

# Chapter 2

## Tissue-Specific KO of ECM Proteins

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### Summary

Nearly 20 years after its first description, gene targeting and generation of transgenic mice by homologous recombination in embryonic stem cells still are cutting edge tools for the postgenomic era. Understanding the function of the large number of genes encoding extracellular matrix proteins and their cellular receptors appears a daunting task that can very much profit from a genetic approach. The generation of new mutant alleles remains essential to define the different biochemical properties of such proteins. While in the past, gene targeting represented a complex procedure, restricted to few laboratories, recent breakthroughs, such as the publication of the mouse genome sequence and the perfection of recombineering techniques in bacteria, made generation of transgenic mice faster and easier. This chapter will thus focus on the recent advances in gene-targeting technology with a special eye on the study of genes involved in cell adhesion and migration.

**Key words:** Gene targeting, Cre-lox, Conditional mutagenesis, Mutagenesis, Recombineering, Adhesion, Extracellular matrix.

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### 1. Introduction

#### 1.1. Gene Targeting

The analysis of phenotypes caused by null and mutant alleles is a very powerful means to understand gene function *in vivo*. Thanks to the gene-targeting technology in ES cells, the genome of a mammalian organism such as the mouse can be artificially modified by precise alterations. The system exploits the ability of ES cells to be cultured and manipulated *in vitro* without losing their totipotency (1, 2). Mutations in specific genes can be achieved by *in vitro* selection of ES cell clones in which the locus of interest has been targeted by homologous recombination (3, 4). The peculiar property of being totipotent, allows ES cells, once injected in the cavity of a blastocyst, to contribute to

the formation of all cell types of a chimeric embryo. Whenever a chimeric mouse possesses ES-derived germ cells, the mutation can be propagated to its offspring. Heterozygous mice are then mated to generate the homozygous mice needed for phenotypic analysis.

### **1.2. Conditional Gene Targeting**

Conditional knock out technology allows the creation of inducible mutations in a tissue specific manner and at a precise developmental stage. Whereas the phenotype caused by germ-line mutations can be biased by epigenetic adaptation, induction of gene alteration in differentiated cells can result in clearer effects. Moreover, via this method, it is possible to study the consequences of ablating genes essential for cell survival (5), identifying functions for distinct splice variants (6), or tracking different gene functions during different developmental stages (7). The technique is based on the introduction of two or more short sequence tags recognized by particular recombinases able to catalyze recombination and excision of the sequence between the two recognition sites. Two recombinase systems are currently used for this purpose: Cre/loxP from bacteriophage P1 (8) and Flp/FRT (9) from *Saccharomyces cerevisiae*. Both recombinases can recognize 34 bp consensus sequences; cut the intervening sequence and rejoin the extremities. Inducible gene targeting can thus be achieved by mating a mouse in which important sites in the locus of interest have been flanked by *recombinase recognition sites* with a transgenic mouse that expresses the *recombinase* in a restricted pattern (8, 10, 11). Similarly, the conditional allele can be silenced by infection with a virus that transduces the *recombinase* gene (12). In this way, a variable percentage of cells ranging from 10 to 100% (13) can be induced to undergo a controlled DNA rearrangement only when and where the recombinase is expressed.

### **1.3. Gene Targeting of ECM Proteins**

Gene targeting has been widely used to study the function of ECM genes (14) and these experimentally induced mutations greatly extended the knowledge derived from the analysis of natural-occurring mutations (15). Interestingly, several of the knock-out mouse strains closely reproduce phenotypes of human hereditary disorders (16–19). For instance fibrillin-1 mutations recapitulate the lethal form of Marfan syndrome (20, 21); fibulin-5 null mice reproduce the clinical signs of cutis laxa (22, 23), and collagen VI null mice develop Betlem myopathy (24). Moreover, comparison of knockout of specific laminin chains demonstrates a role for the different isoforms during development and in particular tissues (25).

Conditional knockouts also allowed clarifying the specific function of extracellular matrix receptors in different tissues. Integrin  $\beta_1$  conditional knockout in the skin demonstrated an important role for

$\beta_1$  integrin in the maintenance of the basement membrane and in the organization of the different layers of the hair follicle. Moreover, mice lacking  $\beta_1$  integrin in skeletal muscles die at birth with severe muscle defects (26).

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## 2. Materials

### 2.1. Generation of Constructs

1. Neomycin resistance cassettes:
  - Plasmid *PL452 loxP-PGK-EM7-NeobpA-loxP* with strong PGK (eukaryotic)–EM7 (prokaryotic) promoters driving the neomycin resistance gene in eukaryotic and prokaryotic cells.
  - Plasmid *PL451 FRT-PGK-EM7-NeobpA-FRT-loxP*. The FRT sites will be used to delete the Neo cassette in mice carrying the floxed gene crossing them with a strain carrying the FRT recombinase.
2. Bacterial strains EL350 DH10B [ $\lambda$ cl857 (cro-bioA < > araC-PBADcre)] (27), TOP 10 plus (Invitrogen Corp., Carlsbad, CA).
3. BAC clone can be ordered from Geneservice (<http://www.geneservice.co.uk>).
4. Kanamycin or ampicillin or chloramphenicol.
5. LB: 10 g bactotryptone (Difco, Detroit, MI), 5 g bacto-yeast extract (Difco), 5 g NaCl. Fill to 1 L with deionized water, adjust pH to 7.0, and autoclave. LB can be stored for a long time at room temperature (RT). Turbid, contaminated media must be discarded.
6. LB agar: Add 15 g agar (Sigma, St. Louis, MO) to LB, autoclave, and allow medium to cool to 50°C before adding antibiotics and pouring plates. Plates can be stored for 1 month at 4°C. Dry the plates in 37°C incubator overnight before use.
7. Taq amplification kit Promega, WI, USA.
8. T4 ligase.
9. Gene Pulser<sup>TM</sup> (Bio-Rad, Richmond, CA).
10. Electroporation cuvette 0.1 cm Biorad.
11. QIAGEN gel extraction kit QIAquick, Hilden cat. no. 28706.
12. QIAGEN Plasmid Midi Kit (cat. no.12143).
13. Arabinose (Sigma A-3256).

**2.2. Definition of the Probe**

1. Restriction enzymes and buffers (store at  $-20^{\circ}\text{C}$ ).
2. T7 sequencing kit (Pharmacia, Uppsala, Sweden).

**2.3. Isolation of Feeder Cells**

1. Mice expressing a neomycin (or hygromycin or puromycin) resistance gene.
2. C57BL6 female mice.
3. 70% ethanol.
4. Sterile dissecting equipment.
5. 10 $\times$  phosphate-buffered saline (10 $\times$  PBS): 80.06 g NaCl, 2.01 g KCl, 14.42 g  $\text{Na}_2\text{HPO}_4$ , 2.04 g  $\text{KH}_2\text{PO}_4$ . Fill to 1 L with DDW and autoclave (store at RT).
6. Trypsin/EDTA solution: 10 $\times$  stock (Invitrogen Gibco, cat. no. 15400-054) (store at  $-20^{\circ}\text{C}$ ) diluted to 1 $\times$  with 1 $\times$  PBS (aliquot and store at  $-20^{\circ}\text{C}$ , store aliquots in use at  $4^{\circ}\text{C}$ ).
7. Feeder medium: DMEM with Glutamax-1 (Invitrogen Gibco, cat. no. 61965-026) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Gibco, cat. no. 10270-106) and with penicillin/streptomycin (Invitrogen Gibco, cat. no. 15140-122) at the final concentration of 100 U/mL and 100  $\mu\text{g}/\text{mL}$ , respectively.
8. Freezing medium: 70% DMEM, 20% FBS, 10% DMSO (Sigma D2650).
9. ES medium: DMEM with Glutamax-1 (Invitrogen Gibco, cat. no. 61965-026) + Na-pyruvate (Invitrogen Gibco, cat. no. 11360-039) supplemented with 20% FBS (FBS needs to be tested for ES cell use, or can be bought from Hyclone already tested, cat. no. SH30071.03), 0.1 mM 2-mercaptoethanol, 5 mL 100 $\times$  nonessential amino acids (NEA) (Invitrogen Gibco, cat. no. 11140-035), and 1,000 U/mL leukemia inhibitory factor (LIF) (ESGRO from Chemicon, cat. no. ESG1106).
10. Mycoplasma PCR Primer Set (Stratagene, cat. no. 302008).

**2.3.1. Electroporation and Selection**

1. Feeder medium (*see Subheading 2.3*).
2. Feeder cells (*see Subheading 2.3*).
3. Restriction enzymes and buffers.
4. Chloroform.
5. Phenol/chloroform: mix 1 volume of Phenol (equilibrated with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 1 volume of chloroform.
6. 3 M Na-acetate pH 5.2, adjust pH with glacial acetic acid (can be stored at RT).
7. 100% ethanol.

8. 70% ethanol.
9. Trypsin/EDTA (*see Subheading 2.3*).
10. ES cells.
11. ES medium (*see Subheading 2.3*).
12. 1× PBS (*see Subheading 2.3*).
13. Burkert's chamber.
14. Mouse Embryonic Stem Cells Nucleofector kit cat. no. 502VPH1001 (Amara GmbH, Cologne, Germany).
15. Nucleofector™ II (Amara GmbH).

### 2.3.2. Picking and Freezing of Resistant Clones

1. ES medium (*see Subheading 2.3*).
2. G418 (Geneticin) (Gibco or Sigma).
3. Feeder cells (*see Subheading 2.3*).
4. Feeder medium (*see Subheading 2.3*).
5. 24-Well plates (Falcon, Los Angeles, CA).
6. 96-Well plates (Falcon).
7. Trypsin/EDTA (*see Subheading 2.3*).
8. Stereomicroscope.
9. Cryovials (Sarstedt, Germany).
10. Cryovial rack (Sarstedt).
11. 1× PBS (*see Subheading 2.3*).
12. Freezing medium (*see Subheading 2.3*).
13. Dry ice.

### 2.3.3. Identification of Homologous Recombinants

1. Lysis buffer: 100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/mL proteinase K (Sigma, USA, P2308). Keep proteinase K stock solution (10 mg/mL) at -20°C and always add freshly.
2. Isopropyl alcohol.
3. Restriction enzymes and buffers.
4. DNase-free bovine serum albumin (BSA) (New England Biolabs, Beverly, MA).
5. Agarose and ethidium bromide.
6. 10× TBE-buffer: 108 g Tris-base, 55 g boric acid, 9 mL 0.5 M EDTA pH 8.0, adjust to 1 L with deionized water (can be stored at RT).
7. Nylon membrane: for example, Amersham Hybond-XL cat. no. RPN203S (GE Healthcare, UK).
8. Denaturation solution: 1.5 M NaCl, 0.5 M NaOH (can be stored at RT).

9. 20× SSC: 175.3 g NaCl, 88.2 g Na-citrate, adjust pH to 7.0, autoclave (can be stored at RT).
10. Hybridization plastic bags.
11. Church buffer: 500 mL 1 M NaPi, 330 mL 20% SDS, 1 mL 0.5 M EDTA, 10-μL sheared salmon sperm DNA, 10 g bovine serum albumin (BSA). Fill to 1 L with deionized water (can be stored at RT).
12. Random priming labeling kit for DNA. For example, Amersham Rediprime II (GE Healthcare, UK).
13. <sup>32</sup>P-CTP (GE Healthcare, UK). The half-life of <sup>32</sup>P is approximately 14.3 days. Care should be taken when handling radioactive isotopes. Refer to local safety rules.
14. Wash solution 1: 2× SSC, 1% SDS (can be stored at RT).
15. Wash solution 2: 0.4× SSC, 1% SDS (can be stored at RT).
16. Autoradiography film.

## **2.4. Generation of Mutant Mouse Lines**

### *2.4.1. Generation of Vasectomized Males*

1. Mice for vasectomy: 8- or more week-old FVB males.
2. Avertin 100% stock: Dissolve 10 g 2,2,2-tribromoethyl alcohol (Fluka, Switzerland, cat. no. 90710) in 10 mL tert-amyl alcohol. For use, dilute the stock solution to 2.5% in PBS. Store both stocks and use solutions at 4°C wrapped in aluminium foil to protect them from light.
3. 75% Ethanol.
4. Surgical equipment: fine dissection scissors; two pairs of watchmaker #5 forceps (sometimes manually sharpened); blunt, fine-curved forceps; serrefine clamp (1.5 in. or smaller); surgical silk or catgut suture with curved needle (e.g., size 10), 1-mL syringes with 26-gage hypodermic needle.

### *2.4.2. Preparation of Needles for Microinjection*

1. Injection glass needles: with (Narishige #GD-1, Japan) and without (Narishige #G-1) internal filament.
2. Diamond glass cutter.
3. Needle puller (Narishige).
4. Microforge with 0.22-mm-thick platinum wire (Narishige, Japan).
5. Micropipette grinder (Narishige).
6. Teflon tube linked to a syringe.
7. 10% hydrofluoric acid (Sigma).
8. 100% ethanol.

### *2.4.3. Mouse Matings*

1. C57B6 mice.
2. Vasectomized males.
3. CBA × C57B6 F1 females.

#### 2.4.4. Isolation of Blastocysts

1. C57B6 females.
2. 70% ethanol.
3. Surgical equipment (*see Subheading 2.4.1*).
4. Stereomicroscope.
5. Flush medium: High glucose DMEM, buffered with 20 mM HEPES pH 7.4.
6. 10-mL syringe.
7. 0.60 × 30 mm syringe needle.
8. Transfer pipette.
9. ES medium (*see Subheading 2.4.1*).

#### 2.4.5. Preparation of ES Cells for Microinjection

1. ES cells.
2. Feeder cells.
3. 6-cm tissue-culture dishes.
4. ES medium (*see Subheading 2.3*).
5. 1× PBS (*see Subheading 2.3*).
6. Trypsin/EDTA (*see Subheading 2.3*).
7. 10-mL sterile tubes.

#### 2.4.6. Microinjection of ES Cells

1. Microinjection setup: microscope with Hoffman or Nomarski optics (e.g., Olympus, Japan, or equivalent). Left and right, water-driven micromanipulators (Narishige, Japan). Two 10-mL syringes, each linked to a metal glass capillary holder (Narishige, Japan) via a silicon tube.
2. Injection chamber: lid of a 3-cm tissue-culture dish with a hole in the middle (about 1 cm in diameter).
3. Vaseline without any additives.
4. Siliconized coverslip: rinse the coverslips in chloroform 2% dimetildiclorosilane for 30 s and air-dry.
5. M2 medium: 94.66 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl<sub>2</sub>, 1.19 mM KH<sub>2</sub>PO<sub>4</sub>, 1.19 mM MgSO<sub>4</sub>, 4.15 mM NaHCO<sub>3</sub>, 20.85 mM HEPES, 23.28 mM sodium lactate, 0.33 mM sodium pyruvate, 5.56 mM glucose, BSA 4 g/L.
6. Petri dish (Falcon, Los Angeles, CA).
7. Ice.
8. Dimethylpolysiloxan (Sigma, cat. no. DMPS-5X).

#### 2.4.7. Embryo Transfer

1. Microinjected blastocysts.
2. Pseudopregnant female mouse.
3. Avertin (*see Subheading 2.4.1*).
4. Two stereomicroscopes.
5. Optic fibers illuminators.

6. Surgical equipment (*see Subheading 2.4.1*).
7. Transfer glass pipette.

## **2.5. Mating of Chimeras**

1. Adult chimeric males.
2. C57B6 females.
3. Adult 129 females.

## **2.6. Genotyping Offspring**

### **2.6.1. Southern Blot Analysis of Tail DNA**

1. 20–30-day-old mice.
2. Ear-clips (National Band and Tag Co.).
3. Rotary wheel.
4. Tail buffer-PK: 1 mM Tris-HCl, pH 7.5, 1 mM EDTA, 250 mM NaCl, 0.2% SDS, and 0.1 mg/mL of freshly added Proteinase K (Sigma, cat. no. P0390).
5. Phenol/chloroform (Phenol, Sigma cat. no. 77613 and Chloroform, Sigma cat. no. C2432).
6. Chloroform.
7. Isopropyl alcohol.
8. Sterile DDW.

### **2.6.2. DNA Preparation for PCR Analysis**

1. Lysis buffer: PCR buffer X1 and 0.1 mg/mL proteinase K in DDW.
2. Thermomixer.
3. Sterile DDW.
4. *Taq* DNA polymerase (for example, Promega, WI, USA, cat. no. M8305).
5. Agarose and ethidium bromide.

## **2.7. Generation and Analysis of Double KO ES Cells**

1. ES cells.
2. ES medium (*see Subheading 2.3*).
3. Feeder cells (*see Subheading 2.3*).
4. 9-cm tissue-culture dishes (Falcon).
5. G418 (Geneticin).

## **2.8. Analysis of Differentiation Abilities of Homozygous ES Cells in Embryo Bodies and Teratomas**

### **2.8.1. Generation of ES Cells-Derived Embryoid Bodies**

1. ES cells.
2. ES medium (*see Subheading 2.3*).
3. Feeder cells (*see Subheading 2.3*).
4. Feeder medium (*see Subheading 2.3*).
5. 1× PBS (*see Subheading 2.3*).
6. Trypsin (*see Subheading 2.3*).
7. Burkert's chamber.
8. 9-cm Petri dish.

### 2.8.2. Induction of ES Cells-Derived Teratomas

1. ES cells.
2. ES medium (*see Subheading 2.3*).
3. Feeder cells (*see Subheading 2.3*).
4. Feeder medium (*see Subheading 2.3*).
5. 1× PBS (*see Subheading 2.3*).
6. Trypsin (*see Subheading 2.3*).
7. Sterile tubes (Falcon).
8. Burkert's chamber.
9. 1-mL syringe.
10. Avertin (*see Subheading 2.8*).

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## 3. Methods

### 3.1. Generation of Constructs

The first step to produce a knock-out or a conditional knock-out mouse is to obtain a targeting vector in which the genomic locus to be mutated is subcloned in a cloning vector and then modified adding loxP sites in specific positions (*see Subheading 1.2*). Thanks to the sequencing of mouse genome and the commercial availability of BAC clones carrying known genomic sequences, it is possible to easily obtain BAC clones containing the gene of interest. To target 129sv ES cells it is important to order BAC clones from pure 129SV background in order to avoid decrease in recombination efficacy due to DNA polymorphism (28).

Constructs for conditional knockouts should usually contain at least two recombinase recognition sites that flank a DNA segment, which once deleted, leads to gene inactivation. For this purpose, recombinase recognition sites can be placed in noncoding regions that flank one or more exons (**Fig. 1**). The presence of the selection cassette flanked by recombinase recognition sites allows eliminating heterologous DNA from the targeted locus, leaving a bona fide functional allele.

The targeting vector can be obtained with a conventional approach, i.e., using restriction endonucleases and DNA ligase, to cut and insert loxP sites and selection markers. However, this approach is complicated by the length of the genomic DNA to be handled and the mutation strategy is limited by the position of the restriction sites present in the sequence. A second possibility is to exploit homologous recombination in *E. coli* (27). This approach allows to quickly insert loxP sites and selectable markers anywhere in the DNA locus. Homologous recombination system in *E. coli* takes advantage from phage-encoded proteins like *exo*, *bet*, and *gam*. *Exo* encodes exonuclease producing 3'

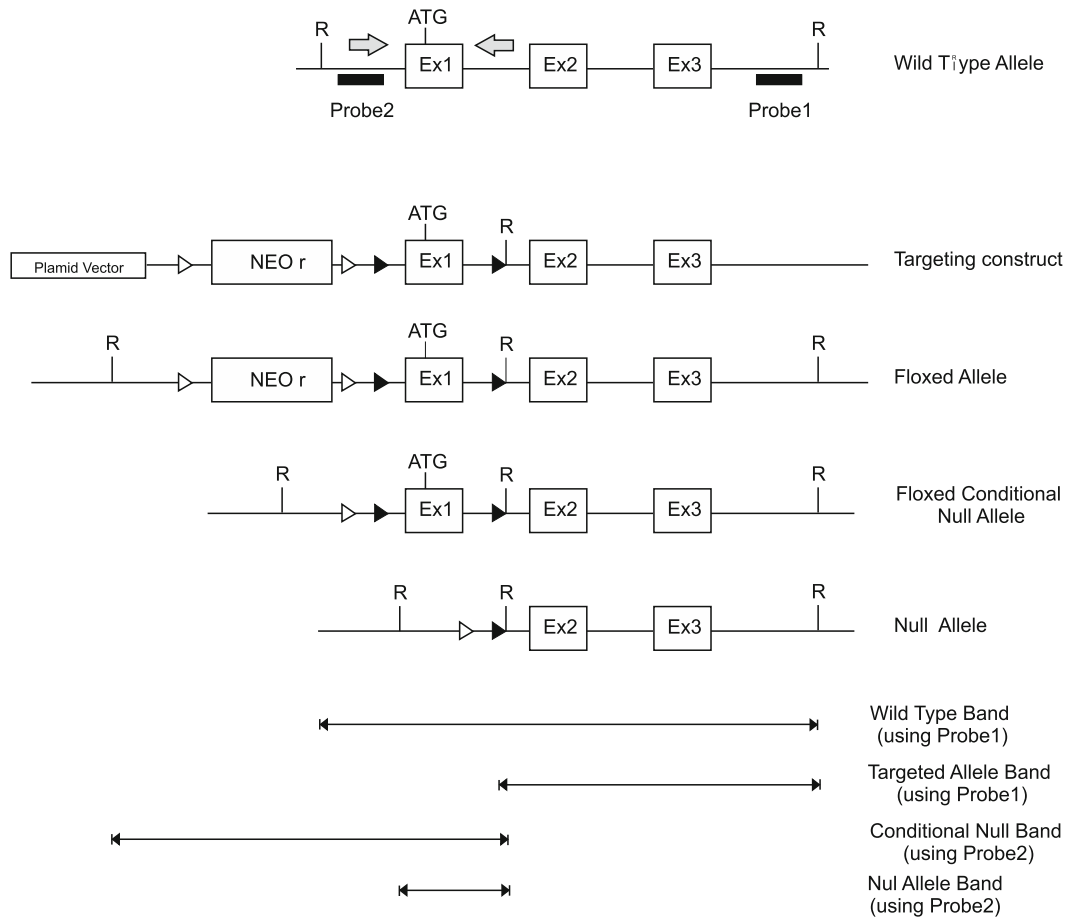


Fig. 1. Example of conditional gene targeting construct. The wild-type allele is replaced by a targeting vector in which the Neo resistance cassette is flanked by two FRT sites (*white triangles*) and the first coding exon (exon1) is flanked by two *loxP* sites (*filled triangles*). Transient expression of *Frt* allows excision of Neomycin resistance cassette in ES cells. The null allele will be obtained crossing floxed homozygous mice with Cre expressing mice. R indicates a restriction enzyme site. After digestion with R, probe 1 allows the identification of homologous recombinant clones, while probe 2 allows the identification of the null and the conditional null alleles. Gray arrows indicate the position of a forward and a reverse primer that can be used for genotyping of the mouse offspring (see Subheading 3.6.2).

single-strand overhangs; bet encodes pairing proteins, and gam inhibits the RecBCD exonuclease activity of *E. coli* that destabilize linear dsDNA.

To obtain homologous recombination, DNA has to be electroporated in the EL350 bacterial strain (27) containing an integrated defective prophage carrying the recombination genes *exo*, *bet*, and *gam*. The expression of these genes is undetectable at 32°C and can be induced at 42°C. Moreover, the defective prophage contains also a Cre gene under the control of an arabinose-inducible promoter that will be used to excise the neomycin resistance gene (see Subheading 3.1.3).

The procedure consists of the following steps (**Fig. 2**):

1. Subcloning of a portion of genomic DNA carried by the BAC (10–15 kb) in pBluescript:
  - (a) Amplification by PCR of two homology arms and cloning in pBluescript.
  - (b) Electroporation of this first construct in EL350 together with the BAC clone.
  - (c) Selection and characterization of the recombinant clone.
2. Insertion of the first loxP site:
  - (a) Insertion of a floxed Neo cassette by homologous recombination.
  - (b) Excision of the floxed Neo cassette by arabinose-induced expression of Cre recombinase in EL350.
  - (c) Selection and characterization of the clone.
3. Insertion of the second loxP site and of the Neo cassette flanked by FRT sites:

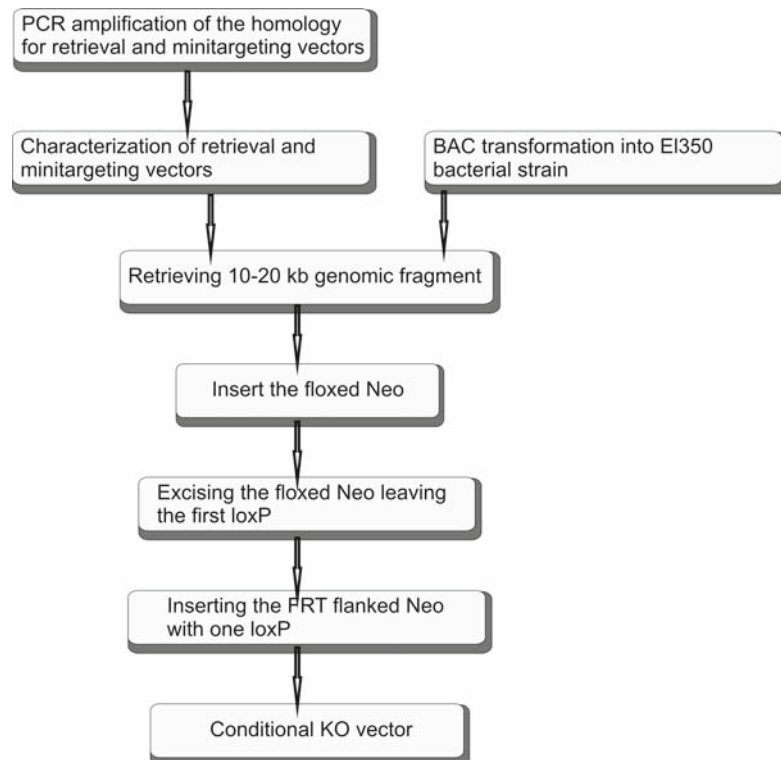


Fig. 2. Flow chart showing the steps necessary for the generation of the conditional knockout construct by homologous recombination in bacteria.

- (a) Insertion of a Neo cassette flanked by FRT sites by homologous recombination.
- (b) Selection and characterization of the final conditional knock-out vector.

For each of these different steps EL350 bacteria are made electrocompetent with distinct protocols (*see Subheadings 3.1.1–3.1.4*).

*3.1.1. Preparation of Electrocompetent Cells for BAC or Plasmid DNA Electroporation*

The following protocol is suitable for production of electrocompetent EL350 cells for BAC and plasmid DNA electroporation as needed in **Subheading “Cloning the Retrieval Minivector”**.

1. Grow EL350 cells ON in 5 mL of LB broth in a Falcon 14-mL polypropylene round-bottom tube at 32°C with shaking.
2. Collect the cells ( $OD_{600} = 1.2$ ) by centrifuging at 2700 g (0°C) for 5 min in falcon tube.
3. Resuspend cell pellets in 888  $\mu$ L of ice-cold water.
4. Transfer cells into a 1.5-mL Eppendorf tube (on ice) and centrifuge using a bench-top centrifuge for 15–20 s at room temperature.
5. Place the tubes on ice, and aspirate the supernatant fluid.
6. Repeat the process two more times.
7. Resuspend, finally, the cell pellet in 50  $\mu$ L of ice-cold water.
8. Transfer to a precooled electroporation cuvette (0.1-cm gap).
9. Add 1  $\mu$ L of BAC DNA (100 ng) or plasmid DNA (1.0 ng) and mix well.
10. Perform electroporation was using a BIO-RAD electroporator under the following condition: 1.75 kV, 25  $\mu$ F with the pulse controller set at 200. Set the time constant at 4.0.
11. Add then 1.0 mL of LB to each cuvette.
12. Incubate at 32°C for 1 h.
13. Spread cells on plates with the appropriate antibiotics.

*3.1.2. Preparation of Electrocompetent Cells for Retrieving the Sequence of Interest from the Selected BAC Clone*

The following protocol is suitable for production of electrocompetent EL350 cells for electroporation of a plasmid in EL350 that already contain the BAC clone in order to obtain homologous recombination and retrieve the genomic sequence of interest as described in **Subheading “Retrieving the Sequence of Interest”**.

1. Inoculate EL350 cells containing BAC of interest (prepared in the previous step) into 5 mL of LB broth in a Falcon 14-mL polypropylene round-bottom tube and grown at 32°C overnight with shaking.

2. Transfer, the next day, 1.0 mL of the overnight culture to 20 mL of LB.
3. Incubated for 2 h with shaking at 180 rpm.
4. When the cells have reached  $OD_{600} = 0.5$  transfer, 10 mL of the cells to a new flask and shake in a 42°C water bath for 15 min.
5. Put the cells into wet ice and shake the flask to make sure that the temperature of the flask dropped as fast as possible.
6. Left the flask in wet ice for another 5 min.
7. Transfer the cells to 25-mL glass centrifuge tubes and spun at 2700 g (0°C) for 5 min.
8. Resuspend cells in 888 µL of ice-cold water and transferred to a 1.5-mL Eppendorf tube (on ice) and wash three times with ice-cold water as described above.
9. Resuspend, finally, the cell pellet in 50 µL of ice-cold water, and add 1–2 µL, 10–50 ng DNA, of the purified plasmid fragment and electroporate as described above in **Subheading 3.1.1** (*see Note 1*).

*3.1.3. Preparation of Frozen EL350 Electrocompetent Cells for Cotransformation BAC DNA and Lox Targeting Vectors*

The following protocol is suitable for production of electrocompetent EL350 cells for the cotransformation of BAC and lox targeting vectors as described in **Subheadings “Targeting the First Lox Site in the Sequence of Interest”** and **“Cloning the Second Targeting Minivector”**:

1. Grow ON EL350 cells in two Falcon 14-mL polypropylene round-bottom tube with 5 mL of LB broth at 32°C with shaking.
2. Add 10-mL overnight culture of EL350 to 500 mL of LB broth in a 2-L flask.
3. Place the culture in a water bath shaker at 32°C until  $OD_{600} = 0.5$  (~2.0 h).
4. Transfer the flask to a 42°C water bath shaker and incubated for 15 min.
5. Put immediately the flask into an ice slurry and shake for 5 min by hand to make sure the temperature dropped as fast as possible.
6. Put the flask on ice for an additional 10 min.
7. Collected cells at 4,000 rpm at 0°C for 5 min and wash three times with sterile ice-cold water and once with sterile cold 15% glycerol in water.
8. Resuspend cells were in 4 mL of ice-cold 15% glycerol in water.
9. Aliquot 50 µL of the cells to pre-cooled Eppendorf tubes (80 tubes total) and stored at –0°C.

*3.1.4. Preparation of Frozen EL350 Electrocompetent Cells Induced for Cre Expression*

10. Thaw the frozen cells at room temperature and quickly put on ice.
11. Transform the purified targeting cassette, 100 ng in 1  $\mu$ L and the template plasmid DNA (10 ng in 1  $\mu$ L) vector using a BIO-RAD electroporator as described previously.
  1. Add a 10 mL overnight culture of EL350 cells to 500 mL of LB broth in a 2-L flask.
  2. Placed the culture in a water bath shaker at 32°C until OD<sub>600</sub> = 0.4 (2.0 h, 180 rpm).
  3. Add 5 mL of 10% l(+) arabinose in H<sub>2</sub>O to the culture to a final concentration of 0.1% and shake at 32°C for another hour.
  4. Collect cells and wash and froze cell pellets as described above.

*3.1.5. Retrieving the Sequence of Interest from the Selected BAC Clone*

Cloning the Retrieval Minivector

The aim of this step is to subclone 10–15 kb of the genomic DNA carried by the BAC in pBluescript using homologous recombination in bacteria.

1. Amplify the two homologous arms (around 300 bp) corresponding to the two ends of the sequence to be retrieved using the BAC clones as template. Include sites for restriction enzymes in the amplification primers to permit directional cloning of the PCR products into pBluescript. (for the left arm: in the forward primer insert sequence for the enzyme “A,” in the reverse primer for the enzyme “C”; for the right arm: in the forward primer insert sequence for the enzyme “C,” in the reverse primer the enzyme “B”).
2. Digest the amplified fragments with the appropriate restriction enzymes (A and C for the left arm and C and B for the right arm), purify them using a gel extraction kit.
3. Ligate the two amplified arms with pBluescript DNA linearized with the appropriate restriction enzymes (in the example “A” and “B”).
4. Transform competent frozen cells (Top10 plus), prepared as described in **Subheading 3.1.1**.
5. Plate on LB plate plus ampicillin.
6. Grow 12 single colonies ON.
7. Extract and digest DNA to check the presence of the correct retrieval vector.
8. Inoculate the colony carrying the correct construct in 100 mL LB broth and grow overnight.
9. Purify DNA from bacteria.
10. Linearize the retrieved vector, using the restriction enzyme “C.”
11. Purify digest vector DNA on agarose gel slice the band and extract it.

### Retrieving the Sequence of Interest

1. Electroporate the purified linearized retrieval vector into electrocompetent EL350 cells (prepared as described in **Subheading 3.1.2**) containing the BAC clone of interest.
2. Plate the electroporated cells on ampicillin containing LB agar plates.
3. Test the right retrieved sequence by restriction.

### 3.1.6. Targeting the First Lox Site in the Plasmid Containing the Subcloned Sequence of Interest

#### Cloning the First Targeting Minivector

The aim of this step is to introduce a loxP site into the targeting vector, by introducing the floxed Neo cassette by homologous recombination in bacteria.

1. Amplify two arms (300 bp) homologous to the left and the right site where has to be inserted the first lox site. Engineer the PCR primer pairs to contain restriction sites to allow for the directional cloning of the left homology arm, the floxed Neo gene and the right homology arm, into pBluescript. Place also in one of these primers a restriction site useful to analyze the recombined DNA in ES cells (left arm: in the forward primer insert the sequence for the enzyme “D” and in the reverse for “E”; right arm: in the forward primer insert the sequence for the enzyme “F” and in the reverse for “G”).
2. Isolate the floxed Neo cassette from the plasmid PL425 digesting it with enzyme “E” and “F.”
3. Purify the floxed Neo cassette on agarose gel, slice the band, and extract it.
4. Ligate the two homologous arms and the purified floxed Neo cassette in pBluescript linearized with enzymes “D” and “G.”
5. Select for the first lox containing targeting vector plating on Ampicillin and Kanamycin plates (the kanamycin resistance is given by the Neo gene that is under the control of a hybrid promoter able to drive its expression both in bacterial and mammalian cells).
6. Extract DNA from 12 colonies.
7. Check for a correct DNA with restriction enzymes.
8. Inoculate the good colony in 100 mL LB broth and grow ON.
9. Purify plasmid DNA.
10. Isolate the Neo cassette with, at the right and the left ends, the two homologous arms by digestion with restriction enzymes (in the example “D” and “G”) and purify it from agarose gel.

### Targeting the First Lox Site in the Sequence of Interest

1. Coelectroporate the purified Neo cassette with the flanking homologous arms in electrocompetent EL350 cells prepared as described in **Subheading 3.1.3**.

2. Select for the colonies targeted with the first lox sequence plating on Ampicillin and Kanamycin containing plates.
3. Test the lox Neo targeted clones by restriction.
4. Prepared DNA from the selected colony.

*3.1.7. Excising the Floxed Neo Leaving the First Lox Site*

The aim of this step is to remove the Neo gene by expressing the arabinose-induced Cre recombinase in EL350, so to leave the first loxP site in the targeting vector.

1. Electroporate 1 ng of lox Neo targeted DNA into 50  $\mu$ L of Cre expressing arabinose induced frozen competent cells as described in **Subheading 3.1.4**.
2. Add 1.0 mL of LB broth to the electroporation cuvette.
3. Plate 10–100  $\mu$ L of the cells on an ampicillin plate and 100  $\mu$ L on a kanamycin plate and incubated at 32°C overnight: the ampicillin plate should have 10–100 colonies, and no colonies should be present on the kanamycin plate. The Cre enzyme induces recombination between the two lox sites removing the Neo gene and leaving a lox site (*see Note 2*). Check that positive clones do not grow on kanamycin.
4. Pick up 12 colonies from ampicillin plate and grow them in LB ON.
5. Extract DNA.
6. Check for restriction map.
7. Choose a correct colony and use its plasmid DNA for coelectroporation in the next step.

*3.1.8. Targeting the Second Lox Site in the Sequence with the First Lox*

The aim of this step is to introduce into the subcloned DNA a second loxP site and a Neo resistance cassette to be used as selection marker both in bacteria and in mouse ES cells. This can be achieved using a Neo cassette under the control of a hybrid promoter. The cassette has also to be flanked by two FLP recombinase recognition sites (FRT) and followed by a loxP site. For further details refer to (27). The excision of the Neo cassette can be obtained by expression of FLP recombinase either by ES cell transient transfection or by crossing of mice with transgenic strains (29).

*Cloning the Second Targeting Minivector*

1. Amplify two arms (around 300 bp) homologous to the left and the right sites where has to be inserted the second loxP site. Engineer the PCR primer pairs to contain restriction sites to allow for the directional cloning of the homology arms, together with the *Neo* cassette, into pBluescript. Place also in one of these primers a restriction site useful to analyze the recombined DNA in ES cells (example, the left arm: in

the forward primer insert the sequence for the enzyme “D” and in the reverse for “E”; right arm: in the forward primer insert the sequence for the enzyme “F” and in the reverse for “G”) (*see* **Note 3**).

2. Ligate the two homologous arms, the Neo cassette, containing a loxP and two FRT sites (obtained from plasmid PL451 digested with enzyme “E” and “F”) in pBluescript linearized with enzymes “D” and “G.”
3. Select for the lox targeting vector plating on plates Ampicillin and Kanamycin.
4. Isolate the Neo cassette flanked by the two homologous arms by digestion with restriction enzymes (in the example “D” and “G”).

#### Targeting the Second Lox Site

1. Coelectroporate the Neo cassette obtained in **Subheading 3.1.8, step 4** with the subcloned genomic DNA in electrocompetent EL350 cells prepared as described in **Subheading 3.1.3**.
2. Select for the clones targeted with the second lox sequence plating on Ampicillin and Kanamycin plates.
3. Test the lox Neo targeted clones by restriction.
4. Extract DNA from a colony carrying the correct construct.

#### 3.2. Definition of the Probe

To initially establish a gene-targeting strategy, a probe that permits the identification of the mutants by Southern blot analysis must be isolated. Genomic DNA is interspersed by repetitive elements, which give high backgrounds in hybridization analysis. It is therefore essential to test several fragments of the BAC clone to isolate a probe encoding a single genomic sequence. The probe must generate a diagnostic signal that distinguishes the targeted allele from the wild-type counterpart. This result can be obtained by finding a restriction enzyme that cuts outside the construct and generates a fragment that encompasses the probe. In the best situation, this same enzyme cuts the targeted allele inside the resistance cassette and generates a fragment that is shorter than the wild-type (**Fig. 1**). Generation of shorter segments is recommended because the identification of a recombinant clone will not be confused by the presence of partially digested DNA. If the search for a probe with the above features fails, it will be necessary to change the strategy outlined above and find alternatives such as extending the restriction map to new enzymes and considering other exons, deletions, or resistance cassettes. Because cloning steps involved in construct preparation are often complex and time consuming, it is wise to start to build the construct only if a good probe has been found.

### 3.3. Manipulation of ES Cells

ES cells to be used for knock-out experiments must be kept in an undifferentiated state. ES cells are small and round, with a large nucleus and few cytoplasm; they strongly adhere to each other and grow as aggregates. They are very sensitive to cell-culture conditions and, if not properly handled, tend to spontaneously differentiate in various cell types. Good ES cell colonies can be judged by microscopic analysis: they must be a multilayered aggregate that shows a clear cut, shiny boundary. On the contrary, differentiated ES cell colonies lose the glossy perimeter, tend to flatten, and/or to darken in the middle. Whenever such colonies are detected, it is advisable to discard the culture. The totipotency of the cells is correlated to the number of passages in culture and it is known to strongly decrease after 30 cycles of trypsinization/freezing. ES cell must be fed every day and, in certain critical concentrations, even twice a day. Colonies are, in fact, sensitive to density on the culture dish and as soon as they touch each other, cells start to differentiate. Thus, ES cell colonies must be split when they reach about 50–75% of confluency. It is important to passage colonies as a single cell suspension: if not properly dissociated they form large aggregates that are very prone to differentiation. Several ES cell lines are currently available and most require a specific technique handling. Consistent results have been obtained using the R1 (30) and the E14 (1) ES cell lines. They grow on the top of a feeder layer of primary embryonic fibroblasts in a medium containing LIF. These cell culture conditions assure very high levels of chimerism and an optimal rate of germ line transmission.

#### 3.3.1. Isolation of Feeder Cells

Feeder cells provide a basal level of LIF production and a number of yet unidentified factors that sustain ES cells growth in the totipotent state. Feeder cells are derived from the carcass of a 14-days-old embryo. To allow selection of recombinant ES clones with antibiotics, embryos must derive from a transgenic mouse that expresses the proper resistance gene.

1. Mate a male homozygous for the resistance cassette transgene (*see Note 4*) with a C57BL6 female.
2. The following day, check for the presence of the vaginal plug, a whitish, solid sperm residue that indicates that the female mated during the night (*see Note 5*). Separate these females and keep them until needed.
3. After 14 days, sacrifice the pregnant females by cervical dislocation.
4. Thoroughly wet the animal in 70% ethanol and put it under a sterile hood above a paper towel.
5. Cut the skin with sterile scissors and carefully expose the abdomen. Grasp with fine forceps one end of the uterine

horn and free with scissors the uterus together with embryos from mesometrium and cervix. Immediately transfer embryos (still inside the uterus) in a 10-cm bacterial culture Petri dish filled with sterile PBS.

6. With scissors separate, each implantation site from the other. With fine forceps, carefully free embryos from the uterine wall, yolk sac, and placenta. Repeatedly wash them in several Petri dishes filled with fresh sterile PBS, until bleeding stops.
7. With fine scissors, cut and discard the head. Open the abdomen with fine forceps and remove all internal organs.
8. Hold the carcass with forceps above a Falcon tube filled with trypsin (1 mL/embryo) and mince it with fine scissors to very small pieces that are let to fall into the trypsin solution.
9. Incubate the suspension at 37°C for 15 min. Break tissue pieces by pipetting up and down with a large gage pipette (e.g., 10-mL pipette). Incubate again at 37°C for 15 min.
10. Thoroughly, dissociate cell clumps by pipetting up and down with a small gage pipette (e.g., a 2 mL or a Pasteur pipette).
11. Fill the falcon tube with feeder medium and let it stand for 5 min to let large aggregates sink. Transfer the supernatant to a fresh falcon tube and centrifuge it at  $120 \times g$  for 5 min. Discard the supernatant and dissolve the pellet with 2 mL/embryo of feeder medium. Seed 1 mL into a 15-cm cell-culture Petri dishes filled with 14 mL of feeder medium (two Petri dishes/embryo).
12. Grow to confluency without changing medium in a 37°C, 5% CO<sub>2</sub> incubator. Wait for another 3 days. Wash plates with 15 mL PBS and incubate for 5 min inside the incubator with 2 mL of trypsin. Resuspend detached cells with 2 mL of feeder medium. Reseed the plate with 0.5 mL of cell suspension.
13. Pellet cells in a Falcon tube. Discard supernatant and irradiate the cell pellet with 6,000 rad to inhibit cell division.
14. Resuspend the irradiated pellet with freezing medium (3 mL/Petri dish). Aliquot 1 mL of cell suspension per cryovial and freeze in a box kept in frozen carbon dioxide. Keep frozen stocks either at -80°C or in liquid nitrogen.
15. Add 14 mL of feeder medium to reseeded cells and repeat from **step 11** for a maximum of two times.
16. To check for sterility, thaw frozen aliquots at 37°C. Add cells to 5 mL of feeder medium in a 10-mL sterile tube. Centrifuge 5 min at  $120 \times g$ . Decant supernatant, resuspend pellet, and seed it in a 10-cm cell-culture Petri dish. Culture cells for few days and then test supernatant for mycoplasma infection.

17. Frozen feeder fibroblasts can be thawed and plated in advance or just together with ES cells (in the presence of ES medium). To obtain a confluent layer of irradiated cells, thawing one frozen vial normally gives enough cells to cover the surface of a 10-cm diameter Petri dish. All other areas should be calculated using that rule of thumb.

### 3.3.2. Electroporation and Selection

Before transfection, ES cells must be expanded and kept growing minimizing passages. The construct is transfected into ES cells by electroporation. This method assures that in most cases only one copy of the exogenous DNA is inserted. In an average transfection experiment,  $2 \times 10^7$  cells (the content of a full 10-cm Petri dish) are transfected with 30  $\mu\text{g}$  of DNA.

1. Using feeder medium, plate irradiated fibroblasts (about nine cryovials) onto nine 10-cm tissue-culture dishes. Change to ES medium when cells are adherent and spread.
2. Linearize 30  $\mu\text{g}$  of the construct digesting with a single cutter enzyme (usually *NotI*) that cleaves at the boundary between one homology arm and the vector. Keep a final DNA concentration of at least 50  $\text{ng}/\mu\text{L}$ . Incubate 1 h at the suitable temperature, using 30 or more enzyme units.
3. Extract the reaction mixture once with an equal volume of phenol/chloroform. Spin at maximum speed for 5 min. Collect the supernatant and extract it once with chloroform. Spin at maximum speed for 30 s. Save the supernatant.
4. Add to the supernatant 1/10 of the volume of 3 M Na acetate pH 5.2 and two volumes of 100% ethanol. Mix thoroughly. A white DNA precipitate particle must appear. Using a yellow tip, transfer the precipitate in a sterile screw cap tube containing 1 mL of 70% ethanol/DDW. Keep in ice until needed.
5. Wash ES cells two times with PBS. Add 1 mL of warm trypsin. Incubate 5 min at 37°C. After adding 2 mL of ES Medium, dissociate colonies by pipetting up and down, avoiding the production of bubbles.
6. Dilute cells to 10 mL and count them using a Burker's chamber. Cells with large cytoplasm derive from the feeder layer and should thus be omitted from the count.
7. Spin  $2 \times 10^7$  cells in a sterile 10-mL tube 5 min at  $120 \times g$ . Resuspend 5 mL of PBS. Repeat this step twice.
8. Spin again and resuspend in PBS to reach a final volume of 600  $\mu\text{L}$ .
9. Spin the DNA precipitate for 30 s. Discard the supernatant under sterile conditions and let the pellet dry out for few min inside the hood. Resuspend DNA in 200  $\mu\text{L}$  PBS.

10. Mix the cell suspension with DNA. Using a Pasteur pipette, transfer the mixture into a sterile cuvette for electroporation. Electroporate cells at 3  $\mu$ F and 0.8 kV. The time constant should correspond to 0.1 ms. Electroporation leads to about 50% of cell death.
11. Quickly and carefully transfer electroporated cells into 8.5 mL of ES Medium. Mix and distribute 1 mL of transfected cells to each feeder plate.

### 3.3.3. Picking and Freezing of Resistant Clones

Twenty-four hours after electroporation, medium is changed to Selection Medium. In case a neomycin resistance cassette is used, cells can be selected by the addition of G418 antibiotic powder to ES Medium at a concentration of 400  $\mu$ g/mL (*see Note 6*). In these conditions, resistant colonies appear within 5–6 days after transfection (*see Note 7*). In a typical experiment, enough recombinant clones can be detected in about 2–300 picked colonies. Not all colonies grow at the same rate, therefore picking and freezing steps can take 2–3 days each.

1. Thaw one cryovial of feeder cells at 37°C. Dilute cells in 10 mL of feeder medium. Spin 5 min at  $120 \times g$ . Decant supernatant and resuspend cells in 25 mL of feeder medium. Aliquot 1 mL of cell suspension into each well of a 24-well dish. Repeat this step for ten or more (depending on the number of colonies that are to be picked) 24-well dishes. Let fibroblasts adhere and spread overnight.
2. Just before starting to pick the colonies, change medium of 24-well dishes to ES Medium.
3. With a 5-mL pipette, transfer two drops of trypsin into each well of a 96-well microtiter dish. Warm at 37°C.
4. Using a stereomicroscope under a laminar flow hood, gently scrape a colony with a P200 pipette equipped with a sterile yellow tip. Suck the cell aggregate in a maximum volume of 10  $\mu$ L and transfer it in a 96-well filled with trypsin. Repeat this step for 12 colonies (*see Note 8*).
5. Incubate the 96-well dish at 37°C for 5 min. Open one 24-well dish with feeder and the 96-well dish under the hood.
6. With a fresh, sterile yellow tip, collect about 100  $\mu$ L of ES Medium from a feeder cells-containing well. Add it to a trypsin well. Dissociate the trypsinized colony by gentle pipetting. Carefully transfer the cell suspension to the same well from which ES Medium was taken. Make a few air bubbles to mark the well. Repeat this step for all trypsinized colonies (*see Note 8*).
7. Repeat **steps 4–6** until enough colonies have been picked.

8. The day after, change to fresh ES Medium all wells that received a colony.
9. Wait 3–4 days for ES cells to expand. As soon as the number and the size of colonies are suitable for sibling, cells from each well can be frozen.
10. Mark cryovials with progressive numbers. Mark the same numbers on the bottom of each well to be passaged.
11. Set an empty cryobox into dry ice. Put labeled cryovials into the holding device.
12. Wash all marked wells of a 24-well dish with 1 mL PBS. Add two drops of trypsin into each well and incubate 5 min at 37°C.
13. Collect 900  $\mu$ L of freezing medium with a P1000 equipped with a fresh, sterile blue tip. Thoroughly resuspend trypsinized cells of one well by gentle pipetting. Retrieve only about 600  $\mu$ L of cell suspension and transfer it into the cryovial with the corresponding number. Place the cryovial into the box in dry ice. Repeat this step for all trypsinized wells.
14. Fill each well to maximum with feeder medium to dilute DMSO, which can eventually be toxic for cells.
15. Repeat **steps 12–14** until all clones have been frozen.
16. The day after freezing, it is extremely important to change medium to feeder medium.

*3.3.4. Identification of  
Homologous  
Recombinants*

1. When medium inside a well turns to yellow, wait an additional day. Then discard the medium and add 500  $\mu$ L of Lysis buffer. Keep at 37°C until all wells have been kept with Lysis buffer for at least one night.
2. Add 500  $\mu$ L of isopropyl alcohol and shake overnight at room temperature.
3. Label Eppendorf tubes correspondingly to lysed clones. Add 100  $\mu$ L of sterile DDW to each tube.
4. Prepare a glass rod by flaming the tip of a Pasteur pipette. Collect with this instrument the white DNA precipitate that formed on the bottom of a 24-well dish well. Disperse the DNA in the water of the corresponding tube. Clean the glass rod in sterile DDW and dry it with a paper towel. Repeat this step for the precipitates of all different clones.
5. Let DNA dissolve overnight at 56°C. Store genomic DNA at 4°C.
6. Digest 15  $\mu$ L of genomic DNA with the suitable restriction enzyme in 30  $\mu$ L of a final reaction volume containing 0.3  $\mu$ L BSA, 3  $\mu$ L buffer, 30–40 U enzyme. Incubate overnight in an oven at 37°C.

7. Load digested DNA on a 0.8% agarose gel in  $1\times$  TBE,  $1\text{ }\mu\text{g/mL}$  ethidium bromide. Separate for 6–8 h at 3 V/cm.
8. Photograph gel on an UV-table together with a ruler.
9. Blot the digested DNA onto Nylon membrane in alkaline conditions.
10. Mark the position of the wells on the nylon membrane with a pencil. Neutralize the membrane twice in  $2\times\text{SSC}$ .
11. Place filters in a plastic bag containing the minimum amount of Church buffer to thoroughly wet them. Prehybridize for 1 h at  $65^{\circ}\text{C}$ . Radioactively label the probe by random priming following the instructions provided in the kit. Hybridize filters overnight at  $65^{\circ}\text{C}$  in Church buffer containing at least  $1\times 10^6$  cpm/mL of  $^{32}\text{P}$ -labeled probe.
12. Wash filters at  $65^{\circ}\text{C}$  in 250–500 mL of hot Washing Solution 1 and 2 for 30 min each.
13. Expose with an autoradiography film until clear bands are visible.

### **3.4. Generation of Mutant Mouse Lines**

Chimeric mice are generated by microinjection of mutant ES cells into the cavity of a blastocyst (31) (*see Note 9*). Chimeras are then mated to generate an offspring that carries the ES cells genotype. To establish these techniques, an animal care facility must be available and treatment of mice must proceed in agreement to local laws regulating in vivo experimentation.

#### **3.4.1. Generation of Vasectomized Males**

1. Anesthetize a male mouse by intraperitoneal injection of 0.5 mL of diluted Avertin solution. Wash skin of lower abdomen (at the level of the top of the legs) with 75% ethanol and make a 1-cm cut with sharp scissors (1.5-cm large). Similarly cut the muscle of the body wall, avoiding the fat pad surrounding the genitals.
2. With bent blunt forceps, gently push the scrotum to move the right testicle into the abdominal cavity (until the white testicular fat pad appears at the edge of the incision). Expose the testicle by pulling the white fat pad. Note that around the testicle, the white coiled epididymis prolongs in a wider tube: the vas deferens.
3. Pierce with the tip of the forceps the thin membrane linking the vas deferens to the testicle and blood vessel. Apply two stitches around the freed tube, at a distance of about 5 mm from each other. With scissors, cut the vas deferens between the two stitches. By gently grasping the fat with forceps, reposition the testicle inside the abdomen. The same procedure is repeated for the left testicle.

4. Separately stitch two or three times the muscle and skin, then put the mouse alone back into a fresh cage. Vasectomized mice can be used 15–30 days after surgery. Testing for residual breeding capacity is advisable.

#### 3.4.2. Preparation of Needles for Microinjection

Two needles are needed: one is used to hold the blastocyst and the other to suck and inject cells (**Fig. 3**). Making such microinjection needles is a laborious task. It is therefore advantageous to prepare sets of these tools in advance and store them until needed.

##### Preparation of Holding Capillaries

1. Heat the middle of a tube on a flame until the glass starts to melt. Quickly move out of the flame and pull the extremities of the tube by hand.
2. Select capillaries with a 2–3-cm long, 0.1-mm-wide tip.
3. With the help of a diamond tip, cut sharp and flush the end of the tip.
4. Adjusting the tip near the heating filament of the microforge, slowly melt the glass until the opening narrows to 1/4 of the original diameter.
5. Placing the capillary orthogonal to the heating filament bend the last 3–4 mm of the tip to form a 30° angle.

##### Preparation of Microinjection Capillaries

1. Using a puller, pull tubes so that they form a 1-cm long 1- $\mu$ m wide tip.
2. Make a small ball of melted glass on the tip of the heating filament of the microforge.

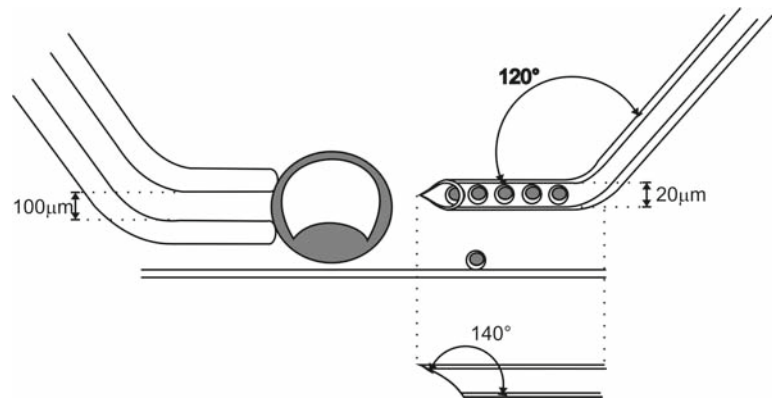


Fig. 3. Lateral view of the injection chamber. On the *left*: holding capillary used to block a blastocyst (*dark gray*). Edges of the glass walls have been rounded by flaming. The opening can vary from 50 to 100  $\mu$ m. On the *right*: injection capillary holding some ES cells. The opening should be not smaller than the diameter of a cell (about 20  $\mu$ m). A view of the tip from the above (as seen on the microscope) is shown below. Note the shape of the tip. Both holding and injecting capillaries should be bent (about 120°) so that they remain parallel to the bottom of the injection chamber.

3. Place the tip of the pulled capillary on the filament. Heat to a moderate temperature (when the ball gets a reddish glow) until the needle slightly fuses with the glass ball. Immediately switch the heating off. The capillary breaks leaving a sharp and flush opening (*see Note 10*).
4. Place capillaries with 20–30  $\mu\text{m}$ -wide tips onto the grinding wheel at an angle of about 20–45°. Leave until a beveled end is formed.
5. Connect the needle to a Teflon tube linked to a syringe and wash it by sucking in and out at least three times with the following solution: 10% hydrofluoric acid, DDW 1, DDW 2, DDW 3, 100% ethanol.
6. Make a sharp fine tip at the beveled end. Heat the glass ball on the microforge filament to a moderate temperature. Gently push the needle against the ball and quickly withdraw it. The tip fuses with the glass ball and makes a sharp spike.
7. Place the capillary orthogonal to the heating filament and with the opening toward the operator. Bend the last 3–4 mm of the tip to form a 30° angle.

#### 3.4.3. Mouse Matings

Blastocysts are obtained by natural mating of a relatively large number of C57B6 mice. The presence of the vaginal plug the morning following mating is considered 0.5 days postcoitum (dpc). Microinjected blastocysts are transferred to the uteri of a pseudopregnant female. To help embryos recover from the trauma of injection and to ensure a high rate of births, recipient females are one day delayed with respect to blastocysts.

1. Mate, at late afternoon, about 25 C57B6 males with two C57B6 females each (*see Note 11*).
2. The following morning check for plugs (*see Note 5*) and keep pregnant females in a separate cage.
3. At late afternoon of the same day, mate vasectomized males with 2 CBA  $\times$  C57B6 F1 females each.
4. The following morning check for plugs (*see Note 5*) and keep pseudopregnant females in a separate cage.

#### 3.4.4. Isolation of Blastocysts

1. To isolate blastocysts, sacrifice by cervical dislocation C57B6 females at 3.5 dpc.
2. Flush hair with 70% ethanol. Open the belly with scissors and expose the uterus below bowels.
3. Put the mouse under the stereomicroscope. Cut cervix (the single tube where uterine horns join) with sharp, fine, and curved scissors. Grasp the cervix with forceps and gently lift the uterine horns. With fine curved scissors, cut mesometrium and vessels along the uterine horns, and also being sure to

avoid hurting the muscular wall. Free the uterus by cutting at the uterotubal junction. Place the uterus in a drop of flushing medium.

4. With forceps, hold a uterine horn near the uterotubal junction. Using scissors, with the other hand open the tip by making a cut parallel to the horn. Repeat this procedure for the other horn. Place the uterus in a fresh drop of flushing medium.
5. Place the uterus in a dry, sterile watch glass under the stereomicroscope. With one hand, use forceps to hold the cervix. With the other hand, hold a 10-mL syringe filled with flushing medium. Insert the needle in the cervix toward one horn. Flush with about 0.5–1 mL, tightening the muscular wall around the needle with forceps. If the uterine tube does not let liquid out, repeat the procedure in **step 4**.
6. Collect blastocysts under the stereomicroscope by sucking them into a transfer pipette. Transfer blastocysts in a watch glass containing ES Medium and keep them in the incubator until needed.

#### *3.4.5. Preparation of ES Cells*

1. Plate ES cells and feeder on a 6-cm large tissue-culture dish 2–3 days before microinjection. Change ES Medium every day. ES cell colonies should reach an optimal density (75–90% confluency) without being passaged.
2. Wash cells two times with 5 mL PBS. Add 0.5 mL trypsin. Incubate 5 min at 37°C.
3. Add 3 mL ES Medium. Carefully dissociate colonies by pipetting up and down. Transfer the cell suspension to a 10-mL sterile tube. Spin for 5 min at 150 g.
4. Resuspend pellet in 5 mL ES Medium. Discard about 4 mL and spin the rest for 5 min at 120 × g.
5. Discard the supernatant leaving just a drop medium. Resuspend cells by gently flickering the tube with fingers.

#### *3.4.6. Microinjection of ES Cells*

Blastocyst injection requires an inverted microscope equipped with phase contrast or Nomarski's optics. Two micromanipulators are set at the left and the right sides of the microscope's stage. Each micromanipulator controls movements of a glass capillary. One needle (on the left) is used to hold the blastocyst and the other (on the right) to collect and inject cells. Each capillary is held through a hollow metal rod connected via silicon tubing to an air-filled syringe. Sucking and blowing of cells and embryos is controlled only by a gentle action on this device.

1. With Vaseline grease, fix a siliconized coverslip to the injection chamber.

2. Put a large drop of M2 medium on the coverslip and, using the transfer pipette, add blastocysts at the upper left corner. In a similar way, add the suspension of ES cells in stripes along the drop.
3. Cool the stage of the microscope to about 10°C by placing on its top a glass Petri dish filled with ice. Put the injection chamber on the cooled stage.
4. Connect capillaries to their metal holder and syringe. Fill the pipettes with M2 medium (*see* **Note 12**).
5. Adjust holding and injection pipettes on micromanipulators. Put capillaries in focus. Pipettes should be carefully turned until they show a straight horizontal orientation.
6. Overlay the M2 drop with dimethyl polysiloxan.
7. Collect about 150 healthy ES cells. Healthy ES cells are round and of medium size. They must have a smooth surface and a bright nucleus. Feeder cells can be easily avoided by their larger size and spiked shape.
8. Using the holding pipette, gently suck a blastocyst. Moving the injection needle, turn the blastocyst until a thin depression between two cells is in focus. Hold the embryo in this position by delicate sucking (**Fig. 3**).
9. By a rapid horizontal movement, insert the injection needle into the blastocyst cavity. Inject about 15 cells. Gently withdraw the glass capillary and place the injected blastocyst on the lower right corner. After injection, blastocysts may collapse.
10. Repeat from **step 8** until cells are exhausted then repeat from **step 7** until all blastocysts were used.

#### 3.4.7. Embryo Transfer

After microinjection, blastocysts should recover in the incubator for at least 1 h. Generally, embryos can be transferred as soon as they start to re-expand to form again their cavity. The number of transferred blastocysts for each pseudopregnant female can vary from 7 to 12.

1. Anesthetize a pseudopregnant female mouse by intraperitoneal injection of 0.5 mL of diluted Avertin solution. Put the mouse with the head facing 12 o'clock under the stereomicroscope equipped with optic fibers illuminators.
2. Disinfect the skin of the back and cut with scissors at about 1–2 cm above the hind leg. Wipe off hairs with a paper napkin. Detach the cut skin from the underlying muscle and displace it around to localize the underneath ovary (a red-dish ball surrounded by a white fat pad). Expose the ovary by cutting the above body wall muscle, avoiding blood vessels (red) and nerves (white). Hold the fat with blunt-ended

curved forceps and gently force the uterine horn out of the cavity.

3. On a second stereomicroscope, prepare the transfer pipette by filling it with a series of small air bubbles followed by ES Medium. Cautiously suck 3–6 embryos, minimizing the liquid in-between. Add a very small last air bubble at the tip. Store the transfer pipette undisturbed nearby the first stereomicroscope.
4. Using the fine forceps in one hand, hold the uterine horn near its apical end and gently stretch it outside the abdominal cavity. With the other hand, quickly grab the syringe needle and puncture the uterine wall avoiding the rupture of blood vessels. Insert the needle so that it reaches the lumen of the uterine horn without piercing again the musculature. Using this same hand, remove the needle and take the mouth pipette, possibly avoiding to leave the binoculars. Locate the hole again and insert the transfer pipette. Gently blow embryos until air bubbles are seen to move inside the uterus. Slowly withdraw the pipette (*see Note 13*). If liquid is expelled from the hole, quickly suck it into the pipette (*see Note 14*).
5. Put the ovary and uterus back inside the abdomen. Using surgical catgut thread, apply a few stitches to the muscle and to the skin.
6. Repeat the same procedure from **step 2** on the other uterine horn.
7. When finished, put the mouse back to the cage and keep it warm by covering it with straw.

### **3.5. Mating of Chimeras**

To check for germ line transmission, adult chimeric males are mated to 2–3 adult C57B6 females. Transmission of the ES cell genotype is evidenced by the birth of agouti pups. The agouti coat color, characteristic of the 129 mouse strain (from which ES cells derive), is dominant over the black coat color shown by C57B6 mice. The higher the chimerism is, the better the chances for germ line transmission. If a chimera generates at least three litters of black pups, it is presumably not able to transmit the ES cell genotype. When chimeras show about 100% of ES cell contribution, it can happen to obtain agouti pups only. Transmission of the mutant allele should show a normal Mendelian distribution among the agouti offspring. Mice obtained by this mating strategy possess a mixed 129 and C57B6 genetic background. An easy way to obtain heterozygous animals of pure 129 inbred strain is to cross the chimera with an adult 129 female. In this case, the litter obtained shows only the agouti coat color. To identify 129 inbred animals, it is necessary to test the genotype

for the presence of the mutant allele. Mice that bear the mutation virtually derive from the mating of two 129 inbred mice and being of pure 129 genetic background, they can be used to generate a 129 inbred mouse colony.

### 3.6. Genotyping of Offspring

Agouti pups must be genotyped to assess whether they are heterozygous for the mutation. For this purpose, genomic DNA must be extracted from tail biopsies and analyzed for the presence of the targeted allele. The high reliability of Southern blot techniques makes this the system of choice for identification of heterozygotes in the very first litters. Faster but more prone to errors is PCR analysis, a procedure that is then suitable for genotyping of assessed mutant mouse strains.

#### 3.6.1. Southern Blot Analysis of Tail DNA

1. Label 20–30-days-old mice by applying numbered ear clips. Mark number, sex, and mouse coat color in a notebook for further reference.
2. Cut the tail with sharp, strong scissors at about 1 cm from the tip and collect the tissue sample in a labeled sterile 1.5-mL tube. Dissolve tail tissue by incubation on a rotary wheel with 0.5 mL of tail buffer-PK overnight at 56°C.
3. Add 0.5 mL of phenol–chloroform. Incubate samples for 10 min on the rotary wheel at RT, then spin them at maximum speed on a bench centrifuge for 5 min.
4. Collect the DNA containing supernatant, avoiding precipitates at the interface, with a 1-mL disposable tip (cut to make the opening wider). Add the supernatant to a fresh tube containing 0.5 mL of chloroform. Incubate samples again on the rotary wheel for 10 min, then spin them for 5 min at maximum speed.
5. Collect the DNA containing supernatant as in **step 4** and add it to a fresh tube containing 0.5 mL of isopropyl alcohol. Invert the tube a few times and pellet the white DNA precipitate to the bottom by a short spin. Carefully eliminate the alcohol and air-dry the DNA for a couple of minutes.
6. Add to the DNA pellet 100 µL of sterile DDW and resuspend it overnight at 56°C. Store the DNA solution at 4°C.
7. Analyze the DNA samples as described in **Subheading 3.3.4, steps 6–13**. Using the probe defined for the screening of targeted ES clones, homozygous and heterozygous mice show one or two bands, respectively.

#### 3.6.2. DNA Preparation for PCR Analysis

1. Design a forward primer upstream the floxed exon and reverse primer downstream the floxed exon, in this way distinct diagnostic fragments will be obtained for wild type and floxed or null allele (**Fig. 1**). Best results are obtained with 20-mers having at least a 50% GC content.

2. Label mice as in **Subheading 3.6.1, step 1**. Collect no longer than 2–3 mm of the tip of the tail in a 1.5-mL tube containing 50  $\mu$ L of lysis buffer.
3. Incubate samples at 60°C shaking in the Thermomixer for 2–3 h. Smash remnants of tail using a fresh yellow tip for each tube. Inactivate proteinase K by 6-min incubation at 95°C. Spin at maximum speed for 5 min. Dilute 5  $\mu$ L of the supernatant into 10  $\mu$ L of sterile DDW.
4. Set up a PCR reaction following standard procedures (32) using 5  $\mu$ L of the diluted sample in a final volume of 50  $\mu$ L (*see Note 15*).
5. Amplify using the following cycle profile:
  - Ten cycles 95°C, 30 s
  - 65°C–1°C each cycle, 30 s
  - 72°C, 30 s
  - 25 cycles 94°C, 30 s
  - 55°C, 30 s
  - 72°C, 30 s
6. Check the reaction on 2% agarose gel. Amplification of one band with both combinations of oligonucleotides indicates heterozygosity. Samples showing a band with either one of the two couples of oligos are to be considered wild-type or mutant homozygous depending on the length of the diagnostic fragment detected and on the presence of the wild-type or mutant allele specific oligonucleotide in the productive reaction.

### **3.7. Generation and Analysis of Double KO ES Cells**

Knocking-out genes essential for embryonic development leads to lethality before birth and in many cases precludes the analysis of gene function in adult tissues. To overcome this limitation, it is necessary to generate *Cre/lox* conditional mutagenesis. A simpler alternative is to isolate ES cells homozygous for the mutation, which can be used to generate chimeras. The analysis of the potential of these double mutant cells to contribute to the formation of different organs may give essential information on gene function in established tissues (33). Homozygous ES cells can be generated by electroporation of a second targeting construct that bears a different selection cassette (e.g., hygromycin in place of neomycin resistance gene). Alternatively, cells bearing a null allele generated by *Cre*-mediated excision of the selection marker gene (**Fig. 1**) can be transfected again with the same construct. In these two situations, about 50% of the recombinant clones should show by Southern blot hybridization a homozygous-specific pattern of bands. The simplest method, however, consists of growing heterozygous ES cells in a medium containing very high levels of selection drug (34) (*see Note 16*).

1. Thaw heterozygous ES cells carrying a neomycin resistance cassette on one allele.
2. Grow and expand on feeder to obtain at least  $10^5$  cells.
3. Plate ES cells on ten 9-cm tissue-culture dishes with  $10^4$  cells each.
4. The next day, change medium with ES medium supplemented with 4 mg/mL G418.
5. Change medium every day. Check for dying cells. If cells do not start to die in 4–5 days, it is likely that the technique will not succeed.
6. Wait for 6–7 days to start to see colonies. Treat resistant clones as described in **Subheading 3.6.3**
7. Analyze the genotype as described in **Subheading 3.6.4**

#### 3.7.1. Analysis of ES Cell Contribution in Chimeric Mice

Genetic differences between ES and host embryo derived cells can be exploited to measure contribution of the injected cells in a chimeric tissue. Allelic variants of glucose phosphate isomerase (GPI) enzymes are often utilized for these measurements because they show distinct electrophoretic mobility on a cellulose acetate plate. The C57B6 derived GPI-B presents a higher electrophoretic mobility compared to the 129 derived GPI-A (*see Note 17*).

1. Dissect a small piece of tissue (10 mg or less) from the chimeric mouse.
2. Lyse the sample in 100–200  $\mu$ L of extraction buffer using a small pestle. Lysates can be stored indefinitely at  $-20^{\circ}\text{C}$ .
3. Mark the upper side of the plastic back of the plate. Soak the Titan III plate in Supre Heme buffer avoiding the formation of air bubbles inside the cellulose acetate.
4. Dilute the sample to the desired concentration and load 8  $\mu$ L on the Super Z well plate.
5. Recover the Titan III plate and blot it dry between two paper towels. Fix the plate on the loading device with the cellulose on the top and so that samples will be loaded near the marked side.
6. Collect some sample by pressing the applicator inside the wells. Blot the applicator on tissue paper. Reload in the same way the applicator and finally press it against the cellulose acetate plate.
7. Fill buffer tanks of an electrophoresis device with Supre Heme buffer. Place a piece of filter paper in both buffer tanks so that they do not touch each other.
8. Overlay the plate onto the two pieces of filter paper so that the cellulose side faces the bottom of the chamber. In this

way, the plate is electrically connected to the buffer tanks. Take care to place the marked side near the anode. Stabilize the extremities of the gel with 5–8 glass slides. Run (from anode to cathode) at 200 V, 4°C for 3 h.

9. Place the Titan III slab onto a glass plate. Melt the agarose and cool 10 mL at 55°C. Add 200  $\mu$ L of each developing reaction component, mix well and pour it over the cellulose acetate plate. Leave the reaction in the dark for 2–15 min, depending on the concentration of samples.
10. Place the plate in stop solution as soon as the bands reach the desired intensity.
11. Photograph immediately.

### ***3.8. Analysis of Differentiation Abilities of Homozygous ES Cells in Embryo Bodies and Teratomas***

In addition to the study of chimeric tissue formation, the differentiation abilities of ES cells can also be tested by in vitro differentiation assays and by the induction of ES-derived teratomas. The extraordinary potential of ES cells to differentiate in vitro can be utilized to study particular aspects of mutant phenotypes that cannot be easily approached in vivo models. The case of embryonic lethal phenotypes provide the typical situation in which the study of the developmental abnormalities can be greatly extended with biochemical and cellular studies on differentiated homozygous cells. Analysis of the differentiation state at different time-points and comparison of mutant and wild-type cultures may give essential clues on the effects of the mutation (35). Several protocols have been developed to differentiate cultured ES cells and most of them are specialized to generate a particular cell type. Nonetheless, ES cells can be aggregated in vitro to form so-called embryo bodies in which various tissues with distinct embryonic origin start to form. These cell aggregates can then be grown for several days and the formed tissues may be analyzed with the classical tools of biochemistry and cell biology. An alternative method to generate ES cells-derived differentiated tissues, consists of injecting ES cells ectopically in syngeneic male mice (for example, under the skin). In these conditions, ES cells can form a teratoma, a noninfiltrating, benign tumors containing large numbers of highly differentiated cells often organized in epithelia, glands, vessels, and even nerves.

#### ***3.8.1. Generation of ES Cells-Derived Embryoid Bodies***

1. Grow ES cells with standard methods to obtain at least  $10^6$  cells.
2. Wash cells two times with PBS and incubate with trypsin for 5 min at 37°C. Add ES medium and disperse aggregates by gently pipetting cells up and down. Add ES medium if needed and let feeder cells attach to the culture dish for 30 min in the incubator.

3. Gently wash the plate to collect nonadherent ES cells and save the supernatant in a sterile tube. Count the cells with a Burcker's chamber. Dilute cells to 500–1,000 in 20  $\mu$ L.
4. Pipette on the inside the lid of a sterile 9-cm Petri dish several 20  $\mu$ L drops of cells. Fill the Petri dish with 5–10 mL of PBS. Gently turn the lid upside down and close the Petri dish so that cells are held in suspension in the hanging drops.
5. Wait for 2 days until aggregates of ES form. Collect aggregates (embryoid bodies) by washing the lids with EF medium. Plate aggregates on bacterial culture Petri dishes in EF medium (supplement of serum to 20% may be required).
6. Embryoid bodies can be grown in suspension up to 30 days. Alternatively, they can be trypsinized and cells can be plated in tissue-culture dishes.

### 3.8.2. Induction of ES Cells-Derived Teratomas

1. Grow ES cells with standard methods to obtain at least  $10^8$  cells.
2. Wash cells two times with PBS and incubate with trypsin for 5 min at 37°C. Add ES medium and disperse aggregates by gently pipetting cells up and down. Add ES medium if needed and let feeder cells attach to the culture dish for 30 min in the incubator.
3. Gently wash the plate to collect nonadherent ES cells and save the supernatant in a sterile tube. Count the cells with a Burcker's chamber.
4. Centrifuge  $10^7$  cells for 5 min at  $120 \times g$ . Resuspend the pellet in PBS.
5. Spin cells down and resuspend the pellet in 300  $\mu$ L. Load the cell suspension in a 1-mL syringe.
6. Anesthetize a 129 male mouse and inject cells subcutaneously.
7. The tumor should be clearly visible after 15–20 days. When it has reached the desired dimension, excise it and treat it for histological analysis. As with embryoid bodies, teratomas can be collected in sterile conditions and trypsinized in order to culture differentiated cells.

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## 4. Notes

1. Using excessive amounts of DNA would lead to some undigested DNA and cause severe background after transformation.

2. We used the following antibiotic concentrations in our experiments: kanamycin and chloramphenicol, 12.5  $\mu\text{g}/\text{mL}$  for BACs, 25  $\mu\text{g}/\text{mL}$  for multicopy plasmids; ampicillin, 25  $\mu\text{g}/\text{mL}$  for BACs, 100  $\mu\text{g}/\text{mL}$  for pBluescript.
3. The restriction enzyme sites can be the same used in the preparation for the first mini targeting vector but not necessarily.
4. A viable homozygous knock-out mouse is often suitable for such purpose. The expression of a resistance gene (usually the *neo* gene) is guaranteed by the fact that the mutant ES cells, from which the mouse line has been derived, had been selected in a similar way.
5. Plugs are unstable and easily lost during the day that follows mating. It is therefore important to check for plugs early morning and not later than 11 a.m. To ease inspection, it is useful to use a blunt, sterile probe such as a flame-sealed tip of a Pasteur pipette.
6. In commercial preparations of G418, only a fraction of the total weight is the real active compound. The routine use of high concentration (400  $\mu\text{g}/\text{mL}$ ) of crude powder has proven to be adequate to avoid testing of different antibiotic batches.
7. Colonies should be picked as soon they start to be detectable by eye inspection. Choosing the picking time is a critical step: whereas waiting too long can result in differentiation of colonies, retrieval of small aggregates supplies too few cells for the subsequent expansion.
8. It is extremely important to put each colony in a separated well. Keeping track of the number of used yellow tips can help to avoid mistakes.
9. Obviously, it is essential to test that the floxed allele is fully functional before starting the conditional deletion experiments.
10. Keep the glass ball clean. Wipe debris away with a piece of cloth. By accumulating glass debris, the ball eventually changes its size and subsequently the temperature at which it can fuse with the needle.
11. Keep females well distributed in more than one cage. In this way, females will not show a synchronized oestrus, thus enhancing the chance of finding individuals able to mate.
12. Making a few small air bubbles in the thinner part of the injection needle can strongly increase the control over cell sucking and blowing.
13. Do not generate high pressure in the pipette. In this way, transfer cannot be controlled and often results in the loss of

the embryos. If the pipette becomes clogged, remove from the uterus and gently wash it in ES Medium, paying attention not to lose the blastocysts.

14. Immediately after embryos are blown into the uterine cavity, the muscular wall sometimes contracts and expels the transferred liquid together with injected blastocysts. It is, therefore, useful to suck into the transfer pipette the liquid that tends to overflow from the hole. In case embryos are expelled, they can be recovered in the pipette.
15. Initially test DNA of a proven heterozygous animal in two different reaction tubes each containing a separate combination of the two couples of oligonucleotides (common, wild-type, or common, mutant allele). To simplify the procedure, test whether all three oligonucleotides can work in one same reaction.
16. The success rate of this technique can vary depending on the nature of the targeted locus and must therefore be empirically tested.
17. The GPI enzymes are homodimers and because dimerization occurs inside the cell, chimeric tissues show only two distinct bands. Three bands can be seen in particular situations such as in skeletal muscle extracts. Myofibers containing nuclei of distinct genotype can generate all three combinations of subunits.

## References

1. Evans MJ, Kaufman MH. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature*; 292:154–156
2. Martin GR. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A*; 78: 7634–7638
3. Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. (1985) Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature*; 317:230–234
4. Thomas KR, Capecchi MR. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell*; 51:503–512
5. Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K. (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science*; 265:103–106
6. Baudoin C, Goumans MJ, Mummery C, Sonnenberg A. (1998) Knockout and knockin of the beta1 exon D define distinct roles for integrin splice variants in heart function and embryonic development. *Genes Dev*; 12: 1202–1216
7. Nagy A, Moens C, Ivanyi E, et al. (1998) Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr Biol*; 8:661–664
8. Kuhn R, Schwenk F, Aguet M, Rajewsky K. (1995) Inducible gene targeting in mice. *Science*; 269:1427–1429
9. Dymecki SM. (1996) FLP recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc Natl Acad Sci USA*; 93:6191–6196
10. Kilby NJ, Snaith MR, Murray JA. (1993) Site-specific recombinases: tools for genome engineering. *Trends Genet*; 9:413–421
11. Rajewsky K, Gu H, Kuhn R, et al. (1996) Conditional gene targeting. *J Clin Invest*; 98:600–603
12. Akagi K, Sandig V, Vooijs M, et al. (1997) Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res*; 25: 1766–1773

13. Betz UA, Vosschenrich CA, Rajewsky K, Muller W. (1996) Bypass of lethality with mosaic mice generated by Cre-loxP-mediated recombination. *Curr Biol*; 6:1307–1316
14. Aszodi A, Legate KR, Nakchbandi I, Fassler R. (2006) What mouse mutants teach us about extracellular matrix function. *Annu Rev Cell Dev Biol*; 22:591–621
15. Morrison-Graham K, Weston JA. (1989) Mouse mutants provide new insights into the role of extracellular matrix in cell migration and differentiation. *Trends Genet*; 5:116–121
16. Aszodi A, Pfeifer A, Wendel M, Hiripi L, Fassler R. (1998) Mouse models for extracellular matrix diseases. *J Mol Med*; 76:238–252
17. Fassler R, Schnegelsberg PN, Dausman J, et al. (1994) Mice lacking alpha 1 (IX) collagen develop noninflammatory degenerative joint disease. *Proc Natl Acad Sci USA*; 91: 5070–5074
18. Mundlos S, Olsen BR. Heritable diseases of the skeleton. (1997) Part II: Molecular insights into skeletal development-matrix components and their homeostasis. *FASEB J*; 11:227–233
19. Bruckner-Tuderman L, Bruckner P. (1998) Genetic diseases of the extracellular matrix: more than just connective tissue disorders. *J Mol Med*; 76:226–237
20. Pereira L, Andrikopoulos K, Tian J, et al. (1997) Targeting of the gene encoding fibrillin-1 recapitulates the vascular aspect of Marfan syndrome. *Nat Genet*; 17:218–222
21. Judge DP, Biery NJ, Keene DR, et al. (2004) Evidence for a critical contribution of haploinsufficiency in the complex pathogenesis of Marfan syndrome. *J Clin Invest*; 114: 172–181
22. Nakamura T, Lozano PR, Ikeda Y, et al. (2002) Fibulin-5/DANCE is essential for elastogenesis in vivo. *Nature*; 415:171–175
23. Yanagisawa H, Davis EC, Starcher BC, et al. (2002) Fibulin-5 is an elastin-binding protein essential for elastic fibre development in vivo. *Nature*; 415:168–171
24. Bonaldo P, Braghetta P, Zanetti M, Piccolo S, Volpin D, Bressan GM. (1998) Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy. *Hum Mol Genet*; 7:2135–2140
25. Miner JH, Yurchenco PD. (2004) Laminin functions in tissue morphogenesis. *Annu Rev Cell Dev Biol*; 20:255–284
26. Schwander M, Leu M, Stumm M, et al. (2003) Beta1 integrins regulate myoblast fusion and sarcomere assembly. *Dev Cell*; 4:673–85
27. Liu P, Jenkins NA, Copeland NG. (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res*; 13:476–484
28. te Riele H, Maandag ER, Berns A. (1992) Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc Natl Acad Sci U S A*; 89:5128–5132
29. Rodriguez CI, Buchholz F, Galloway J, et al. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat Genet*; 25:139–140
30. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA*; 90:8424–8428
31. Hogan B, Beddington R, Costantini F, Lacy E. Manipulating the Mouse Embryo. A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1994
32. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989
33. Hirsch E, Iglesias A, Potocnik AJ, Hartmann U, Fassler R. (1996) Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. *Nature*; 380:171–175
34. Mortensen RM, Zubiaur M, Neer EJ, Seidman JG. (1991) Embryonic stem cells lacking a functional inhibitory G-protein subunit (alpha i2) produced by gene targeting of both alleles. *Proc Natl Acad Sci USA*; 88: 7036–7040
35. Sasaki T, Forsberg E, Bloch W, Addicks K, Fassler R, Timpl R. (1998) Deficiency of beta 1 integrins in teratoma interferes with basement membrane assembly and laminin-1 expression. *Exp Cell Res*; 238: 70–81

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