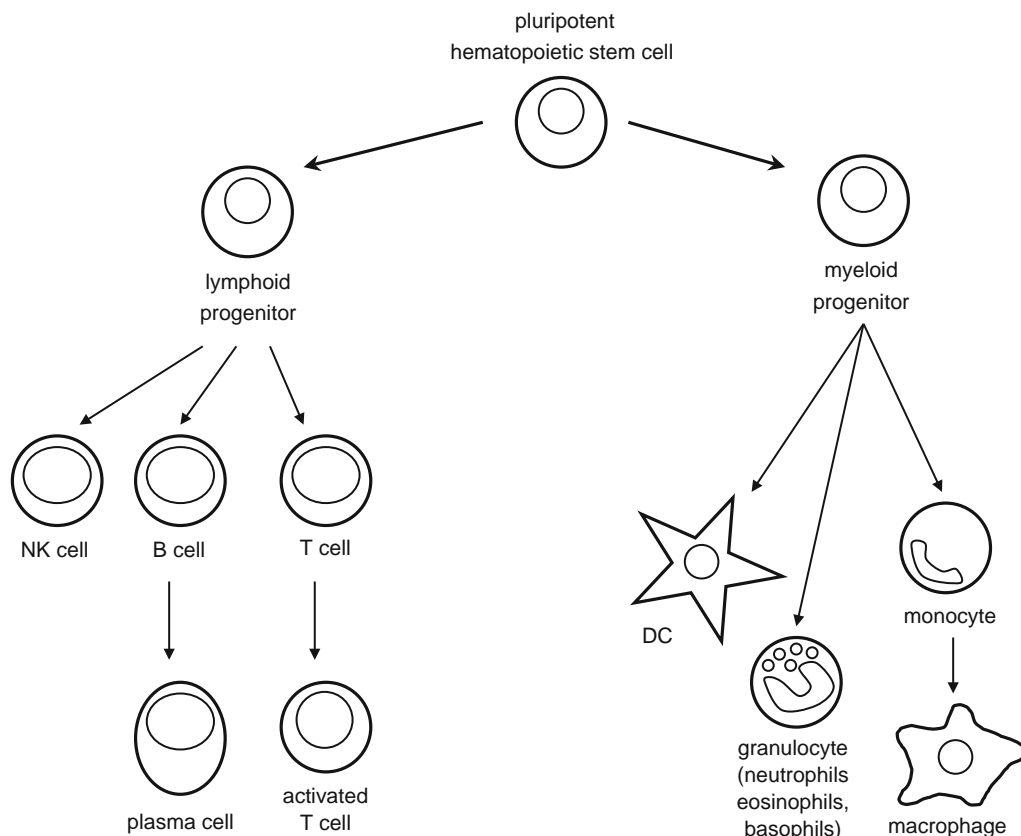
Fig. 1. Hematopoietic stem cell-derived lineages.

choice, establishing a new hematopoietic system, including new monocytes and tissue macrophages.

The bone marrow microenvironment is a complex structure, where 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 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 the 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 due to hemorrhages and septicemia (3).

However, following the administration of donor bone marrow after TBI, repopulation of the marrow occurs and the recipient can survive. Two distinct phases of engraftment can be distinguished. The initial rescue of the hematopoietic system is mediated by already committed progenitors, the final long-term recovery owes to the establishment of pluripotent stem cells in the marrow (4). Four days after the 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 the use of specific integrins and chemokines. By the seventh day after transplantation, the bone marrow cell population is completely restored. Peripheral blood cell counts will return to normal levels after 4–6 weeks.

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 (major histocompatibilty complex (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 to adapt 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 not only in the replacement of the monocyte/macrophage system in the mice, but also in the replacement of the additional white blood cells. The second part of the protocols in this chapter 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 cells and B cells in vitro (5). Among others, these include the methods to measure 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 biological 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 the 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 are 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 that makes 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 individually ventilated cages (IVC).
3. Autoclaved water.
4. Neomycin solution (Gibco).
5. Polymyxin B Sulphate solution (Gibco).
6. X-ray apparatus.
7. Mouse container (to irradiate the mice in).
8. Scissors.
9. Forceps.
10. Down-flow (cell culture) cabinet.
11. Ethanol.
12. Phosphate-buffered saline (PBS).
13. 19 G, 25 G, 27 G needles.
14. RPMI1640 + 2% fetal calf serum (FCS) + 5 U/ml heparin.

2.2. T Cell Transfers	1. Complete RPMI or DMEM with 5% FCS.
2.2.1. Preparation of Single Cell Suspensions from Spleen	2. 60×15 mm petri dishes.
	3. Scissors and forceps.
	4. 6 ml syringes with 19 G 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 mM), 37.2 mg Na_2EDTA (0.1 mM); Add 800 ml H_2O and adjust pH to 1 N HCl. Finish by adding H_2O to 1 L and filter sterilize through a 0.2 µm filter, and 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: Hanks Balanced Salt Solution (HBSS) without Ca⁺⁺, Mg⁺⁺, and phenol red containing 10% heat-inactivated FCS and 20 mM HEPES.

4. Goat antimouse IgG-coated magnetic beads. (Dynabeads M-450, or sheep antirat IgG-coated magnetic beads Dynabeads M-450).

5. Magnetic separation device (Dynal).

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 (two pairs of 10 cm half-curved).

6. 9 mm wound clips and clip applier.

7. Pentobarbital.

8. 1 ml syringe with 27-G needle.

3. Methods

3.1. Bone Marrow Transplantation

3.1.1. Preparation of Acceptor Mice

Seven days before the bone marrow transfer:

1. Recipient mice are transferred to filter-top or IVC cages.

2. Mice are fed a regular chow diet.

3. Mice are put on water to which neomycin (100 mg/L) and polymyxin B sulphate (60,000 U/L) are added, until

4 weeks after the transfer. The drinking water is replaced with fresh bottles twice a week.

One day before the bone marrow transfer:

4. In a cross flow, mice are transferred from their cages to an irradiation box (which can be a plastic box suitable for five to ten mice).

5. Mice are subjected to a lethal dose of TBI using a röntgen source (10 Gy) (see Note 1).

6. After the irradiation, mice are transferred, in the cross flow, back to their 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 G 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 G needle, transfer it to a new tube and then pass it through a 25 G needle.

11. Count the cells.

12. Spin the cells down at $250 \times g$, 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 a cross flow cabinet and inject the irradiated mice intravenously with 100 µl of cell suspension using a 27 G needle (see Note 4).

2. Six 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

The following protocol 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 of 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 G needle.

5. Expel suspension into a 50 ml polypropylene centrifuge tube through a 100 μm nylon sieve. Wash petri dish with 4 ml of medium.

6. Centrifuge 10 min at $200 \times g$. Discard supernatant.

7. Resuspend pellet in Red Blood Cell lysing buffer, 5 ml per spleen.

8. Incubate 5 min at room temperature with occasional shaking.

9. Add 5 ml of medium and centrifuge at $200 \times g$, and discard supernatant.

10. Resuspend pellet in medium and centrifuge at $200 \times g$, and 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 vs. T cells or CD4 T cells vs. 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 isolations 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 the 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. Once the optimum antibody concentration has been determined, prepare a 10× antibody stock solution.

2. Resuspend splenocytes in coating medium at a concentration of 2×10^7 cells/ml.

3. From the 10× antibody stock solution, add appropriate volume (i.e., one-tenth 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 $150 \times g$. 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 antimouse 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 approximately 5 min. Aspirate washing fluid. Repeat this procedure once.

6. Resuspend washed beads in an equal volume of coating buffer that was originally pipetted 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

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 direct against MHC class II (I-A or I-E, accessory cells, and B cells), B220 (B cells), CD3 (all T cells), CD4, and CD8 (see Notes 7 and 8).

3.2.3. Thymectomy

Development and maturation of T cells is dependent on the thymus. Therefore, the 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 the 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 the 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 its 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 (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 are 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 both the 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 wound and warm mouse with heat lamp.

13. The mouse should recover from surgery and anesthesia in about 60 min.

3.2.4. Adoptive Transfer of 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, full hematopoietic stem cell replacement is established, meaning that all hematopoietic cells of the recipient are of donor-origin. However, sometimes, replacement 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. Also other genetic differences, such as the absence or the presence of a specific gene in the donors, can 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 lymphnodes 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 lymphnodes, and >90% in the thymus.

7. T cells from mouse spleen and lymphnodes 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. In Table 1 a list of hybridomas available from the ATCC is given with antigen specificities and corresponding commonly used mouse strains.

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# TIB 207, CD8: ATCC# 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 the rearrangement of T cell receptor and immunoglobulin genes is lacking resulting in the

Table 1
Monoclonal antibodies against mouse I-A/I-E (2)

Primary specificity	Cross-reactive specificity	Hybridoma	Isotype	ATCC#
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

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 the rearrangement of T cell receptor and immunoglobulin genes. Consequently, these mice 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 requires 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.

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Useful Websites

10. http://www.protocol-online.net/index.htm (A collection of protocols for biomedical research including immunological research.)

11. http://tvmouse.compmed.ucdavis.edu/virtualNecropsy/ (A virtual mouse necropsy on the web including a schematic overview of the location of the various lymphnodes.)

12. http://www.rodentia.com/wmc/ (Everything about (mutant) mice.)

13. http://www.invitrogen.com/site/us/en/home/brands/Dynal.html (Vendor of immunomagnetic beads.)

14. http://www.jax.org/ (Vendor of (mutant) mouse strains.)

15. http://www.atcc.org/ (American Type Culture Collection.)

Chapter 20

Adenovirus-Mediated Gene Transfer

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Abstract

Recombinant adenoviruses are attractive vectors for short-term expression in mouse liver and primary cell lines. Various versatile vector systems have been developed which can be used for the reliable production of recombinant adenoviruses. This protocol describes the entire process for the production of recombinant adenoviruses using the AdEasy system. This protocol will give a practical step-by-step description from the cloning of the gene of interest until the *in vivo* administration in mice. The entire process will take about 8 weeks to complete.

Key words: Recombinant adenoviruses, Adenovirus, AdEasy, pAdEasy, Viral transduction, Transduction

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 enables a very 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 biological effects of the transgene product should be detectable within this relatively short period of transgene expression. However, it has been demonstrated for many physiological processes, including, for example, lipoprotein metabolism and blood coagulation that this period of gene expression is sufficient to determine the effect of overexpression or downregulation 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 either encode an intracellular protein, a membrane protein, or a secreted protein. Alternatively, the expression of a dominant variant of a gene enables analysis in the presence of a functional endogenous gene. In this setting, the dominant 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. In addition to these overexpression strategies, adenoviral-based RNAi vectors have been developed which can be used for specific (inducible) gene knockdown both *in vivo* and *in vitro* (1, 2). Recombinant adenovirus that expresses shRNA has been successfully used to induce gene silencing in the mouse liver (3).

In the following sections, the different steps necessary to go from a candidate gene to injection of the adenoviral vector into a mouse is 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 adenoviral vectors and the retargeting of adenoviral vectors to infect tissues other than the liver.

2. Materials

2.1. 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: PmeI, PacI, BstXI.
4. High efficiency electro-competent *Escherichia coli* BJ5183 cells.
5. 2.0 mm electroporation cuvettes.

6. Electroporator.

7. L-Broth (LB) medium.

8. LB-agar plates containing 50 µg/ml kanamycin.

2.2. Generation of Infectious Adenovirus

1. 293 or 911 cell line.

2. Tissue culture medium: DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml Penicillin, 100 µg/ml Streptomycin, 20 mM Biotin, 20 mM Glutamine.

3. 6-well tissue culture dishes.

2.3. Expansion of Adenoviral Vectors

1. 293 or 911 cells and tissue culture medium (see Subheading 2.2).

2. Various sizes tissue culture flasks.

3. Phosphate-buffered saline (PBS) with calcium and magnesium (PBS++).

4. Horse serum (HS).

5. PBS++ with 2% HS (PBS/HS).

6. Tissue culture medium with 2% HS.

2.4. 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.

3. Beckman SW41 (swingout) rotor and Ti70 (fixed angle) rotor or equivalent.

4. 10× TD buffer: 1.37 mM NaCl, 50 mM KCl, 7.3 mM Na_2HPO_4, 250 mM Tris–HCl, pH 7.8, autoclaved.

5. 200× Ca^{2+}/Mg^{2+}: 0.18 M $CaCl_2$, 0.1 M $MgCl_2$, autoclaved.

6. Dialysis buffer: 1× TD with 1× Ca^{2+}/Mg^{2+} in ddH_2O.

7. 10× sucrose solution in H_2O (50%), filter sterilized.

8. 10× salt buffer: 1.4 M NaCl, 49 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.8 filter sterilized.

9. Sucrose dialysis buffer: 1× sucrose solution with 1× salt buffer in ddH_2O.

10. Slide-A-Lyzer cassettes (Pierce, MWCO: 10,000, volume: 0.5–3 ml).

2.5. Titration of Adenoviral Vector Preparations

1. PBS/HS (see Subheading 2.3).

2. 2× agar solution: 1.7% Agar Noble in 40 mM HEPES pH 7.2, autoclaved.

3. 2× F-15 medium: double concentration medium made from powder, add sodium pyruvate (final concentration 2 mM) and sodiumbicarbonate (final concentration 3 g/L), filter sterilized.

2.6. Detection of Replication Competent Adenovirus

1. Forward primer: 5′-GGGTGGAGTTTGTGACGTG-3′ (=RCAf1).

2. Reverse primer: 5′-TCGTGAAGGGTAGGTGGTTC-3′ (=RCAr2).

3. 10× PCR buffer without $MgCl_2$.

4. Taq polymerase.

5. $MgCl_2$: 25 mM.

6. Proteinase K: 20 mg/ml.

7. dNTPs: 10 mM (2.5 mM per nucleotide).

8. tRNA: 10 mg/ml.

9. H5*dl*7001 or wild type Ad5, proteinase-K treated.

10. pJM17 DNA 10 ng/μl.

11. $T_{10}E_1$: 10 mM Tris–HCl, pH 7.4, 1 mM EDTA.

3. Methods

Adenoviruses were first isolated as causal agents for adenoid inflammation in children, and 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 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 Coxacky Adenovirus Receptor (CAR). Adenoviruses contain some 36 kb of double-stranded 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 are derived 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 a review on adenovirus biology, see (4).

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 are 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 (5) or bacteria (6). 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. in the Vogelstein laboratory and termed the AdEasy system was proven to be very reliable and practical in our hands and is described below (6). Alternative methods for the generation of adenoviral vectors have been described to be successful (7–12) but are not discussed in this paper. In addition, several commercial systems that eliminate the need for homologous recombination in bacteria or mammalian cells have been developed. The Adeno-X™ system sold by clontech makes use of two extremely rare restriction enzyme sites (PI-*Sce* I and I-*Ceu* I) for the transfer of the expression cassette from the shuttle vector to the adenoviral vector. Invitrogen sells the Virapower™ adenoviral expression system. Shuttling of the expression cassette from the shuttle vector to the adenoviral destination vector is mediated by the bacteriophage lambda-based recombination system (Gateway®). For both systems, vectors have been developed for the expression of proteins or shRNA molecules. As these vectors are only commercially available and provided with detailed technical support, they are not discussed in this paper.

3.1. Generation of Adenoviral Vectors by Homologous Recombination in Bacteria: The AdEasy System

The general outline of the AdEasy system is as follows: the gene of interest is first cloned into a shuttle vector. Four types of shuttle vector are available, with and without CMV promoter/poly-adenylation site, and with and without separate CMV-driven green fluorescent protein (GFP) gene. The resultant (kanamycin-resistant) plasmid is linearized by digesting with 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.[1] Recombinants are selected for kanamycin resistance, and

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[1]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.
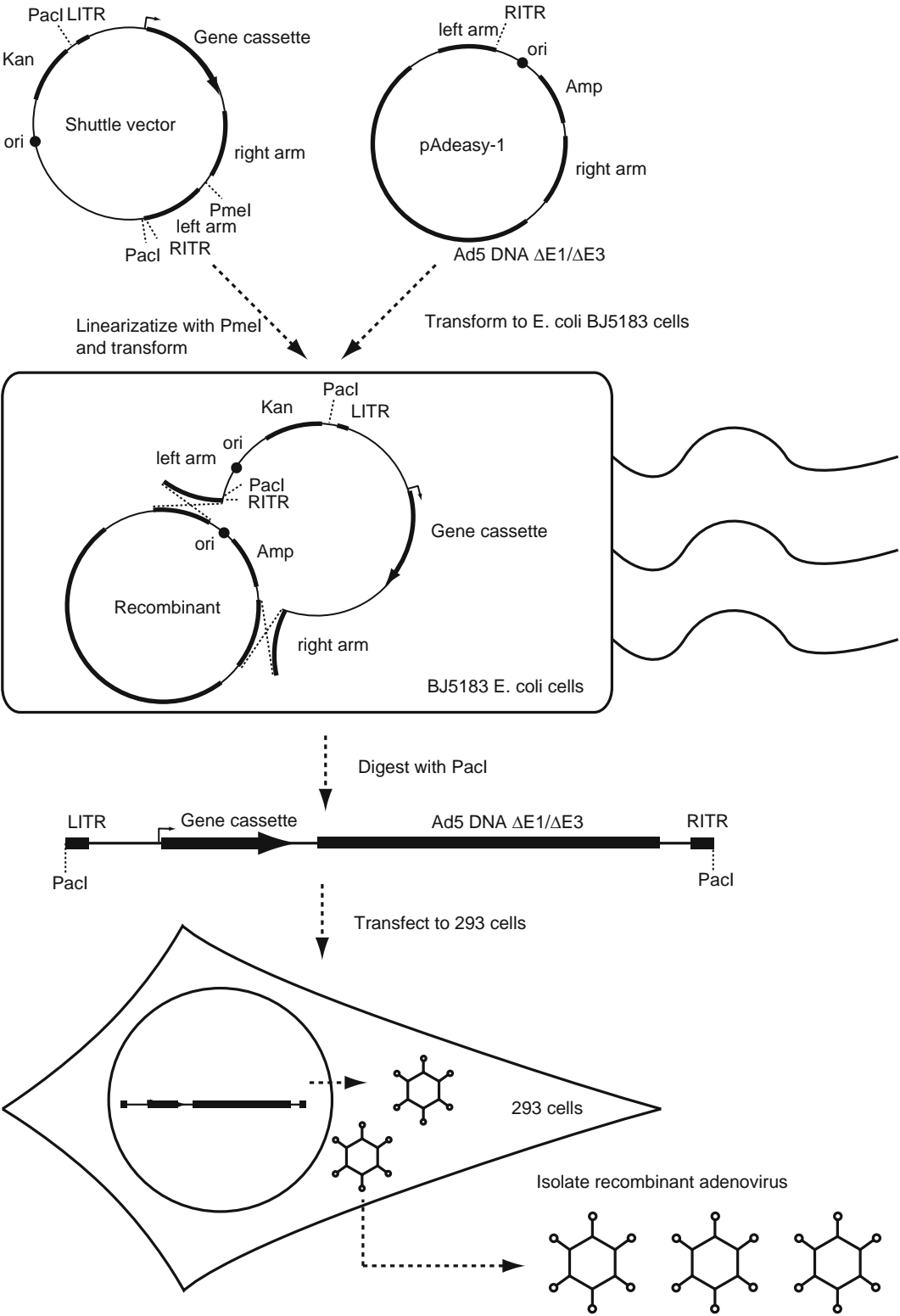
recombination confirmed by restriction endonuclease analyses. The use of *E. coli* BJ5183 cells for recombination is critical for the efficient recombination to occur. Alternatively, one can make use of AdEasier cells. AdEasier-1 cells are BJ5183 derivatives which already contain the AdEasy-1 plasmid (13). In order to generate recombinant vectors ready to transfer into 293 cells, one simply has to transform the AdEasier-1 cells with a shuttle vector containing the gene of interest, and then proceed as with the standard AdEasy system. 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 AdEasy system can be obtained from the Vogelstein laboratory (http://www.coloncancer.org/adeasy.htm) or purchased from Stratagene (http://www.stratagene.com). The Vogelstein laboratory maintains an excellent Website for additional information, help, and protocols: http://www.coloncancer.org/adeasy.htm.

The E1A expressing 293 cell line is widely available or can be purchased through ATCC (http://www.atcc.com), and 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 (14). 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). More recently, the PER.C6 cell line was developed (15). These cells contain the adenovirus serotype 5 E1A- and E1B-encoding sequences under the control of the human phosphoglycerate kinase promoter, but lack sequence overlap with matched adenoviral vectors. The yields from these cells are similar to those obtained from 911 and 293 cells, but are completely devoid of replication-competent adenovirus (RCA). PER.C6 cells are also able to grow in serum-free medium greatly reducing culturing costs (16). This cell line can be purchased from CruCell B.V, Leiden and is in particular of interest if clinical grade adenoviral batches are desired.

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.

Fig. 1. Schematic overview for the production of recombinant adenoviral particles. The gene cassette is cloned in the Shuttle vector and cotransformed with pAdeasy-1 in BJ5183 *E. coli* cells. Following recombination, the plasmid is isolated, linearized with *Pac*I, and transfected in 293 cells. Recombinant adenoviral particles are subsequently released from the cells and concentrated by means of cesium chloride purification.

2. Linearize the newly constructed plasmid with PmeI (make sure digestion is complete and gel-purify the fragment, see Note 1).

3. Ethanol-precipitate and resuspend the DNA in ddH$_2$O.

4. Prepare adenoviral backbone plasmid DNA (pAdEasy-1) resuspended in ddH$_2$O (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 cuvettes at 2.5 kV, 200 W, and 25 mF in a BioRad Gene Pulser (these settings should give a time constant between 4–5 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 mini-prep DNA according to standard procedures. Note that the smaller colonies often represent the recombinants.

10. Screen clones by analyzing their supercoiled sizes on agarose gels, comparing them to pAdEasy-1 and shuttle vector controls.

11. Subsequently, digest DNA from positive clones with BstXI. If the insert does not harbor BstXI sites, the 8.2 kb DNA-fragment found in pAdEasy-1 digested with BstXI 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 and DNA sequencing. Although sequence runs are typically shorter compared to sequence runs on smaller plasmids, reads between 400 and 600 bp are obtained in most cases from clean silica column-based DNA isolations.

13. Before the production of virus, it is highly recommended to verify correct mRNA and protein expression from pShuttle-CMV by transient transfection in 293 cells. This is possible as pShuttle contains the necessary elements for transient expression in mammalian cells.

3.1.2. Generation of Infectious Adenovirus

1. Grow 911 or 293 cells to approximately 80% confluency on a 6-well plate (~1 × 10^6 cells per well).

2. Transfect recombinant adenoviral vector DNA, digested with PacI, ethanol-precipitated, and resuspended in ddH$_2$O 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). (20–30% of the cells should be fluorescent.)

4. Harvest the well on day 12 posttransfection by vigorous pipetting (five to seven adenovirus-plaques with cytophatic effect (CPE) 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 (~2 ml) to infect 1 well of a 24 well-plate with 293 or 911 cells (~4 × 10^5 cells).

6. Transfected cells are monitored for GFP expression and collected on day 7 postinfection.

7. The obtained viral lysate (~1 ml) is used for titration in a plaque-assay (see Subheading 3.4.1) 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 1,000 infectious viral particles per cell. Adenovirus infection is lytic and two options are available to harvest the amplified virus. (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 (17). (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. Note that the number of repeated rounds of infection should be followed accordingly and kept to a minimum in order to reduce the chances of producing RCA (see below).

1. The lysate that is obtained from a single well of a 24-well plate (Subheading 3.1.2, step 7) is used to infect 50–75 cm² 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′ at $100 \times g$ in a table top centrifuge).

6. Aspirate nearly all 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′ at $1,000 \times g$ in a table top centrifuge).

9. Remove virus-containing supernatant. This centrifuged crude lysate can be stored at –20°C until further processing.

After titration of the prep, repeat **steps 1–7** on three to five 175 cm² plates and subsequently on ten to thirty 175 cm² plates. If tissue culture incubator space is a problem, 500 cm² triple flask with a volume comparable to that of a 175 cm² flask can be used.

3.3. Purification of Adenoviral Vectors

The centrifuged 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/cm³) to a clear ultracentrifugation tube and overlay with 4 ml of CsCl ($d = 1.2$ g/cm³).

2. Carefully overlay the CsCl block gradient with the centrifuged virus-containing supernatant (up to 4 ml of supernatant).

3. In an ultracentrifuge, spin for ≥2 h at an average speed of $110,000 \times g$. Use a swinging bucket rotor.

4. Aspirate the white band(s) that is located roughly at the interface between the two densities using an 18G needle attached to a 2-ml syringe.

5. Transfer the solution to a quick-seal ultracentrifugation and completely fill the tube with CsCl ($d = 1.33$ g/cm³). Seal the tube.

6. Spin overnight in an ultracentrifuge, at $170,000 \times g$ in a fixed angle rotor.

7. Aspirate the lowest white band using an 18G 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 1× 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 1× TD buffer, add fresh, cold, 1× Sucrose buffer and dialyze for an additional 3 h, at 4°C.

10. Remove virus solution from the dialysis cassette using an 18G 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 two to three 30 µl aliquots for titration of the virus (see Subheadings 3.4.1 and 3.4.2).

3.4. Analysis of Adenoviral Vector Preparations

It is recommended that at all stages of virus amplification the infectious titer of the virus is 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. As an alternative approach, the end-point dilution assay (see Subheading 3.4.2) can be successfully used on 293 cells for the determination of the infectious titer. Note that viral titer determination by the plaque assay or limited dilution assay can only be performed in cell lines that express the E1 proteins. 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 AdEasy vectors enables this event to occur. Once RCA is present in a preparation, it will quickly overgrow the recombinant replication incompetent virus. Besides being a safety concern to the investigator, it should be noted that RCA may cause severe side effects when injected in mice.

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 pipetting 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 -9

(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 2× 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 2× 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 – or pfu/ml) = (number of plaques in highest dilution) × $10^{(-dilution\ factor)}$.

3.4.2. End-Point Dilution Assay (Kärber Method)

In contrast to the plaque assay, the end-point dilution assay can be applied to determine the infectious titer in 293 cells. Instead of counting the number of plaques per well, the number of wells containing CPE is scored.

1. Seed 293 cells in 96-well plates at a density of 10^4 per well in 100 µl growth medium. Incubate for 24 h.

2. Prepare tenfold serial dilutions of your virus in DMEM without serum. Start with a 1:100 dilution. The appropriate rate for testing is 10^{-3} to 10^{-10} and thus covers eight rows of the 96-well plate.

3. Add 100 µl diluted virus to each well in columns 1–10 (columns 11 and 12 serve as a control for cell viability), and incubate for 10 days in a humidified CO_2 at 37°C.

4. Score each individual well for CPE using normal light microscopy. The noninfected control wells serve as a negative control example for scoring. The test is valid only if the following criteria are met:

The noninfected wells should not show CPE, the 10^{-3} dilution should be positive for all wells and the $10E^{-10}$ dilution should be devoid of CPE.

5. Calculate the fraction of CPE-positive cells for each row (number of CPE positive cells divided by 10).

6. Calculate the the sum of the fraction of CPE positive cells (S) (see example below and use a value of 1 for the 10^{-1} and 10^{-2} dilutions).

7. Calculate the tissue culture infectious dose 50% (TCID50) using the Kärber formula. $TCID50 = 10^{d+(S-0.5)}$. The d stands for log(base 10) dilution interval. Thus, when using tenfold dilutions, $d = 1$.

8. Calculate the TCID50/ml. If you had cells with 200 μl medium: $TCID50/ml = TCID50 \times 1,000/200$

9. $Pfu/ml = TCID50/ml/5.0119$ (TCID50 test gives a 0.7 log (=5.0117) times higher titer compared to the plaque assay). (Table 1)

3.4.3. 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 the ratio up to 10,000 has 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).

In undiluted samples, an OD_{260} of 1 equals 5×10^{11} of total virus particles in the cuvette.

Table 1
Example to calculate the infectious titer using the end-point dilution assay

Dilution	Fraction CPE positive cells
10^{-8}	$10/10 = 1$
10^{-9}	$10/10 = 1$
10^{-10}	$10/10 = 0.4$
10^{-11}	$8/10 = 0$
10^{-12}	$3/10 = 0$
10^{-13}	$0/10 = 0$
Sum	$2.4 + 7$ (dilutions 10^{-2} and 10^{-1}) $= 9.4$
TCID50	$=10^{(1+9.4-0.5)} = 7.94 \times 10^9$
TCID50/ml	$=7.94 \times 10^9 \times 1,000/200 = 3.97 \times 10^{10}$
Pfu/ml	$=3.97 \times 10^{10}/5.0119 = 7.92 \times 10^9$

*3.4.4. Detection
of Replication Competent
Adenovirus*

It is extremely important to screen any virus batch for the presence of RCA. RCA is defined as adenovirus that can replicate independently in normal human cells. In most cases, RCA arises 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 and the E1 region ends up in the recombinant vector in *Cis*-configuration, thus alleviating the need for E1 in trans (for a review on RCA, see (18)). We have developed a sensitive method for the detection of RCA, using PCR (19) (also, see Notes 4 and 5).

1. Preparation of the virus:
   (a) Use 45–90 µl of a freeze/thaw isolate (Subheading 3.2, step 9). Note that the uncentrifuged crude lysate from adenoviral vectors grown on 293 cells may not be used, since the (abundant) 293 genomic DNA harboring the E1 region will give a false positive signal. In this case, use 1–2 µl of CsCL-purified adenovirus (Subheading 3.3, step 10).
   (b) Add 10× PCR buffer to 1× final concentration
   (c) Add 1 µl proteinase K, incubate 16 h at 42°C
   (d) Boil 5′ to inactivate proteinase K
   (e) Centrifuge 5′ at 12,000 × *g* and retain supernatant.

2. Preparation of control samples: make a serial dilution H5*dl*7001 or wild type Ad5 virus in $T_{10}E_1$, resulting in 700, 70, 7, and 0.7 pfu/µl.

3. Prepare 1× PCR mix, and the various PCR reactions according to the pipetting schemes shown below:

PCR settings

5′ 94°C	
40″ 94°C	32 cycles
45″ 63°C	
50″ 72°C	
8′ 72°C	
Hold 4°C	

4. Add one-sixth volume of 6× DNA loading buffer, and load 20 µl of each PCR reaction on an agarose gel. RCA-band: 615 bp (Ad5 nucleotides 52–693). (Tables 2 and 3.)

Note that only if the diagnostic band (615 bp) in the reaction with 0.7 pfu is visible, the assay can be considered optimal.

Table 2
Composition of the 1× PCR reaction mixture

Component	1× PCR mix (μl)
10× 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

The amounts are given for analysis of one sample

Table 3
Setup of the PCR reactions for the detection of RCA

	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
1× PCR-mix	45	45	45	45	45
Water	4	4	4	4	0

The positive control (pos ctrl) can be any virus containing the E1 region
A negative (water) control should also be included

3.5. In Vitro Application of Adenoviral Vectors

The expression and function of the gene or shRNA cassette cloned into the adenoviral vector is generally first analyzed *in vitro*. As an initial analysis before taking this step, a cell lysate from the producer cell line may also be analyzed (given the prerequisite that transgene expression is driven from a promoter that is active in the 293 producer cell line, such as the CMV promoter).

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) (primary) 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. Surprisingly, mouse primary hepatocytes are an order of magnitude more susceptible compared to the frequently used hepatocarcinoma cell line HepG2 (which is of human origin). As a starting point, an MOI of 10 and 100 can be used on primary hepatocytes and HepG2 cells, respectively. In order to determine an optimal MOI, the "Track" series of vectors that are part of the AdEasy 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 more pure 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, etc.) the virus dose that will result in liver toxicity will vary. It is useful to monitor liver enzymes (such as ALAT or ASAT) 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, >99% of the injected virus will be cleared from the circulation. Gene expression that peaks at days 4 and 5 is significantly decreased at day 8 and generally is detectable up to day 12–14 depending on the half-life of the expressed mRNA and protein. The rapid decline in gene expression seems mostly related to downregulation of the transcriptional activity of the CMV promoter. Immune responses to infected cells that have leaky expression of adenovirus backbone genes also contribute to downregulation of gene expression. At high doses, peptides from the viral coat proteins are also exposed at MHC class 1 molecules on the cell surface of hepatocytes (20). This sensitizes the hepatocyte to perforin-mediated cell lysis induced by cytotoxic T lymphocytes. Other protective responses have been described as well (4), but are beyond the scope of this paper.

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 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.

As an alternative method, the adenovirus may be injected through the retro-orbital venous sinus. Both methods have been proven to be equally effective for the injection of various drugs (21).

3.7. Developments in Adenovirus-Mediated Gene Transfer. Different Generations 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 (22, 23). 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 30°C due 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 30°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 (24, 25). Other second-generation systems are based on additional deletions in the E4 region (26, 27). The E4 is expressed in a trans-setting within the producer cell line and transactivated by E1. Prolonged expression of E4 is toxic and inducible cell lines have been generated in order to maintain the viability of the cell line. An additional type of second-generation system is based on the deletions in all the early regions (E1–E2–E3 and partially E4) (28). Although initially promising, second-generation systems are

often of limited use as the viral yields are significantly lower compared to the first-generation vectors.

Helper dependent or gutted adenoviral vectors or now referred to as third-generation vectors that lack all endogenous adenoviral genes (29). 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 flanked by a functional packaging signal. The 36 kb of space that is available for the transgene is filled with stuffer DNA in this "gutted" vector.

The helper dependent vector is amplified on a 293 or 911 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 functional packaging signal will result in preferential packaging of this vector into the virion. Similar systems based on the FLP/frt and attB-attP-PhiC31 recombination systems have also been reported (30, 31). Using helper dependent vectors long-term expression has been demonstrated (32).

Although much effort has been put in the development of adenoviral systems with a prolonged expression, adeno-associated virus is now the viral system of choice for long-term expression. Novel vectors based on a self-complementary system show enhanced expression, low immunogenicity and expression periods over 1 year (33, 34). Although adeno-associated virus can integrate at a specific locus in chromosome 19, this feature is removed from most vectors for safety reasons, and the viral genome remains present episomally. Despite all these advantages, adeno-associated viral systems have several disadvantages over adenoviral vector systems. High viral titers are difficult to obtain and the encapsidation limit for self-complementary adeno-associated virus is around 3.3 kb making the expression of many genes impossible.

3.8. Altering Tissue-Specificity of Adenoviral Vectors

Recombinant adenovirus vectors can be generated in a relatively short period of time can be amplified to high titers and 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 (35, 36). Initially, the knob of the fiber protein binds a cellular receptor called CAR (37).

Subsequently, the pentonbase binds αVβ1 or αVβ3 integrins via a 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 (38), added to the C-terminus of the fiber-knob (39, 40), and inserted into the hexon protein (41). 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 (42–44). Also, the fibers of the CAR-binding adenoviral vectors have been replaced by fibers from adenoviruses belonging to different serotypes and recognizing other receptors (45–48). 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 (49).

4. Notes

1. Probably, the most critical step in the AdEasy system is the recombination of the shuttle vector with the adenoviral backbone plasmid in the BJ5183 bacteria. Digestion of the shuttle vector with PmeI 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. As mentioned above, AdEasier greatly enhances the odds of successful recombination. AdEasier-1 cells are BJ5183 derivatives which already contain the AdEasy-1 plasmid (13). These cells can be prepared by the transformation of pAdEasy-1 into competent BJ5183. Thereafter, the transformants are cultured and made electrocompetent. AdEasier cells are also commercially available from Stratagene. Before the production of homemade electrocompetent cells, it is important to pick individual clones to verify that no unwanted detrimental rearrangements have occurred as the BJ5183 strain has a high frequency of homologous recombination.

2. It is recommended that at all stages of virus amplification the infectious titer of the virus is 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 will result in massive cell death due to viral toxicity.

3. 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.

4. Once RCA is detected in a virus preparation, it is pertinent to stop amplification and perform plaque purification. RCA may be harmful for the investigator or the mice to be transduced. Plaque purification 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 pipette 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 the formation of RCA, novel E1A expressing cell lines have been developed that lack overlap between the E1A region that is present in the cellular genome and the recombinant adenovirus and thus prohibit recombination between these two sequences (15, 50).

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