

Mouse Knockout Models of Hypertension

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Summary

Gene-targeting technology allows the planned alteration of any gene in the mouse genome and has been very successfully employed to study the function of numerous gene products in a complete animal. The method includes the design of a suitable targeting construct, its transfection into pluripotential embryonic stem cells, selection for cells in which one allele of the endogenous gene has been exchanged for the construct by homologous recombination, and the transfer of these cells into host blastocysts. The blastocysts are then transferred into the uterus of a pseudopregnant foster mother giving birth to chimeras, which are bred to yield heterozygous and finally homozygous mutant mice for the targeted gene.

Key Words: Embryonic stem cells; homologous recombination; gene targeting; knockout mouse; electroporation; double selection; gancyclovir; functional genomics; Chimera; pseudopregnancy.

1. Introduction

The genomes of humans and mice have been completely sequenced and about 30,000 genes have been detected in both organisms (**1–3**). The challenge is now to elucidate the function of all these genes. The most powerful way to perform such “functional genomics” is gene targeting, allowing the targeted overexpression, deletion, or mutation of a gene product in a mouse and thereby studying its function in the context of all other unaffected genes. During the 15 yr in which this technology has been available, several thousand genes have been knocked out, i.e., they have been functionally ablated by gene targeting. Among these are numerous genes important for blood pressure regulation and the pathogenesis of hypertension (for review, *see refs. 4 and 5*). More than 80% of all genes remain to be characterized by this technology.

Besides the complete ablation of a gene, gene targeting also allows the introduction of subtle mutations into a gene and the conditional, i.e., tissue- or time-dependent knockout of a gene. The technologies developed for these pur-

poses will not be described in this chapter. The reader is referred to recent reviews in this field (6–8). This chapter will focus on the classical knockout technique, allowing the complete ablation of a gene.

Gene targeting technique is based on two lines of research that were developed in the early 1980s and converged a few years later (9). At this time, Martin Evans had developed cell lines from the inner cell mass of mouse blastocysts that were immortal and pluripotent, i.e., they could differentiate into all tissues of a developing embryo in vitro and gave rise to teratocarcinomas when injected into adult mice. Moreover, when these so-called embryonic stem (ES) cells were introduced into recipient blastocysts, they took part in embryonic development and a chimeric mouse was born, in which ES cells had populated all tissues including the germline. Consequently, in the offspring of these mice, one haploid genome of some animals was entirely derived from the ES cells. Thus, genetic alterations in ES cells could be transferred into the genome of a living animal after two mouse generations.

In parallel, the groups of Oliver Smithies and Mario Capecchi had independently developed systems to introduce mutations in genes in cultured cells by exploiting the phenomenon of homologous recombination. Cells use this process during meiosis to exchange genetic material between homologous chromosomes. For this, the two homologous chromosomes recognize each other by a not yet fully understood process and align. Then, pieces of homologous DNA are exchanged between the chromosomes by double crossover of the DNA strands. Capecchi and Smithies found that they only had to introduce several kilobases of chromosomal DNA into cells to allow this process to happen. These relatively short DNA fragments also aligned with their homologous region on the cellular chromosome, and by double crossover parts in the center of the homology region on the introduced DNA could be exchanged and ended up in the chromosome. Thus, they could freely decide how to change a gene of interest by putting a mutation into the central part of the homology region and letting the homologous recombination machinery introduce this mutation into the chromosome. However, there was a major drawback. This process was very inefficient: it occurred in only about one of 1000 transfected cells. Thus, it was not practicable, although not impossible (10), to use classical transgenic technology, i.e., the microinjection of such DNA constructs into fertilized oocytes (*see* Chapter 3) in order to introduce targeted mutations into the mouse genome.

But the ES cells just described by Martin Evans opened up a way to do this with relative ease, because they allowed a selection step, in which millions of cells were used as starting material and after which a few survivors carried the intended genetic alteration. These surviving clones could then be used to inject

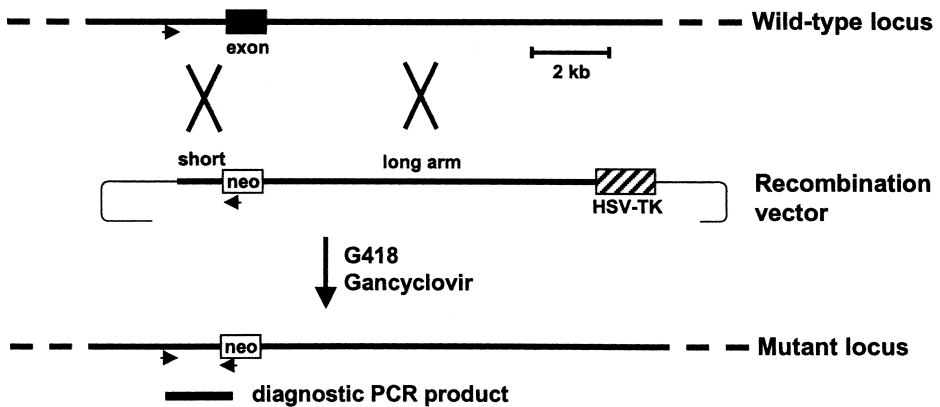


Fig. 1. Gene targeting by double selection technique. Two fragments of the gene of interest (thick lines) are cloned into a plasmid vector (thin lines) together with a neomycin resistance (*neo*) and a Herpes simplex virus thymidine kinase gene (*HSV-TK*). The linearized construct is transfected into ES cells and the cells are selected by G418 (a neomycin analog) and Gancyclovir (which kills *HSV-TK* expressing cells). Cells that have inserted the construct in their genome by homologous recombination have lost the *TK* gene and survive double selection.

blastocysts and to develop mice carrying the targeted mutation in all cells of the body via an intermediate chimeric step. The double-selection technique developed by Capecchi (*II*) is still the most widely used method for gene targeting, and its principle will therefore be described below.

The targeting constructs contain a neomycin resistance (*neo*) gene in the center of the homology region and a Herpes simplex virus (*HSV*) thymidine kinase (*TK*) gene outside of this region (**Fig. 1**). The constructs are introduced into ES cells by electroporation. Most of the cells that take up the foreign DNA insert it randomly into their genome as in classical transgenic technology. In this case, the *TK* gene enters a chromosome and remains in the genome of the cell. In very few cells, homologous recombination happens and the *neo* gene is integrated into the gene of interest, but the *TK* gene gets lost in this process. Thus, correctly targeted ES cells survive the following double selection with neomycin (or G418) and gancyclovir, which is phosphorylated by *HSV-TK* in cells and used for DNA and RNA synthesis leading to chain termination and death of *TK*-expressing cells. In contrast, cells with random transgene integration express the *TK* gene and are sensitive to gancyclovir, and cells that remained untransfected also die under double selection, since they lack the *neo* gene. Thus, the only survivors are ES cells that carry the intended genotype, at

least in theory. In practice, sometimes more than 90% of the surviving clones show random integration of the targeting construct, because of the fact that the TK gene can also be inactivated during random insertion into the genome by partial deletion, mutation, or by the silencing influence of neighboring DNA regions. Nevertheless, the efficiency is high enough to find clones with relative ease in which homologous recombination has occurred and which, therefore, carry the intended genetic alteration. Such clones are identified either by Southern blot detecting DNA fragments specific for the targeted locus, or more conveniently by polymerase chain reaction (PCR). To allow the efficient use of such a diagnostic PCR, the targeting constructs are mostly not symmetrically designed, i.e., the two fragments of homologous DNA flanking the neo gene are of different lengths. One “arm” is short (max 1000 bp) and the other arm is long (min 4 kb) (**Fig. 1**). For the diagnostic PCR, one primer is designed in the region on the gene of interest immediately upstream of the short arm but outside of the homology region on the targeting construct and another one on the neo gene. They are only able to give rise to a PCR fragment when their binding sites are brought close together during homologous recombination.

The positively identified clones are then used to generate gene-targeted mice by blastocyst injection and breeding of the resulting chimera (**Fig. 2**). In the majority of cases the experiment is still not finished when the first animals with the targeted mutation are born from the chimera, because they are mutated on only one allele. In these heterozygous mutants the intact allele is mostly dominant and the phenotype of the mutation is not obvious. Thus, these animals have to be bred, and after another mouse generation, homozygous “knock-out” animals are born, which carry the targeted mutation on both homologous chromosomes and therefore exhibit the phenotype of the genetic ablation. Although this is not the subject of this chapter, the work needed to detect and describe this phenotype should not be underestimated. It mostly surpasses the time needed to generate the animal model and needs elaborate embryological and/or physiological methodology.

One major drawback of the gene targeting technology is its availability only for the mouse, whereas the rat would be the preferred animal model for cardiovascular research. This limitation is due to the lack of germline-competent ES cells for any other species besides the mouse. All attempts to establish such cells for rats have been unsuccessful (**12–14**). Furthermore, nuclear transfer technology, which allows generation of germline mutations in animals other than mice (**15,16**) has not yet been established in the rat (**17**). Only recently, random mutagenesis using ethylnitrosurea (ENU) followed by a facilitated yeast-based screen for mutations in a gene of interest has allowed for the first time the ablation of a specific gene in the rat (**18**). However, this technology requires the screening of hundreds of rats and creates animals with multiple

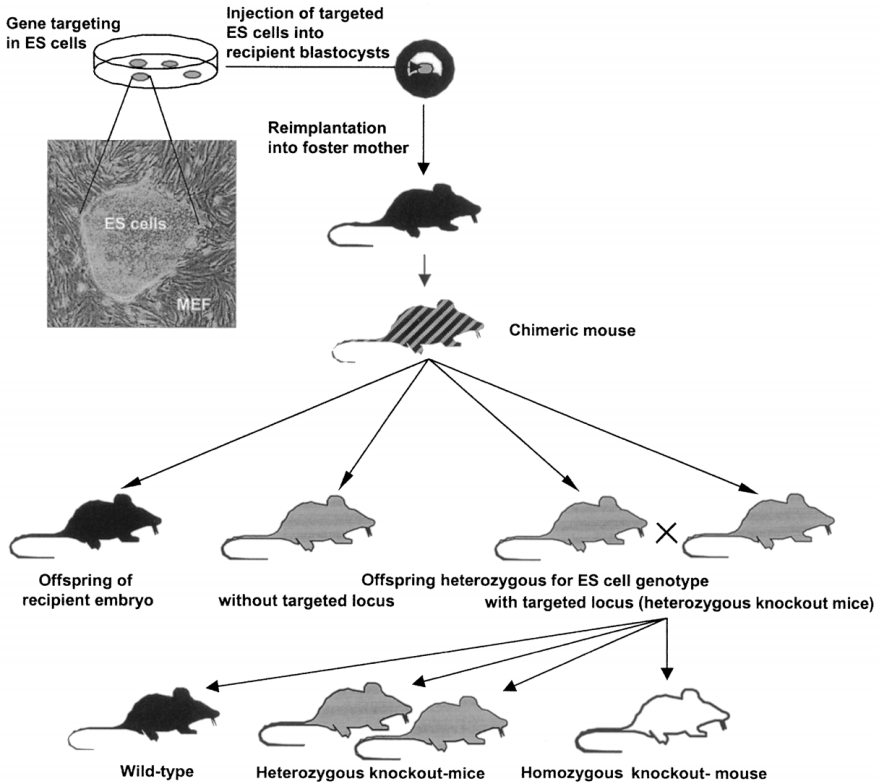


Fig. 2. Generation of knockout mice. After successful gene targeting in ES cells, which grow as three-dimensional clumps on mouse embryo fibroblasts (MEFs), the cells are injected into a host blastocyst, which is then transferred into the uterus of a pseudopregnant foster mother. The resulting chimeras are bred and, if the cells have populated the germline, give heterozygous knockout offspring. Interbreeding of those mice yields homozygous knockout mice.

additional mutations in the genome, with unpredictable influences on the phenotype. Antisense RNA (19) and recently also RNA interference (20) in combination with classical transgenic technology, has been employed successfully to blunt expression of genes in rats, but the general applicability of these methods is questionable. Thus, there is still the need to establish gene targeting technology in the preferred animal model for hypertension research, the rat.

2. Materials

2.1. Equipment

1. Tissue culture hood.

2. Tissue culture incubator at 37°C with 5% CO₂ and saturated-water atmosphere.
3. Inverted microscope, fitting into the tissue culture hood.
4. Inverted microscope with Nomarski optic, 10X and 40X objectives, and two micromanipulators on a vibration-free table.
5. Binocular microscope.
6. Pipet puller.
7. Microforge.
8. Electroporator.
9. Liquid nitrogen container.

2.2. Plastic Ware

1. Polystyrene tissue culture dishes: 10 cm (10 mL), 6 cm (4 mL), 12 well (each 2 mL), 24 well (each 1.5 mL), 96 well (each 0.5 mL).
2. Plastic centrifuge tubes: 50 mL, 15 mL.
3. Cryotubes: 2 mL.
4. Plastic pipets: 1 mL, 5 mL, 10 mL, 25 mL.
5. Electroporation cuvettes: 0.4 cm.

2.3. Reagents and Solutions

1. 10X phosphate-buffered saline (PBS): 1.37 M NaCl, 26.8 mM KCl, 80.6 mM Na₂HPO₄·7H₂O, 14.7 mM KH₂PO₄·2H₂O, pH. 7.4 (store at 4°C).
2. Dulbecco's Modified Eagles Medium (DMEM) with high glucose (4.5 g/L), without sodium pyruvate (store at 4°C, replace after 3 wk, prewarm before use).
3. Fetal calf serum (FCS), ES-cell tested (store at -20°C, heat-inactivate the complement system at 56°C for 30 min before use) (*see Note 1*).
4. 100X Pen/Strep (50 U/mL penicillin, 50 U/mL streptomycin) (store at -20°C).
5. 100X L-Glutamine (200 mM) (store at -20°C).
6. 100X MEM nonessential amino acids (store at -20°C).
7. Leukemia-inhibiting factor (LIF, Esgro) (store at -20°C).
8. 100X β-Mercaptoethanol: 7 μL β-mercaptoethanol in 10 mL PBS (10 mM; prepare fresh).
9. Medium for mouse embryo fibroblasts (MEF) (store at 4°C, prewarm before use): DMEM, 10% FCS, 1X Pen/Strep, 1X glutamine.
10. Medium for ES cells (store at 4°C, prewarm before use): DMEM, 15% FCS, 1X Pen/Strep, 1X glutamine, 1X MEM nonessential amino acids, 1X β-mercaptoethanol, 10³ U/mL LIF.
11. Freezing medium (store at 4°C): 40% DMEM, 50% FCS, 10% dimethyl sulfoxide (DMSO).
12. M16 and M2 medium (store at 4°C): M2 and M16 are media optimized for the culture of preimplantation embryos (commercially available, e.g., from Sigma). M16 is not suitable for long-term handling outside the CO₂ incubator because its pH is kept stable by 5% CO₂ in the atmosphere. M2 medium has a stable pH because of HEPES buffer.
13. Gelatine solution: 0.1% in 1X PBS (store at 4°C).

Table 1
Compositions of M16 and M2

Compound	M2	M16
NaCl	94.66 mM	94.66 mM
KCl	4.78 mM	4.78 mM
KH ₂ PO ₄	1.19 mM	1.19 mM
MgSO ₄	1.19 mM	1.19 mM
Na lactate	23.28 mM	23.28 mM
Glucose	5.56 mM	5.56 mM
NaHCO ₃	4.15 mM	25.0 mM
Na pyruvate	0.33 mM	0.33 mM
CaCl ₂	1.71 mM	1.71 mM
HEPES, pH 7.4	20.0 mM	—
Phenol red	1 mg/mL	1 mg/mL
BSA (Bovine Serum Albumin)	4 mg/mL	4 mg/mL

14. Capecchi buffer: 20 mM HEPES-Na, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose, 0.1 mM β -mercaptoethanol.
15. Trypsin/EDTA: 0.05% trypsin, 0.02% EDTA (store at 4°C for up to 2 wk).
16. Mitomycin solution: 1 mg/mL mitomycin C in 1X PBS (store at –20°C).
17. G418 (Geneticin) solution 100 mg/mL in H₂O (store at –20°C).
18. Gancyclovir (Cytovene, Syntex) solution: 200 μ M in H₂O (store at –20°C).
19. Proteinase K solution: 10 mg/mL in H₂O (store at –20°C).
20. DNase I solution: 10 mg/mL in H₂O (store at –20°C).
21. Anesthetic solution: 10% (v/v) ketamine, 0.02 % (v/v) xylazine, 0.9% (w/v) NaCl in H₂O (prepare fresh before use).

2.4. Animals

Male and female (21–23 d old) mice of the C57Bl/6 strain are necessary to get blastocysts, and females as well as vasectomized males of the outbred NMRI strain or F1 hybrids (e.g., C57Bl/6 X DBA) are used to generate foster mothers. All these strains are available from commercial breeders.

3. Methods

3.1. Design and Generation of Targeting Construct

3.1.1. Selection of Targeting Region

The first question that has to be addressed before a gene targeting experiment can be started is which part of the gene of interest should be affected. If functional deletion of the gene is intended, the functionally most important part of the gene should be selected. In rare cases, the decision is easy because

the whole coding region of the gene resides on one exon, such as for most G protein-coupled receptors. However, in the majority of cases, genes comprise several exons and cover tens of kilobases of genomic DNA. Consequently, the researcher has to decide which exons should be deleted in order to inactivate the gene. For enzymes, exons coding for the active site might be selected, for receptors or transcription factors the ligand or the DNA-binding domains, respectively, might be targeted. Alternatively, one of the first exons of a gene may be deleted. Insertion of the neo cassette should lead to missplicing and thereby to a nonfunctional mRNA. In addition, most neo cassettes, if cloned in the same orientation as the arms, contain a transcriptional stop signal ([poly(A) site]) that should not only stop transcription from the promoter driving the neo gene but also target gene transcription (*see Note 2*). This strategy may fail if transcriptional read-through and alternative splicing of the gene leads to mRNAs with partial or even complete restoration of a functional protein coding sequence (*21*). If possible, one of the first coding exons should therefore be selected, the length of which in base pairs cannot be divisible by three. Even if alternative splicing would lead to the restoration of a near-full-length mRNA, the coding frame would be shifted and no functional protein would be generated. The length of the deleted region ideally should be about 1 kb because the neo gene, which is inserted at this site by the targeting procedure, would then restore the length of the genomic region. However, deletions from a few bases up to several kilobases have also been achieved (*22*).

3.1.2. Cloning of Genomic DNA Fragments

When the target region of the gene is selected, the next step is to clone the flanking genomic fragments necessary for the targeting construct. There are two options to do this. Classically, large DNA fragments are isolated from mouse genomic libraries, characterized by restriction mapping, and subfragments are cloned into the targeting vector. More flexible and convenient is the generation of the fragments by long-range PCR using commercially available kits from total genomic DNA. The completeness of the mouse genomic sequence in Genbank (*3*) allows the design of gene-specific primers, thereby determining the exact length and borders of the fragments and to include suitable restriction sites for the following cloning steps (*see Notes 3 and 4*). Two fragments flanking the region to be targeted need to be cloned or amplified by PCR; one should be short (around 1000 bp) and one should be long (4–10 kb) (**Fig. 1**). Thereby, the efficiency of homologous recombination increases with the size of the total homology region (*23,24*). On the other hand, the efficiency of the long-range PCR decreases with the length of the amplified product, and the cloning of the long arm becomes more difficult the longer the

fragments get. Therefore, a short arm of about 1 kb and a long arm of about 5 kb can be envisaged as suitable.

3.1.3. Cloning of Targeting Vector

These two arms are cloned into the targeting vector in the same orientation flanking a neomycin-resistance gene. The neo gene replaces the missing region of the target gene between the two arms. At the other end of the long arm the TK gene is inserted. Most laboratories generating knockout mice have basic targeting vectors with both selectable marker genes flanked by suitable multiple cloning sites. One essential feature of these vectors is that the expression of both marker genes is controlled by promoters active in undifferentiated ES cells, such as the HSV-TK promoter or the promoter for phosphoglycerokinase (PGK). When the construct is planned, it has to be ensured that the complete targeting vector can still be linearized by a restriction enzyme either at the beginning of the short arm or somewhere in the plasmid portion, because linear DNA has to be inserted into the ES cells for successful homologous recombination to take place. Linearization close to the TK gene is not recommended because it leads to an increased chance of mutation of this gene during random insertion and, thus, an augmentation of false clones surviving double selection. For the isolation of the fragments, genomic DNA from ES cells should be used because it has been shown that the targeting efficiency is markedly enhanced for isogenic DNA compared to DNA derived from other mouse strains (24–26). The reasons for this are enigmatic because the exact mechanism of homologous recombination is still a matter of intensive research. A second construct should be prepared in parallel. It contains a longer short arm elongated at its 5' end by 50–200 bp and the neo gene but may lack the long arm and the TK gene. It is used as positive control for the diagnostic PCR to detect ES cell clones with a correctly targeted gene.

3.1.4. Establishment of Diagnostic PCR

The diagnostic PCR to detect correctly targeted ES cell clones must be established before the cells are transfected. The following two primers should be designed: A 5'-primer mapping in the region immediately upstream of the short arm on the gene of interest and the 3'-primer mapping on the neo gene (see **Note 5**). Thus, the primers will yield a PCR product only if the neo gene is inserted into the gene of interest by homologous recombination. The PCR is optimized for annealing temperature and magnesium concentration using serial dilutions of the elongated DNA construct mentioned in **Subheading 3.1.3**. (1 fg to 1 ng) together with 100 ng of genomic ES cell DNA. Only if the PCR gives a reproducible signal after 35 cycles down to 10 fg of the test construct,

which mimics the situation in targeted ES cells clones, can the PCR be considered as sensitive and reliable enough.

3.2. Culture and Transfection of Embryonic Stem Cells

ES cells are particularly sensitive cells. They have special requirements for optimal growth and easily lose their capacity to contribute to the germline when the culture conditions are not optimal or when they are cultured for too many passages. Thus, experiments with ES cells should be started with cells of an early passage number (around 10) after establishment from blastocysts. The majority of ES cell lines have to be grown on feeder cells, mostly mitotically inactivated mouse embryo fibroblasts (MEFs), and need leukemia-inhibiting factor (LIF) in the medium to stay in the pluripotent state.

MEFs produce this factor, but because the secretion may be variable it should also be added to the medium. Undifferentiated ES cells on MEFs grow as three-dimensional clumps with irregularly round and borders shining brightly in the phase-contrast microscope (**Fig. 2**). Single cells are hardly visible in the clumps. If single cell borders are detectable or if flat cells appear at the edge of the colonies, the cells have started to differentiate and should not be used any more.

3.2.1. Isolation, Culture, and Mitotic Inactivation of MEFs

MEFs should be prepared from neomycin-resistant mice, best from heterozygous or homozygous knockouts regardless of which gene is altered in the animals, except for genes affecting very early embryonic development such as LIF.

1. Breed female and check for vaginal plug in the morning.
2. 13–15 d later, kill female, take uterus, and rinse twice with 1X PBS in 10-cm dishes in the tissue culture hood.
3. Take out embryos and remove head, liver, and heart.
4. Mince the remaining embryonic tissue with a razor blade or scalpel in 3 mL of 1X PBS per embryo.
5. Let tissue fragments precipitate in a 15-mL centrifuge tube at 4°C and remove supernatant.
6. Overlay with 1 mL trypsin/EDTA and leave at 4°C for 12–16 h.
7. Remove supernatant and incubate for 30 min at 37°C.
8. Add 10 mL of MEF medium and disaggregate cell clumps by pipeting with a 10-mL plastic pipet.
9. Distribute cells on one or two 10-cm dishes per embryo, add MEF medium to 10 mL, and put in incubator overnight.
10. The next day, wash cells three times with 1X PBS and add fresh MEF medium.
11. When MEF reach near confluency, remove MEF medium, wash twice with 1X PBS, add 2 mL of trypsin/EDTA solution, incubate at 37°C until cells detach (after about 3 min), add 2 mL of MEF medium to stop trypsin action (because of

trypsin inhibitors in the FCS component), dissociate and transfer the cells with a 10-mL pipet to a 15-mL centrifuge tube, and spin down at 120g for 3 min.

- a. Splitting (1:4): Resuspend cell pellet in 4 mL MEF medium and pipet each 1 mL in cell culture dishes with 9 mL MEF. Trypsinization and splitting can be repeated twice before MEFs are used for mitomycin C treatment and ES cell culture.
 - b. For freezing, resuspend cell pellet in 1 mL of freezing medium, transfer into cryotubes, slowly freeze in a plastic container at -80°C overnight, and put for long-term storage in liquid nitrogen. For thawing, quickly warm up cryotube in 37°C , put contents in 15-mL centrifuge tube with 5 mL MEFs medium, spin down (120g, 3 min), resuspend pellet in 3 mL MEF medium, and transfer each 1 mL into 10-cm cell culture dishes with 9 mL of MEF medium.
12. For mitotic inactivation, remove MEF medium from near-confluent MEF, add 5 mL of MEF medium with 50 μL mitomycin C solution, incubate for 3 h at 37°C , remove medium, wash three times with 1X PBS, Trypsinize or freeze MEFs. Mitomycin C is cross-linked to DNA and thereby inhibits mitosis.
13. After splitting or thawing, count mitomycin-treated MEFs in haemocytometer and plate on gelatinized culture dishes or wells at a density of about 4×10^4 cells/ cm^2 . For gelatinization, cover bottom of dish or well with gelatine solution and incubate for at least 30 min at 37°C and afterwards wash twice with 1X PBS.

3.2.2. Culture of ES Cells

1. Thaw a vial of ES cells quickly in 37°C , put contents in 15-mL centrifuge tube with 5 mL ES medium, spin down (120g, 3 min), resuspend pellet in 3 mL ES medium, and transfer each into 10-cm cell culture dishes with MEFs 7 mL of ES medium. Change medium next day.
2. After 2–3 d, the cells need to be split. For this, replace medium 2 h before splitting, then remove the medium again, wash twice with 1X PBS, add 2 mL Trypsin/EDTA solution, incubate at room temperature for 1–3 min, resuspend and dissociate cells with a 2-mL glass Pasteur pipet. Add suspension to 5 mL ES medium in centrifuge tube, spin down at 120g for 3 min, resuspend cell pellet in 2 mL of ES medium, plate on 5 to 10-cm dishes with MEFs and fill up ES medium to 10 mL. Repeat this splitting at least every 48 h.
3. Freezing of ES cells: Instead of replating the cells after trypsinization, transfer them into 15-mL centrifuge tube with 5 mL ES medium, spin down (120g, 3 min), resuspend pellet in 1 mL freezing medium, put in cryotubes, slowly freeze in a plastic container at -80°C overnight, and put for long-term storage in liquid nitrogen.

3.2.3. Transfection of ES Cells and Selection of Gene-Targeted Clones

1. Add fresh medium to a confluent 10-cm dish with ES cells and trypsinize 2–4 h later.
2. Spin down (120g, 3 min) cells and resuspend in Capecchi buffer, count in

hemocytometer.

3. Spin down again and resuspend at 1×10^7 cells in 0.4 mL Capecchi buffer, transfer into electroporation cuvet.
4. Add 20 μg linearized and purified gene-targeting construct in 20–50 μL sterile deionized water and mix with 2-mL plastic pipet.
5. Electroporate the cells at 240 mV and a capacitance of 500 μF , leave at room temperature for 10 min.
6. Add 1.5 mL ES medium to the cuvet, plate cells on three 10-cm dishes with neomycin-resistant MEFs in 10 mL ES medium.
7. 2 d later, change ES medium and start selection with 250 $\mu\text{g}/\text{mL}$ G418.
8. 1 d later, change to ES medium with 250 $\mu\text{g}/\text{mL}$ G418 and 2 μM gancyclovir and change selection medium daily, because dead cells need to be removed quickly to avoid ES cell differentiation induced by cell debris (*see Note 6*).
9. Seven to ten days later, between 50 and several hundred ES cell colonies should be visible on each dish.
10. Prepare 96-well plate with MEFs on gelatine in ES cell medium.
11. Prepare 96-well plate with 50 μL trypsin/EDTA per well and 96 numbered PCR tubes.
12. Replace medium on colonies carefully with 5 mL of 1X PBS and put on microscope under the tissue culture hood.
13. Pick morphologically normal-looking clones with a plastic tip on a 20- μL pipet and transfer each into one well with trypsin/EDTA. To avoid too extended contact of the ES cells with trypsin and EDTA, stop after 12 clones, incubate 96-well plate for 2 min at 37°C, and stop the trypsin action by addition of 150 μL ES cell medium to each well (the serum in the medium contains a trypsin inhibitor). Resuspend cells of each well, put 100 μL of the suspension into the PCR tube with the corresponding number and 100 μL in the corresponding well with MEFs on the other 96-well plate, which is then returned directly to the incubator. Repeat the procedure for the next 12 clones until 96 clones are picked.
14. For DNA isolation and diagnostic PCR, spin down cells in PCR tubes (700g, 5 min), remove supernatant, add 20 μL H_2O and vortex. Then heat up to 90°C for 10 min, cool down to 55°C, add 2 μL proteinase K solution, centrifuge briefly and incubate for 55°C for 1 h (or 37°C over night). Finally, inactivate proteinase K at 90°C for 10 min, centrifuge (18,000g), transfer 5 μL of supernatant into a fresh PCR tube, and run a diagnostic PCR to detect correct targeting (*see Note 7*).

3.2.4. Generation and Breeding of Chimeras

1. For blastocyst isolation, 21–23 d old female C57Bl/6 mice are superovulated by injection at noon with five IU PMSG (pregnant mare's serum gonadotropin) and 46 h later with five IU hCG (human chorionic gonadotropin). They are then mated and the following morning the mice are checked for vaginal plugs.
2. 3 d later (3.5 d postcoitum), the mice are killed by cervical dislocation, the uterus is isolated, freed of fat tissue, and put into a Petri dish with 1X PBS. The two uterine horns are cut at the cervix and transferred into a dry 10-cm dish. After

ligation at the ovary side with forceps, 0.3 mL of M2 medium is flushed through each horn from the ovary side using a syringe with a G27 needle. Blastocysts are collected with a mouth pipet under a binocular microscope, transferred into M16 medium, and stored in a tissue culture incubator until use.

3. Micropipets are prepared from 1.5-mm borosilicate glass capillaries using a capillary puller, a microforge and a micropipet grinder (*see Note 8*). The holding pipet should have an inner diameter of 20–30 μm and an outer diameter of 100–150 μm and its tip should be smoothened by melting in the microforge. The injection capillary should have an outer diameter of 15–20 μm and an inner diameter slightly larger than the size of an ES cell. At the tip of the injection capillary a 45° bevel and a protruding needle should be generated by the use of the micropipet grinder and the microforge, respectively. Both pipets should be bent in the microforge to allow parallel movement of the tip relative to the surface of the injection chamber. The pipet is coated with 10% polyvinylpyrrolidone (PVP) in M2 medium immediately before use.
4. Pipet 200–500 ES cells into 30 μL of M2 medium covered with mineral oil on the bottom of the injection chamber on the inverted microscope with the micromanipulators. Add 20–30 blastocysts with the mouth pipet. Catch one blastocyst with the holding pipet and fix it with the inner cell mass near the opening of the holding pipet. Load 12–15 ES cells into the injection pipet and inject them into the cavity of the blastocyst (*see Note 9*). After removal of the pipet, the blastocyst will collapse. After injection, which should not take more than 30 min, transfer blastocysts into fresh M16 medium and incubate for 1–2 h in the tissue culture incubator.
5. For uterus transfer of blastocysts, induce pseudopregnancy in a female by mating with vasectomized male and check next morning for vaginal plug. Mostly, outbred (e.g., NMRI) or F1 hybrid (e.g., C57Bl/6XDBA) females are used as recipients. Two days later (d 2.5 postcoitum), anesthetize female by intraperitoneal injection of 17 μL per gram of body weight of anesthetic solution. Make an incision of 0.8 cm on the back on one side of the spine below the ribs, grasp the fat pad attached to the uterus with forceps and take out the uterus. Make a small hole into the uterus near the oviduct with a G27 needle. Load the mouth pipet with an air bubble followed by 15 blastocysts, and flush the blastocysts through the hole into the uterine lumen until the air bubble has reached the tip of the pipet; avoid releasing the air bubble into the uterus. Let the uterus slide back and close the wound with clips. Repeat on the other side.
6. Seventeen days later pups are born and, when the fur is visible a few days later, the degree of chimerism is deducible from the coat color. Because most ES cell lines have a male karyotype, male chimeras with high coat color chimerism have the highest chance of producing ES-cell-derived offspring, recognized by the pure agouti coat color. Fifty percent of these mice are heterozygous, i.e., they carry the mutation in one allele of the targeted gene. According to Mendel's laws, breeding of these mice will generate 25% homozygous knockout mice, with the mutation in both alleles recognized by PCR or Southern blot, which detects the

absence of the target region. If the offspring contains less than the expected 25% homozygous mice, prenatal or perinatal lethality must be assumed and an embryonic phenotype of the genetic alteration should be considered.

4. Notes

1. Different batches of FCS should be tested for plating efficiency and toxicity before use by plating 1000 ES cells in 6-cm dishes with MEF and LIF and both 10% and 30% FCS. After 7 d, count colonies and check for differentiation. There should be no less colonies with 30% FCS than with 10% FCS, and the colonies should look undifferentiated.
2. If the neo gene is inserted in the opposite orientation to the arms of the gene of interest in the targeting construct, it may completely inhibit target gene expression by a yet enigmatic mechanism (27).
3. Avoid long stretches of repetitive DNA in the arms.
4. The generation of the homology regions by long-range PCR is suitable only for gene ablation experiments. If conditional gene targeting is intended, mutations induced by the thermostable polymerases, particularly in exons, may render the PCR products useless.
5. Sometimes several primer pairs have to be tested for the diagnostic PCR.
6. If medium becomes yellow, change it up to twice daily.
7. Before microinjection of positive clones, verify the correct genotype by Southern blot.
8. Alternatively, quite suitable pipets can be purchased from commercial suppliers
9. When the pipet is clogged, flush it with mineral oil and recoat with PVP solution.

Acknowledgments

The author would like to thank Vanessa Merino, Natasha Alenina, Cibele Campos Cardoso, Cécile Cayla, Tanja Schmidt, and Claudia Wilhelm for helpful discussions.

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Methods and Protocols

Fennell, J.P.; Baker, A.H. (Eds.)

2005, XVI, 502 p. 98 illus., Hardcover

ISBN: 978-1-58829-323-7

A product of Humana Press