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Shree Ram Singh
Vincenzo Coppola *Editors*

Mouse Genetics

Methods and Protocols

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Preface

Although evolution has separated mice and humans about 75 million years ago, they still share an incredible level of anatomical, physiological, and especially genomic resemblance. Modeling human disease in mice offers numerous advantages over other mammalian animal models because they are small, easy to breed, available in large number of inbred strains, and their genome is fully sequenced. Most of all, early stage mouse embryos are conducive to in vitro manipulation, and many embryonic stem (ES) cell lines have shown robust homologous recombination and germline transmission in existence. All these elements have allowed researchers to develop innovative technologies that efficiently edit the mouse genome in vivo. It is now possible to engineer targeted alterations such as gene knockout and knockin or elegant conditional gene modification, which allows temporal-spatial regulation and cell lineage tracing. Other genetic technologies such as Recombinase-Mediated Cassette Exchange (RMCE), which can generate allelic series of mutants and mutagenesis, can also be obtained by use of transposons. Over the years, almost all human diseases including cancer, diabetes, obesity, cystic fibrosis, arthritis, and heart and neurodegenerative diseases have been modeled in mice.

Mouse Genetics: *Methods and Protocols* provides selected mouse genetic techniques and their application in modeling varieties of human diseases. The chapters are mainly focused on the generation of different transgenic mice to accomplish the manipulation of genes of interest, tracing cell lineages, and modeling human diseases. Composed in the highly successful *Methods in Molecular Biology series* format, each chapter contains a brief introduction, a list of necessary materials, systematic methods, and a notes section, which shares tips on troubleshooting to avoid known pitfalls.

We hope that *Mouse Genetics*: *Methods and Protocols* would provide fundamental techniques and protocols to geneticists, molecular biologists, cell and developmental biologists, students, and postdoctoral fellows working in the various disciplines of mouse biology and modeling human disease.

We would like to thank Prof. John M. Walker and the staff at Springer for their invitation, editorial guidance, and assistance throughout the preparation of the book for publication. We also would like to express our sincere appreciation and gratitude to the contributors for sharing their precious laboratory expertise with the mouse community. Finally yet importantly, we would like to thank our family members for their continued support.

Frederick, MD, USA ***Shree Ram Singh, Ph.D.***
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Part I

Mouse Genetics Techniques

Chapter 1

Development of Pronuclear Injection-Based Targeted Transgenesis in Mice Through Cre–*loxP* Site-Specific Recombination

Masato Ohtsuka

Abstract

Microinjection of DNA into the pronuclei of zygotes is the simplest and most widely used method for generating transgenic (Tg) mice. However, it is always associated with random integration of multiple copies of the transgene, resulting in unstable, low, or no transgene expression due to positional effects and/or repeat-induced gene silencing. In addition, random integration sometimes disrupts an endogenous gene that can affect the phenotypes of Tg mice. Our recently developed pronuclear injection-based targeted transgenesis (PITT) method enables the integration of a single-copy transgene into a predetermined genomic locus through Cre–*loxP* site-specific recombination. The PITT method enables stable and reliable transgene expression in Tg mice and is also applicable for generating knockdown mice. Therefore, the PITT method could represent next-generation transgenesis that overcomes the pitfalls of conventional transgenesis.

Key words Transgenic mouse, PITT, Pronuclear injection, Targeted transgenesis, Transgene expression, Cre–*loxP*, *Rosa26*, Knockdown mouse

1 Introduction

Transgenic (Tg) mice have been widely used for analyzing gene function and for creating animal models of human disease. The most commonly used method to generate Tg mice is microinjection of a DNA solution into the pronuclei of fertilized eggs [pronuclear injection (PI)]. Although this is the simplest method and has been utilized for more than three decades since Gordon et al. [1] first generated Tg mice, the PI method has some limitations such as an inability to control the transgene integration site and copy number. These drawbacks often lead to unexpected and unstable transgene expression in Tg mice, probably due to positional effects and/or the repeat-induced gene silencing [2–4]. In addition, random transgene integration sometimes results in disruption of an endogenous gene, which may cause unexpected phenotypes.

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Therefore, production and analysis of multiple Tg mouse lines are always required for obtaining reliable results. Such a task is laborious, costly, and time consuming. Although embryonic stem (ES) cell-mediated targeted integration of transgenes can overcome these drawbacks, it is more costly and time consuming than PI-based transgenesis.

Recently, we developed a novel targeted transgenesis method that enables the targeted integration of a single-copy transgene into a predetermined genomic locus through simple PI [5]. We have termed this technology as PI-based targeted transgenesis (PITT). This method is based on Cre–*loxP* site-specific recombination. Cre (causes recombination) is an enzyme derived from bacteriophage P1 that binds to a 34 bp region of *loxP* [locus of crossing over (x), P1] and catalyzes recombination between two *loxP* sequences. The *loxP* sequence consists of an 8-bp spacer region, which determines direction, between two perfect 13-bp inverted repeats. The Cre–*loxP* system has been widely used as a tool for modifying the genome of many model organisms, including mice. One of the most frequently utilized applications of the Cre–*loxP* system is the excision of the floxed DNA fragment from the genome of conditional knockout mice. In contrast, the PITT method utilizes the Cre–*loxP* system for insertion of the DNA of interest (DOI) into the genome (Fig. 1). For this purpose, we use the mutant *loxP* sequences JT15 [left element (LE)] and JTZ17 [right element (RE)] in the inverted-repeat region [6] because LE/RE double-mutated *loxP*, produced as a result of insertion through JT15/JTZ17 recombination, is not recognized by the Cre enzyme with high affinity, promoting the integration reaction over the excision reaction. We also used the spacer-region mutant lox2272 [7] in addition to JT15 and JTZ17 for efficient targeted integration [8].

Almost all previous reports on Cre–*loxP*-based Tg mice production have employed the Cre–*loxP* recombination reaction in ES cells [9]. In this study, we aimed to develop a targeted transgenesis method through Cre–*loxP* recombination in zygotes rather than ES cells. To accomplish this, we first established seed mice lines containing JT15 and lox2272 at the *Rosa26* or the *H2-Tw3* locus by ES cell-based gene targeting [5]. These seed mice are maintained as homozygotes and their fertilized eggs are used for injection to generate targeted Tg mice. The donor vector containing JTZ17 and lox2272 in addition to the DOI is co-injected into the eggs along with a Cre expression plasmid or *iCre* (codon-improved Cre recombinase) mRNA [10]. As a result, Cre–*loxP* recombination in zygotes may integrate the DOI into the tagged *Rosa26* or *H2-Tw3* locus (Fig. 1). We applied the PITT method for the generation of a number of gene-overexpressing Tg mice such as various fluorescent Tg mice [11]. In addition, we report the successful knockdown of the *tyrosinase* gene using the PITT method [5].

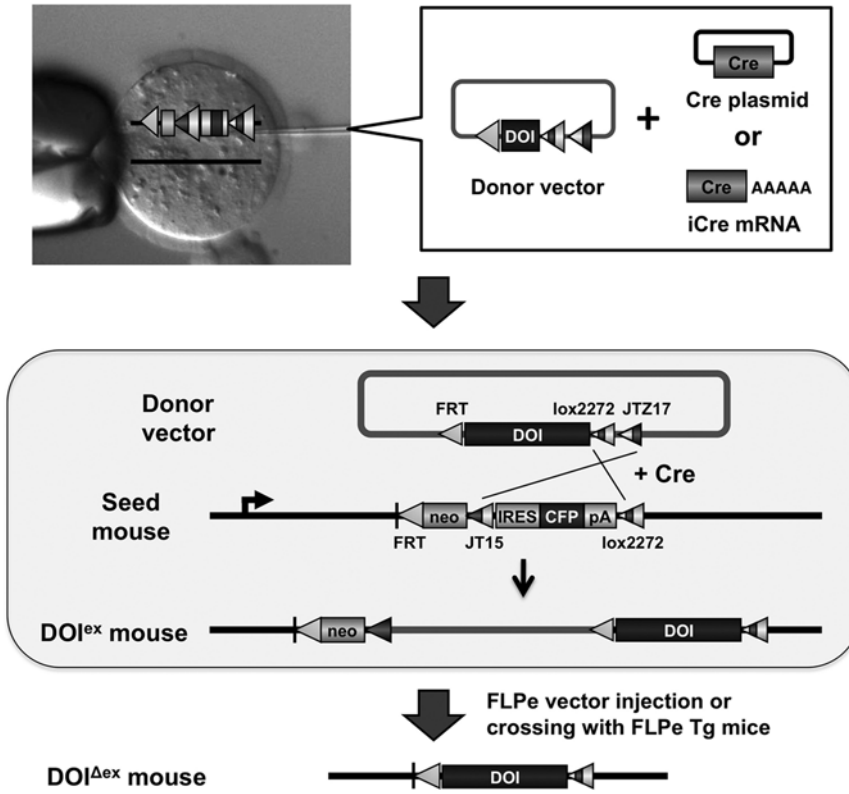

Fig. 1 Schematic diagram of pronuclear injection-based targeted transgenesis (PITT). The donor vector is co-injected with Cre expression plasmid or *iCre* mRNA into the pronuclei of fertilized eggs obtained from seed mice. In case of injection with *iCre* mRNA, cytoplasmic injection and pronuclear injection are performed. As a result of site-specific recombination between mutant loxPs after catalysis by the Cre enzyme, one copy of the donor vector is integrated into the predetermined genomic locus (e.g., *Rosa26* or *H2-Tw3*) and DOI^{ex} mouse is generated. Extra sequence containing vector sequence and selection marker (neo) is removed at the FLP–FRT recombination step, resulting in the generation of the DOI$^{\Delta ex}$

Our previous data confirmed that the expression of transgene tends to be highly reproducible and stable [11, 5]; therefore, only one germline-transmissible line is sufficient for maintenance and analysis. This is advantageous from the viewpoint of animal welfare because it reduces the number of mice required. Therefore, PITT could become widely used as a next-generation transgenesis method in the near future.

2 Materials

2.1 Vector Construction and DNA/mRNA Preparation

1. Donor vector: The vector contains mutant *loxP* sequences, such as JTZ17 and lox2272, in addition to the FRT sequence, which is used for removal of extra sequences (Figs. 1 and 2).

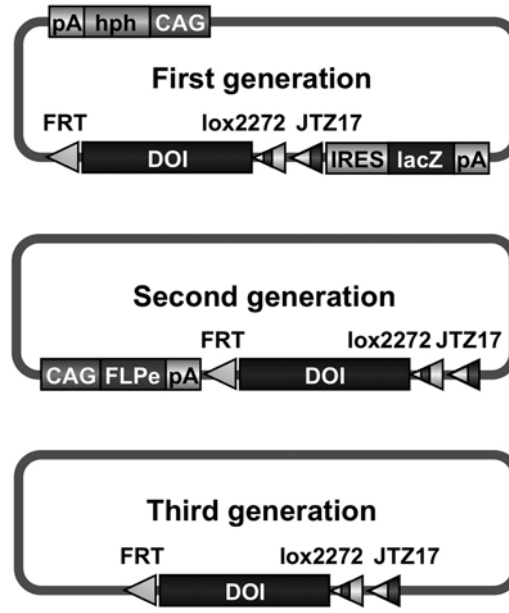

Fig. 2 Basic structure of donor vectors. Three types of donor vectors (first-, second-, and third-generation vectors) are currently available [15, 5, 12]. Notably, there are some variants for each type of vector. For example, *eGFP* is used instead of *lacZ* in some first-generation vectors. Furthermore, the location and direction of the FLPe expression cassette differ in some second-generation vectors

Many donor vectors containing several cloning sites are currently available (*see* **Note 1**).

2. KOD-Plus (TOYOBO).
3. Restriction enzymes: Restriction enzymes such as *Age*I, *Bsr*GI, and *Fse*I [New England BioLabs (NEB)] are used.
4. Low-melting agarose gel (SeaPlaque GTG Agarose; Lonza).
5. 10 mM Tris/0.1 mM EDTA (pH 8.0; modified TE).
6. Alkaline phosphatase, calf intestinal phosphatase (CIP; NEB).
7. Phenol, saturated with TE buffer.
8. Ethanol (Wako).
9. 3 M Sodium acetate (NaOAc) buffer solution (pH 5.2; Nacalai Tesque).
10. Quick Ligation Kit (NEB).
11. Competent cells: DH5alpha (TaKaRa).
12. LB medium (MP Biomedicals): A pouch of powder is dissolved in 1 L of water and autoclaved at 121 °C for 20 min.
13. Cre expression vector: pCAG/NCre.
14. HiSpeed Plasmid Midi Kit (Qiagen).

15. Isopropanol (2-propanol; Wako).
16. Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen).
17. pBBI: This vector is used for synthesis of *iCre* mRNA [12].
18. RNaseA (100 mg/ml; Qiagen).
19. 13 % Polyethylene glycol (PEG8000)/1.6 M NaCl.
20. Phenol/chloroform/isoamyl alcohol (25:24:1, v/v; pH 7.9).
21. Chloroform.
22. Nuclease-free water (not DEPC treated; Ambion).
23. mMESSAGE mMACHINE T7 Ultra Kit (Ambion).
24. MEGAclear Kit (Ambion).
25. EmbryoMax Injection Buffer (Millipore).
26. Ultrafree-MC filter (HV, 0.45 μm pore size; Millipore).
27. pCAGGS–FLPe (Gene Bridges): This should be prepared in cases in which the extra sequence is removed by PI of FLPe plasmid.

2.2 Mice

1. Seed mice: The mice harbor mutant loxP sequences (JT15 and lox2272) and the FRT sequence at the *Rosa26* or the *H2-Tw3* locus. They are maintained as homozygotes in a mixed genetic background (C57BL/6 and 129/Ola) [5].
2. Wild-type, 8-week-old, female mice [e.g., B6D2F1 (BDF1), a hybrid of C57BL/6N and DBA/2N]: Used for obtaining superovulated eggs (CLEA Japan, Inc.).
3. Pseudo-pregnant mice: We usually use adult female ICR mice (CLEA Japan, Inc.).
4. Vasectomized mice: We usually use adult male ICR mice (CLEA Japan, Inc.).
5. FLPe Tg mice (RIKEN BRC; #RBRC01835): To be used in case the extra sequence is removed by crossing FLPe Tg mouse [13].

2.3 Superovulation and Egg Cultivation

1. eCG (Peamex; Nihon Zenyaku Kogyo).
2. hCG (Puberogen; Novartis Animal Health).
3. M2 medium (Sigma).
4. Hyaluronidase (Sigma).
5. HTF medium (ARK Resource).
6. Liquid paraffin (Nacalai Tesque).
7. Petri dishes (BD Falcon).

2.4 Microinjection

1. CO_2 incubator (SANYO Electric Co).
2. Micropipette Puller (Sutter Instruments).
3. Inverted microscope combined with micromanipulation system (Olympus).

Table 1 Examples of primers used for genotyping

Name	Primer name shown in Fig. 4	Sequence (5′-3′)	Donor vector used in the experiment	Primer-binding region
M274	a-R	CGATGGAAAATACTCCGAGGC	–	*Rosa26* genomic region
#166	b-F	ACCCGTGATATTGCTGAAGAG	–	Neomycin-resistant gene
M159	b-R	GCAAGGCGATTAAGTTGGGTAAC	First generation	lacZ
M070	b-R	AACGACAGGAGCACGATCATG	Third generation	pBR322 vector region
M194	c-F	AAGAAGGCACATGGCTGAATATC	First generation	lacZ
M132	c-R	CATTTGTGGGCTGTTTACCAAC	First generation	polyA sequence downstream of lacZ gene
M273	d-F	TCAGTAAGGGAGCTGCAGTGG	–	*Rosa26* genomic region
M515	–	GCTGTTTCACTGGTTATGCG	–	Cre gene
M581	–	GCGAGTTGATAGCTGGCTG	–	Cre gene
M587	e-F1	CACCTAAGGTCCTGGTTCGTC	Except for second generation	FLPe gene
M580	e-R1	TCCCAGATCGTTTCACCCTC	Except for second generation	FLPe gene
M632	e-F2	GCAGATAAGGGAAATAGCCACAG	Second generation	FLPe gene
#142	e-R2	AAGAAGACAGGGCCAGGTTT	Second generation	IRES region

There are several variants of each generation vector and some primers shown above may not work with some donor vectors

2.5 Genotyping

1. Allele-In-One Mouse Tail Direct Lysis Buffer (Allele Biotechnology).
2. Heating block (TAITEC).
3. Primer sets (*see* Table 1).
4. Taq polymerase (TaKaRa).
5. Thermal cycler (GeneAmp PCR System 9700; Applied Biosystems Inc).

3 Methods

3.1 Construction of Donor Vector

Many types of cloning vectors are currently available for construction of the donor vector (Fig. 2; *see* **Note 1**). Each vector contains cloning sites such as *Not*I, *Asc*I, and *Pac*I, depending on the vector. The DOI should be positioned between FRT and lox2272. If the DOI expression is controlled by the ubiquitous CAG promoter, *eGFP* cDNA in the pAMF vector, which has been used for generation of the eGFP Tg mouse, can be replaced by DOI cDNA. In our case, a cDNA has been cloned into the *Age*I and *Bsr*GI (or *Fse*I) sites of pAMF [5]. The PITT method can also be used for the generation of artificial microRNA-based knockdown mice. MicroRNAs are designed using the BLOCK-iT RNAi Designer website.

1. When using polymerase chain reaction (PCR)-amplified DOI for cloning, amplify DOI using KOD-Plus.
2. Digest an expression cassette or a DOI fragment and donor vector with suitable restriction enzymes.
3. Electrophorese the DNA fragments in a low-melting agarose gel.
4. After excising the gels containing DNA fragments under LED light, add 2 volumes of modified TE to the samples and freeze at −80 °C for more than 20 min.
5. After thawing the samples at room temperature, spin down them and transfer the supernatants to new tubes. Dephosphorylate the donor vector fragment with CIP at 37 °C for 40 min.
6. Purify the DNA fragments by phenol extraction and subsequent ethanol precipitation.
7. Mix the vector fragment and the DOI and ligate using Quick Ligase (5 min at room temperature).
8. Transform the ligation mixture into *E. coli*-competent cells, spread onto an LB plate containing ampicillin (100 μg/ml), and culture at 37 °C overnight.
9. Pick colonies and confirm that they contain the correct clones by performing PCR, restriction digestion analysis, and sequencing.

3.2 Preparation of Donor Vector and Cre Expression Vector

Plasmids are prepared using the Qiagen HiSpeed Plasmid Midi Kit according to the manufacturer's protocol with some modifications.

1. Grow *E. coli* containing the vector overnight in 200 ml LB broth containing ampicillin (100 μg/ml) at 37 °C in a shaking incubator.
2. Centrifuge the broth for 15 min at 3,500 rpm. Discard the supernatant.

3. Resuspend the pellet in ice-cold P1 buffer (6 ml) containing RNaseA (100 μg/ml) and vortex it.
4. Add P2 buffer (6 ml) and mix by inverting five times. Incubate for 5 min at room temperature.
5. Add ice-cold P3 buffer (6 ml) and mix it by inverting five times.
6. Pour the lysate into the QIAfilter Cartridge and incubate for 10 min at room temperature. Filter the lysate into the pre-equilibrated HiSpeed Tip.
7. Wash the HiSpeed Tip with buffer QC (20 ml) followed by elution of the DNA with buffer QF (5 ml).
8. Add isopropanol (3.5 ml), mix, and incubate for 5 min at room temperature.
9. Filter the mixture through the QIAprecipitator and wash the DNA by filtering with ethanol (2 ml). Dry the membrane of the QIAprecipitator by passing air through it.
10. Elute the DNA into the collection tube by adding TE buffer.
11. Add an equal amount of TE-saturated phenol, vortex, and centrifuge at 12,000 rpm for 5 min.
12. Transfer the aqueous phase into a new tube and add 0.1 volume of 3 M NaOAc (pH 5.2) and 2.5 volume of ethanol.
13. Precipitate the DNA by centrifugation at 12,000 rpm for 10 min at room temperature and wash the pellet once with 70 % ethanol.
14. Air-dry the pellet for 10 min and resuspend it in 50–100 μl of modified TE. Place the DNA sample at 4 °C overnight.
15. Quantify the plasmid concentration using PicoGreen (*see* **Note 2**). Plasmids are stored at –20 °C until use.

3.3 Preparation of iCre mRNA

In addition to the Cre plasmid DNA-based injection strategy, cytoplasmic injection of *iCre* mRNA is also effective for PITT. Targeted integration efficiency is higher with *iCre* mRNA-based PITT (approximately 20 %) [12] than that with Cre plasmid-based PITT (4–5 %) [9]. pBBI, a plasmid for *iCre* mRNA preparation, is constructed by replacing the *eGFP* cDNA in pcDNA3.1EGFP-poly (A83) [14] with the *iCre* coding sequence [10]. Because this plasmid already contains a polyA tail region, the polyA tailing procedure following in vitro transcription can be omitted.

1. Treat the pBBI (30 μg) prepared in Subheading 3.2 with RNaseA (10 μg/ml) at 37 °C for 20 min.
2. Add equal volume (100 μl) of 13 % PEG/1.6 M NaCl and incubate on ice for 1 h.
3. Centrifuge the sample at 12,000 rpm for 5 min at 4 °C and wash the pellet with 70 % ethanol.

4. Resuspend the pellet in modified TE, extract the solution with phenol/chloroform/isoamyl alcohol (pH 7.9), and precipitate the plasmid DNA with ethanol.
5. Resuspend the pellet in 20 μl of modified TE.
6. Digest the plasmid DNA obtained in **step 5** with *Xba*I (120 U) by incubation at 37 °C for 3.5 h in a total volume of 120 μl. Check for complete digestion by agarose gel electrophoresis using 1/240 volume of the sample.
7. Add water (290 μl) and 3 M NaOAc (pH 5.2; 40 μl) to the sample obtained at **step 6** and extract the solution with 450 μl of phenol/chloroform/isoamyl alcohol (pH 7.9).
8. Re-extract the DNA with chloroform and precipitate with 870 μl of ethanol.
9. Rinse the pellet with 70 % ethanol and resuspend it with 23 μl of nuclease-free water.
10. Quantify the plasmid DNA concentration using 1 μl of the linearized plasmid.
11. Combine all the in vitro transcription reagents included in the mMESSAGE mMACHINE T7 Ultra Kit and 5 μg of the linearized plasmid in a total volume of 100 μl, according to the manufacturer's protocol.
12. Incubate the mixed sample at 37 °C for 3 h, add 5 μl of TURBO DNase I, and incubate for 15 min at 37 °C.
13. Dispense the sample into two tubes (52.5 μl each) to purify the mRNA using the MEGAclear Kit.
14. Add elution solution (room temperature) to 100 μl in each tube, followed by the addition of binding solution concentrate (350 μl).
15. Add ethanol (250 μl) to each sample and centrifuge at 16,000 rpm for 20 s.
16. Add wash solution (500 μl) and centrifuge at 16,000 rpm for 20 s. Perform this step twice.
17. After complete removal of the wash solution by additional centrifugation of the sample at 16,000 rpm for 1 min, place a filter cartridge onto a new tube.
18. Apply preheated elution solution (50 μl) and centrifuge the sample at 16,000 rpm for 1 min. Perform this step twice.
19. Combine the two samples into one tube (total volume of 200 μl) and add 20 μl of 5 M ammonium acetate (included in the kit) and 550 μl of ice-cold ethanol.
20. After incubating the sample at −20 °C for more than 30 min, precipitate the mRNA by centrifugation at 16,000 rpm for 15 min at 4 °C.

21. Rinse the sample with 70 % ethanol, dry the pellet, and add 50 μl of nuclease-free water (included in the kit).
22. Quantify the mRNA concentration by UV absorbance, and check the RNA quality by 1 % agarose gel electrophoresis.
23. After diluting the purified mRNA to 450 ng/μl, dispense it to new tubes (5.0 μl per tube) and store at −80 °C until use (*see* **Note 3**).

3.4 Preparation of Fertilized Eggs for Microinjection

Fertilized eggs can be obtained either by natural mating or by in vitro fertilization (IVF). In our experience, IVF enables us to obtain a number of developmentally synchronized zygotes and to greatly reduce the required number of male mice; however, it causes a slight reduction in the rate of pups obtained/embryos transferred compared with the natural mating strategy [15].

3.4.1 Natural Mating

1. Intraperitoneally inject 5 IU of eCG into female mice (homozygous seed mice or BDF1) at 17:00 h.
2. Intraperitoneally inject 5 IU of hCG 48 h later (at 17:00 h). Mate the females to the homozygous male seed mice.
3. Identify the females with vaginal plugs on the following morning.
4. Sacrifice the plugged females. Remove the oviducts into dishes containing M2 medium.
5. Transfer the oviduct to M2 medium containing 300 μg/ml hyaluronidase and introduce the egg–cumulus cell complex into the medium by teasing the ampulla of the oviduct with a 26-gauge needle. Separate the oocytes from cumulus cells by pipetting.
6. Wash the eggs twice with M2 medium.
7. Incubate the eggs in M2 medium in a 5 % CO_2 incubator until use.

3.4.2 IVF

1. Intraperitoneally inject 5 IU of eCG into female mice (BDF1 or homozygous seed mice) at 19:00 h.
2. After 48 h (at 19:00 h), intraperitoneally inject 5 IU of hCG.
3. The following morning (at 8:00 h), about 30 min before egg collection, dissect the cauda epididymides from the homozygous male seed mouse.
4. Cut the epididymides and transfer the sperm into a dish containing HTF medium (300 μl) using forceps. Incubate the sperm in a 5 % CO_2 incubator for 1–1.5 h to allow capacitation.
5. Sacrifice the females, dissect the oviducts, and place into dishes containing M2 medium.

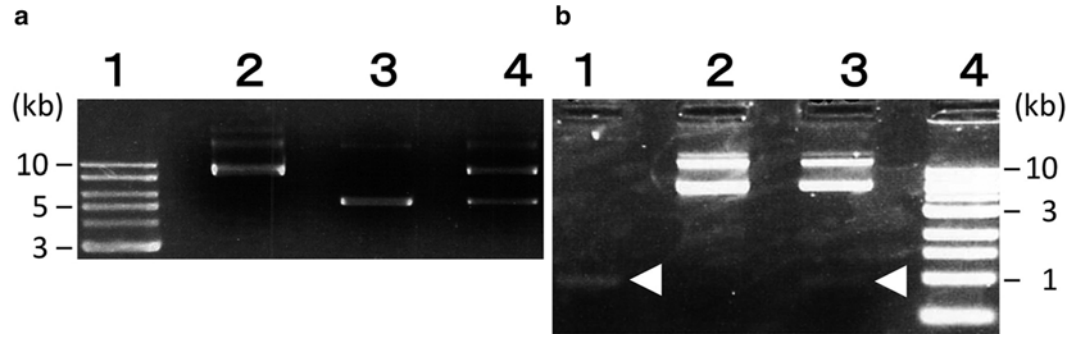

Fig. 3 Quality check of injection solutions by gel electrophoresis on 1 % agarose gels. (**a**) Lane 1, 1-kb DNA ladder marker; lane 2, 3 μl of donor vector solution (20 ng/μl); lane 3, 3 μl of Cre plasmid solution (10 ng/μl); lane 4, 3 μl of donor vector solution (10 ng/μl) and Cre plasmid (5 ng/μl) mixture. (**b**) Lane 1, 3 μl of *iCre* mRNA solution (2 ng/μl); lane 2, 3 μl of donor vector solution (20 ng/μl); lane 3, 3 μl of donor vector solution (10 ng/μl) and *iCre* mRNA (1 ng/μl) mixture; lane 4, 1-kb DNA ladder marker. *Arrowheads* indicate bands corresponding to the *iCre* mRNA

6. Introduce the egg–cumulus cell complex into the medium by teasing the ampulla of the oviduct with a 26-gauge needle. Transfer the egg–cumulus cell complex to HTF drops (250 μl) and cover with liquid paraffin in a Petri dish.
7. Add the pre-cultured sperm to the HTF drop containing oocytes (at 9:00 h) and incubate in a 5 % CO_2 incubator to allow IVF. Under this schedule, injection is initiated at 16:00 h.

3.5 Preparation of Injection Solution

This step is very important because the concentrations of the donor vector and Cre (plasmid DNA or mRNA) affect the success rate of producing PITT-derived Tg mice (*see* **Note 4**). In our case, we found that 5 and 1 ng/μl are the best for Cre plasmid and *iCre* mRNA, respectively (Fig. 3). Notably, targeted integration efficiency is markedly reduced, but the survival rate improves, when 2.5 ng/μl of Cre plasmid is used (data not shown). Linearization of the donor vector and Cre plasmid is not required, and the circular form should always be used because integration is dependent on the Cre–*loxP* site-specific recombination.

3.5.1 Cre Plasmid Injection

1. After thawing the frozen stock solution of the donor vector and the Cre plasmid pCAG/NCre (200–2,000 ng/μl), vortex the solution gently, and centrifuge at 12,000 rpm for 3 min to precipitate any possible contaminating debris.
2. Carefully remove at least 2 μl of each stock solution (from near the surface of the solutions) and transfer into new tubes. Dilute to concentrations of 10–20 ng/μl for the donor vector and 10 ng/μl for the Cre plasmid with EmbryoMax injection buffer.

3. After gentle mixing of the diluted samples by vortexing or pipetting, centrifuge at 12,000 rpm for 3 min.
4. Carefully remove 20 μl of the diluted donor vector and Cre plasmid (from near the surface) and mix them to give a final concentration of 5–10 ng/μl donor vector and 5 ng/μl Cre plasmid in 40 μl of total volume. Use this injection solution immediately or store below –20 °C until use (*see* **Note 5**).

3.5.2 iCre mRNA Injection

1. Dilute the donor vector with EmbryoMax injection buffer to a concentration of 20 ng/μl by the procedure described above.
2. After thawing the frozen stock solution of the *iCre* mRNA (5 μl of 450 ng/μl solution in our laboratory), mix the solution gently by tapping the tube, and centrifuge at 14,000 rpm for 3 min at 4 °C.
3. Dilute 4 μl of the stock solution with EmbryoMax injection buffer to a concentration of 20 ng/μl.
4. Filter the *iCr*e mRNA using an Ultrafree-MC filter (12,000 × *g* for 2 min at 4 °C) to avoid clogging the injection needle (*see* **Note 6**).
5. Dilute the filtered mRNA with EmbryoMax injection buffer to a concentration of 2 ng/μl and then centrifuge at 14,000 rpm for 3 min.
6. Carefully remove 20 μl each of the diluted donor vector and *iCre* mRNA (from near the surface) and mix. The resulting solution (40 μl total) has final concentrations of 10 and 1 ng/μl of donor vector and *iCre* mRNA, respectively. Use the injection solution immediately or store below –20 °C until use (*see* **Notes 5** and **7**).

3.6 Injection

Pronuclear injection of the donor vector/Cre plasmid is essentially the same as that used for Tg mice production. We have employed two alternative procedures for donor vector/*iCre* mRNA injection into zygotes. In method 1, injection is performed by a one-shot insertion of a needle into the zygote. Briefly, injection is first performed toward the male pronucleus using the Olympus micromanipulator system and then toward the cytoplasm prior to withdrawal of the injection needle from the zygote. In method 2, the injection is completed by a two-shot insertion of a needle into the zygote. Briefly, injection is first performed toward the male pronucleus and the pipette is removed and reinserted toward the cytoplasm. In our experience, injection of an excess volume of the DNA/mRNA mixture into zygotes is often harmful to the development of early embryos, particularly homozygous embryos. Therefore, it is recommended that the optimal volume of the DNA/mRNA mixture be determined prior to the microinjection experiments.

3.7 Embryo Transfer

Embryo transfer is based on the method used for production of Tg mice (*see* **Note 8**). Briefly, two-cell embryos developing 1 day after microinjection are transferred to oviducts of pseudopregnant females that have been mated with vasectomized males. These females are allowed to survive and deliver their pups.

3.8 Genotyping of the Targeted Tg Mice

Template DNA for genotyping can be prepared from either ear or tail samples biopsied from 21-day-old or older mice. We usually use ear punching for identification of individual mouse.

1. Transfer pieces of the isolated samples to microtubes (*see* **Note 9**).
2. If necessary, determine sample fluorescence under a fluorescence microscope.
3. Add 40–50 μl of Allele-In-One Mouse Tail Direct Lysis Buffer.
4. Incubate the samples in a 55 °C heating block for 3 h or overnight.
5. Incubate the samples in an 85 °C heating block for 45 min.
6. Perform PCR with appropriate primer sets (Table 1 and Fig. 4a; *see* **Note 10**) under optimized PCR conditions. Each sample (1 μl) can be directly subjected to PCR as the template.

3.9 Removal of Extra Sequence

The extra sequence can be removed either (1) by injection of an FLP expression plasmid (e.g., pCAGGS–FLPe) into the pronuclei of zygotes obtained from DOI^{ex} mice or (2) by crossing DOI^{ex} mice with FLPe Tg mice [13] to obtain $DOI^{\Delta ex}$ mice (Fig. 1; *see* **Note 11**). Elimination of the extra sequence is confirmed by PCR-based genotyping as shown in Fig. 4b (*see* **Note 12**).

4 Notes

1. Three types of donor vectors, namely first-, second-, and third-generation vectors, are available (Fig. 2) [5, 15, 12]. The first-generation vectors contain a hygromycin B phosphotransferase (*hph*) gene expression cassette, enabling drug selection in ES cells after transfection. The cassette can be removed by FLP treatment after targeted integration is completed [5]. The second-generation vectors contain an FLPe expression cassette instead of the *hph* expression cassette. Because FLPe expression allows self-removal of extra sequences, further FLP treatment can be omitted [15]. The third-generation vectors contain neither the *hph* nor the FLPe expression cassettes; therefore, their size is much smaller compared with that of the first- and second-generation vectors. This property may be beneficial for effective targeting because reduction of vector size has been reported to be correlated with improved targeted

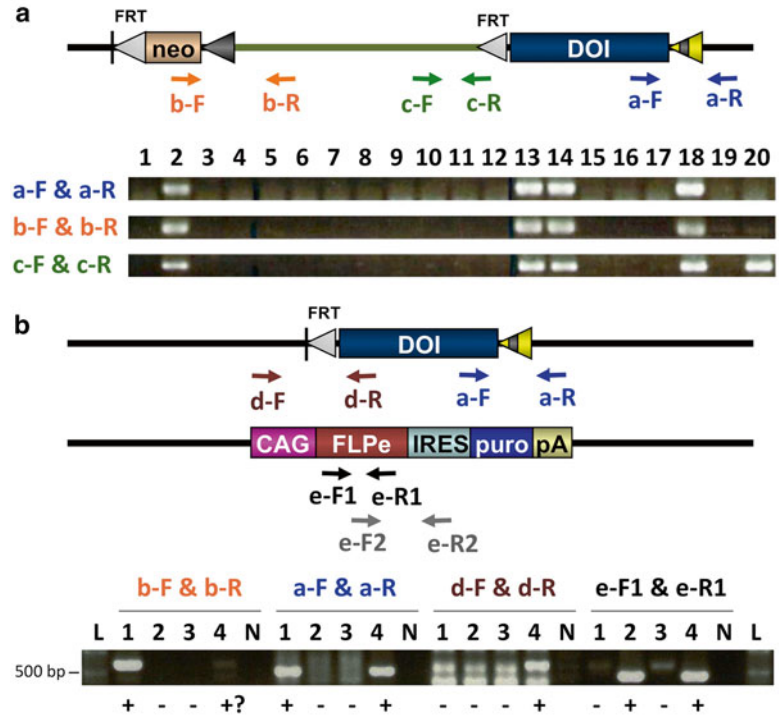

Fig. 4 Genotyping of the PITT mice. (**a**) PCR screening of PITT founder mice (DOI^{ex}). *Arrows* indicate the PCR primer sets used for detection of correct integrants. Primer sets a-F and a-R and b-F and b-R are used to amplify the junction regions and primer set c-F and c-R is used to amplify the internal sequence of the donor vector, including DOI. Both genomic junction and internal sequences should be amplified in the PITT founders (lane numbers 2, 13, 14, and 18), whereas only the internal region is amplifiable in the pup with random integration (lane number 20). Sequences of primers a-R, b-F, b-R, c-F, and c-R are shown in Table 1. (**b**) PCR screening of $DOI^{\Delta ex}$ and FLPe transgenic mice. *Arrows* indicate the PCR primer sets used for detection of pups without the extra sequence ($DOI^{\Delta ex}$ allele; *upper*) and for detection of the FLPe transgene (*lower*). The primer set d-F and d-R is used for identification of the $DOI^{\Delta ex}$ allele, whereas the primer set e-F1 and e-R1 (e-F2 and e-R2 when using a second-generation vector) is used for detection of the FLPe transgene. Sequences of primers d-F, e-F1, e-R1, e-F2, and e-R2 are shown in Table 1. Gel electrophoresis of PCR products amplified from genomic DNA of four pups, obtained from a cross between a DOI^{ex} mouse (generated after introduction of the third-generation vector) and an FLPe Tg mouse, is shown below. The genotyping results of each PCR reaction are shown below the gel image. Visible bands from one pup (lane number 4), amplified using both b-F and b-R (very weak band) and d-F and d-R primer sets, suggest genetic mosaicism of the DOI^{ex} and $DOI^{\Delta ex}$ alleles. The primers a-F and d-R are designed based on the sequence of the DOI. Lane L, 100-bp ladder; lane N, negative control

integration efficiency [16]. The first-generation vector is derived from high-copy pUC119, whereas the second- and third-generation vectors are derived from low-copy pBR322. Therefore, lower plasmid yields are to be expected in *E. coli* cells transformed with the second- and third-generation vectors than in those transformed with the first-generation vector. These vectors are available on request.

2. Determination of precise vector concentrations is important because viability of embryos and PITT efficiency are sensitive to the concentrations of injected DNA. We sometimes quantify the DNA using several methods (e.g., NanoDrop).
3. A total of approximately 190 μg of *iCre* mRNA can be obtained at this scale.
4. In our experience, the amount of Cre produced in eggs is critical for embryo survival. Therefore, we recommend exploring the optimal concentration of Cre plasmid or *iCre* mRNA by assessing the survival rates of injected eggs after culturing them till the blastocyst stage (see Supplementary Tables S3 and S4 in Ohtsuka et al. 2010, 2013). Once the optimal concentration is determined, the same batches of Cre can be used under the same experimental conditions for all microinjection experiments.
5. Check the quality of the prepared injection solution by agarose gel electrophoresis (Fig. 3). We usually use freshly prepared injection solution for each injection experiment. In case that an injection solution has to be frozen, storing it for more than 2 weeks and repeated freezing–thawing should be avoided.
6. Aliquot the filtered *iCre* mRNA (20 ng/μl; 12 μl/tube) and store at −80 °C for future injections.
7. It is desirable to check the stability of the *iCre* mRNA injection solution (DNA/mRNA). In our experience, the *iCre* mRNA in the injection solution remains intact even after storage at room temperature for 3 days.
8. Egg transfer is performed by transferring ten eggs per oviduct. Therefore, a total of 20 eggs are transferred to a recipient female mouse.
9. Immediately proceed to the next step or freeze the samples below −20 °C.
10. In addition to the donor vector integration, insertion of the Cre plasmid is also checked by the primer set M515 and M581 (Table 1) [5]. In our experience, no simultaneous integration of the donor vector (target integration) and Cre plasmid occurs, and random integration of the donor vector is less frequent than its targeted integration.
11. Tg mice exhibiting highly reproducible and stable transgene expression can be obtained after this removal procedure.

This step can be omitted when using a second-generation vector (Fig. 2; *see* **Note 1**), although approximately 40 % of founders may still have the extra sequence [15]. In this case, the extra sequence can be removed by employing standard procedures (i.e., FLPe plasmid injection or mating with an FLPe Tg mouse). The primer set e-F2 and e-R2 should be used for detection of the FLPe transgene because the primer set e-F1 and e-R1 amplifies both an FLPe gene included in the second-generation donor vector and the FLPe transgene in the FLPe Tg mouse (Table 1 and Fig. 4b).

12. Mice that have been treated with FLP include those with only the $DOI^{\Delta ex}$ allele and those with both the $DOI^{\Delta ex}$ and DOI^{ex} alleles (mosaic mice). The latter are positive in PCR using both the primer sets b-F and b-R and d-F and d-R (Fig. 4b), and mice containing only the $DOI^{\Delta ex}$ allele in all cells can be obtained in the next generation.

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Chapter 2

Generation of Conditional Knockout Mice

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Abstract

Conditional knockout mouse models are powerful tools to examine the biological and molecular function(s) of genes in specific tissues. The general procedure to generate such genetically engineered mouse models consists of three main steps. The first step is to find the appropriate genomic clone of the gene of interest and to design the cloning and Southern blot strategies. The second step is the cloning of the gene-targeting vector with all its essential components including positive and negative selection cassettes and the insertion of *LoxP* sites. Although conventional methods are still being widely used for DNA cloning, we describe in this book chapter the use of λ Red phage-based homologous recombination in *Escherichia coli* to capture the genomic DNA of the gene of interest and to assemble the gene-targeting vector. This new method provides several advantages as it does not require the presence of restriction sites within the gene of interest to insert *LoxP*-flanked DNA fragments. In the final step, the gene-targeting vector is transferred into embryonic stem (ES) cells, and successfully targeted ES cell clones are injected into mouse blastocysts to generate conditional knockout mice.

Key words Conditional knockout, Mouse model, Cre/LoxP, Homologous recombination, Subcloning

1 Introduction

Gene targeting techniques and the generation of knockout mouse models are powerful tools for studying the biological and molecular function(s) of genes in vivo. Conventional knockout mice, in which critical parts of the genes of interest have been eliminated, were the first models created by relatively simple cloning strategies of the target vector. The vector is electroporated into embryonic ES cells, and the endogenous gene is replaced with the corresponding region of the targeting vector through homologous recombination. These first-generation gene target models provided mechanistic insight into essential functions of a gene during pre- and postnatal development. In about 30 % of cases, however, the conventional deletion of genes causes embryonic lethality. Another shortcoming of conventional knockout mice can be secondary

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developmental defects in other organs or tissues that are indirectly affected by a gene deletion. For example, a primary defect in hormone-producing organs such as the pituitary gland or ovary can give rise to secondary phenotypes in effector organs such as the mammary gland. Although some of these primary and secondary phenotypes can be discriminated using a combination of gene deletion and transplant models, advances in BAC recombineering and site-specific recombination technologies such as Cre/*LoxP* and Flp/*Frt* systems have become the standard procedures for the generation of conditional knockout mouse models to study the primary function(s) of genes in specific tissues.

The conditional deletion of the target gene can be achieved by crossing two different mouse strains. One of these strains is the *floxed* mouse line that carries one or two copies of the "floxed" (i.e., flanked by *LoxP* sites) alleles of the gene of interest. The second strain is a transgenic mouse line expressing the site-specific recombinase Cre under a tissue-specific promoter. In the offspring, the gene deletion occurs through site-directed excision of the *floxed* allele within the cells that express Cre recombinase. Since the first report of a conditional knockout by the laboratory of Klaus Rajewsky in 1994 [1], a myriad of strains carrying floxed alleles and transgenic mouse lines that express Cre under tissue-specific promoters have been generated. Many of these lines are available from the Jackson Laboratory (http://www.jax.org/) and other repositories such as that of the NCI in Frederick, MD (http://mouse.ncifcrf.gov/). Using an interferon-responsive promoter to drive Cre recombinase, the Rajewsky lab also pioneered the development of inducible gene knockouts [2]. Subsequently, Luc St-Onge and Priscilla Furth in the laboratory of Peter Gruss expressed Cre recombinase under the control of a tetracycline (tet)-responsive promoter [3]. In this system, the temporal expression of Cre and excision of the target gene are controlled by the tet-regulated transactivator (tTA or rtTA), which can be expressed ubiquitously or in a cell type-specific manner. Another elegant approach to achieve an inducible and tissue-specific deletion of genes is the use of transgenic lines that express a tamoxifen-dependent Cre recombinase (Cre-ERT2) in selected cell types [4].

Conventional cloning methods are still widely used to generate gene-targeting vectors and conditional knockout mouse models. The availability of suitable restriction enzymes within the locus of interest and the sizes of inserted DNA fragments are common limitations of conventional cloning strategies. A newer method developed in the laboratory of Drs. Copeland and Jenkins [5, 6], which is based on the homologous recombination function of the λ Red phage in *Escherichia coli*, has pioneered the subcloning of large genomic fragments and the generation of targeting vectors. This bacteriophage-based technique allows recombination of homologous sequences as short as 40–50 bp to as large as 10–20 kb.

During the initial design of the targeting vector, this methodology can be used to capture a gene of interest from a genomic bacterial artificial chromosome (BAC) library into a plasmid. This unique feature of the technique does not require the presence of restriction enzyme sites within the gene of interest to insert *LoxP*-flanked DNA segments.

The use of the retrieving plasmid and mini targeting vector construction described in this book chapter is based on the primary reports by Liu et al. [6] and Malureanu [7]. The technical description in this chapter focuses primarily on designing a subcloning strategy and methodologies using the λ Red phage system to generate conditional targeting vectors. In addition to the design of vectors containing positive and negative selection markers, the targeting strategy outlined here allows the elimination of the selection cassette (i.e., PGK-neomycin) from the targeted locus in ES cells or mice using Flp recombinase.

2 Materials

2.1 Bacterial Strains

The bacteria strains SW102, SW106, and SW105 [5] are available from Frederick National Laboratory for Cancer Research at National Cancer Institute (http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx). All three bacterial lines are a modified DH10B strain carrying a defective λ phage. They all carry a fully functional galactose operon with a deletion of *galK*, which allows efficient BAC recombineering. SW106 and SW105 cells also contain L-arabinose-inducible Cre or Flp, respectively, which mediate recombination of the *LoxP* or the *Frt* site in the bacteria (Table 1).

2.2 Plasmids

Plasmids PL253, PL452, and PL451 [6] are also available from Fredrick National Laboratory for Cancer Research (Table 2). PL253 is a pBluescript-based plasmid for retrieving the target gene sequences from a BAC clone. This retrieving vector has an *MC1*

Table 1
Bacterial strains for bacteriophage-based recombineering

Strain	Genotype	Antibiotic resistance
SW102	DH10B[λ*cl*857(*cro-bio*A)<>*tet*]	None
SW106	DH10B[λ*cl*857 (*cro-bio*A)<>*araC-PBADcre*]	None
SW105	DH10B[λ*cl*857 (*cro-bio*A)<>*araC-PBADflpe*]	None

Table 2
Plasmids for recombineering

Strain	Genotype	Antibiotic resistance
PL253	Modified *MclTK*	Ampicillin
PL452	*LoxP-Pgk-em7-NeobpA-LoxP*	Ampicillin
PL451	*Frt-Pgk-em7-NeobpA-Frt-LoxP*	Ampicillin

promoter-driven thymidine kinase (*TK*) cassette for use in negative selection of ES cells. PL452 plasmid contains a neomycin (neo) resistance cassette flanked by two *LoxP* sites. PL451 carries a neo cassette flanked by two *Frt* sites and one *LoxP* site. Neo genes in both of the plasmids are driven from a prokaryotic promoter (*em7*) and a eukaryotic promoter (*Pgk*) (*see* **Note 1**). pBluescript (pSK+) is utilized for cloning of *LoxP* sites carrying homologous regions of the target gene as a mini targeting vector.

2.3 Bacterial Artificial Chromosomes

A targeted gene sequence is inserted into the retrieving vector, PL253, from a BAC clone. BAC libraries generated from AB2.2 ES cell DNA (129S7 mouse strain) are available from BioScience [8]. BAC libraries from the C57BL/6 mouse strain can be obtained from the BACPAC resources center at Children's Hospital Oakland Research Institute (http://bacpac.chori.org/). For efficient gene targeting, it is recommended to use isogenic DNA, i.e., genomic clones from a BAC library that are genetically identical or similar to the ES cells.

2.4 Reagents Needed

1. Restriction enzymes (New England Biolabs).
2. Miniprep, Gel extraction, and Maxiprep kits (Qiagen).
3. GoTaq® DNA Polymerase (Promega).
4. TOPO® TA Cloning kit (Invitrogen) (optional; to clone Southern probe).
5. Random Primed DNA labeling kit (Roche).
6. DNA Denaturizing and Neutralizing solution (Quality Bio).
7. dCTP (alpha ^{32}P) (Perkin Elmer).
8. QuikHyb (Agilent technologies).
9. GeneScreen Plus® Hybridization Transfer Membrane (Perkin Elmer).
10. DH5α™ Competent cells (Invitrogen).
11. L(+) Arabinose (Sigma).
12. Expand Long Range PCR, dNTPack (Roche).

3 Methods

3.1 Designing of Cloning and Southern Blot

The first step in generating conditional knockout mice is to design the cloning and Southern blot strategies and to find appropriate BAC clones. In order to choose the most critical exon(s) for deletion, there are many considerations such as intron-exon spacing, presence of exons of other genes in the opposite strand, and protein domain. These issues have been described in detail elsewhere [9–11] and are not being discussed here. There are many online resources for searching the sequence of your target gene and BAC clones. For demonstration purposes, the Ensemble website was used to find the BAC clone that contains the sequence from the gene of interest. The probe for the Southern blot was tested using mouse genomic and BAC DNA.

3.1.1 Designing of Cloning and Southern Blot Strategy

1. Go to the Ensembl website (http://www.ensembl.org/index.html).
2. Access "Mouse" in the genome section and search for the gene of interest.
3. Select the appropriate gene when the list appears.
4. Click "Sequence" in the Gene tab to download the sequence of the target gene.
5. The exon details can be found by clicking on "Exons" in Transcript tab.
6. Draw a strategy map of the targeted gene. Locate where the *LoxP* sites should be inserted, and where the probe should bind and identify restriction enzyme sites for Southern blot analysis as represented in Fig. 1 (*see* **Note 2**).

3.1.2 Selecting BAC Clones Across a Desired Gene Locus

1. In the Ensemble website (http://www.ensembl.org/index.html), access "Mouse" in the genome section and search for the gene of interest.
2. Select the appropriate gene when the list appears and open "Location."
3. Click "Configure this page," "Custom Data" tab, and then "Attach DAS," to select BAC clones.
4. Go to "Configure Region Image" tab and click "Sequence and Assembly," and then "Clones."
5. "Enable" the option for mouse BAC clones and close the panel to accept the changes.
6. A Contig map and list of BAC clones should appear.
7. Select the BAC clones that cover the targeted gene.

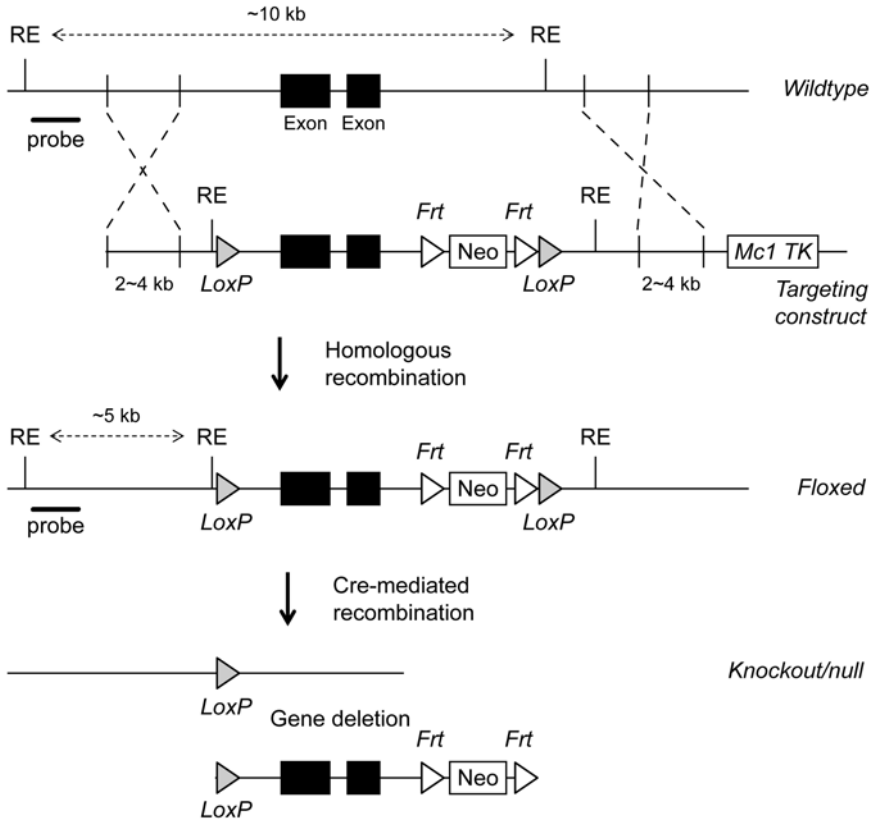

Fig. 1 Gene targeting and generation of a conditional knockout (*floxed*) allele of the gene of interest. Targeting strategy to flank the exons of the gene of interest with *LoxP* sites. The probe for Southern blot analysis is designed to be between 400 and 800 bp. RE indicates appropriate restriction enzyme sites that cover the exons being flanked by *LoxP* sites. It is recommended to choose the RE that creates a DNA digest of about 10 kb in size for the *wild-type* and about 5 kb for the *floxed* allele. The targeting vector is constructed by placing a PGK-neo selectable marker flanked by Frt sites. The RE in the 5′ end of the first *LoxP* site can be generated in the later process. Cre recombination deletes the exons that are flanked by the *LoxP* sites

3.1.3 Mini-Prep of BAC Clone

1. Culture a BAC clone in 5 ml of LB media containing the appropriate antibiotic at 37 °C and agitate at ~225 rpm overnight (*see* **Note 3**).
2. Centrifuge the cultured bacterial cells at 6,000 × *g* for 1 min.
3. Resuspend the pelleted cells with 250 μl Buffer P1 from the Qiagen Miniprep kit, then add 250 μl Buffer P2, and invert thoroughly. Mix 350 μl Buffer N3 and mix by inverting. Centrifuge at >13,000 × *g* for 10 min and collect supernatant.
4. Add 750 μl isopropanol, mix by inverting, and incubate at room temperature for 10 min (*see* **Note 4**). Centrifuge at >13,000 × *g* for 10 min to precipitate the BAC DNA, wash with 70 % ethanol, allow to dry until ethanol has evaporated, and then reconstitute with 100 μl TE (pH 8.0). DNA concentration should be around 1 μg/μl.

3.1.4 Designing Probes and Southern Blot Analysis Using Mouse Genomic and BAC DNA

1. Design ~20–30 bp primers to amplify ~400–800 bp of probe for Southern blot (*see* Fig. 1).
2. Perform PCR on BAC DNA with the primers using a Taq polymerase. Extract the PCR product from an agarose gel using the Gel Extraction kit and clone into the TA vector following the instructions from the TOPO® TA Cloning kit. Analyze the sequence of the probe using M13 forward and reverse primers. The probe can be isolated from the TA vector with EcoRI or the appropriate restriction enzyme (*see* **Note 5**). Alternatively, a PCR product generated using BAC DNA can be used as a probe after gel purification (instead of cloning into TA vector).
3. To test whether the probe works in a Southern blot assay, digest 10–15 μg of genomic DNA from a wild-type mouse or ES cell DNA (preferably from the strain that will be used for targeting) with the appropriate restriction enzymes (*see* **Note 6**). To use as a control, digest ~1 μg of the BAC DNA and dilute the digested BAC DNA to obtain equimolar concentration with genomic DNA. A control lane on the Southern blot using the digested BAC DNA should serve as an ideal positive control.
4. Run the restriction digested DNA on a 0.7 % agarose gel, denature the DNA in the gel using denaturizing solution, neutralize the DNA with neutralizing solution, and then transfer the DNA onto a charged nylon membrane.
5. Fix the DNA to the blot by UV-cross-linking.
6. Label the probe with ^{32}P following the instructions from a Random Primed DNA labeling kit.
7. Pre-hybridize the blot with a hybridization buffer such as Agilent's QuikHyb at 68 °C in a hybridization oven for at least 1 h.
8. Add the probe directly to the hybridized buffer and incubate at 65 °C overnight.
9. Wash the membranes with 2×, 1×, and 0.5× SSC buffer containing 0.1 % SDS for 30 min each and check the radiation level after each washing. If the level is too strong after the 2× wash, then wash with 1×, then 0.5×, and so on until the level is around 0.5 mR/h.
10. Expose the blot to X-ray film at −80 °C for 48–72 h.

3.2 Construction of the Targeting Vector

The first step is to transfer the genomic region of interest (includes left and right homology arms) from the BAC DNA into the retrieving vector PL253 that carries an *Mc1* promoter-driven thymidine kinase (*TK*) cassette used for negative selection of the ES cells (Fig. 2). Next, in the retrieving vector, the first *LoxP* sequences

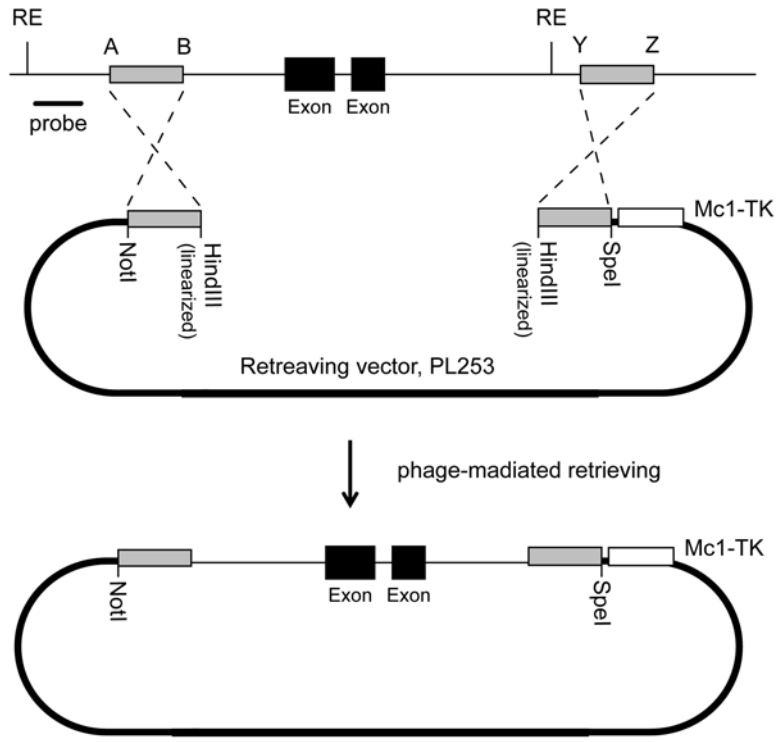

Fig. 2 Subcloning the fragment of the gene of interest from a BAC into the PL253 *MCL-TK*-containing retrieving vector by λ phage-based homologous recombination. The two homologous arms (*gray boxes*) are amplified from the BAC DNA using the primers A/B and Y/Z, and cloned into PL253. The plasmid is linearized with HindIII and to induce recombination with the BAC DNA

are inserted into the appropriate area and the neomycin selection cassette is removed by arabinose-mediated Cre recombination after positive selection. Then, the second *LoxP* site is transferred into the targeting gene (Fig. 3). The λ phage-based recombination is utilized for these cloning steps.

3.2.1 Subcloning the Genomic Region from the BAC into a Retrieving Vector

To begin the subcloning process, the homologous regions from both the 5′ and 3′ ends of the target gene in the BAC plasmid are amplified by PCR and these PCR fragments are cloned into the retrieving vector PL253 (Fig. 2). Both the BAC plasmid and the vector are electroporated into SW102 competent cells where the PL253 captures the target gene by λ phage-based recombination.

1. Select 200–500 bp regions at the 5′ and 3′ ends of the target gene for homologous recombination into the PL253 retrieving vector. Design PCR primers that not only amplify the homologous regions but also add specific restriction sites that are utilized for inserting homologous recombination arms into the retrieving vector. Table 3 shows which restriction enzymes can be included with specific primers (*see* **Note 7**).

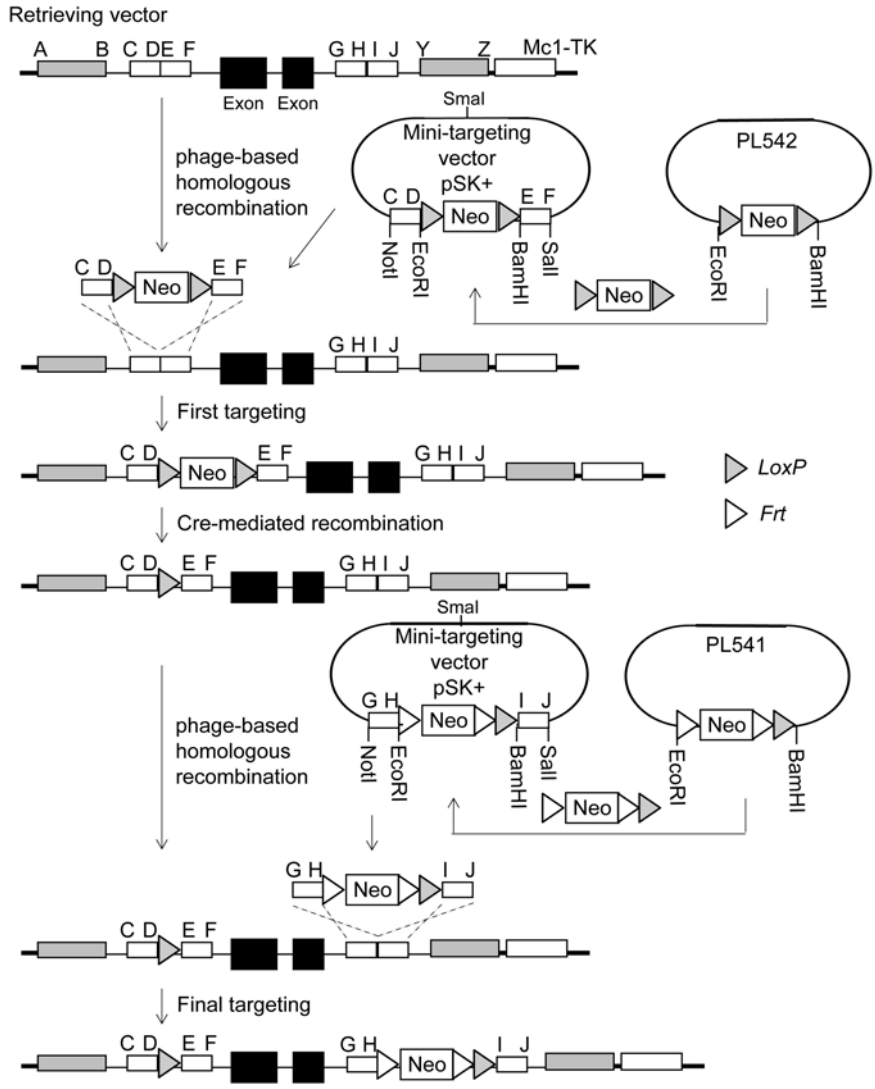

Fig. 3 Constructing a conditional knockout allele. The mini targeting vector is constructed by ligation with two homologous arms amplified from the BAC DNA and a floxed neomycin resistance cassette (*gray arrows*: *LoxP* site). The retrieving vector and the excised targeting cassette are recombined in the competent cells, SW106. Then, the neo cassette, flanked by *LoxP* sites, is deleted by arabinose-induced Cre recombinase. The second *LoxP* site is inserted as well as the first site

2. Perform PCR of the BAC plasmid (Subheading 3.1.3) with primers A/B and Y/Z, and digest the appropriate PCR products with either NotI or SpeI and HindIII restriction enzymes. These fragments (AB and YZ) are ligated using T4 DNA ligase into the PL253 that has been linearized with NotI and SpeI and gel purified.
3. Transform the ligation mixture into DH5α cells and culture the cells on an LB plate that contains ampicillin (100 μg/ml).

Table 3
Unique restriction enzyme sites contained in the primers for homologous recombination arms

Primer	Restriction enzymes
A	NotI
B	HindIII
Y	HindIII
Z	SpeI

4. To confirm the insertion of homologous arms with correct orientation, perform a colony PCR with the following primer sets: M13 forward primer B, primer A/primer Z, and primer Y/M13 reverse primer. Additionally, the entire insert sequence can be analyzed by utilizing the M13 forward and reverse primers. Next, linearize the retrieving vector with HindIII and gel purify.
5. Transform the original BAC clone into the recombinant bacterial strain, SW102. Next, culture the cells in 5 ml LB media at 32 °C overnight. The next day, transfer 1 ml of the culture into 10 ml of LB media and culture for a few hours until the cell density reaches an OD_{600} of 0.6. Collect the competent cells by spinning at 1,800 × *g* and wash with cold water (4 °C) three times. Resuspend the cell pellet with 100 μl of water and mix with 5–15 ng of BAC plasmid. The BAC DNA should then be electroporated into the cells in a precooled electroporation cuvette (0.1 cm gap) under the following condition: 1.75 Kv, 25 μF with the pulse controller set at 200 Ω. The cells are then cultured in 1 ml of SOC media for 1 h, and spread (1–100 μl) onto LB plates containing 12.5 μg/ml chloramphenicol. Incubate plates at 32 °C overnight. The bacterial colonies should carry the BAC clone.
6. Pick a colony from the plate and culture in LB media until the cell density reaches an OD_{600} as described above. To activate the λ Red system, heat-shock the 10 ml of the bacterial culture in a shaking water bath at 42 °C for 15 min. Allow the cells to cool down on ice and wash with 4 °C water. Electroporate the HindIII linearized retrieving vector (5–15 ng) that was obtained in **step 4** of this section into the competent cells as described above. After culturing the cells for 1 h in SOC media, spread the cells on an LB plate containing ampicillin and incubate at 32 °C overnight. Most of the colonies should contain the PL253 vector that retrieved the target gene. Non-heat-shocked bacteria would be a good negative control for the λ phage-based recombination (*see* **Note 8**).

7. To eliminate unrecombined plasmids, purify the DNA from the bacteria, transform it into DH5α cells, and select with ampicillin. The positive cells are confirmed by colony PCR and through unique restriction enzyme digestion. Once the positive cells are confirmed, purify the retrieving vector for use in Subheading 3.2.2, **step 3**.

3.2.2 Insertion of LoxP Sites into the Targeting Vector

This section discusses the insertion of the *LoxP* sites into the targeting vector (Fig. 3). The *LoxP* sites are first cloned into pBluescript surrounded by about 300 bp of homologous sequence from the original BAC clone; these constructs are called mini targeting vectors. The first *LoxP* site containing the *LoxP-neo-LoxP* sequence is inserted into the retrieving vector, using the λ phage-based recombination system. The neomycin resistance cassette is then removed by Cre recombination in the SW106 competent bacteria, which have a functional arabinose-inducible Cre recombinase. After removing the neomycin cassette, the second *LoxP* site, comprising the *Frt-neo-Frt-LoxP* sequence, is subsequently inserted into the retrieving vector.

1. To subclone the mini targeting vectors, homologous recombination sequences first need to be amplified from the original BAC clone. Design primers that will amplify about 300 bp from both the 5′ and 3′ regions around the proposed *LoxP* site. Similar to Subheading 3.2.1, **step 1**, these primers should contain unique restriction enzyme sites at the 5′ end as described in Table 4. Include a restriction enzyme site in primer D that is utilized in Subheading 3.1.4, **step 3**, to aid in Southern blot analysis (*see* **Note 9**). The PCR products are then digested with the appropriate restriction enzymes and purified using a gel extraction kit.
2. To release the *LoxP-Neo-LoxP* or the *Frt-Neo-Frt-LoxP* cassettes, plasmids PL452 (first *LoxP*) and PL451 (second *LoxP*) are digested with EcoRI and BamHI restriction enzymes.

Table 4
Unique restriction enzyme sites in the primers for the mini targeting vectors

Primer	Restriction enzymes
C, G	NotI
D, H	EcoRI
E, I	BamHI
F, J	SalI

The purified PCR fragments and the corresponding digested *LoxP* site are inserted with T4 DNA ligase into NotI- and SalI-digested pBluescript. After transformation of the ligation products into DH5α cells, the orientation of the mini targeting vectors should be confirmed by colony PCR and restriction enzyme digestions. The *LoxP*/homologous arm fragment is released from the mini targeting vector using NotI, SalI, and SmaI (*see* **Note 10**); gel purify the fragment using a gel extraction kit.

3. Transform the retrieving vector (Subheading 3.2.1, **step 7**) into SW106 electro-competent cells as described in Subheading 3.2.1, **step 5**. Culture 10 ml of the transformed competent cells (*see* Subheading 3.2.1, **step 5**) until the cell density reaches an OD_{600} as described in Subheading 3.2.1, **step 5**. To activate the λ Red system, heat-shock 10 ml of the bacterial culture in a shaking water bath at 42 °C for 15 min. Cool the cells on ice and wash them with cold water. Mix 1 ng of the fragment containing the first *LoxP* (*LoxP-Neo-LoxP)* cassette with the competent cell suspension. Perform electroporation using an electroporation cuvette as described above. After culturing the cells for 1 h in SOC media, spread the cells on an LB plate containing ampicillin and kanamycin (100 μg/ml and 40 μg/ml, respectively) and incubate at 32 °C overnight. The positive cells that grow should contain both ampicillin and kanamycin resistance genes from the retrieving vector and the *LoxP* insert. Non-heat-shocked bacteria would be a negative control for the λ phage-based recombination since it does not carry kanamycin resistance. The recombination can be confirmed with colony PCR using primer sets that start outside of the recombination arms and go into the *Pgk-em7-Neo* sequence. To eliminate unrecombined DNA in the cells, transform the purified vector into DH5α cells and culture on an LB plate containing both ampicillin and kanamycin (*see* Subheading 3.2.1, **step 7**).
4. To remove the neomycin resistance cassette, culture SW106 competent cells until the cell density reaches an OD_{600}. Add 100 μl of 10 % L(+) arabinose into 10 ml of the culture (final concentration, 0.1 %) and culture at 32 °C for 1 h. After washing the cells with cold water, the vector carrying the first *LoxP* site is transformed into the cells by electroporation. The cells are cultured for 1 h in SOC media and spread on both ampicillin and kanamycin plates (*see* **Note 11**). After overnight incubation at 32 °C, there should be colonies only on ampicillin plates. Perform PCR using primers C and F on the bacterial colonies to confirm that the neomycin resistance cassette is deleted by arabinose-mediated Cre recombination.

5. The last step for the subcloning is inserting the second *LoxP* site. Either of the competent cells, SW106 or SW105, can be used to introduce the *LoxP* site into the subcloning vector following the protocol in Subheading 3.2.2, **step 5**. SW105 cells have an Flpe gene under the control of arabinose to delete the neomycin resistance cassette. Note that an Flpe-induced recombination is not required in this protocol, but it is important to analyze the sequence within the region that has been modified in this final construct.

3.3 Gene Targeting in ES Cells

The subcloned vector is linearized at its 5′ end using NotI or appropriate restriction enzyme and electroporated into ES cell lines. ES cells from mouse strain 129 were used in this subcloning process since the BAC clones were generated from AB2.2 ES cells [8]. The transformed cells are selected with G418 (200 μg/ml) to screen for neomycin-resistant colonies. The gene targeting into mouse ES cells is described in detail elsewhere [12]. The selected colonies are confirmed for homologous recombination by long-range PCR on both sides in which one primer is outside of the homologous arm and the other binds to the targeting cassette near respective *LoxP* site. Then, the PCR-positive colonies are further analyzed by Southern blot as described previously. The targeted ES cells should show a large-size band as the *wild-type* allele and a small-size band as a conditional knockout (*floxed*) allele.

3.4 Blastocyst Injection and Germline Transmission

The targeted ES cells are injected into the blastocoel of 3.5-day-old mouse blastocysts. For this purpose, host embryos are chosen that can be distinguished from the coat color contribution from the ES cells. For example, C57BL/6 embryo (black) donors are injected with 129 ES cells (agouti). Currently black and albino B6 ES cells have been established that offer a wide variety of choices so that the mutants generated can be under a pure genetic background. The injected embryos are surgically transplanted into the uterine horns of recipient females. If the injected ES cells become part of growing embryos, the offspring will have typical chimeric patches of coat color derived from host embryos and the ES cells. The chimeric mice are crossed with wild-type mice to determine germline transmission, i.e., whether the mutation in ES cells transmits to the offspring. This is characteristic of pups that exhibit the coat color from the ES cells. For example, germline offspring exhibit an agouti coat color, which is derived from agouti 129 ES cells, and black pups are derived from blastocysts of C57BL/6 host. The germline mutation mice carry a heterozygous *floxed* allele. These F1 mice must be crossed to each other to yield homozygous mice. If the neomycin resistance cassette causes embryonic lethality in the homozygous state, then the neomycin cassette can be deleted in the germline of transgenic mice expressing Flp recombinase (see Jackson Laboratory for availability of strains in the desired genetic background).

4 Notes

1. *PGK* permits expression of the neomycin resistance cassette in mammalian cells, whereas *Em7* allows for expression of the cassette in bacterial cells.
2. In this schematic, the expected size of the genomic region captured from a BAC clone to a targeting vector should be around 10 kb and homologous recombination arms in both 5′ and 3′ prime ends should be 2–4 kb each. These sizes will allow for high-efficiency homologous recombination from targeted vector in mouse ES cells.
3. The bacterial cells can be stored in LB media containing 10 % glycerol at −80 °C.
4. Plasmids >50 kb, such as BACs, elute less efficiency from silica than smaller plasmids. Therefore, precipitation of DNA using isopropanol instead of using the columns from the Miniprep kit is suggested.
5. The sequence of the multi-cloning site on the TA vector that captures the probe can be found on the Invitrogen website (http://www.invitrogen.com).
6. It is ideal to choose restriction enzymes for Southern blot analysis that can generate about 10–15 kb fragment of your gene of interest, labeled in the figure as the *wild-type* (10 kb) allele. These restriction enzymes can be engineered into the homologous arms for the first *LoxP* site and are utilized in Subheading 3.2.2, **step 1**, to create a smaller fragment (~5 kb) labeled as the *conditional knockout* allele.
7. An additional 3–4 random nucleotides should be added at the 5′ termini of the primers to help in the restriction enzyme digestion of the PCR products. Each primer should contain the random nucleotides, the restriction enzymes, and then 20–25 sequence-specific nucleotides from the 5′ end.
8. There should be at least ten times less non-heat-shocked colonies than colonies that have been heat-shocked.
9. The appropriate restriction enzyme site should be incorporated in between EcoRI and the actual primer sequences recognizing the targeted gene.
10. The *LoxP* site with homologous arms is almost the same size as the mini targeting vector backbone (about 2 kb each). There is a SmaI restriction site in the middle of the vector backbone that can be used to create smaller fragments to more easily separate the *LoxP* part from the vector backbone on the gel.

11. The cells that have deleted the neomycin resistance cassette should lose kanamycin resistance. Therefore, the kanamycin plate would be used as a negative control for the recombination.

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Chapter 3

Generating Mouse Lines for Lineage Tracing and Knockout Studies

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Abstract

In 2007 Capecchi, Evans, and Smithies received the Nobel Prize in recognition for discovering the principles for introducing specific gene modifications in mice via embryonic stem cells, a technology, which has revolutionized the field of biomedical science allowing for the generation of genetically engineered animals. Here we describe detailed protocols based on and developed from these ground-breaking discoveries, allowing for the modification of genes not only to create mutations to study gene function but additionally to modify genes with fluorescent markers, thus permitting the isolation of specific rare wild-type and mutant cell types for further detailed analysis at the biochemical, pathological, and genomic levels.

Key words Mouse, Gene targeting, Embryonic stem cell culture, Pluripotent, Chimera, Microinjection, Germline transmission, Cre-recombinase

1 Introduction

> Mice are gentle. Mice are sweet. Mice have tiny hands and feet. Mice are nice [1].

Mice have long made it into the heart of children as one of their favorite humanized book characters while often simply being regarded as pest by accompanying adults. With the discovery of recombinant DNA technology, however, mice have proven themselves as one of the greatest allies of medical scientists given their vertebrate status, housing and breeding efficiency, and not to forget the advantage of a known genome and ease of manipulation thereof. To date nearly 14,000 entries can be found for gene-targeted mouse lines and more than 1,800 for Cre-recombinase-expressing mouse lines (for example ref. 2 and references therein). Several biotechnology companies and research institutes devoted themselves to develop high-throughput methodologies to disrupt virtually every gene in mouse ES cells often at the same time inserting a reporter gene allowing to assay for gene expression.

Shree Ram Singh and Vincenzo Coppola (eds.), *Mouse Genetics: Methods and Protocols*, Methods in Molecular Biology, vol. 1194, DOI 10.1007/978-1-4939-1215-5_3,

Nowadays the numbers of available mouse lines are huge and should by all means be explored before embarking on a new project. However, available lines are not always tailored to a researcher's particular need. For these cases, we provide methods as a guideline, which have been successfully used many times in our hands.

2 Materials

2.1 Construct Design for Reporter Gene-Targeted Wild-Type and Knockout Lines

2.1.1 General Considerations

Given the cost of generating genetically engineered animals it cannot be emphasized enough how important it is to spend time researching readily available genome databases from NCBI (http://www.ncbi.nlm.nih.gov), MGI (http://www.informatics.jax.org/genes.shtml), UCSC (http://genome.ucsc.edu), or MGD (http://www.nih.gov/science/models/mouse/resources/mgd.html) to name a few for DNA sequence information regarding the gene of interest (from now on referred to as *GeneX*) and the existence of alternatively spliced transcripts or possible protein isoforms as well as potential downstream AUG start codons. Genomic areas containing repetitive elements should be avoided. The use of DNA isogenic to the embryonic stem (ES) cells should increase the targeting efficiency; this can be achieved by selecting BAC clones from libraries of the same genetic background as the ES cell or by PCR amplification employing error-proof and long-range Taq polymerases.

2.1.2 BAC Modification

Generally, bacterial artificial chromosomes (BAC) can be retrieved from the BACPAC Resources Center at Children's Hospital Oakland Research Institute (CHORI). Suitable primers for BAC screening, modification, and subcloning can be designed using Primer 3 (v. 0.4.0) software (http://frodo.wi.mit.edu/primer3/). The Gene Bridges Quick and Easy BAC Modification kit, the Gene Bridges Quick and Easy BAC Subcloning kit, and the Gene Bridges Quick and Easy Conditional Knockout Kit (loxP/Cre) can be used for genetic modifications through the Red/ET recombineering technology following the manufacturer's protocol.

2.1.3 Choosing a Selection Cassette

A typical selection cassette should include a strong ubiquitously expressed promoter active in eukaryotic cells and an antibiotic resistance gene suitable for selection in the eukaryotic system (e.g., neomycin, hygromycin). For the option of the future removal of this cassette (recommended), it should be flanked with the recognition sites for either Cre or FLPe recombinases.

2.1.4 Adding a Reporter Gene

While more labor intensive, cell labeling by stable insertion of a reporter gene into the genome has advantages over transient cell labeling given that the label will not be lost with increasing cell

divisions or in case of dye injection will not accidentally leak into a neighboring cell. However, the type of cell labeling and the type of reporter gene should always be carefully chosen based on the intended downstream application. For expression analysis of weakly expressed genes *lacZ* or *placental alkaline phosphatase (PLAP)* is suitable yet they will require tissue fixation. The half-life of a given reporter should also be taken into consideration, as a long half-life may contribute to imprecise information about the gene expression of a specific population of cells. Also, the desired subcellular localization of the reporter might vary for different studies; for example, plasma membrane labeling could be of interest to study cell migration properties while nuclear labeling might be more suitable for cell sorting and cell lineage tracing [3]. For any in vivo analysis like fluorescence-activated cell sorting (FACS) or time-lapse imaging *enhanced green fluorescence protein (EGFP)* gene and derivatives are the reporter of choice owing to the brightness and photo stability [4–6]. For quantitative evaluations of gene expression a close to stoichiometric expression of *GeneX* and the reporter is important. This can be facilitated by generating a fusion protein or by including an internal ribosome entry side (IRES) or a viral 2A peptide into the construct design; for further detail *see* ref. 7. Below are two examples of possible reporter selection cassettes:

F2A-EGFP-FRT-PGKgb2-Neo-FRT

IRES-LacZ -loxP-PGKgb2-Neo-loxP

These cassettes can be used for generating a gene knockout or facilitating in vivo gene expression analysis when creating a phenotypically wild-type allele, depending on the site of insertion.

2.1.5 Further Considerations for Cell Lineage Tracing

Cell lineage analysis requires the ability to label a distinct cell population of interest and all their progenitors. This can be achieved as a "turn on and stay on" event or as a "pulse labeling event." In both cases Cre or FLPe recombinase driven by a well-characterized enhancer will allow for the excision of a recombinase recognition site-flanked DNA-stop sequence, upon which removal a reporter gene is expressed. The latter approach requires an inducible system generally controlled by drug administration, which can fine-tune the timing during which this enhancer is actively driving recombinase expression [8].

Mice with Inducible Cre or FLPe Recombinase Expression

Jackson Laboratories and others offer a wide range of Cre-recombinase (http://jaxmice.jax.org/list/xprs_creRT1801.html) or FLPe-recombinase-expressing mouse lines (http://jaxmice.jax.org/list/xprs_FLP1807.html).

Available Reporter Mice

Jackson Laboratories and others offer a wide range of reporter lines specific for Cre-mediated (http://jaxmice.jax.org/list/xprs_creRT1805.html) or FLPe-mediated (http://jaxmice.jax.org/list/xprs_FLP1811.html) recombination.

2.2 Materials for Mouse Embryonic Stem Cell Culture

1. ES cells: R1 (129/Sv×129/Sv-CP)F1 or V6.4 (C57BL/6 J× 129/SvJae) or equivalent, many available from ATCC or other institutions (*see* **Note 1**).
2. ESM: DMEM (Gibco, Invitrogen) supplemented with 15 % heat-inactivated ES-grade fetal bovine serum (FBS) (Gibco, Invitrogen), 0.1 mM β-mercaptoethanol (Gibco), 4 mM L-glutamine (Gibco), 40 μg/ml gentamicin (Gibco, Invitrogen), and 500 U/ml LIF (ESGRO, Chemicon).
3. Trypsin (0.05 %): Gibco, Invitrogen.
4. Irradiated mouse primary embryonic fibroblast (PEF): Applied StemCell Inc.
5. Irradiated DR4 feeder: Applied StemCell Inc.
   Geneticin/G418: Gibco (*see* **Note 2**).
6. Freezing medium: 7 ml DMEM (Gibco), 2 ml heat-inactivated FBS (Gibco), 1 ml DMSO; mix well and then filter through a 0.2 μm syringe filter.
7. P1000/P200/P20/P10 and multichannel pipette aids: Gilson or equivalent with respective filter tips.
8. Disposable reagent reservoirs (25 ml): Thermo Scientific.

2.3 Materials for DNA Extraction

1. Proteinase K: 20 mg/ml in H_2O (store at –20 °C).
2. Proteinase K digestion buffer (PKDB): 50 mM Tris pH 7.0–8.0, 5 mM EDTA, 1 % SDS, 0.2 M NaCl.
3. Maxtract (1.5 ml): Qiagen.
4. Phenol/Chloroform: Ambion.

2.4 Materials for Southern Blot Screening

1. PCR DIG Probe Synthesis Kit: Roche.
2. QIAquick PCR purification spin columns: QIAGEN.
3. DIG Easy Hyb buffer: Roche.
4. Nylon membrane: Amersham Hybond-XL.
5. 20× SSC: NaCl 175.3 g, sodium citrate 88.2 g; dissolve in H_2O to 1,000 ml, adjust to pH 7.0, and then autoclave.
6. 5× MAB: Maleic acid 29 g, NaCl 22 g, NaOH ~20 g; dissolve in 500 ml H_2O, adjust to pH 7.5, and then autoclave.
7. MABT: MAB with 0.3 % Tween20.
8. 10× Blocking solution: Dissolve 100 g blocking powder into 1,000 ml 1× MAB, autoclave, and keep 50 ml aliquots at –20 °C.
9. Anti-DIG antibody: Roche Anti-DIG-AP FAB fragments.
10. Detection buffer: 0.1 M Tris–HCl/0.1 M NaCl, pH to 9.5.
11. CDP-Star "ready-to use": Roche.
12. Stripping buffer: 0.2 M NaOH/0.1 % SDS.

2.5 Efficient Generation of Germline Transmitting Chimeras (See Note 3)

1. M2 medium: Sigma.
2. M16 medium: Sigma.
3. Embryo culture tested mineral oil: Sigma.
4. Dissecting Stereomicroscope: Leica.
5. Inverted microscope unit: Leica.
6. ES injection needle: Transfertip® Eppendorff.
7. Embryo holding needle: Vacutip Eppendorff.
8. 35 mm Easy Grip cell culture dish: BD Falcon.
9. Embryo donor: CD-1® Mouse (Crl:CD1(ICR)) available from Charles River or similar.
10. Recipient pseudopregnant female: (C57BL/6×CBA)F1/J available from JAX or similar.
11. Dumont#5 Fine forceps: Fine Science Tools.
12. Graefe forceps: Fine Science Tools.
13. Extra fine Graefe forceps: Fine Science Tools.
14. Extra fine Bonn scissors: Fine Science Tools.
15. Micro Serrefine: Fine Science Tools.
16. Needle Holder: Fine Science Tools.
17. Needles with Suture Thread: Fine Science Tools.
18. Wound clip applicator: Fine Science Tools.
19. Wound clips: Fine Science Tools.
20. Affigel Blue Gel beads: Biorad.
21. 2.5 % Avertin: Sigma Aldrich (diluted in 0.9 % NaCl).

3 Methods

3.1 Construct Design for Reporter Gene-Targeted Wild-Type and Knockout Lines

3.1.1 Basic Strategy for a Gene Knockout Construct Design

To be successful in achieving a gene knockout it is necessary to assure that no functional truncated protein can be generated by alternative splicing, exon skipping, or alternative start codon usage based on existing and available genetic information. At the same time excessive deletion of genomic sequences should be avoided as it becomes more and more evident that intronic regions often contain elements relevant for transcriptional control or noncoding RNA that might be involved in gene transcript regulation [9].

1. Research existing genome databases for DNA sequence information regarding the sequence of *GeneX* and identify suitable restriction sites for the generation of the long homology arm (LA) and short homology arm (SA).
2. Research existing databases for the existence of alternatively spliced transcripts or downstream AUG codons (e.g., Ensembl: http://www.ensembl.org).

3. Retrieve (if necessary several) BAC clones that will include the intact DNA sequence surrounding the ATG start codon, ideally with several kilobases (kb) flanking the ATG start codon of *GeneX*, and thoroughly verify the correct identity of the clone by sequencing or restriction digests.
4. Design your construct in a way that the insertion of the reporter cassette will disrupt the production of the translation product of *GeneX*, namely protein GeneX^{+}. This is most easily achieved by inserting the reporter cassette at a nucleotide position that would correspond to a few amino acids C-terminal to the ATG start site of translation. The resulting truncated protein GeneX^{-} of just a few amino acids would be unlikely to have any biological function [10–13].
5. Design PCR primers for the amplification of appropriate 50 bp homology arms to flank a reporter selection cassette.
6. BAC modification can be performed according to the manufacturer's protocol of the Gene Bridges Quick and Easy BAC Modification kit. In brief, transform the *pRed/ET* plasmids into DH10B (*E. coli* strain) containing the required BAC clone and use chloramphenicol (12.5 μg/ml) and tetracycline (3 μg/ml) for selection.
7. Transform the reporter/selection cassette of choice into an *E. coli* clone containing the *pRed/ET* plasmids.
8. Select for a clone with successful homologous recombination by antibiotic resistance and colony PCR.
9. Ensure the absence of mutations by sequencing the PCR products of the inserted cassette with multiple overlapping primer sets.
10. Subclone mutation-free, successfully modified BAC clones into a minimal vector using the Gene Bridges Quick and Easy BAC Subcloning kit using the same *Red/ET* recombineering technology as per the manufacturer's protocol.
11. Choose grabbing arms from a repeat-free region, with a short arm of at least 1.5 kb and a long arm of at least 7 kb.
12. Add a unique restriction site at one end of the grabbing arms to facilitate plasmid linearization for future electroporation.
13. Identify positive recombinants (subclones) by kanamycin (20 μg/ml) and ampicillin (100 μg/ml) selection and colony PCR.
14. Use restriction enzyme digestion to check positive clones for the correct BAC modification.
15. Eliminate any clones with mutations by sequencing the PCR products of the modified region with multiple overlapping primer sets.

16. Linearize mutation-free subclones via the unique restriction site introduced in **step 12**.
17. Electroporate into mouse ES cells.

3.1.2 Basic Strategy for Designing a Wild-Type Allele with a Reporter Gene Knock-In

To maintain a wild-type allele phenotype while inserting a reporter gene under the control of the endogenous promoter of *GeneX* it is imperative to assure that the protein product of *GeneX* remains functionally normal. While there is no 100 % guarantee for this, the best strategy is to target the 3′UTR of *GeneX* with a suitable reporter/selection cassette, immediately downstream of the *GeneX* stop codon. It needs to be pointed out that the 3′UTR may contain regulatory elements controlling *GeneX* or other genes. It is therefore important to exploit existing database information to identify and avoid the disruption of such elements.

1. Research existing genome databases for DNA sequence information regarding the sequence of *GeneX* and identify suitable restriction sites for the generation of the long homology arm (LA) and short homology arm (SA) in the 3′UTR.
2. Research existing databases for the existence of alternatively spliced transcripts or known regulative elements in the 3′UTR possibly affecting the expression of *GeneX* or other genes.
3. Retrieve (if necessary several) BAC clones that will include the intact 3′UTR sequence of *GeneX*, ideally with some sequence upstream of the stop codon, and thoroughly verify the correct identity of the clone by sequencing or restriction digests.
4. Design your construct in a way that the reporter cassette is located just 3′ to the TGA stop codon of *GeneX*. As the reporter cassette is equipped with a bicistronic mechanism, the production of EGFP will follow the production of GeneX at essentially a 1:1 ratio (Fig. 1). While the distance from the TGA stop codon is not critical, it is essential that the reporter cassette is inserted well 5′ to the polyadenylation site. If this is not precisely known, it is prudent to place the cassette insertion site within several bp 3′ to the TGA stop codon [9–14].
5. Follow **steps 5–17** of Subheading 3.1.1.

3.1.3 Considerations Regarding Cell Lineage Tracing

In a way similar to what has been described for homologous recombination targeting strategies generating mice for gene ablation or reporter gene expression, mice can be engineered to express Cre or FLPe recombinase. Alternatively, the sequence encoding these recombinases can be placed under the control of a well-characterized tissue-specific enhancer and a transgenic mouse line can be generated via pronuclear injection into mouse oocytes (for details *see* Section E, Production of Transgenic Mice in Manipulating the Mouse Embryo [15]). Since DNA integration into the genome will occur at random, it is advisable to generate and characterize

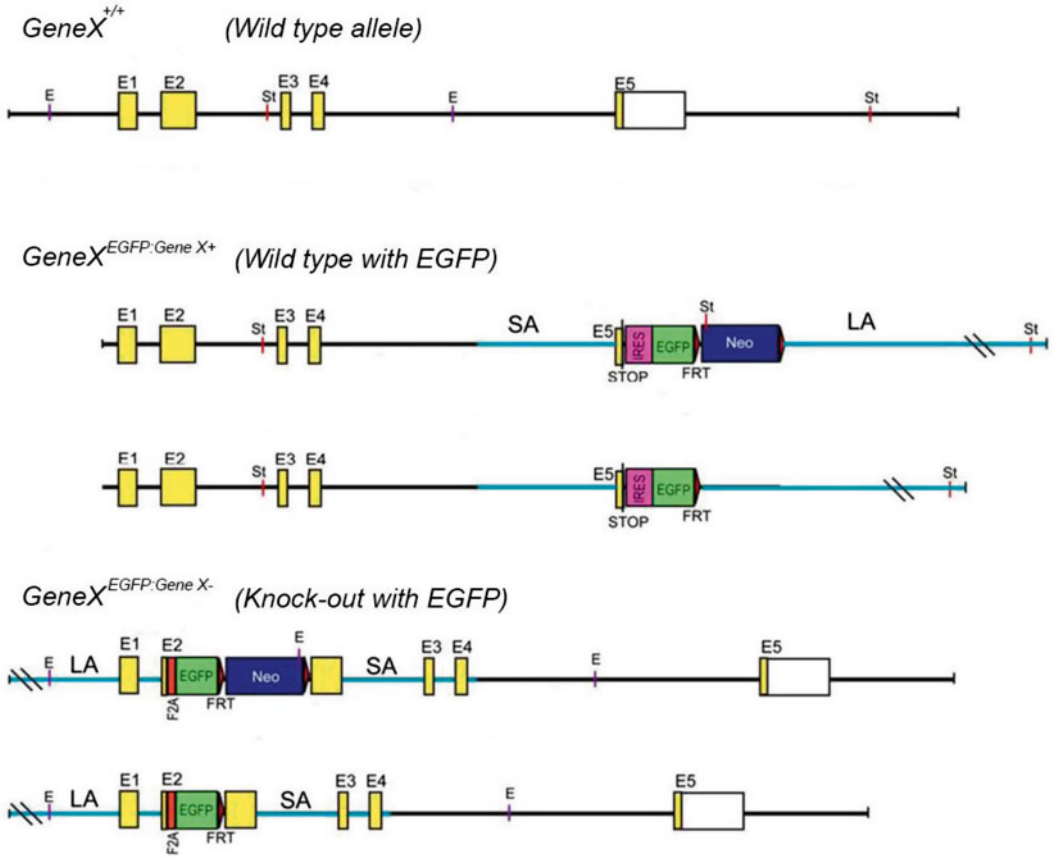

Fig. 1 Schematic of *EGFP* gene-targeting constructs. Targeting constructs for *GeneX*$^{\text{EGFP:GeneX+}}$ and *GeneX*$^{\text{EGFP:GeneX–}}$. The genomic organization of *GeneX* is represented in a *line diagram. Yellow boxes* represent the exons, *black solid lines* represent intronic regions, *white boxes* represent the untranslated regions (UTR), *pink boxes* represent the internal ribosome entry site (IRES), *orange boxes* represent the F2A peptide sequence, *red triangles* represent the FRT sites, *green boxes* represent EGFP, and *blue boxes* represent the *Neo* cassette [9–11, 14]

several founder lines, ensuring stable time- and tissue-specific expression as anticipated. A large number of well-characterized Cre- and FLPe-recombinase-expressing lines, constitutive, tissue specific, or inducible, are already available and should be explored for their suitability before embarking on generating a new line.

Cell Lineage Tracing

For cell lineage analysis mice expressing Cre or FLPe recombinase in a time- or a tissue-specific manner are mated with stop-reporter lines. Stop-reporter lines are mice that carry a DNA-stop signal flanked by the respective recombinase recognition sites (loxP or FRT) preventing expression of a downstream reporter gene which is often but not necessarily under the control of an ubiquitously expressed promoter like ROSA26 [16]. Once the recombination

event occurs the stop signal gets excised and the reporter is expressed and as such labeling the cell in which the event occurred and all of its progenitors.

Inducible Cell Lineage Tracing

Sometimes the *Cre* or *FLPe recombinase* gene has been fused to the human estrogen receptor ligand-binding domain and optimized modifications thereof known as ER^{T2} [17–19]. This allows for selective activation of the recombinase upon availability of the ligand tamoxifen. Tamoxifen can be administered via food or as oral gavage when dissolved in sunflower oil or equivalent.

3.2 Targeting Mouse Embryonic Stem Cells

3.2.1 Time Course

1. Week 1:

   Wednesday: Plate wild-type (wt) feeder (5 × 10 cm dish).

   Thursday: Thaw mES cells (1 vial per 2 × 10 cm dish).

   Friday: Change medium.

2. Week 2:

   Monday: Change medium of mES cells, plate DR4 feeder (10 × 10 cm plates), and trypsinize mES cells from week 1 (Thursday) into 3–4× 10 cm plates to allow them to reach 70–80 % confluence the next morning.

   Tuesday: Trypsinize and electroporate.

   Wednesday: Change medium (G418 selection (10 ml)).

   Friday: Change medium (G418 selection (15 ml)).

3. Week 3:

   Monday: Change medium (G418 selection (10 ml)).

   Wednesday: Change medium (G418 selection (10 ml)) and plate wt feeder for 2× (3× 96) and 2× (4× 24) well plates.

   Thursday: Change medium on feeder, pick 2× 96-well plate (−gelatin/−feeder), which will be immediately broken up into 2× (3× 96-well) replica plates (+gelatin/+feeder). This is very time consuming!

   Friday: Change medium of 2× (3× 96-well) and 2× (4× 24-well) plates.

4. Week 4:

   Monday: Check replica plates, freeze the two better ones down, and transfer third plate into 24-well plates. This is very time consuming!

   Tuesday to Friday: Check cells and add PKDB to each vial that reaches ~70–80 % confluence.

5. Week 5 and onward:

   DNA extraction and Southern blot screening.

   Recover positive clones, verify by Southern blotting and karyotyping, and send for microinjection.

3.2.2 General Mouse ES Cell Culture Maintenance

Grow all mES cells on gelatinized plates (0.1 %) containing irradiated mouse primary embryonic fibroblast (PEF) also known as feeder. Cells were grown in ES media (ESM) at 37 °C in 5 % CO_2. Add fresh media on alternate days and passage cells with 0.05 % trypsin every few days once they reach 80–90 % confluence.

*3.2.3 Plating of DR4 Feeder (See **Notes 4** and **5**)*

1. Prepare gelatinized 10 cm dishes with 10 ml ESM each.
2. Retrieve one vial from −150 °C and thaw in 37 °C water bath.
3. Prepare 1× 15 ml Falcon tube with ~7–8 ml ESM and add thawed cells to it.
4. Spin for 3 min at 1,000 × *g*.
5. On average, a 0.5 cm pellet from the bottom of the tube should be enough cells for 8–10 plates.
6. Aspirate supernatant, resuspend in 5 ml ESM, and then aliquot 0.5 ml per plate.
7. Next day: Change medium.

*3.2.4 Plating of Wild-Type Feeder (See **Note 6**)*

1. Prepare 6× 96-well and 8× 24-well gelatinized plates.
2. Retrieve frozen feeder vials and thaw in 37 °C water bath.
3. Prepare 2× 15 ml Falcon tubes with ~7–8 ml medium and add thawed cells to it.
4. Spin for 3 min at 1,000 × *g*.
5. On average, a 0.5 cm pellet from the bottom of the tube should be enough cells for 8–10 plates.
6. Aspirate supernatant and resuspend cells well in 4 ml medium each.
7. Add 19 ml medium in disposable reagent reservoir and add 1 ml of the resuspended wt feeder (repeat until all feeder is used up), and mix cells and medium constantly.
8. Aliquot (with multichannel pipette) as follows (12 ml per plate):

   ~450 µl per 24-well/100 µl per 96-well; prepare a total of 160 ml.

   Try adding the cell/medium mix to the side wall or all will end up in the center.
9. Next day: Change medium.

3.2.5 Electroporation

1. One day before the electroporation, in the morning, trypsinize the mES cells from 2× 10 cm plates into 3–4× 10 cm plates to reach about 70–80 % confluence on the next day. Maybe change medium in the evening.
2. Next day—electroporation day: Rinse and wash plates two to three times with PBS (*see* **Note 7**).

3. Trypsinize cells with 1–1.5 ml 0.05 % trypsin for 2–3 min at 37 °C and transfer with a Gilson-P1000 into a 15 ml Falcon tube with ~8 ml ESM.
4. Spin for 3 min at 1,000 × *g*.
5. Aspirate supernatant and resuspend in 1 ml ESM.
6. Cell count:
   (a) Dilute 50 µl of cells from **step 5** with 450 µl ESM (10× dilution).
   (b) Load 10 µl on hemocytometer for cell count.
   (c) Hemocytometer shows 9 big squares with 16 small squares each.
   (d) Count the cells in the 4 small corner squares = *n* cells.
   (e) Calculate: Total number of cells = (n/4) × 10 (dilution factor) × 10^4/ml.
7. Respin and adjust the concentration to 10^7 cells (R1 ES cells) or 5×10^6 cells (V6.4 ES cells) per 500 µl.
8. Label electroporation cuvettes:
   (M): medium/(+): Cells + DNA/(−): Cells no DNA.
9. Spin down DNA (*see* **Note 8**).
10. Mix 600 µl cells well with 12 µg DNA in a 15 ml Falcon tube.
11. Transfer 500 µl cells with DNA to (+) cuvette, 500 µl cells w/o DNA to (−), and 500 µl medium to the third (M); avoid bubbles between the electrodes!
12. Electroporate at 125 µFarads/0.4 kV (*see* **Note 9**).
    (a) Pulse medium (M) first; the color should be darker purple on one side and lighter pink on the other side after the pulse.
    (b) Proceed with cells (+) and (−).
13. Let the cuvette sit at RT for 5–10 min.
14. Add cells from (+) into 9 ml ESM.
15. Change media of the 10× 10 cm DR4 feeder plates from the day before (*see* 3.2.3).
16. Add different amounts of (+) cells from **step 14** to nine of the DR4 plates:
    0.3 ml/0.5 ml/0.8 ml/1.0 ml/1.0 ml/1.2 ml/1.5 ml/2.0 ml/rest.
17. Add cells from (−) to tenth DR4 plate.
18. Incubate overnight.

3.2.6 G418 Selection

1. Change ESM medium to ESM with 10 ml of 350 μg/ml G418 per 10 cm plate.
2. Grow under selection for 7–10 days, and change medium every other day.

*3.2.7 Picking Colonies into Three Replica Plates After G418 Selection (See **Note 10**)*

1. Check the previously prepared 2× 3× 96-well feeder plates for contamination, and then change medium as follows: To feeder plate 1 add 200 μl fresh ESM to each well. For feeder plates 2 and 3—wait until **step 6**(**c**).
2. Label empty 96-well plates and be aware of their orientation to avoid a mix-up later (*see* **Note 11**).
3. Take one selection plate with isolated clones from the incubator and change medium to PBS.
4. Make a small black dot on the glass plate of the dissection stereomicroscope. Move one "clone to pick" over the black dot, and then scrape it off with a Gilson-P20 and filter tips while sucking it up in a total volume of ~10 μl PBS (*see* **Notes 12** and **13**).
5. Transfer to 96-well plate (referred to as plate "0") (*see* **Note 14**).
6. Once 2–3 rows are picked start breaking the clones up (*see* **Note 15**).
   (a) Add 90 μl trypsin to each well with a multichannel pipette and then incubate for 2–3 min at 37 °C.
   (b) In the meantime take plates 1–3 out of the incubator.
   (c) Aspirate medium of plates 2 and 3 from rows corresponding to those with picked colonies on plate "0" (do not allow feeder to become dry).
   (d) Trypsinize picked clones by pipetting up and down vigorously, and then transfer into plate 1 according to the position of plate 0.
   (e) Wash wells of plate 0 well with ESM from plate 1 and transfer everything into plate 1; there mix well and transfer 100 μl each into plates 2 and 3.
7. Return plates 1–3 into incubator; if possible take a new selection plate and repeat **steps 4**–**7** for the next 2 rows, etc.
8. Change medium the next day to 250 μl/well.

3.2.8 Freezing of 96-Well Plates

1. Take the two best ~70–80 % confluent 96-well plates for freezing and clearly label them on the side.
2. Always prepare fresh freezing medium, mix well, and filter through a 20 μm syringe filter into reservoir (*see* **Note 16**).
3. Take plates out of the incubator and aspirate old medium with multichannel suction device.
4. Aliquot 70–90 μl freezing medium into 96-well plate with multichannel pipette, and avoid bubbles.

5. Wrap plates individually with cling wrap and then aluminum foil and label clearly on top and side.
6. Fill a ~5 × 10 cm Styrofoam box with paper towels around the plate to allow for slow freezing, add lid, and tape close, secure with rubber bands.
7. Move into −80 °C, and then after 2 days into −150 °C.

*3.2.9 Expanding 96-Well Plate for DNA Extraction (See **Notes 17** and **18**)*

1. Check for contamination of previously prepared 4× 24-well feeder plate.
2. Change medium (two plates at the time so that feeder does not dry out).
3. Return plates # 2/3/4 into incubator.
4. Trypsinize two rows at a time of the 96-well plate using a multichannel pipette.
5. Aspirate medium and then add 70 μl fresh trypsin to each well (only two rows at the time! No PBS wash)—incubate for 2–3 min at 37 °C.
6. Using only alternate positions on a multichannel pipette start with clones 1/3/5/7/9/11 followed by 2/4/6/8/10/12 followed by 13/15/17/19/21/23 followed by 14/16/18/20/22/24 to shift them from a 96-well into the 24-well plate. To do so:
   (a) Pipette up and down, stir and scratch cells to break up the clone, transfer, and then wash the well several times with medium.
   (b) When transferring into 24-well make sure that pipette and wells are well aligned.
   (c) When transferring put 24-well plate at an angle and pipette up and down several times to mix in order to avoid all cells being stuck in the center.
7. Return the plate to the incubator, repeat **steps 4**–7 with row 3 + 4, etc., and then move on to plate 2.
8. Check medium/cell confluence daily; if 70–80 % confluence is reached add 0.5 ml PKDB to this well and return to incubator. After 2–3 days all wells should have reached this point.
9. Proceed with DNA extraction.

3.2.10 DNA Extraction

1. Pipette PKDB-treated sample into Maxtract 1.5 ml tube.
2. Add an equal volume of phenol:chloroform.
3. Agitate on shaker for ≤30 s.
4. Spin at 13,200 × *g* for 5 min at RT.
5. Transfer the top aqueous phase into a new 1.5 ml centrifuge tube.
6. Add 1.5 ml of 100 % EtOH and mix by inverting.

7. Spin at 13,200 × *g* for 5 min at RT and discard supernatant.
8. Add 1 ml of 70 % EtOH, and mix by inverting.
9. Spin at 13,200 × *g* for 5 min at RT, discard supernatant, and air-dry pellet until it turns clear (*see* **Note 19**).
10. Resuspend fully in 50 µl H_2O (overnight) and then measure DNA concentration.

*3.2.11 Probe Generation for Southern Blot Screening (See **Note 20**)*

1. Start with a small-scale trial of labeled versus unlabeled probe.
2. Scale up to 10× for actual labeled probe.
3. Clean the probe with QIAquick PCR purification spin columns according to instructions.
4. Elute probe with 50 µl nuclease-free water (preheated to 50 °C) and measure concentration.

3.2.12 Screening by Downward Southern Blot

1. Define a set of restriction enzymes that will cut the DNA in a way that the targeted and the nontargeted allele can be clearly distinguished. Ideal fragment sizes lie between 5 and 15 kb with ideally at least 1–2 kb difference.
2. Define an internal and an external probe for hybridization with the help of online software programs like Repeat mask (http://www.repeatmasker.org) and Primer 3 (http://frodo.wi.mit.edu/primer3/).
3. Use PCR stripe tubes for setting up the restriction enzyme digest for 7–10 µg DNA in a total volume of 35–40 µl and digest overnight at 37 °C.
4. Add 7–8 µl 6× loading dye and run on a 0.8 % agarose gel in 1× TAE buffer without ethidium bromide (EtBr) overnight at 35 V (>10,000 bp) or 20 V(1,500–10,000 bp) depending on the fragment size and gel chamber.
5. Prepare three evenly high piles of C-fold paper towels in a tray for blotting, then cover with ~10 unfolded paper towels, add two layers of thin Whatman paper, and flatten with a heavy weight (*see* Fig. 2).

Fig. 2 Schematic illustration of a typical setup for a downward Southern blot. The denatured gel is placed on a stack of c-fold towels followed by two layers of 3 mm Whatman filter paper and a positively charged nylon membrane [9, 12–14]

6. After the overnight run stain the gel for 30 min with EtBr in 1× TAE (0.1 mg/ml) under slight agitation.
7. Take a photo with the molecular weight ladder next to a ruler to calculate the fragment size.
8. Transfer the gel to 0.5 M NaOH for at least 2× 45 min each. For DNA fragments of more than 12 kb, depurination with 0.25 M HCl can be performed for 10 min before proceeding with NaOH denaturation.
9. Proceed to cutting the gel according to the expected fragment sizes, and keep gel in NaOH all the time so that it will not dry out.
10. Cut membrane (Amersham Hybond-XL) liberally (only touch with forceps, no gloves) and place it on the Whatman paper from **step 5**.
11. Place the gel bubble free on top of the membrane with the loading side facing up and gently press it down. Remove bubbles and then wet the top with 0.5 M NaOH.
12. Wrap the setup gently with cling wrap to prevent it from drying out and put a light balanced weight on it.
13. Every 30 min to 1 h, wet the top surface with 0.5 M NaOH, and then allow for transfer overnight.
14. Mark wells with pencil and trim membrane (keep excess membrane for step (e) in **Note 21**).
15. Remove gel in 5× SSC (optional: stain gel with EtBr to check on transfer efficiency) and then rinse membrane with 5× SSC for 2× 5 min.
16. Preheat 500 ml Roche DIG Easy Hyb Solution.
17. Add pre-hyb solution (Roche DIG Easy Hyb Solution) and membrane to the hyb-bottle, seal with Parafilm, and prehybridize at 42 °C for at least 4 h (*see* **Note 21**).
18. Replace pre-hyb solution with probe/hyb solution and hybridize at 42 °C for 6–16 h under agitation or rotation.
19. Remove membrane from bottle and wash in container with 2× SSC/0.1 % SDS for 5 min at RT (*see* **Note 22**).
20. Wash 2× for 15 min with 0.5× SSC/0.1 % SDS at 55–60 °C depending on the PCR annealing temperature of probe when generated (*see* **Note 23**).
21. Rinse with 1× MAB or 1× MABT for 5 min at RT.
22. Block membrane with 1× blocking solution (BS) for 30 min at RT (*see* **Note 24**).
23. Spin anti-DIG antibody (AB) for 5 min at 4 °C with max speed, and then dilute AB 1:10,000 in fresh 1× BS.
24. Replace BS (from **step 22**) with AB in BS (from **step 23**).

25. Incubate for 30 min at RT.
26. Wash membrane with 1× MABT for 2× 15 min.
27. Equilibrate membrane with 20 ml detection buffer (DB) for 5 min at RT.
28. Place the membrane in a polyethylene bag and squeeze out excess DB.
29. Cover membrane with 1 ml CDP-Star chemiluminescent AP substrate (1 ml/100 cm^2) and incubate for 5 min at RT.
30. Squeeze out excess CDP-Star, seal bag, and incubate membrane for 10 min at 37 °C.
31. Expose sealed membrane to X-ray film and develop X-ray film after ~1 h.
32. Stripping:
    (a) Wash for 2× 15 min at 37 °C with stripping buffer (0.2 M NaOH/0.1 % SDS).
    (b) Rinse for 2× 5 min with 2× SSC, and then store in 2× SSC at 4 °C.
33. Re-probe with new probe/hyb solution overnight.

3.2.13 Thawing of Positive Clones from 96-Well Plate After SB Confirmation

1. Remove Styrofoam box from –80 °C, take one replica plate for thawing, and put the second one immediately into –150 °C for long-term storage.
2. Put plates in incubator and move every 2 min to a warm spot.

   After about 8–9 min, check if they start thawing, and then proceed to add ESM (*see* **step 5**).
3. Identify and then verify the positive wells and circle them.
4. Gently add pre-warmed ESM to the identified wells to facilitate the thawing with a Gilson-P200; do not flush away the cells from the bottom.
5. Suck off ESM/freezing media of the identified wells without touching the bottom of the well.
6. Wash away the freezing medium 2× with ESM (*see* **Note 25**).
7. After second wash aspirate medium as dry as possible without loosing the cells, then add 200 μl ESM, and incubate overnight.
8. Prepare 1× 96-well feeder plate for the following day and (depending on the number of positive clones) 2 or more 24-well feeder plates.
9. Next day: Trypsinize identified thawed clones into a new 96-well plate with feeder and change medium the next day; from there expand the clones into 24-well, etc.
10. Change medium of the 96-well feeder plate.

11. Label the well according to how many clones will be transferred and add ~140 μl ESM to these wells.
12. Aspirate medium of the thawed, positive clone, then add 50–70 μl trypsin, and incubate for 2–3 min.
13. Break up the colonies by pipetting and transfer to the correspondingly labeled well of the new 96-well plate (*see* **step 11**) (*see* **Note 26**).
14. Next day: Change medium.
15. Next day: If
    (a) The clones grow slow, or there is just one colony, trypsinize again and transfer to a new 96-well (new row on the same plate).
    (b) The clones grow well, transfer to 1 (or 2 wells) on a 24-well plate (with feeder).
    If they are split in 2× 24-well, one can be frozen down as backup.
16. From 24-well, expand to 6-well and then to 10 cm dish (all with feeder) (*see* **Note 27**).
17. Freeze the 10 cm dish into 4–6 vials for storage until a microinjection slot is available.
18. Ideally send some cells for karyotyping prior to microinjection.

3.2.14 Freezing Down Clones from 24 (or 6)-Well Plates (or 10 cm Dish)

1. A 70–80 % confluent 6-well can be frozen down in 1–2 tubes, a confluent 24-well in 1 tube, and a confluent 10 cm dish in 4–6 tubes.
2. Prepare fresh freezing medium.
3. Wash 24-well with PBS, and then add ~300 μl trypsin for 3 min.
4. Transfer into 15 ml falcon tube and spin for 3 min at 1,000 × *g*.
5. Aspirate supernatant, then resuspend in 500 μl freezing medium, and transfer to cryotube.
6. Transfer to slow freezing aid, store overnight at −80 °C, and then transfer to −150 °C.

3.3 Efficient Generation of Germline Transmitting Chimeras

As a general consideration each pseudopregnant female should receive between 10 and 16 "healthy" chimeric embryos during reimplantation; the final number can vary depending on the total number of embryos and recipient females available at a given session. Ideally, if more than one ES clone needs to be injected, enough embryos should be injected per ES clone to allow for the reimplantation of at least two recipient pseudopregnant females per clone. If necessary, all of the steps of the protocol below can be handled by a single person and generally should allow for the injection of four different ES clones per week.

3.3.1 Mating Scheme

The following mating scheme is designed for two microinjection sessions per week (on Thursday and Friday): We prefer natural mating over superovulation for mES cell microinjection [20].

1. For the 2-cell to 8-cell (2C-8C) embryo production mate 15 ICR stud males (7–24 weeks) with 2 females (6–12 weeks) each from Monday to Wednesday and check plugs each following morning. Females with a vaginal plug on Tuesday will provide 8C embryos for the Thursday injection, females with a vaginal plug on Wednesday will provide 2C embryos for the Thursday injection or 8C embryos for the Friday injection, and females with a vaginal plug on Thursday will provide 2C embryos for the Friday injection. If C57BL/6J is used as a host strain, the number of mating pairs should be at least doubled to guarantee the availability of enough embryos.
2. As recipient pseudopregnant females we recommend using F1(CBA/B6) females between 6 and 12 weeks of age; they have proven themselves as excellent mothers and easy to manipulate during oviduct transfer; as males we generally use vasectomized ICR. On Wednesday mate 15 vasectomized males each with two F1(CBA/B6) females for the reimplantation on Thursday; on Thursday mate 15 vasectomized males with two F1(CBA/B6) females for the reimplantation on Friday. If insufficient pseudopregnant females are available on the day of the reimplantation, the embryos can be cultured overnight and reimplanted the following morning. Plugged females are caged as groups of five until they are needed for the injection/reimplantation procedure. Unused pseudopregnant females should be sequestered for 10 days before they are re-mated.

3.3.2 Preparations for Embryo Harvest

1. On either morning of the microinjection day 2 ml of M16 medium is filtered through a 0.2 μm syringe filter into a sterile Eppendorf tube.
2. To avoid an accidental mix-up of ES clones one 35 mm Easy-Grip tissue culture dish is prepared for each targeting construct as follows: The plate is labeled with the clone name, and then a thin line is drawn with a marker at the outside bottom of the dish separating the dish into an “uninjected” and “injected” halves. Two microdrops of ~30 μl filtered M16 medium are placed on the uninjected half and three similar microdrops on the injected half of the dish. This asymmetric orientation is not necessary but helpful to prevent a mix-up of injected and uninjected embryos. The microdrops are gently overlaid with mineral oil to prevent evaporation; the dish is then placed into a humidified incubator with 5 % CO_2 and maintained at 37 °C until needed (*see* **Note 28**).
3. Use a 0.2 μm syringe filter to filter approximately 2 ml M2 medium into the bottom of one 35 mm Easy-Grip tissue culture dish for the collection of the oviducts.

4. Depending on the number of oviducts harvested and the skills in flushing filter another 2–5 ml of M2 medium and set aside at RT.
5. Place the lid of a 10 cm non-surface-treated sterile petri dish upside down under a stereomicroscope.
6. Cut the needle of a Hamilton syringe to a length of 1.5–2 cm before removing the filament. Remove the filament and gently bend the needle 0.5 cm from the end at an 80–90° angle. If well maintained this needle can be used for a long time.

3.3.3 Harvesting 2-8C Embryos

1. On either morning of the microinjection day the designated plugged ICR females are euthanized according to IACUC-approved responsible ethical animal research guidelines.
2. Oviducts of all ICR females are dissected free of ovaries, uterus, and fat using small blunt-ended, curved, and serrated forceps and small scissors and collected in the dish with filtered M2 media.
3. Each oviduct is transferred to the upside-down 10 cm dish lid using fine Dumont #5 forceps. With practice all oviducts can be aligned on the dish to allow for a more time-efficient flushing.
4. A 1 ml syringe is filled with filtered M2 medium and the prepared needle from a Hamilton syringe is firmly attached.
5. Under a stereomicroscope, the infundibulum of each oviduct is located using Dumont #5 forceps and the tip of the Hamilton needle.
6. The needle tip is gently inserted into the infundibulum and held in place by gently squeezing the Dumont #5 forceps against the wall of the oviduct.
7. A gentle flush of approximately 0.1 ml of the filtered M2 medium is generally sufficient to retrieve the 2C-8C-stage embryos from the oviduct. The flushed oviduct is discarded and the procedure is repeated until all oviducts are flushed.
8. Embryos from each pool are aspirated with a fine fire-polished glass microcapillary, washed through several microdrops of filtered M2 medium, counted, and assessed for suitability. Only healthy-looking embryos with an intact zona pellucida will be used for microinjection. Often a mixture of 4C-stage embryos to early blastocysts can be recovered from E2.5 pregnant females. As long as the embryos appear healthy all stages are equally suitable for microinjection.
9. The selected embryos will be washed in the first microdrop of M16 medium on the "uninjected" half of the previously prepared dish, then transferred to the second microdrop, and stored in the CO_2 incubator until the time of injection.

3.3.4 Preparing ES Cells for Microinjection

It cannot be emphasized enough that the quality of the ES cells is absolutely crucial for a successful outcome of the microinjection. Ideally, cells have been karyotyped and were never exposed to suboptimal culturing conditions. Given the cost of a microinjection procedure, ES clones should be chosen very carefully.

1. The evening before the ES cells are injected the cells are plated at a range of 3–6 different concentrations on an either gelatin- or feeder-covered 24-well plate using ESM. Prior to the injection, a morphology check will identify the most suitable well.
2. Rinse with 1 ml PBS prior to adding 60 μl of 0.05 % trypsin for no more than 3 min at 37 °C. Excessive exposure to trypsin will cause the cells to lyse during the subsequent microinjection procedure, giving rise to very sticky conditions in the injection droplet, which by all means should be avoided.
3. The trypsin is inactivated by adding 1 ml of ESM and the cells are mechanically dislodged into single cells by gently pipetting them up and down with a Gilson-P1000, transferred to a 15 ml Falcon tube containing 4 ml ESM, and spun for 3 min at 1,000 × *g*. When aspirating the supernatant care is required to avoid losing the very small cell pellet.
4. Wash the cell pellet with 5 ml PBS and spin again for 3 min at 1,000 × *g*.
5. Resuspend cells in 100–500 μl M2 medium with a Gilson-P200 and transfer to a 1.5 ml Eppendorf tube.
6. Place cells on ice until the microinjection procedure. Cells can be stored on ice for several hours and still give rise to high-percentage germline-transmitting chimeras.

3.3.5 Setting Up for ES Cell Microinjection

Microinjections are performed with a Leica inverted microscope unit with a commercially available ES cell injection needle controlled by Cell Tram Vario and a blunt-ended holding needle controlled by Cell Tram Air both from Eppendorf or any equivalent setup. Refrigeration is not required when injecting pre-blastocyst-stage embryos.

1. Capillary forces are employed to fill the tip of both needles with filtered M2 medium prior to placing them into the needle holder of the microinjection unit.
2. A 500 μl drop of filtered M2 medium is placed in the center of an upside-down lid of a 35 mm Easy-Grip tissue culture dish. The dish is gently tapped on the bench to flatten the M2 drop and then covered with mineral oil.
3. Great care is taken to place the angled tip of the holding and injection needle parallel to the bottom of the dish and parallel

to each other; this step is crucial for a smooth and time-efficient injection session.

4. The cells are removed from the ice and resuspended by gently tapping the bottom of the tube. About 10 μl from the center of the cell resuspension are transferred with a Gilson-P20 to the top half of the M2 drop.
5. Once the cells have settled to the bottom of the dish the holding and injection needle are lowered into the M2 drop near the bottom of the dish.
6. Embryos are added. If the embryos are healthy to begin with keeping them in the injection droplet for up to 1 h during the injection procedure did not seem to negatively impact on the outcome of the injection.

3.3.6 ES Cell Microinjection of 2C-8C-Stage Embryos

1. One embryo at the time is firmly aspirated by the holding pipette.
2. Up to 100 ES cells are aspirated into the injection pipette. Only healthy-looking round-shaped ES cells of medium size should be picked, if necessary using a 40× or a 63× objective.
3. Using high magnification while simultaneously focusing on the zona pellucida, the inner diameter of the holding needle, and the tip of the injection needle one embryo after the next is injected by gently pushing the needle through the zona and releasing on average ten ES cells into the open space between the zona and the blastomeres [20].
4. Care should be taken when injecting 2C-stage embryos to avoid harming the blastomeres, which appear more prone to lyse after accidental contact with the needle tip and then those of 4C-8C-stage embryos.
5. Once all embryos are injected they are washed in the first M16 drop of the "injected" side, transferred to the second drop, and placed back into the incubator until reimplantation.

3.3.7 Reimplantation of Injected Embryos via Oviduct Transfer

It is important to have a high-quality dissecting stereomicroscope with an octopus light source available. Oviduct transfer into E0.5 pseudopregnant recipients is suitable for all stages of preimplantation embryos. Ideally a second dissecting stereomicroscope is available side by side to allow for a smooth embryo transfer.

1. Prepare a fresh upside-down lid of a petri dish with two drops of filtered M2 medium per clone and one extra drop for blue beads and place it under a dissecting stereomicroscope.
2. Wash the embryos in one drop of filtered M2 medium, and then align them in the second drop alongside two blue beads for each oviduct. Avoid any mix-up of embryos from different clones. Ideally, 5–8 embryos should be reimplanted into each oviduct.

3. Recipient F1(CBA/B6) females plugged that morning by a vasectomized male are deeply anesthetized with 2.5 % avertin according to the animals' body weight.
4. Clean the bench area carefully with 70 % EtOH, then place a deeply anesthetized female sidewise on a c-fold towel on an adsorbent pad, and gently wipe down her flank with 70 % EtOH. Shaving is not necessary.
5. Make a small incision into the skin between the femur and the spine with sharp scissors. Gently dislocate the skin from the underlying body wall around the incision site using scissors.
6. Make a small cut into the abdominal wall using a fresh pair of small, sharp scissors. To do so, hold the body wall with small curved Graefe forceps with teeth.
7. Use small, serrated, curved Graefe forceps to locate the ovary and hold on to the associated fat pad.
8. Gently pull ovary, oviduct, and ~0.5 cm of the uterus through the opening of the body wall. Use a small Serrefine clip to hold it in place.
9. Transfer the female on the c-fold towel under the second dissecting stereomicroscope, adjust the octopus light source for optimal vision, and focus on the ovary/oviduct.
10. Use two straight Dumont #5 ultrafine forceps to rupture the bursa. Avoid rupturing any vessels if possible.
11. It is imperative to assure a swollen ampulla. The recipient female is not at an appropriate stage of her pregnancy if the ampulla is not swollen and should be euthanized instead.
12. To locate the infundibulum use two straight Dumont #5 ultrafine forceps and very gently look through the oviduct coil; it is generally located in proximity to the ovary. Make sure that it is positioned so that the needle can enter easily.
13. Aspirate the injected embryos from the M2 drop with a glass capillary pipette in the following order: Medium—small air bubble—small amount of medium—small air bubble—one blue bead—embryos—one blue bead.
14. Use one straight Dumont #5 ultrafine forceps to support the infundibulum, then insert the glass capillary into the infundibulum, gently blow the embryos into the oviduct until the air bubbles and blue beads can be seen in the ampulla, and then gently retract the capillary. Blow all excess medium out and gently wipe the capillary with a kimwipe or it will clog.
15. Transfer the female on the c-fold towel back to the absorbent pad and gently push the ovary/oviduct/uterus back through the opening in the body wall.

16. Use a suture needle holder and a needle with attached suture to close the body wall. One stitch is generally sufficient. Use a wound clip applicator and 1–2 wound clips to close the skin. Care should be taken not to include the body wall.
17. Repeat the procedure for the second flank.
18. Return the female to a preheated clean cage and observe regularly. Provide a water bottle and nesting material.
19. Pups will be born 19–20 days later (*see* **Notes 29–31**).

4 Notes

1. When starting with a new line it is advisable to test for their germline transmission capability prior to genetic modifications.
2. Prepare and aliquot a stock of 50 mg/ml and store at −20 °C.
3. In most countries, it is mandatory to have an IACUC protocol covering all animal-related procedures in place before embarking on this part of the project.
4. DR4 is only needed while under G418 or other antibiotic selection.
5. Feeder needs at least 5 h to attach to the plate before it can be used.
6. Wt feeder does not necessarily need ESM; it can grow with 10 % FBS in DMEM.
7. One plate should be enough if 70–80 % confluent; freeze down the rest for future use. Take note of the passage number.
8. DNA concentration should be higher than 1 μg/μl and at least a total of 25 μg.
9. The time constant should be around ~2.0–3.0 ms.
10. Procedure takes ~5 h.
11. Pick a total of 2× 96-well plates per electroporation.
12. Only pick clones with appropriate morphology (*see* Fig. 3).
13. Do not misplace the clone by blowing PBS out too forcefully.
14. Make sure that no mix-up of wells occurs.
15. Breaking up the clone thoroughly and an equal distribution between the three replica plates in **step 6**(e) are VERY IMPORTANT.
16. 10 ml is required per one 96-well plate.
17. Use up old existing feeder plates for DNA extraction first (can be 1 week old).
18. At the time of DNA extraction wells should be 70–80 % confluent to guarantee enough DNA for 2–3 rounds of Southern

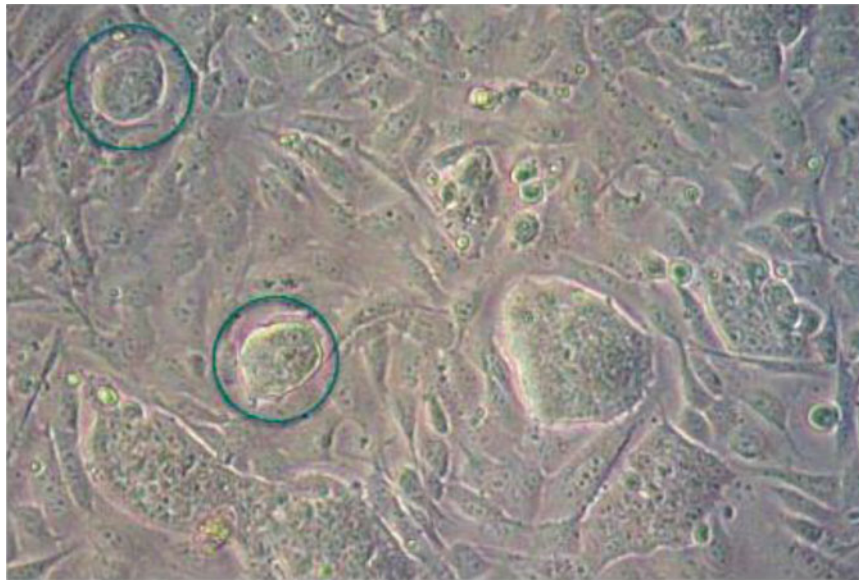

Fig. 3 Mouse ES cells (R1) in culture on wild-type feeder cells. Two morphologically healthy-appearing colonies with "sharp cliff" edges are circled in green [11, 12]

blotting (SB) while avoiding dead cells with degraded DNA (which will result in SB bands with a smear).

19. Over-dried pellets are difficult to resuspend.
20. Use PCR DIG probe synthesis kit from Roche according to instructions.
21. New probes often give background signal. To avoid background:
    (a) During the pre-hybridization, pre-warm 25 ml of Roche DIG Easy Hyb solution.
    (b) Use 25 ng probe per 1 ml Roche DIG Easy Hyb Solution.
    (c) Heat probe in 1.5 ml Eppendorf tube for 10 min at 99 °C in a thermoblock.
    (d) Quickly chill on ice and add to pre-warmed Roche DIG Easy Hyb solution from (a) (probe/hyb solution).
    (e) Place excess membrane from **step 14** in Subheading 3.2.12 in bottle and add pre-warmed probe/hyb solution from (d).
    (f) Incubate alongside the pre-hybridizing membranes

    or if a probe/hyb solution is reused heat for 10–20 min at 68 °C before the end of the pre-hybridization time.

22. Probe/hyb solution can be reused and stored at −20 °C.
23. Increasing temperature or lowering SSC concentration increases the stringency.
24. For 200 ml 1× BS dilute 10× BS as follows: 20 ml 10× BS + 180 ml 1× MAB; use 100 ml each for **steps 22** and **23** in Subheading 3.2.12.
25. Avoid flushing too hard or the cells will come off the bottom of the well; do not touch the bottom of the well when aspirating the medium or cells get lost.
26. It can take up to 3 weeks and five trypsinizations until they recover.
27. While expanding from 24-well to 6-well, split each clone into 1× 24-well (for SB reconfirmation) and 1× 6-well.
28. It is advisable to maintain a clean incubator and to verify the CO_2 sensor frequently.
29. This procedure using high-quality ES cells will routinely give rise to high-percentage germline-transmitting chimeras, generally 100 % chimerism by coat color [20].
30. If the microinjection does not provide this desired result, one should improve the skills of the reimplantation technique, practicing with uninjected embryos first or questioning the quality of the ES cell clone.
31. If the ES cells are of low quality or aberrant several scenarios are possible:
    (a) Firstly, the cells can contribute to the animal, but the animal will be abnormal and often cannibalized right after birth by the mother. In this case only low-percentage chimeras and litters of small size will be detected.
    (b) Secondly, the cells cannot readily contribute to an animal; in this case only low-percentage chimeras will be found (if any) yet the litter size will roughly reflect the number of reimplanted embryos assuming proficient skills at reimplanting.

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Chapter 4

Generation of an Allelic Series of Knock-In Mice Using Recombinase-Mediated Cassette Exchange (RMCE)

Anton J.M. Roebroek and Bart Van Gool

Abstract

Molecular genetic strategies applying embryonic stem cell (ES cell) technologies to study the function of a gene in mice or to generate a mouse model for a human disease are continuously under development. Next to (conditional) inactivation of genes the application and importance of approaches to generate knock-in mutations are increasing. In this chapter the principle and application of recombinase-mediated cassette exchange (RMCE) are discussed as being a new emerging knock-in strategy, which enables easy generation of a series of different knock-in mutations within one gene. An RMCE protocol, which was used to generate a series of different knock-in mutations in the *Lrp1* gene of ES cells, is described in detail as an example of how RMCE can be used to generate highly efficiently an allelic series of differently modified ES cell clones from a parental modified ES cell clone. Subsequently the differently modified ES cell clones can be used to generate an allelic series of mutant knock-in mice.

Key words Knock-in, Mutation, Recombinase-mediated cassette exchange, RMCE, Site-specific recombinase, FRT, Flp, LoxP, Cre

1 Introduction

1.1 Knock-Ins

Molecular genetic strategies applying ES cell technologies to study gene function in mice are until today predominantly directed towards (conditional) inactivation of a gene. Indeed inactivation of a gene is a valuable strategy to unravel the function of a gene via analysis of a loss-of-function mutant or to generate mouse models for inherited diseases caused by deficiencies. However, targeted manipulation via homologous recombination in ES cells, which is the basis of targeted gene inactivation or knock-out, can also be used to introduce tailor-made modifications of a particular gene. For example, the protein-coding region of a gene can be replaced, e.g., by sequences encoding a reporter protein or encoding a homologous protein of a different species. Also smaller modifications like introduction of small tags and point mutations can be introduced into the protein-coding region of a gene. Such tailor-made modifications allow more subtle analysis

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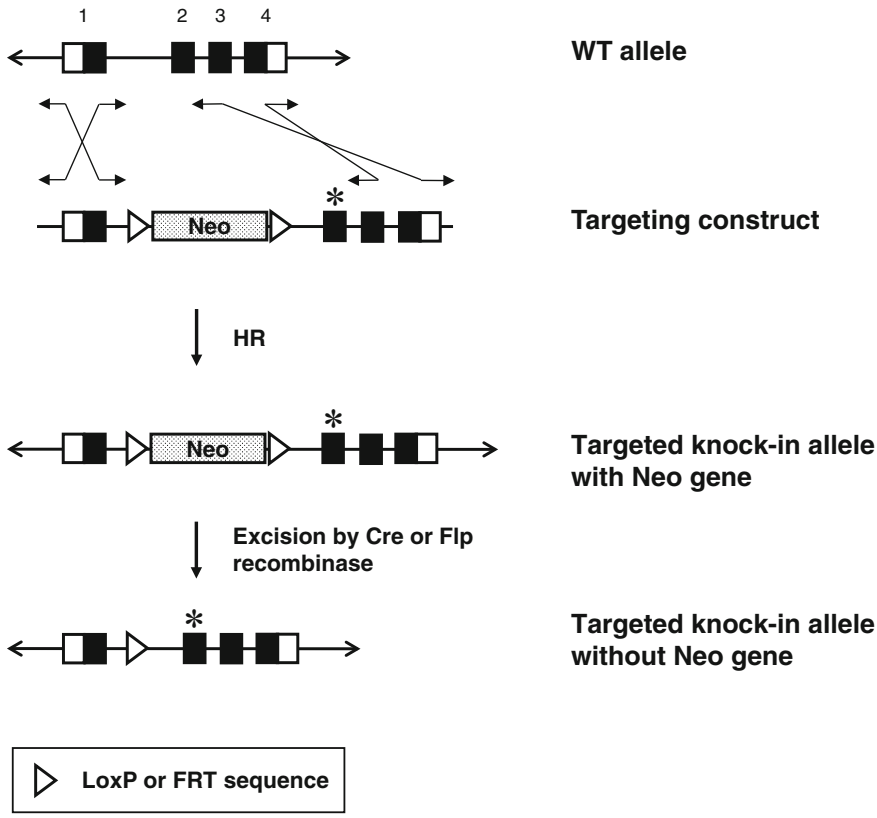

Fig. 1 Classical knock-in strategy introducing a point mutation. Schematic representation of a gene, existing out of 4 exons (*black boxes* represent the protein-coding sequences in exons 1–4), which should be targeted with a point mutation in exon 2 (e.g., modification of an encoded amino acid residue indicated by *) and a Neo gene flanked by two LoxP or two FRT sequences (indicated by *open triangles*) present within intron 1 sequences. After homologous recombination (HR) in the flanking sequences (indicated by *crossed double arrows*), the targeted gene contains both the Neo gene and the modified exon 2. After removal of the Neo gene by Cre or Flp in vitro in ES cells or in vivo in the mouse, the resulting targeted gene encodes the desired modification. In intron 1 only a small LoxP or FRT sequence is left behind, which is not expected to interfere with the expression of the targeted gene

of the function of the gene. In principle, the strategy and the methods applied to generate a knock-in are not very different from the ones to be followed to generate a targeted locus allowing conditional inactivation using the LoxP/Cre and/or FRT/Flp systems. An example of such a straightforward approach to generate a knock-in allele carrying a point mutation is depicted in Fig. 1. Besides the desired point mutation, the targeting construct should contain centrally a selectable marker gene for positive selection. Since the presence of such marker gene within a gene could interfere with the expression of the modified gene, the selection marker gene should be positioned between two LoxP or two FRT recombination target sequences allowing its removal either already in vitro in the ES cells themselves

or later on in vivo by the activity of Cre or FLP site-specific recombinases. Please note the similarity of this aspect with conditional knock-outs, where the selection marker gene also needs to be removed from the modified allele to avoid possible interference. Upon excision of the marker gene only a small insertion of a single LoxP or FRT sequence remains behind, usually at a position in an intron where it is expected not to interfere with the expression of the targeted gene carrying the point mutation.

Successful application of a knock-in approach is likely to unravel new insights into the function of a particular gene. Novel answers, however, usually result in additional questions, which might require the introduction of additional modifications in the same gene. To obtain a series of allelic modifications of the same gene, the knock-in targeting procedure should be carried out repeatedly using targeting constructs with different modifications. Thus, the laborious and time-consuming procedures for targeting a construct by homologous recombination into the gene in ES cells and subsequent isolation and characterization of many clones in order to identify correct ones need to be performed repeatedly. A knock-in strategy using recombinase-mediated cassette exchange (RMCE) might then be a suitable, time-saving alternative for repeated application of classical knock-in strategies. RMCE refers to a highly efficient exchange of an initial cassette—introduced by homologous recombination into the gene of the so-called parental ES cell clone—by other cassettes encoding different modifications of the gene. The advantage of RMCE is that the parental ES cell clone can be used repeatedly in the highly efficient RMCE exchange procedure as is explained in detail below.

1.2 Principle of RMCE

Recombinase-mediated cassette exchange (RMCE) methods were initially designed as elegant strategies using site-specific recombinases (ΦC31 and its recombination target sequences attB and attP; Cre and recombination target sequence LoxP; Flp and recombination target sequence FRT) to knock-in sequences at preferred targeted loci avoiding undesired co-integration of prokaryotic plasmid backbone sequences (reviewed by [1–4]). These RMCE methods are based upon the replacement of cassettes flanked by two non-interacting or heterotypic recombination target sites, which cannot be recombined with each other by the corresponding recombinase. The different systems can in principle be used for similar applications. In this chapter about the application of RMCE to generate knock-ins, the focus is upon the use of the Flp recombinase in combination with heterotypic FRT sequences as was worked out initially by Bode and colleagues [5–7]. For Flp, several mutant FRT recombination target sequences have been developed by introducing nucleotide substitutions in the 8 bp spacer sequence in between inverted 13 bp repeats that make up these recombination target sequences. It should be noted that the 48 bp sequence

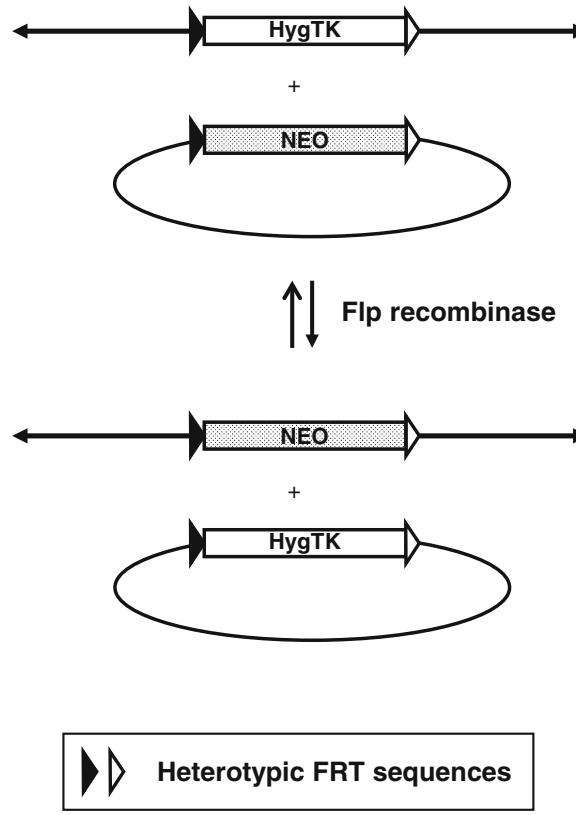

Fig. 2 Principle of recombinase-mediated cassette exchange (RMCE). By the activity of transiently expressed Flp recombinase a HygTK gene cassette flanked by heterotypic FRT sequences present in a genomic sequence is exchanged in a double-recombination event for a Neo gene cassette flanked by the same heterotypic recombination target sequences present in a circular plasmid. In principle, the exchange is bidirectional and is expected not to have a preferred direction. However, after selection against the presence of HygTK by ganciclovir and/or selection for the presence of Neo by G418 only cells resulting from the intended exchange will survive

of FRT contains two copies of a 13 bp (inverted) repeat, intervened by an extra nucleotide at one side. These mutant heterotypic FRT recombination target sequences recombine only with an identical target sequence, whereas recombination between two different heterotypic recombination target sequences does not happen. The principle of RMCE using heterotypic LoxP or FRT sequences is depicted in Fig. 2. A double recombination between identical heterotypic recombination target sequences results in exchange of cassettes carrying different selection maker genes, one (HygTK) present in the genome, and the other (Neo) on a circular plasmid. In principle, the exchange is bidirectional and is expected not to have a preferred direction. However, because the exchange is

occurring in vitro in, e.g., ES cells by transient expression of the Flp recombinase, selection for (G418 for the presence of Neo) or against the presence of a selection marker (ganciclovir against the presence of HygTK) can be applied to obtain the desired outcome. Only cells resulting from the desired exchange will survive selection, as ultimately cell division will result in dilution and final disappearance of the circular DNA molecules together with the plasmid encoding the transiently expressed Flp recombinase.

1.3 Potential Applications of RMCE

Potential applications of RMCE for the generation of several different knock-in alleles of a putative gene are depicted schematically in Fig. 3. The initial step, preceding the actual application of RMCE for the generation of knock-in mice, is the introduction by homologous recombination of an exchangeable cassette in ES cells replacing parts of the gene to be targeted. The exchangeable cassette contains a HygTK selection marker gene, which allows positive selection by hygromycin B in the initial targeting step and negative selection by ganciclovir in the RMCE step later on. After targeting of the cassette flanked by heterotypic FRT sequences this generated parental ES cell line can subsequently be used repeatedly to generate different knock-in alleles of the gene by an exchange of the HygTK cassette for different sequences. In the targeting construct, the 5′-end of the HygTK cassette flanked with the heterotypic FRT sequences is preferably introduced in an intron, whereas its 3′-end should be positioned in a more downstream intron or even downstream of the last exon replacing an internal or a 3′-end part of the gene, respectively. The FRT sites, which remain present in the locus after the exchange, should not be present in the protein-coding region. Furthermore, in the next step, the actual RMCE step, the exchange plasmid must contain the appropriate knock-in sequences and preferably an excisable selection maker gene flanked by LoxP sequences. In this RMCE step, the parental ES cell line is electroporated with a mixture of a plasmid for transient expression of Flp and the exchange plasmid. Next, selection should be applied in order to obtain the desired ES cell clones derived from the correct exchange. A theoretical example for application of RMCE as depicted in Fig. 3 shows a gene with 5 exons. Exon 2 (encoding the initiation codon) to the last exon (encoding the stop codon and polyadenylation site) are replaced via homologous recombination by the HygTK cassette flanked by the heterotypic FRT sequences (Fig. 3a). In the next RMCE step, this cassette can be replaced by knock-in sequences encoding a point mutation in exon 5, resulting in functional restoration of the gene, but modified (Fig. 3b). Alternatively, sequences encoding a specific splice variant ablating a particular exon (e.g., exon 4), sequences encoding a fusion protein in which protein domains of the gene are replaced by protein domains of another gene, or sequences encoding

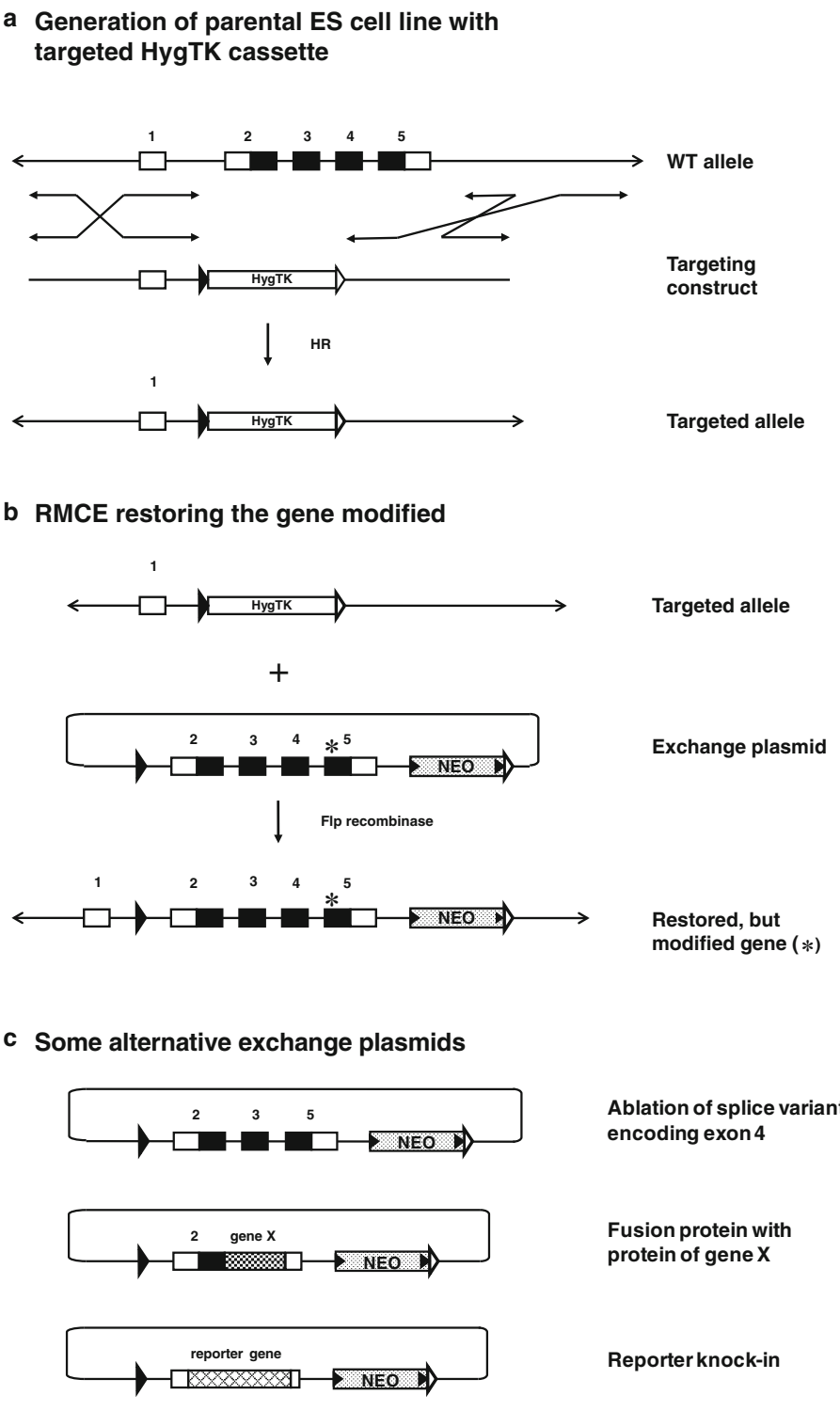

Fig. 3 Examples of potential applications of RMCE. Application of RMCE for the generation of different knock-ins is schematically depicted here for a theoretical gene existing out of five exons (*black boxes* represent the protein-coding sequences in exons 2–5). (**a**) Generation of a parental ES cell line suitable for RMCE via homologous recombination (HR) between the gene and a targeting construct. In the targeting construct part of the

a completely different protein-coding sequence (e.g., GFP reporter gene) could be knocked in by RMCE (Fig. 3c). Unless the excisable selection marker gene is positioned downstream of the gene, it will be necessary to remove it in order to avoid interference with the expression of the modified gene itself. In principle, the targeted gene could also be restored by wild-type sequences with extra LoxP sequences enabling conditional inactivation later on. Of course it is obvious that the size and the complexity of the gene of interest define the nature of the initial targeting construct and exchange plasmids. Small genes could be removed completely and eventually restored with modified versions. In case of large genes, it might be necessary to replace only a part of the gene and to focus on modifications of that particular part of the gene (e.g., particular protein domains encoding signaling motifs, a catalytic domain, etc.). Application of this RMCE method, however, should only be considered if multiple modifications are envisaged, because it always requires two consecutive steps of ES cell modification. On the other hand, a parental ES cell line, in which essential parts of the gene are replaced by the HygTK cassette, is usually suitable for the generation of a classical germ-line knock-out, leaving open the possibility for future modifications by application of RMCE.

In the RMCE examples in Fig. 3, the focus is on the modification of the protein-coding region. However, an RMCE-based knock-in approach can also be used to introduce multiple modifications in regulatory elements like enhancers and promoters.

1.4 Successful Application of RMCE in the Lrp1 Gene

Our lab successfully applied RMCE using Flp/FRT in ES cells in order to generate *Lrp1* knock-in mice [8, 9]. The objective was to generate knock-in mice with inactivating mutations in protein domains/signaling motifs of the lipoprotein receptor LRP1, a multifunctional endocytic receptor. Because of the large size of this gene (about 100 kbp, 89 exons) the RMCE knock-in strategy was designed to target only the 3′ end exons (exon 76–89), encoding the domains/motifs of interest, as schematically depicted in Fig. 4. To reduce the complexity of plasmid construction a combination of genomic

Fig. 3 (continued) gene is replaced by the HygTK cassette flanked by heterotypic FRT sequences (*large open* and *black triangles*). (**b**) Reintroduction of the initially replaced sequences by RMCE restoring the gene, which encodes now a modification in exon 5 (e.g., insertion, deletion, or modification of an amino acid residue or a protein domain indicated by *). A Neo selection marker gene (flanked by LoxP sequences for removal later on, *small black triangles*) precedes the 3′-end FRT sequence in the exchange plasmid to allow positive selection for cells with the intended exchange. (**c**) A limited number of alternative exchange plasmids are shown as illustration for the usefulness of the parental ES cell line for the generation of an allelic series of ES cells with different modifications. Adapted from Roebroek et al. (2003), Copyright © Humana Press [Methods in Molecular Biology, 209, 2003 (Transgenic mouse: Methods and Protocols, chapter 10, pg. 197, Fig. 3)]

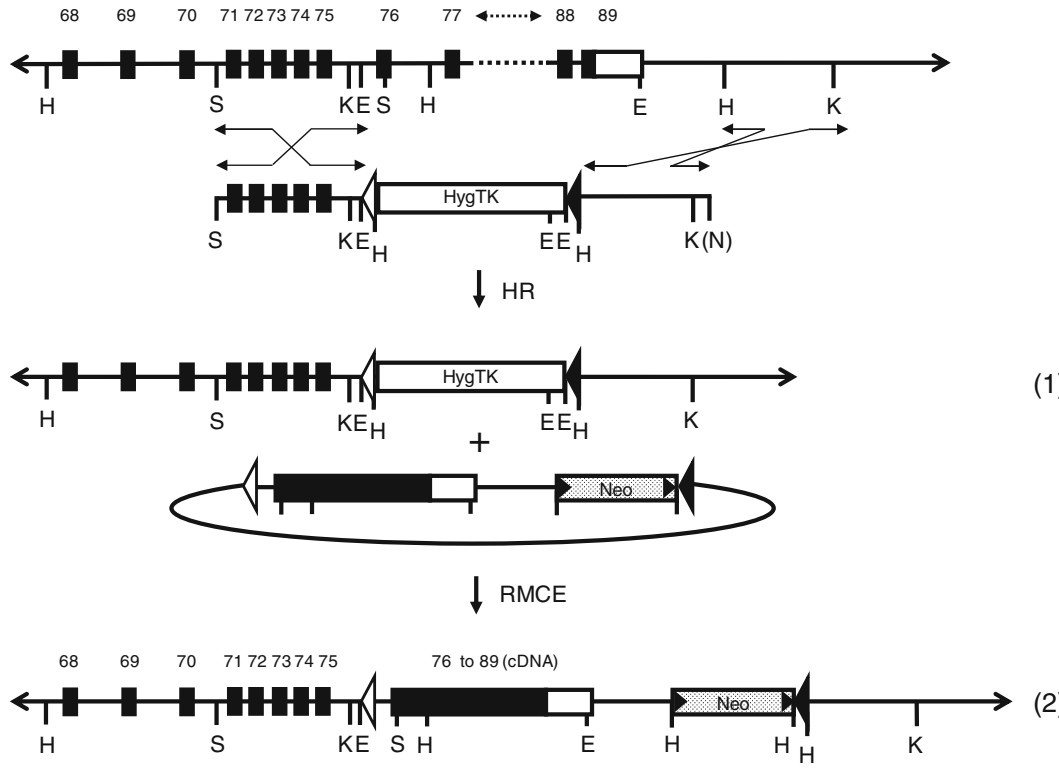

Fig. 4 Application of RMCE in the *Lrp1* gene. By homologous recombination (HR) the 3′-end part of the *Lrp1* gene (3′-part intron 75 to sequences downstream of exon 89) is replaced by an exchangeable cassette encoding a HygTK selection marker gene and flanked by heterotypic FRT sequences (*large open* and *black triangles*) (1), which can subsequently be replaced via RMCE with initially removed *Lrp1* coding sequences (genomic and cDNA sequences) restoring the *Lrp1* gene, either wild type or mutant (2). For selection purposes, a Neo expression cassette flanked by LoxP sequences (*small black triangles*) was included in the exchange plasmid, downstream of the *Lrp1* sequences. Restriction enzyme sites: E = EcoRI, H = HindIII, K = KpnI, N = NotI, S = SacI. Adapted from Roebroek et al. [8], Copyright © American Society for Microbiology [Molecular and Cellular Biology, 26, 2006, 605–616, Fig. 1b, doi: 10.1128/MCB.26.2.605-616.2006]

and cDNA sequences—the introns 76–88 are no longer present—was used in the exchange plasmids. Reintroduction of wild-type *Lrp1* sequences resulted in a phenotypically normal mouse [8]. So, the modified *Lrp1* allele encoding wild-type LRP1 can be considered as a functional wild-type *Lrp1* allele.

This implies that the other modifications, which introduced actual mutations in the LRP1 protein by the same RMCE method, can indeed be considered as genuine mutations in *Lrp1*. Inactivation of a proximal NPXY motif (NPTY→ AATA, encoded by exon 88) in the intracellular domain of LRP1 resulted in perinatal death due to late fetal liver destruction. Quite the reverse, inactivation of a distal NPXYXXL motif (NPVYATL → AAVAATL, encoded by exon 89) showed initially no phenotype. Finally, inactivation of a cleavage site, which is essential for a mat-

uration step by the endoprotease furin (RHRR → AHAA, encoded by exon 76), revealed only a mild liver phenotype [8]. Comparative analyses of a proximal NPXY and distal NPXYXXL double-mutant mouse showed an earlier lethal phenotype compared to the proximal NPXY mutant. Subsequent analyses of derived mouse embryonic fibroblast (MEF) cell lines demonstrated that inactivation of the proximal NPXY motif impairs early steps in the biosynthesis of mature LRP1, which can only be partly rescued by the distal NPXYXXL motif [9]. Further characterization of the distal NPXYXXL mutant in the background of an LDLR-deficient mouse model for atherosclerosis revealed enhanced postprandial dyslipidemia and atherogenesis pointing towards an atheroprotective role of this motif [10]. In the ApoE-deficient background, another mouse model for atherosclerosis, the NPXYXXL mutant, revealed impaired LRP1 translocation to the plasma membrane and LDLR upregulation correlating with improved dyslipidemia and atherosclerosis [11]. Finally, primary cultures of neuronal cells and mouse brain capillary endothelial cells derived from the NPXYXXL mutant were successfully used to study the involvement of LRP1 in the activation of *N*-methyl-D-ASPARTATE receptors and bidirectional transcytosis of Abeta across the blood-brain barrier [12, 13].

The protocol presented here in the next part of this chapter refers especially to the RMCE step in the procedure as was applied for the generation of several different *Lrp1* knock-in mice [8, 9], as explained in detail above in the text and Fig. 4. For culture of ES cells during application of RMCE in ES cells, most of the general guidelines and protocols used for culture of ES cells during targeting of ES cells by homologous recombination apply also for this special application. Of course, composition of media, culture conditions, use of feeder cells, etc. depend on the ES cells to be used.

2 Materials

2.1 Plasmids

1. The plasmid for targeting the *Lrp1* gene in ES cells by homologous recombination and the exchange plasmids used in the RMCE procedure are described in detail in [8] and schematically depicted in Fig. 4. The HygTK cassette, which was incorporated into the plasmid for targeting of *Lrp1*, was derived from a plasmid described in [6]. This cassette allows positive selection by hygromycin B during the initial targeting step and negative selection by ganciclovir during the RMCE step. The different exchange plasmids contained a Neo resistance gene for positive selection by G418.

2. For transient expression of the Flp recombinase, the Flp expression plasmid pCAGGS-FLPeIRESpuro was used [14].

2.2 ES Cells and Cell Culture

1. For RMCE in *Lrp1* the ES cell line E14 was used [15].
2. The E14 ES cells were grown on Mitomycin C-treated feeder cells.
3. The ES cell culture medium, Glasgow MEM supplemented with nonessential amino acids, sodium pyruvate, glutamine, and 2-mercaptoethanol, contained finally 20 % FCS (fetal calf serum) and 30 % BRL (buffalo rat liver) cell conditioned medium to supply for LIF (leucocyte inhibitor factor).
4. Electroporator (Gene Pulser® electroporation system, Bio-Rad).

3 Methods

The first step to target the exchangeable cassette into the *Lrp1* gene by homologous recombination to generate the parental ES cell line suitable for future application of RMCE is not worked out in detail here, because this is beyond the scope of this chapter. Thus, the procedure described in detail here is limited to the RMCE itself. Information concerning general guidelines and protocols to target and analyze ES cells can be found elsewhere in this volume. It should be noted, however, that this initial targeting step of the *Lrp1* gene was in fact quite inefficient: only 1 out of 300 isolated hygromycin B-resistant clones resulted from proper homologous recombination. The next RMCE step, however, was quite effective, since efficiencies of this exchange step varied from 38 to 61 % for the different exchange plasmids upon G418 selection [8]. This difference in efficiency illustrates the power of application of RMCE in order to generate a series of knock-in mutants compared to classical knock-in approaches starting all over again from the start with targeting for homologous recombination.

The experimental procedure for RMCE in the *Lrp1* gene in E14 ES cells is given below in detail (*see* **Notes 1–3**).

1. Before electroporation, mix 133 μg of pCAGGS-FLPeIRES puro, the Flp vector used for transient Flp expression, together with 40 μg exchange plasmid DNA (both supercoiled circular DNA) in a total volume of 40 μl sterile TE. These large amounts of DNA are similar to the amounts used by Seibler and colleagues in their initial RMCE experiments in ES cells [6].
2. Gently mix 450 μl DMEM medium containing 10 % FCS and a suspension of 5×10^6 parental ES cells, heterozygous for the exchangeable cassette introduced previously by homologous

recombination (# of ES cells = # of total cells – # of feeder cells), with 30 µl of the DNA sample (100 µg Flp expression vector plus 30 µg exchange plasmid) using a pipette.

3. Transfer the mixture to an electroporation cuvette and pulse with an electroporator set to 200 V and 960 µF. These conditions are identical to the conditions used previously to introduce the exchangeable cassette by homologous recombination in the *Lrp1* gene of the E14 ES cells.
4. Seed the entire electroporated cell suspension in a 75 cm^2 culture flask on a feeder layer (3×10^6 mitomycin C-treated feeder cells) in ES cell culture medium.
5. Culture the cells for about 36–48 h (depending on the density and growth of the ES cells) without selection pressure to let the recombination events happen.
6. Due to the transient expression of the Flp recombinase, likely mixed clones are formed, because the bidirectional exchange can of course occur after cell division. To reduce the chance on selecting such mixed clones, trypsinize the ES cells and seed them at a very low density of 2.5×10^5 ES cells per 25 cm^2 culture flask on a feeder layer.
7. After a next 12- to 24-h incubation period, replace the ES cell culture medium by culture medium containing 400 µg/ml G418 to start positive selection for clones resulting from the desired exchange. From this moment on, the ES cells are cultured under continuous selection pressure in the next steps (*see* **Note 1**).
8. After 8–10 days, pick individual G418-resistant clones (about 5–25 surviving clones per flask) and seed them after trypsinization in a 24-well plate on a feeder layer.
9. After sufficient growth of the ES cell clones, pass the clones to two duplicate 24-well plates, one plate with and the other without feeder cells.
10. After sufficient growth of the ES cell clones, freeze down the ES cell clones in the plate with feeder cells for later propagation; use the ES cells in the other plate without feeder cells for DNA analysis.
11. For DNA analysis, use four PCR primer pairs specific for amplification of sequences across the two heterotypic FRT sites before and after the RMCE exchange of cassettes. Additional Southern blot analyses of correct clones can be used to check the outcome of the RMCE exchange in further detail.
12. ES cell clones, which have undergone the desired exchange, can subsequently be used to generate chimeric mice.

4 Notes

1. Potential use of a combination of negative and positive selection: Ganciclovir negative selection (to select against the HygTK gene in the parental ES cell line) alone was never tested during RMCE application in E14 ES cells. In a pilot experiment, however, a combination of G418-positive selection (to select for the Neo gene present after exchange) and ganciclovir-negative selection was applied in the RMCE procedure resulting in 100 % efficiency. Nevertheless, the surviving E14 ES cell clones grew poorly under these conditions. G418-positive selection alone yielded an efficiency of about 50 % without a negative impact on the growth of the resistant ES cells. Subsequently, the double selection was in the actual follow-up experiments no longer applied in order to avoid a potential negative impact on the capacity of the manipulated ES cell lines to go germ line. The high efficiency of G418 selection alone proved to be sufficient to obtain a high yield of desired clones. Several different experiments, in which RMCE was applied, resulted in an overall efficiency between 38 and 61 % of correctly exchanged, selection-surviving ES cell clones, which were subsequently used to generate knock-in mice. If negative selection is not considered in the RMCE procedure, the initial targeting construct might of course carry only a HygB selection marker gene or another selection marker gene, e.g., Puro (puromycin), instead of HygTK.
2. Size of the insert in the exchange plasmid: It should be noted that the RMCE procedure worked equally efficient for another locus than *Lrp1* in these E14 ES cells. Exchange plasmids with inserts up to 12 kbp including a Neo gene between the heterotypic FRT sites could be used efficiently to replace in the parental ES cell line an initial 2 kbp cassette with a HygB selection marker gene between the heterotypic FRT sites (Roebroek et al., unpublished data). This indicates that this RMCE method can be used to insert large fragments into a locus replacing a small initial cassette.
3. Application of RMCE in MEFs: RMCE could also be applied in immortalized MEFs with equal efficiency. These MEFs were derived from homozygous embryos carrying the initial exchangeable HygTK cassette present in the parental ES cell line. The above-described protocol was applied unchanged except that MEFs instead of ES cells, normal DMEM/F12 medium containing 10 % FCS, and no feeders were used. The objective of this application of RMCE in MEFs was to obtain additional homozygous *Lrp1* knock-in MEF cell lines by two consecutive rounds of RMCE replacing both HygTK cassettes by two different cassettes encoding an identical knock-in

mutation, but carrying different selection marker genes. Although two consecutive rounds of RMCE could be performed successfully with high efficiency, the procedure to generate additional knock-in MEFs was no further applied, because the final outcome was nevertheless disappointing due to the apparent tetraploidy of the MEFs, which was obviously introduced during the immortalization process in the course of their generation. This implied the need of four subsequent rounds of RMCE to obtain the desired additional "homozygous" knock-in mutant MEFs, a way which was chosen not to be pursued any further (Roebroek et al., unpublished data).

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Chapter 5

Generating Chimeric Mice from Embryonic Stem Cells via Vial Coculturing or Hypertonic Microinjection

Kun-Hsiung Lee

Abstract

The generation of a fertile embryonic stem cell (ESC)-derived or F0 (100 % coat color chimerism) mice is the final criterion in proving that the ESC is truly pluripotent. Many methods have been developed to produce chimeric mice. To date, the most popular methods for generating chimeric embryos is well sandwich aggregation between zona pellucida (ZP) removed (denuded) 2.5-day post-coitum (dpc) embryos and ESC clumps, or direct microinjection of ESCs into the cavity (blastocoel) of 3.5-dpc blastocysts. However, due to systemic limitations and the disadvantages of conventional microinjection, aggregation, and coculturing, two novel methods (vial coculturing and hypertonic microinjection) were developed in recent years at my laboratory.

Coculturing 2.5-dpc denuded embryos with ESCs in 1.7-mL vials for ~3 h generates chimeras that have significantly high levels of chimerism (including 100 % coat color chimerism) and germline transmission. This method has significantly fewer instrumental and technological limitations than existing methods, and is an efficient, simple, inexpensive, and reproducible method for "mass production" of chimeric embryos. For laboratories without a microinjection system, this is the method of choice for generating chimeric embryos. Microinjecting ESCs into a subzonal space of 2.5-dpc embryos can generate germline-transmitted chimeras including 100 % coat color chimerism. However, this method is adopted rarely due to the very small and tight space between ZP and blastomeres. Using a laser pulse or Piezo-driven instrument/device to help introduce ESCs into the subzonal space of 2.5-dpc embryos demonstrates the superior efficiency in generating ESC-derived (F0) chimeras. Unfortunately, due to the need for an expensive instrument/device and extra fine skill, not many studies have used either method. Recently, ESCs injected into the large subzonal space of 2.5-dpc embryos in an injection medium containing 0.2–0.3 M sucrose very efficiently generated viable, healthy, and fertile chimeric mice with 100 % coat color chimerism.

Both vial coculture and hypertonic microinjection methods are useful and effective alternatives for producing germline chimeric or F0 mice efficiently and reliably. Furthermore, both novel methods are also good for induced pluripotent stem cells (iPSCs) to generate chimeric embryos.

Key words Chimeric mouse, ES cell, iPS cell, 2.5-dpc embryo, Tetraploid embryo, Vial coculturing, Hypertonic microinjection

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1 Introduction

A chimera is an animal that has at least two populations of genetically distinct cells that originated in different embryos, fetuses, or individuals of the same or different species [1]. Authentic embryonic stem, ES, cells (ESCs) are defined by three cardinal properties: unlimited symmetrical self-renewal in vitro, comprehensive contribution to primary chimeras, and generation of functional gametes for genome transmission [2]. Furthermore, fertile ESC-derived mice, developed mainly from ESCs, generated via tetraploid (4n) embryos assembled with ESCs by either well sandwich aggregation or microinjection [3–8], are regarded as the most solid criterion for ESC pluripotency. Additionally, these criteria are also applied to induced pluripotent stem cells (iPSCs). Actually, iPSC-derived mice have been generated [9–12]. To generate chimeras, ESCs could be genetically modified (Mouse Genome Informatics, MGI: http://www.informatics.jax.org/; International Mouse Phenotyping Consortium, IMPC: https://www.mousephenotype.org/).

Since the first chimeric embryos were generated using zona pellucida (ZP)-removed (denuded) preimplantation embryos aggregated together [13], many other methods have been developed to produce chimeric embryos [1]. The techniques have become effected over decades [1, 5, 14–24]. To date, the most popular methods for generating chimeric embryos is well sandwich aggregation between denuded 2.5-day post-coitum (dpc) embryos and ESC clumps or direct microinjection of ESCs into the cavity (blastocoel) of 3.5-dpc blastocysts [1, 5, 20]. Single denuded embryo aggregation or coculturing with ESCs is employed rarely because the efficiency in generating chimeric mice is markedly inferior to that of microinjection and well sandwich aggregation. However, due to systemic limitations and the disadvantages and shortcomings of conventional microinjection, aggregation, and coculturing, two novel methods (vial coculturing and hypertonic microinjection) were developed in recent years at my laboratory [1, 25, 26].

The vial coculturing method, an efficient, simple, inexpensive, and reproducible method for mass production of chimeric embryos, cocultures 2.5-dpc denuded eight-cell embryos and/or compacting morulae with ESCs in 1.7-mL Eppendorf vials (micro test tubes) for ~3 h. This approach has significantly fewer instrumental and technological limitations than existing methods. The resulting chimeras have significant levels of chimerism (including 100 % coat color chimerism) and high germline transmission rates [26].

Previous studies demonstrated that microinjecting ESCs into a subzonal space of 2.5-dpc eight-cell embryos could generate 100 % coat color chimerism [27]. However, this approach is adopted rarely due to the small and tight space between the ZP

and blastomeres, such that one must be extremely careful to avoid damaging blastomeres while microinjecting. Using a laser pulse [21, 28] or Piezo-driven [29] instrument/device to make a hole in the ZP and then introducing ESCs into the subzonal space of 2.5-dpc diploid (2n) embryos have superior efficiency in generating F0 chimeras (100 % coat color chimerism), which are equivalent to ESC-derived mice [28]. However, few studies have used either method due to the extra fine skills and expensive instrument/device needed.

Recently, ESCs microinjected into the large subzonal space of 2.5-dpc embryos in a microinjection medium supplemented with 0.2–0.3 M sucrose very efficiently generated chimeric embryos with high percentages of chimerism and viable, healthy, germline-transmitted chimeric mice including 100 % coat color chimerism [1, 25, 30].

Both vial coculturing and hypertonic microinjection are useful alternative methods for producing germline chimeric or F0 mice efficiently and reliably. Therefore, this chapter uses mouse ESCs as an example to describe the conditions and techniques used to generate mouse chimeras. Furthermore, both novel methods are also good for iPSCs to generate chimeric embryos (unpublished observation).

2 Materials

The following suggested or recommended tools, devices, equipment, instruments, materials, and chemicals can be replaced by other commercially available equivalents unless stated otherwise. All media should be of embryo- or cell-culture grade. All liquid additives and supplements for media are suggested to purchase commercially.

The governing Institutional Animal Care and Use Committee should approve all treatments of mice, including husbandry, experimental treatments, sacrifice, anesthesia, surgery, and euthanasia.

2.1 Mouse

The following mouse facility and mice are needed for superovulation, natural mating, and embryo transfer to generate chimeric mice via ESCs assembled with 2.5-dpc embryos. The number of mice needed largely depends on experimental scale. However, 2n and 4n embryos can be donor embryos. Several commercial companies (http://www.criver.com, http://www.harlan.com, http://www.taconic.com) and a nonprofit organization (http://www.jax.org) provide much mouse-related information.

1. Clean conventional or specific pathogen-free (SPF) mouse facility.
2. Embryo donor female mice (≧4–6 weeks of age or matured; ICR, CD1, or inbreds) [16, 21, 23, 31]: The coat color of

donor embryos should be distinguishable from ESCs. To maintain ~100 females at any time when the mouse experiment is in session.

3. Fertile (stud) male mice (≧8–12 weeks of age until low fertility is found; ICR, CD1, or inbreds): At least eight individuals in each cage are maintained and eight adults in two cages are for replacement (*see* **Note 1**).
4. Vasectomized males (any non-inbred strain; preferably F1 hybrids): At least 8 individuals in each cage are maintained and 12 individuals are maintained in 3 or 4 cages for replacement (*see* **Note 1**).
5. Female recipients (ICR, CD1, or F1 hybrids, preferably experienced mothers 3–6 months of age): The coat color of recipients is better to distinguish from donor embryos. Maintain ~80 females at any time when the mouse experiment is in session.
6. Wild-type sexually matured female mice for mating with chimeric males to test the germline transmission: Maintain ~50 females at any time when the mouse experiment is in session.

2.2 ES Cell and Embryo Culture

For routine culturing of ESCs and embryos, the following items are essential at all times.

1. CO_2 incubator (set 36.8 °C and 5 % CO_2) and CO_2 tank.
2. 50×, 100×, and 200× (optional) phase microscope with adjustable long working distance.
3. Dissection (stereo) microscope with large transparent stage and transmitted light for recovery of embryos as well as reflected illumination for mouse embryo transfer.
4. (Bench-top) centrifuge: The 250 ± 50 × g for 3–4 min is recommended for spinning down cells.
5. Vertical laminar airflow hood: For routine cell treatments and preparation of media.
6. Pipette aid and suction unit (vacuum set: ~10 cmHg).
7. Horizontal laminar airflow hood (optional): For recovering and transferring embryos.
8. Water bath: Set ~25 °C (room temperature, RT) to warm up media or solutions. Set 37 °C for thawing frozen cells.
9. Analytical balance with 1 or 0.1 mg readability (accuracy).
10. Weight scale with 0.1 g readability.
11. pH meter.
12. Osmolality meter (optional).
13. Magnetic stirrer with heating and stir bars.
14. Vortex mixer.

15. Liquid nitrogen tank and accessories for freezing cells.
16. Sterile ultrapure water and double-distilled water (ddH_2O).
17. Fibroblast culture medium for STO, murine embryonic fibroblast (mEF), and other fibroblasts (Table 1).
18. ES cell culture medium: Serum replacement (SR) and FBS ESC media (Table 2).
19. Cell frozen medium: 5 mL DMSO in 45 mL FBS or cell culture media.
20. Cell culture dishes: 35, 60, and 100 mm.
21. 60-mm Petri dish.
22. Long- and short-tip glass Pasteur pipettes.
23. Disposable sterile plastic pipettes: 5, 10, and 25 mL.
24. Pipetman and tips: 10, 20, 100, and 1,000 μL.
25. Ca^{++}, Mg^{++}-free PBS.
26. Low protein-binding bottle top and syringe filters: 0.2 and 0.45 μm.
27. Disposable sterile syringes: 1, 5, and 10 mL.
28. The 0.1 % gelatin (Sigma) solution: 0.1 g/100 mL ddH_2O/bottle. After it is autoclaved, store at 4 °C. The gelatin solution does not need to be pre-warmed before used. Add 0.1 % gelatin solution to cell culture dish (enough to cover the dish surface) and leave the dish at RT for 10–30 min. Aspirate the gelatin solution and allow time for dishes to dry before preparing the feeder layer. The gelatin-coated dishes can be used for days.
29. Mitomycin C (Sigma) stock: 50 μg/0.1 mL Ca^{++}, Mg^{++}-free PBS, or ddH_2O/vial. It is toxic and light sensitive; wear gloves

Table 1
Fibroblast medium for STO, mEF, and other fibroblasts

Fibroblast medium[a]	Concentration	Cat. No., Gibco/Invitrogen	112 mL
D-MEM[b] (4.5 g D-glucose/L, with GlutaMAX™-I)	89.3 %	10569-010/500 mL (4 °C)	100 mL
Penicillin/streptomycin	45 U/mL pen, 45 μg/mL strep	15070-063/100 mL (5,000 U/mL pen, 5,000 μg/mL strep; –20 °C)	1 mL
Fetal bovine serum (FBS)[c]	9.8 %	Tested batches or ES cell grade (–20 °C, dark)	11 mL

[a]pH 7.2 ± 0.1 (adjusted via 1.0 N NaOH), 280 ± 10 mOsm (adjusted via H_2O). Store at 4 °C for a maximum of 4 weeks
[b]Any high-glucose D-MEM can be used. If GlutaMAX™-I is not included, the conventional glutamine should be added and supplemented with equivalent amount every 2–3 weeks
[c]It could be 5 % FBS and 5 % newborn calf serum

Table 2
Knockout serum replacement (KSR) ES cell medium (K ESC M.)

K ESC medium[a]	Concentration	Cat. No., Gibco/Invitrogen	32.00 mL
KnockOut™ (KO) D-MEM	77.19 %	10829-018/500 mL (4 °C)	24.70 mL
KnockOut™ serum replacement (KSR)[b]	20.0 %	10828-028/500 mL (–20 °C, dark)	6.40 mL
Penicillin/streptomycin[c]	15.62 U/mL pen, 15.62 μg/mL strep	15070-063/100 mL (5,000 U/ml pen, 5,000 μg/mL strep; –20 °C)	0.10 mL
GlutaMAX™-I supplement	1.75 mM	35050-061/100 mL (200 mM, –20 °C)	0.28 mL
MEM NEAA solution	1 %	11140-050/100 mL (10 mM, 100×; 4 °C)	0.32 mL
β-Mercaptoethanol[d]	0.10 mM	21985-023/50 mL (1,000×, 55 mM: 4 °C)	0.06 mL (60 μL)
CHO-LIF[e] (or rmLIF)	0.31 % (~1,000 IU/mL)	Homemade (–20 °C) (commercial product)	0.10 mL

[a]pH 7.2 ± 0.1 (adjusted via 1.0 N NaOH), 280 ± 10 mOsm (adjusted via H_2O). Store at 4 °C for a maximum of 2 weeks. Do not heat up the entire bottle of K ESC medium, as KO D-MEM and KSR do not tolerate repeated warming and cooling. Alternatively, the media can be kept at RT in advance before used because the small volume of medium in the 35- or 60-mm dish warms up very quickly in a 37 °C incubator

[b]The KSR can be entirely replaced by N2B27 (*see* **Note 4**). The KSR replaced by FBS is the FBS ESC medium (F ESC M.) (*see* **Note 3**), which is good for 3–4 weeks at 4 °C. The KSR replaced by 19 % KSR and 1 % FBS is the KF ESC M., which is good for ~3 weeks at 4 °C

[c]The dosages are ~30 % of media containing serum. This is because serum proteins tend to bind to a certain amount of the antibiotic added; without these serum proteins, the level of antibiotic may be toxic to certain cells

[d]β-Mercaptoethanol 10 μL in PBS 14.3 mL

[e]Commercially available recombinant LIF is expensive. My laboratory produces and tests routinely my own CHO cells expressed recombinant LIF [59]

and handle with cautions. Aliquot vials stored in a dark box should be wrapped in foil and kept at –20 °C. Working solution: 10 μg/mL fibroblast medium kept for 2–3 h in inactivation culture. After aspirating the mitomycin C solution, wash it with pre-warmed Ca^{++}, Mg^{++}-free PBS four times to remove any trace of mitomycin C. The 0.25 % trypsin-EDTA is used to make a single-cell suspension for feeder preparation or freezing.

30. Preparation of STO or mEF feeder layer (Table 3): Freshly prepared STO feeder could be used within 4 days, and mitotically inactivated feeder cells should be discarded after 10–14 days. For convenience, many inactivated STOs can be frozen and used to prepare the feeder layer right after being thawed.
31. TrypLE™ Express (Gibco Cat. # 12605; dissociate cell culture in FBS and FBS-free media) and/or 0.25 % trypsin-EDTA (dissociate cell culture in FBS media).

Table 3
Amount of fresh inactivated STOs or mEFs needed to prepare the feeder layer[a]

	Surface area, cm^2	No. of inactivated STO needed, $\times 10^5$	No. of inactivated mEF needed, $\times 10^5$	Minimum medium needed, mL
24-Well plate with 15-mm diameter	1.9	1	1.5	0.4
12-Well plate with 22-mm diameter	3.8	2	3	0.7
35-mm Dish	8	4.5	6.5	1.2
60-mm Dish	21	12	17	3
100-mm Dish	55	30	40	7

[a]Because of contact inhibition of growth and packing of growing fibroblast, the number of cells in the same growth area varies. Under normal conditions, a confluent 100-mm dish contains ~7×10^6 STO, which could make 4–6 60-mm dishes, 14–16 35-mm dishes, and 2 100-mm dishes or roughly 2 24-well plates. A confluent 60-mm dish contains ~2.5×10^6 STO, which could make 4–5 35-mm dishes (Table 1). To prepare a mEF feeder layer, the number of cells required is 1.3–1.5 times STO

32. 15-mL Polypropylene conical centrifuge tubes.
33. 1.7-mL Polypropylene (Eppendorf) vials with snap cap.
34. Mouth pipette/tubing assembly (mouth pipette): A mouth pipette assembly consists of an ethanol flame-pulled glass Pasteur pipette, a pipette holder, tubing, and an aspirator mouthpiece. The opening of a pipette tip can be narrowed down and sterilized by passing through a flame. Usually, the external diameter is 150–300 μm and internal diameter is 100–200 μm. For further details, *see* Nagy et al. [32] (*see* **Note 2**).
35. Acrylic racks or any device to hold or secure pipettes.
36. Dissection and surgery tools for mouse: Fine dissection scissors, watchmaker's forceps, forceps with teeth, curved forceps with fine blunt and serrated tips, tweezers, serrefine (serafine) clamps, and suture and/or wound clip applier for dissecting out the uterus horn and oviduct as well as embryo transfer.
37. 20 mM HEPES-buffered KSOM (HK): For embryos flushing and handling outside a CO_2 incubator and stay at RT. The HK can be replaced by M2 or modified PB1 medium [16].
38. Potassium simplex optimized medium contains amino acids (KSOM-AA): Embryo cultivation medium [5, 33]. The KSOM-AA can be replaced by M16 medium [16].
39. Lightweight mineral (paraffin) oil: Embryo-tested (Sigma; density: 0.84 g/mL at 25 °C), stay at RT.
40. Medium-weight mineral oil: Paraffin Liquid/Special reagent for tissue culture (Nacalai Tesque; density: 0.87 g/mL at 20 °C), stored in the dark at RT.

41. Depression glass vessel with cover glass for recovery of flushed embryos (optional).
42. Examination gloves.
43. Surgical mask.
44. 75 % (for sterilization) and 95 % (for cleaning) ethanol.
45. 95 % Alcohol lamp or Bunsen burner.
46. Paper towels, tissues, absorbent cotton, and Kimwipes.

2.3 Preparation of Media for Cells

All media could be laboratory prepared or purchased commercially. The quality of commercial media is typically more consistent and usually generates better results than laboratory-prepared media, especially when cultivating embryos. Therefore, I usually purchase commercially available KSOM-AA for culturing embryos. Unfortunately, the price of commercial media is much higher than that of laboratory-prepared media. Therefore, all media not for embryo culturing could be laboratory prepared from either powder or liquid gradients. The results would be good as that with commercial products. For instance, the ESC media and HK are always prepared in my laboratory.

When preparing media, water quality is one of the most important gradients. Prepare all media using ultrapure water (prepared by purifying deionized water to attain a resistivity of >18 MΩ cm at 25 °C, pyrogens <0.03 Eu/mL, and bacteria <1 cfu/mL; or purchase commercially available ultrapure water for embryo culturing). Low-quality water always causes the poor embryonic development and cell proliferation. Avoiding dust contamination of media and solutions is essential. Thus, using powder-free gloves or clean and 75 % ethanol sterilized bare hands is recommended.

Media for ESCs tend to be exhausted rapidly during culturing; thereafter, acidification is observed obviously. Homemade media with a pH 7.2 ± 0.1 and 280 ± 10 mOsm/kg H_2O are suitable ranges for culturing cells. For media containing FBS, variation in pH and osmolarity is less than that of SR media. Media should be stored at 4 °C for no longer than 3 weeks unless indicated otherwise. Because the half-life of proteins and small molecules in media is ~1 day at 37 °C, culturing media is usually exchanged every other day or daily when cultivating ESCs.

Conventional ESC media usually contain 15–20 % FBS or 10 % FBS plus 10 % newborn calf serum. Unfortunately, serum, a variable biological product, is expensive. Notably, FBS contains not only growth factors that support ESCs but also potential differentiation factors that induce ESC differentiation [34–37]. Therefore, testing and selecting batches of FBS to support the growth of undifferentiated ESCs is necessary [38] (*see* **Note 3**). Otherwise, ESC-grade FBS, which costs more than conventional FBS, can be used. Thereafter, chemically defined KSR [39] was

developed to use instead of FBS. However, KSR is a commercial product, and its formula remains unknown. With its well-defined chemical formula, N2B27 can replace KSR and achieve almost the same deriving and maintaining ESC results [40, 41] (*see* **Note 4**).

The following formulae for fibroblasts (Table 1) and ESCs (Table 2) have been used in my laboratory for years with good to excellent outcomes.

2.4 Preparation of Media for Embryos

The optimum osmolarity for the development of mouse two-cell embryos into blastocysts is 276 mOsm (range, 260–290 mOsm). The optimum pH is near 6.8 (range, 6.4–7.2) [42]. However, due to the evaporation of media (especially a small volume of droplets), homemade media with a pH 7.1 ± 0.1 and 275 ± 5 mOsm are suitable ranges for culturing embryos. Prepared media are filtrated via 0.2 μm for sterilization. Notably, the pH of media usually increases 0.1–0.2 after 0.2-μm filtration.

To cultivate mouse embryos, different media have been developed. To date, KSOM-AA medium is the most popular. The KSOM-AA can be purchased commercially or homemade. However, it is convenient to prepare KSOM-AA and/or HK media from homemade concentrated stock solutions (Tables 4 and 5), as described previously [43], and may freeze aliquots at −20 °C. Thaw ~2 mL KSOM-AA and 10–30 mL HK each time or weekly. The KSOM-AA can be stored at 4 °C for a maximum of 2 weeks, while HK should be used within 3 weeks.

2.5 Mouse Superovulation and Natural Mating

1. Embryo donor female.
2. Stud male.
3. Pregnant mare serum gonadotrophin (PMSG) (FSH analog): 50–100 IU/mL sterile ddH_2O. Aliquots of 0.2–1.0 mL/vial are then stored at −20 or −80 °C until used (good for ≧1 year).
4. Human chorionic gonadotrophin (hCG) (LH analog): 50–100 IU/mL sterile ddH_2O. Aliquots of 0.2–1.0 mL/vial are stored at −20 or −80 °C until used (good for ≧1 year).
5. 1-mL Syringe with 26G (gauge) needles.
6. Examination gloves.
7. Surgical mask.
8. A probe for checking vaginal plugs.
9. 75 and 95 % ethanol.
10. Paper towels, tissues, absorbent cotton, and Kimwipes.

2.6 Collection of Mouse 2.5-dpc Embryos for Generation of Chimeric Embryos

1. HK.
2. KSOM-AA.
3. KKF (KSOM-AA containing 1 % KSR and 1 % FBS; alternative chimeric embryos overnight culture medium) [26, 29]: 0.98 mL KSOM-AA + 0.01 mL KSR + 0.01 mL FBS/vial.

Table 4
Concentrated stocks[a] for preparing HEPES-buffered KSOM (HK) and KSOM-AA media

Stock A, 10× (up to 3 months at 4 °C)	mM	Component	Sigma	g/100 mL
	95.00	NaCl	S 5886	5.5530
	2.50	KCl	P 5405	0.1860
	0.35	KH_2PO_4	P 5655	0.0476
	0.20	$MgSO_4 \cdot 7H_2O$	M 7774	0.0493
	10.00	Sodium lactate	L 7900 (60 % w/w syrup)	1.87 mL
	0.20	D-Glucose	G 6152	0.036
	100 U/mL	Penicillin G	P 4687	0.060
		Streptomycin	S 1277	0.050
Stock B, 10× (up to 2 weeks at 4 °C)	mM	Component	Sigma	g/20 mL
	25.00	$NaHCO_3$	S 5761	0.420
	0.001 g/L	Phenol red · Na (optional)	P 5530	0.001
Stock C, 100× (up to 2 weeks at 4 °C)	mM	Component	Sigma	g/10 mL
	0.20	Sodium pyruvate	P 4562	0.022
Stock D, 100× (up to 3 months at 4 °C)	mM	Component	Sigma	g/10 mL
	1.71	$CaCl_2 \cdot 2H_2O$	C 7902	0.252
Stock E[b], 10× (up to 3 months at 4 °C)	mM	Component	Sigma	g/100 mL
	20.85	HEPES	H 6147	4.969
	0.001 g/L	Phenol red · Na	P 5530	0.001
Stock F[c], 100× (autoclaved, up to 3 months at 4 °C)	mM	Component	Sigma	g/10 mL
	0.011	EDTA · 2Na · $2H_2O$	E 6635	0.0041
Stock G, 200× (frozen)	mM	Component	Sigma G 7513	
	1.00	L-Glutamine	200 mM, 29.2 mg/mL	

[a]Concentrated stocks are 0.2-μm filtrated or autoclaved
[b]Add 50 mL ultrapure H_2O, adjust pH to 7.4, and then add another 50 mL ultrapure H_2O
[c]Adjust 1 mL ultrapure H_2O to pH 8.0, dissolve EDTA, and then add another 9 mL ultrapure H_2O

4. Medium-weight mineral oil.
5. Pipetman and tips: 20 μL.
6. A 60-mm Petri dish for preparation of HK, KSOM-AA, and KKF 20-μL microdroplets (Fig. 1).
7. Mated and plugged female mice.
8. A 95 % alcohol lamp.
9. Dissection microscope.
10. Sterile dissection tools (i.e., fine scissors, forceps, and tweezers for dissecting out the uterine horn and oviduct): Keep all clean tools in 95 % ethanol and burn in ethanol flame right before used.
11. A 1-mL syringe with a blunt 26–32G needle (that with a sharp tip cutoff and rounded with a sharpening stone). A hand-drawn

Table 5
To prepare KSOM-AA and HEPES-buffered KSOM (HK) media from concentrated stocks

Stock	KSOM-AA[a] (20.3 mL)	HEPES-buffered KSOM[b] (100 mL)
Ultrapure H_2O	15.3	77.0
A (10×)	2.0	10.0
B (10×) $NaHCO_3$	2.0 (25 mM)	1.6 (4 mM)
C (100×) Na pyruvate	0.2	1.0
D (100×) $CaCl_2 \cdot 2H_2O$	0.2	1.0
E (10×) HEPES + phenol red	–	8.4
F (1,000×) EDTA · 2Na · $2H_2O$	0.2	1.0
G (200×) L-glutamine	0.1	–
MEM EAA (Sigma M 5550)	0.2	–
MEM NEAA (Sigma M 7145)	0.1	–
BSA (Fr. V, Sigma A 3311) dissolve with gentle and slowly, do not foam	0.1 g	0.5 g[c]

[a]pH 7.1 ± 0.1 (adjusted via 0.5 N NaOH), 275 ± 5 mOsm (adjusted via H_2O). Store at 4 °C for a maximum of 2 weeks
[b]pH 7.1 ± 0.1 (adjusted via 1.0 N NaOH), 275 ± 5 mOsm (adjusted via H_2O). Store at 4 °C for a maximum of 3 weeks
[c]The BSA can be replaced by ~5 % FBS or 0.01 % polyvinyl alcohol (PVA; 0.01 g/100 mL, Sigma P 8136)

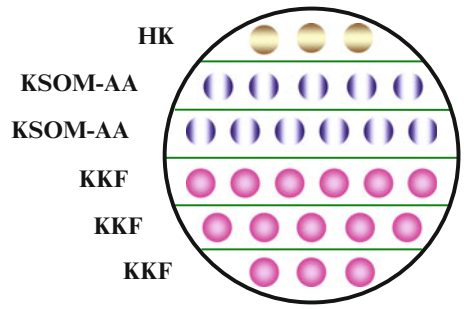

Fig. 1 Schematic illustration of HEPES-buffered KSOM (HK), KSOM-AA, and KKF (KSOM-AA containing 1 % KSR and 1 % FBS) microdroplets (20 μL) on a 60-mm Petri dish covered with medium-weight mineral oil for holding recovered mouse embryos in HK at room temperature (RT) and overnight cultivation of (chimeric) embryos in either KSOM-AA or KKF at 37 °C in a CO_2 incubator

and prepared glass Pasteur pipette with a rubber or a latex bulb could also be used.

12. A 60-mm Petri dish or depression glass vessel (optional) with cover glass for recovery of flushed embryos.
13. Mouse pipette.
14. Acrylic rack.

15. 75 and 95 % ethanol.
16. Paper towels, tissues, absorbent cotton, and Kimwipes.
17. Horizontal laminar airflow hood (optional).

2.7 Enrichment of ES Cells

1. A long working distance inverted microscope with phase-contrast optics (50× and 100×).
2. Vertical laminar airflow hood.
3. CO_2 incubator.
4. (Bench-top) centrifuge.
5. Hemocytometer.
6. 35-mm, 60-mm, and/or 100-mm cell culture dishes.
7. A 60–80 % confluent ESC on ES cell medium with STO or mEF feeders in a 35-mm cell dish cultured for ~2.5 days.
8. K and KF ES cell media.
9. KKF.
10. Ca^{++}, Mg^{++}-free PBS.
11. TrypLE Express.
12. Two 15-mL conical centrifuge tubes or 1.7-mL vials.

2.8 2.5-dpc Denuded Embryos for Vial Coculturing with Enriched ES Cells

1. Dissection microscope.
2. HK.
3. KSOM-AA.
4. KKF.
5. KF ESC medium.
6. Homemade (Table 6) or commercial (Sigma) acidic Tyrode's (AT) solution.

Table 6
Formula of acidic Tyrode's (AT) solution[a]

Component	Sigma	Amount
NaCl	S 5886	0.800 g
KCl	P 5404	0.020 g
$CaCl_2 \cdot 2H_2O$	C 7902	0.024 g
$MgCl_2 \cdot 6H_2O$	M 2393	0.010 g
D-Glucose	G 6152	0.100 g
Polyvinyl-pyrrolidone	P 0930	0.400 g
ddH_2O	–	100 mL

[a]Adjust pH with 8 N HCl to 2.5. After 0.2-μm filtrated, it can be stored in vials (0.4 mL) or tubes at −20 °C for years

7. A 60-mm Petri dish containing 20-μL HK, AT, and KKF microdroplets for embryos before and after ZP removed.
8. A 60-mm Petri dish containing 20-μL HK, KSOM-AA, and/or KKF microdroplets for embryos before and after ES cell coculturing (Fig. 1).
9. Enriched ES cells in KKF in a 15-mL conical centrifuge tube or 1.7-mL vial.
10. Recovered 2.5-dpc embryos in HK droplets at RT.

2.9 Micromanipulation System (Fig. 2)

1. A long working distance inverted microscope with phase-contrast and differential interference-contrast (DIC, optional) optics (50×, 100×, and 200×).
2. Two motor-drive coarse micromanipulators (MM-89; Narishige Group).
3. Two three-axis hanging-joystick oil-hydraulic micromanipulators (MMO-202 ND; Narishige): This micromanipulator features an oil hydraulic hanging-joystick enabling smooth and fine three-dimensional movement with a single lever.
4. Adaptors for micromanipulator and inverted microscope.

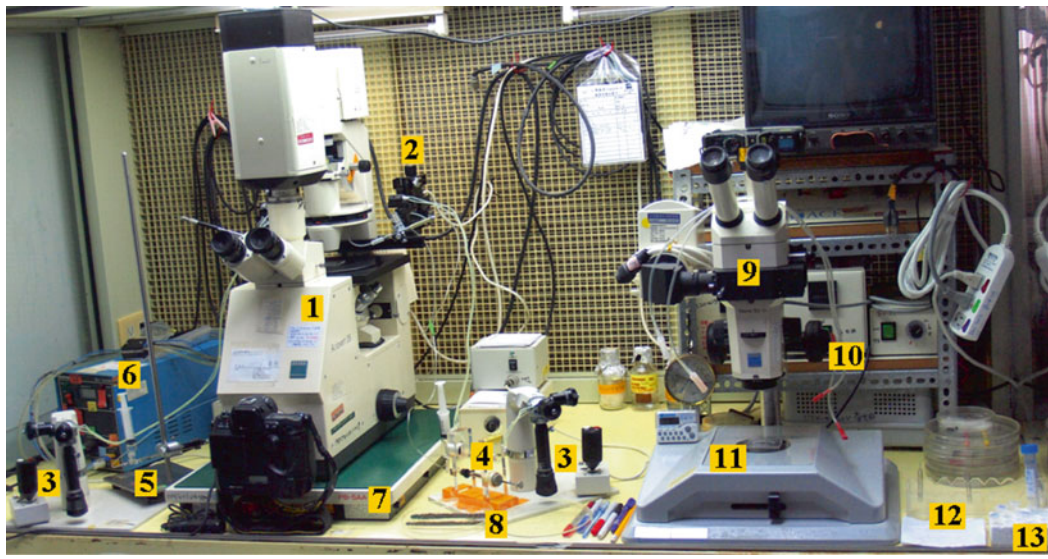

Fig. 2 The micromanipulation system and accessories have been used at my laboratory for more than 10 years. (1) A long working distance inverted microscope. (2) A motor-drive coarse micromanipulator. (3) A three-axis hanging-joystick oil-hydraulic micromanipulator. (4) A 500-μL screw-drive gastight glass syringe. (5) A laboratory-assembled 50-μL micrometer-drive gastight glass syringe. (6) A cooling device. (7) A portable anti-vibration table. (8) Homemade micropipettes secured on synthetic clay bars in a 100-mm cell culture dish. (9) A dissection microscope. (10) Mouth pipettes. (11) Homemade injection chamber. (12) Pipettes placing on an acrylic rack. (13) A 15-mL tube and 1.7-mL vials placing in a Styrofoam holder

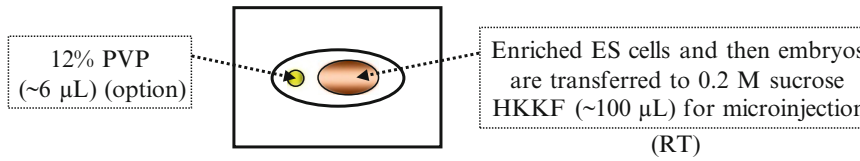

Fig. 3 Schematic illustration of the homemade microinjection chamber. A 45 × 55 mm injection plate with an 18 × 35 mm hole at the center of a ~2-mm-thick acrylic. The hole is adhered with a microscopic cover glass (24 × 50 mm; thickness, 0.13–0.16 mm) using the cyanoacrylate instant glue. The acrylic can be glass, stainless steel, or other rust-free metals

5. A 500-μL screw-drive gastight glass syringe, micrometer-drive Manual Microinjector (Sutter Instrument Co.), or Microinjector (IM-6, IM-9B; Narishige) to control the holding pipette.
6. Holding tubing: Airtight and filled with heavy-weight mineral oil (Teflon Tubing: External diameter, 2.0 mm; internal diameter, 0.9 mm, CT-1; Narishige).
7. A laboratory-assembled 50-μL micrometer-drive gastight glass syringe or Manual Microinjector (Sutter) to control the microinjection pipette.
8. Microinjection tubing: Airtight and filled with lightweight mineral oil (CT-1; Narishige).
9. Tubing and syringe connector.
10. Tubing and pipette connectors (CI-1; Narishige).
11. Three-way valves attached between the tubing and syringe connector, as well as to a 10-mL mineral oil reservoir syringe.
12. Cleaned, particle-free (via ultrasonic), and sterilized (via autoclave) thin-wall glass (capillary) tubing without a filament (external diameter: 1.0 mm, internal diameter: 0.75 mm, overall length: 15 cm, B100-75-15; Sutter) for making injection and holding pipettes after it is pulled.
13. Pipette puller (P-80/PC; Sutter).
14. Microforge attached with a foot switch and equipped with 50× and 100× microscope containing micrometer (MF-79; Narishige).
15. Microgrinder with 50× and 200× microscope (EG-6; Narishige).
16. Two synthetic clay bars in a box to hold the homemade micropipettes.
17. Microinjection chamber (homemade—Fig. 3, lid of a 60-mm Petri dish, a depression slide, or others).
18. (Portable) anti-vibration table (optional).
19. Cooling stage (optional).

2.10 Hypertonic Microinjecting Enriched ES Cells into the Subzonal Space of 2.5-dpc Embryos

1. Micromanipulation system (Fig. 2).
2. Dissection microscope.
3. Acrylic rack.
4. HK.
5. KSOM-AA.
6. KKF.
7. A 60-mm Petri dish containing 20-μL HK, KSOM-AA, and/or KKF microdroplets for embryos before and after microinjection of ESCs (Fig. 1).
8. HKKF (HK containing 5 % KSR and 5 % FBS; blastocyst microinjection medium): 1.8 mL HK + 0.1 mL KSR + 0.1 mL FBS/vial.
9. 0.2 M Sucrose HKKF (500 ± 10 mOsm; hypertonic microinjection medium): 1.5 mL HKKF + 0.103 g sucrose/vial. A 1.5 mL HKKF containing 0.3 M (0.154 g) sucrose can also be used.
10. 1.7-mL Vials.
11. Enriched ES cells in ~20-μL 0.2 M sucrose HKKF in a 1.7-mL vial or 15-mL conical centrifuge tube.
12. Recovered 2.5-dpc embryos in HK droplets (Fig. 1) at RT.
13. 12 % PVP or Sigmacote® (Sigma) (optional): 12 % PVP (polyvinylpyrrolidone; Sigma, MW = 360,000): Transfer 10 mL HK into an 100-mm Petri dish and then spray 1.2 g PVP on the surface evenly. Put into a 4 °C refrigerator for 1 week after sealing the dish lid with parafilm. Sterilization is completed via a 0.45-μm and then a 0.2-μm filter. Aliquot (~20 μL/vial) and store at 4 °C for ~1 year.
14. Microinjection chamber (homemade, Fig. 3).

2.11 Uterine Transfer

1. Chimeric embryos cultured overnight in KSOM-AA or KKF.
2. HK.
3. Weight scale.
4. Avertin (anesthetics for mouse): Add 1.0 g 2,2,2-tribromoethanol (tribromoethyl alcohol; Aldrich) to 1.0 mL tertiary amyl alcohol (2-methyl-2-butanol; Aldrich), and dissolve by swirling in 37 °C. Add 49 mL ddH_2O as the working solution. Aliquot the working solution into brown bottles wrapped in foil. Store at –20 °C or at 4 °C for months [16]. Avertin will recrystallize at low temperatures; therefore, mix by swirling before use (ip 0.15–0.17 mL/10 g body weight) (*see* **Note 5**).
5. Pseudopregnant recipients at 2.5-dpc.
6. Dissection microscope.
7. Small and fine dissection scissors.

8. Two pairs of watchmaker's forceps.
9. Curved forceps, fine blunt with serrated tips.
10. Forceps with teeth.
11. Serrefine clamp.
12. Suture and/or wound clip system.
13. Mouth pipette.
14. Embryo transfer pipettes: 200–300 μm external diameter, 150–200 μm internal diameter (*see* **Note 6**).
15. Acrylic rack.
16. 1-mL Syringes with 26–32G needles.
17. Ear punch, ear tag and applicator, or toe clipping.
18. 50 or 100 W light bulb, warming plate, or heating pad.
19. 95 % Alcohol lamp.
20. 75 and 95 % ethanol.
21. Paper towels, tissues, absorbent cotton, and Kimwipes.

3 Methods

Carry out all procedures under sterilization and at RT unless otherwise specified.

3.1 Superovulation for Natural Mating and Pseudopregnant Mating

Sexually mature ICR×ICR and (ICR×CBA) F1 females used as embryo donors yield good outcomes. Average yield per superovulated and plugged outbred female (e.g., ICR and CD1) is ~20 embryos and an inbred female (e.g., C57BL/6) is ~6 embryos (*see* **Note 7**). Female mice aged 6–30 weeks are superovulated and housed with male studs, which must be individually caged. Place 1–2 females in a cage with a resident male, not vice versa. Check for copulation plugs on the following morning. To maximize the number of plugs, stud males are used once or twice per week. When plugging ratio drops below 50 %, stud males are replaced (*see* **Note 1**).

Most outbred strains (e.g., ICR and CD1) or F1 hybrids (e.g., ICR×CBA) can be used as recipients. Experienced mothers are preferred (*see* **Note 8**).

Table 7 presents a summary of the time schedule for superovulation, mating, chimeric embryo generation, and embryo transfer (*see* **Note 9**). For details, *see* Lee [1].

1. Thaw a vial of PMSG containing sufficient amount for injection of 6–12 superovulated females. Then intraperitoneally (ip) inject with ~0.1 mL PMSG (5–10 IU) for each female between 14:30–16:30 on day 1.

Table 7
Time schedule of superovulation and natural mating to obtain 2.5-dpc embryos for vial coculturing or hypertonic microinjection and pseudopregnant recipients for embryo transfer[a]

Day	Week day			Time[a]	Treatment
1	Friday	Saturday	Sunday	14:30–16:30	Embryo donor females (6–12): PMSG 5–10 IU/0.1 mL, intraperitoneal (ip) injection
3	Sunday	Monday	Tuesday	14:30–16:30	Embryo donor females: hCG 5–10 IU/0.1 mL, ip
				16:00–17:00	Natural mating with studs
4	Monday	Tuesday	Wednesday	08:00–10:00	Check vaginal plugs/embryo donors
				16:00–17:00	Natural estrus females (6–12) mated with vasectomized males
5	Tuesday	Wednesday	Thursday	08:00–10:00	Check vaginal plugs/recipients
6	Wednesday	Thursday	Friday	08:00–10:00	Isolation of 2.5-dpc embryos
				10:30–14:00	Hypertonic microinjection/vial coculturing[b]
7	Thursday	Friday	Saturday	08:00–16:00	Uterine horn transfer of chimeric embryos to 2.5-dpc pseudopregnant recipients
7	Thursday	Friday	Saturday	08:00–10:00	Isolation of 3.5-dpc blastocysts[c]
				10:30–14:00	Conventional blastocyst microinjection
				14:00–17:00	Microinjected blastocysts transferred to uterine horns of 2.5-dpc pseudopregnant recipients
23	Sunday	Monday	Tuesday		Pups born after ETed for 17 days

[a]Day (14 h)/night (10 h) rhythm: Light period from 05:00 to 19:00 (*see* **Note 9**)
[b]Two skillful persons working together as a team doing microinjection is recommended. One skillful technician can do vial coculturing routinely
[c]If many checked donors and recipients exist, conventional microinjection can be carried out on the day following vial coculturing or hypertonic microinjection

2. On day 3, at 46–50 h (14:30–16:30) after PMSG injection, thaw a vial of hCG and inject the same females with ~0.1 mL hCG (5–10 IU).
3. Then place the females directly to fertile studs for mating. Use 1–2 females per stud.

4. Check for copulation plugs on the following morning. Plugged females carry 0.5-dpc embryos at this time. At ~16:30, 6–12 females in natural estrus are selected and mated with vasectomized males. Usually, 1–2 females are placed in a cage with one resident vasectomized male.
5. Check for copulation plugs on the next morning. Plugged females indicate 0.5-dpc status.

3.2 Collecting 2.5-dpc Embryos

Before sacrificing the embryo-donor mice, prepare a 60-mm Petri dish containing droplets of HK, KSOM-AA, and KKF overlaid with medium-weight mineral oil (Fig. 1) and keep at RT until use.

1. At ~09:00, the 2.5-dpc embryo-donor mice are sacrificed via cervical dislocation.
2. Lay the mice on their backs and wash them thoroughly in 75 % ethanol.
3. Cut the skin laterally at the midline and wipe the hair away with 75 % ethanol-soaked cotton. Hold the skin below and above the incision firmly with your fingers. Pull the skin toward the head and the tail until the peritoneum is exposed.
4. Cut and open the abdominal cavity, push the intestine toward the head, and locate the uterus.
5. Grasp the cervix and cut across it. Pull the uterine horn upward by cutting away the ligaments and fat, and then dissect from both sides a piece consisting of the uterine horn, oviduct, and ovary.
6. After all tracts recovered and stay on a 60-mm Petri dish lid (or depression glass vessel), trim the tract on a paper towel prior to flushing embryos.
7. Under dissection microscope, cut off ovaries (red spots indicate the ovulation) and collect oviducts separately in 1-mL HK medium on a Petri dish lid. The remaining in one piece is the uterine horn. To make a ~0.3 cm longitudinal cut in the end of each horn. Dissection could be done in a horizontal laminar flow (optional).
8. Repeat **steps 5–7** until all uteri and oviducts are harvested.
9. Pick up one uterus to a new 1-mL HK medium on the 60-mm Petri dish lid and is placed under the 10× dissection microscope. With the help of fine forceps, insert a blunt (and bended) 30–32G needle (could be a hand drawn and flame-polished glass Pasteur pipette with a rubber or latex bulb), attached to a HK-filled 1-mL syringe, into one side of the uterus at the cervical end. Hold the needle in place with fine forceps while pressing the plunger. Uterine swelling indicates successful flushing. Flush each horn with ~0.2 mL of HK medium toward the oviduct. Be careful not to use too much pressure as this

could spray the HK. Repeat this step for another uterus. Repeat this step for other uteri.

10. Use a mouth pipette with an internal diameter of ~150 μm to pick up all flushed embryos. Wash them ~3 times in clean HK medium, then transfer them in an HK droplet in the 60-mm dish (**step 1**), and keep at RT until use.
11. Repeat **steps 9** and **10** to recover embryos from oviducts. Locate the infundibulum, the funnel-shaped oviduct end nearest the ovary. Insert the needle into the infundibulum and the first part of the oviduct and flush while holding the needle in place with fine forceps. Recover, wash, and store embryos in an HK droplet in the 60-mm dish at RT until use.

3.3 Double-Plating Method to Enrich ES Cells

Before conducting experiments to generate chimeric embryos via assembling 2.5-dpc embryos and ESCs, an excellent or at least good ESC line should be acquired and propagated promptly. As a general recommendation, use ESC lines that are from a reliable source, and have demonstrated pluripotency by the high-percentage contribution in chimeras with germline transmission. The ESCs grown on STO or mEF feeder cells exhibit a rapid proliferation (usually pass every 2.5 ± 0.5 days when splitting the cells 1:5–1:10) with excellent three-dimensional (3D) colonies, sharp edges, and only very few flat colonies are the simple and useful criteria of good quality.

For routine culturing and considerations of ESCs, *see* previous book chapters or articles [25, 38, 44, 45] (*see* **Note 10**).

An excellent and quite pure ES single-cell suspension is necessary for either coculturing or microinjection to ensure that cells adhere to the 2.5-dpc denuded embryos or cells introduced to the subzonal space of embryos is ES cells mainly. As most ESCs culture usually on fibroblast feeder layers (*see* **Note 11**), any protocol that eliminates feeder cells and enriches ESCs is beneficial. Previous protocols based on the differential attachment to a dish surface and settle down in tubes of cells have been recommended and widely used [5, 21, 23]. Thereafter, a modified double-plating enrichment method, developed at my laboratory, yielded excellent outcomes (Fig. 4) is adopted at this protocol [26] (*see* **Note 12**). The method is as follows, and takes ~140 min to finish. The prepared enriched cells, mainly ESCs, kept in 2–3 mL KF ESC medium and stored at 4 °C, are usable for several hours.

1. A 60–80 % confluent ESCs grown on a 35-mm cell culture dish (~4×10^6 ESCs; 60-mm dish: ~15×10^6 ESCs), either with 0.1 % gelatin-coated or with feeders, for 1.5–3.0 days is digested to a single-cell suspension. Feeding the ESCs for 1–2 h before trypsinization is optional.
2. The cells are washed with ~1 mL Ca^{++}, Mg^{++}-free PBS once and then digested via ~0.5 mL TrypLE Express in a 37 °C, 5 %

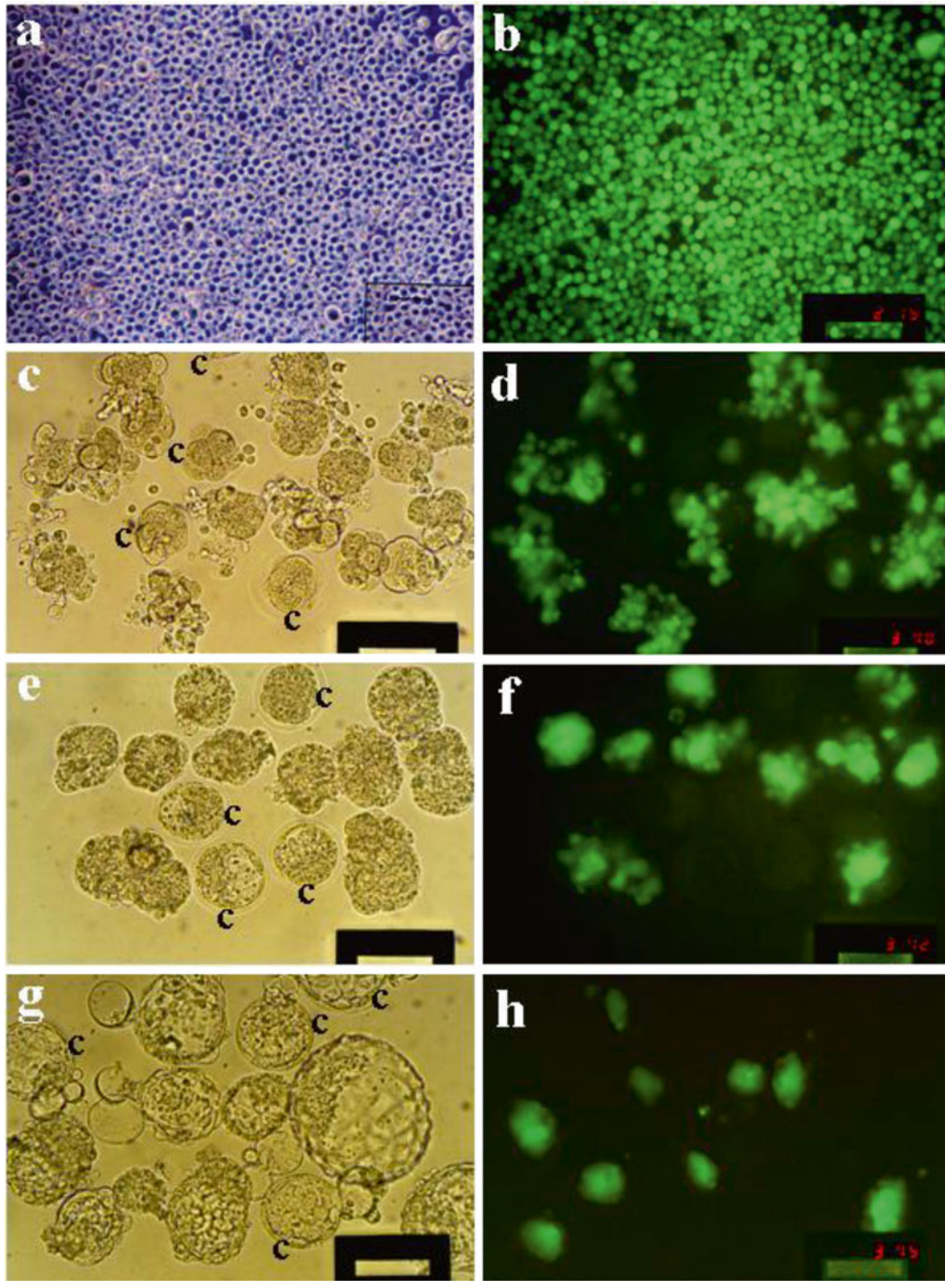

Fig. 4 Enrichment of mouse ESCs and development of denuded embryo-ESC aggregates. (**a**) Attaching and/or attached cells were recovered after the original single-cell suspension of the ESC, ESC 26GJ9012-8-2, cultured on a 100-mm dish in a 5 % CO_2 incubator at 37 °C for 80 min. (**b**) Over 94 % of cells expressed the green fluorescence protein (GFP). (**c**, **d**) After denuded eight-cell embryos, morulae, and ESC 26GJ9012-8-2 cells

CO_2 incubator for ~15 min (0.25 % trypsin needs 4–7 min) until the cells in floating colonies lost contact. This step must be observed carefully under a microscope, especially when done for the first time. In total, 0.5 mL KF ESC medium is added and pipetted up and down by pressing the tip of the pipette onto the bottom of the dish several times until a single-cell suspension is achieved and confirmed under the phase microscope.

3. Approximately 35 % (for microinjection) single-cell suspension is transferred to an untreated 60-mm cell dish with ~3 mL KF ESC medium or 100 % (for vial coculturing) single-cell suspension is transferred to a blank 100-mm cell dish with ~8 mL KF ESC medium. Incubate the culture in a CO_2 incubator for 80 min. The thawed ESCs are also used starting with this step.
4. The medium is removed by aspiration gently. Approximately 3 mL or 8 mL KF ESC medium is added to the 60-mm or 100-mm dish, respectively, which is shaken gently to detach cells (*see* **Note 13**), which are then harvested and transferred to a new 60-mm or 100-mm dish and placed in a CO_2 incubator for another 20 min.
5. The floating cell suspension is transferred to a 15-mL tube and stored at 4 °C for ≧20 min until the next step.
6. In total, approximately 30–50 % supernatant is aspirated and then centrifuged at 250 × g for 3 min.
7. The pellet is resuspended in either KKF to the desired cell concentration (5×10^5 cells/mL) for subsequent vial coculturing. Otherwise, the pellet is resuspended in ~2 mL microinjection medium (HKKF containing 0.2 M sucrose) and then centrifuged at 250 × g for 3 min. The supernatant is aspirated until only 2–4 μL is left. The pellet is loosening up by agitating vigorously the tube several times with your fingers for subsequent hypertonic microinjection.

Fig. 4 (continued) were cocultured in a 1.7-mL vial for 2 h, recovered embryos had adherent green fluorescing ESCs on their surfaces. The left bright and right green fluorescent images show the same view of 14 embryos, including two eight-cell embryos, two compacting morulae with zona pellucida (as the control; **c**), five single-embryo-cell aggregates, three 2-embryo-cell aggregates, and two 3-embryo-cell aggregates (some kind of sandwich aggregation). (**e**, **f**) After culturing aggregates from panels (**c**) and (**d**) overnight in droplets of KSOM-AA containing 1.0 % FBS, the aggregates had cells with surface green fluorescence mingling in the developing compacting and compacted chimeric morulae. (**g**, **h**) After further overnight culturing, chimeric morulae from panels (**e**) and (**f**) developed into chimeric blastocysts displaying green fluorescence cells primarily in the ICM. Scale in panel (**a**) bar = 50 μm. Scale in panels (**b**–**h**): bar = 100 μm. Reproduced with permission from Lee et al. [26]

3.4 Zona Pellucida-Removed Embryos (Denuded Embryos)

The ZP from a six-cell-stage embryo to compacting morula is removed with an acidic Tyrode (AT) solution. The denuded embryos are washed and placed in HKKF at RT for ~20 min, and are then mixed with enriched ESCs for coculturing in 1.7-mL vials for 3 ± 1 h.

1. Prepare a 60-mm Petri dish containing 8 20-μL droplets of HK, HKKF, and AT each. The lightweight mineral oil is covered on droplets.
2. Partition six-cell embryos to compacting morulae in HK droplets. Usually 10–30 embryos (depending on the skill in using the mouth pipette) are placed in one droplet.
3. The HK is sucked dry via the mouth pipette, and the embryos are left in. Approximately 15-μL AT is then added. After seconds when the ZP begins to disappear (observing under a ~25× dissection microscope), the AT is sucked dry; then add HKKF and gently move embryos by mouth pipetting them up and down (*see* **Note 14**). Once the ZP disappears, use HKKF to wash three to four times and keep in the same drop at RT until vial coculturing. Ensure that the ZP-removed (denuded) embryos are well spread out, as they are sticky and tend to aggregate.
4. Repeat **step 3** with the remaining embryos.

3.5 Coculturing of 2.5-dpc Denuded Embryos with Enriched ES Cells in 1.7-mL Vials (Micro Test Tubes)

For laboratories without micromanipulation systems and techniques, I suggest using either the vial coculturing method [26] or well sandwich aggregation method [5, 20, 22] to generate chimeric mice [1]. It should be noted that optimal culture conditions for preimplantation embryos and for ESCs are not the same or even conflict each other so that in making chimeras there is a compromise while the ESCs being adhered or introduced to the embryos [16, 29, 46–48]. However, since the embryo is far more sensitive than ESCs, the culture conditions should be depending on embryos mainly [26]. The following is the vial coculture protocol.

1. In total, ~0.8 mL aliquots of enriched ESCs, from either fresh or thawed, at ~5.0×10^5 cells/mL in KKF medium, are added to clean and sterile 1.7-mL vials with snap caps.
2. After standing for 3–5 min, 10–150 2.5-dpc denuded eight-cell embryos and morulae are blown gently and circularly from beneath the medium surface into each vial via a mouth pipette. This step ensures that denuded embryos are surrounded with ESCs in all directions that the gravity makes the adhesion among them will be strong enough. The coculturing vial is returned to the CO_2 incubator and incubated for 3 ± 1 h.
3. Precipitation in the vial is aspirated into a 35-mm Petri dish gently and mouth pipetted up and down. The denuded

embryos adherent to cells is carefully recovered to HKKF. The recovery rate is more than 90 % [26]. Loose cells on the surface of the embryo-ESC aggregate are removed by washing with a mouth pipette. Aggregates with two or three clustered embryos (adherent with cells) can be separated by gentle pipetting.

4. The embryo-ESC aggregates are washed and transferred to either KKF or KSOM-AA medium droplets under medium-weight mineral oil on Petri dishes (Fig. 1). Two to four aggregates are carefully allocated at different corners of the same droplet to prevent possible adhesion and are cultured overnight in a 5 % CO_2 incubator until transferred to 2.5-dpc pseudopregnant recipients.

3.6 Homemade Micropipettes

The quality of the microinjection pipettes is extremely important to the success and efficiency of microinjection. Both microinjection and holding pipettes can be standard or custom-made expensive commercial products. However, for the cost reason, my laboratory uses homemade pipettes all the times. When a microinjection experiment is on schedule, both pipettes are homemade (need ~0.5 h) on that morning. Unfortunately, suitable equipment and fine skills are needed to make excellent or good pipettes, especially microinjection pipettes. Previous articles have described methods in details and troubleshooting steps are useful references [5, 16, 19, 49].

Bare hands are always used when preparing holding and injection pipettes. For hypertonic microinjecting ESCs into embryos, the internal diameter of injection pipettes is 14–19 μm and the external diameter is 18–25 μm. A holding pipette is heat-polished until an opening of 22–24 μm remains. The external diameter is 60 ± 10 μm.

3.6.1 Making Microinjection Pipette

1. A cleaned, particle-free, and sterilized glass (capillary) tubing with a thin wall (overall length: 15 cm) without a filament is re-sterilized by passing it through an ethanol flame about ten times.
2. Pull out the glass tubing with a parameters-set pipette puller to the correct shape and size.
3. Bring a pulled pipette to the microforge and ensuring that an equatorial position is placed. To break a pipette at the position with 18–23 μm external diameter is via contact with a small bead of glass (diameter ≦150 μm) on the apex of a platinum wire (filament; diameter: ~120 μm) which should be turned on and off very rapidly to avoid melting the pipette.
4. Move the pipette to the microgrinder and bevel the opening (~15 s) which external diameter will increase 1–3 μm. Lift the pipette. All debris attached to the tip must be removed via

a high-speed air from squeezing a dust-blowing ball. Remove the beveled pipette and the tip then is washed and sterilized with 75 % ethanol spray, and thereafter wiped and sucked dry via Kimwipes. The surface of the grinder is cleaned using Kimwipes containing 95 % ethanol.

5. Bring the beveled and cleaned pipette, which has the desired external diameter of 20–26 μm, to the microforge and make a spike at the beveled tip via touching a suitably hot bead of glass on the apex of the filament. If the microinjection pipette does not qualify, re-make it or make a holding pipette.
6. Make a ~30° bend at ~0.5 cm from the tip by placing on the hot bead of glass, such that the final orientation of the spike (away from the glass surface of the microinjection chamber) is almost horizontal when it enters the injection medium.
7. Place and secure the ready-made injection pipette on the right side of the synthetic clay bars in a box until use.

3.6.2 Making Holding Pipette

1. Bring a previously pulled pipette or failed microinjection pipette to the microforge and break the pipette, via glass bead on the filament, such that its external diameter is 70–80 μm.
2. Align both the filament and opening of the pipette. To make the opening and the glass bead have a sufficient distance to allow for filament expansion when turned on. Heat-polish the end of the opening (~2 s) until the diameter is 22–24 μm (the external diameter now is 55–70 μm).
3. Place a ~30° bend ~0.5 cm from the tip via on the hot glass bead.
4. Place and secure the ready-made holding pipette on the left side of the synthetic clay bars in a box until use.

3.7 Hypertonic Microinjecting ES Cells into the Subzonal Space of 2.5-dpc Embryos (Fig. 5)

For a new beginner, one should ask advice and acquire experiences from well-established and skillful laboratories for microinjection. Furthermore, one may follow references for system setup and technique acquisition [16, 19, 43, 50]. A Japan Company provides useful information about microinjection on website (http://products.narishige-group.com/english.html).

For laboratories that have a micromanipulation system and techniques needed to microinject ESCs into 3.5-dpc blastocysts or morulae to generate chimeric mice, the hypertonic microinjection method is a reasonable, simple, and useful alternative. One can perform all tasks the same as in the conventional microinjection protocol, except that the injection medium is supplemented with 0.2 or 0.3 M sucrose.

For microinjection experiments, two skillful persons should work together as a team. In this arrangement, one enriches ESCs and recovers embryos, while another prepares micropipettes and performs the micromanipulation.

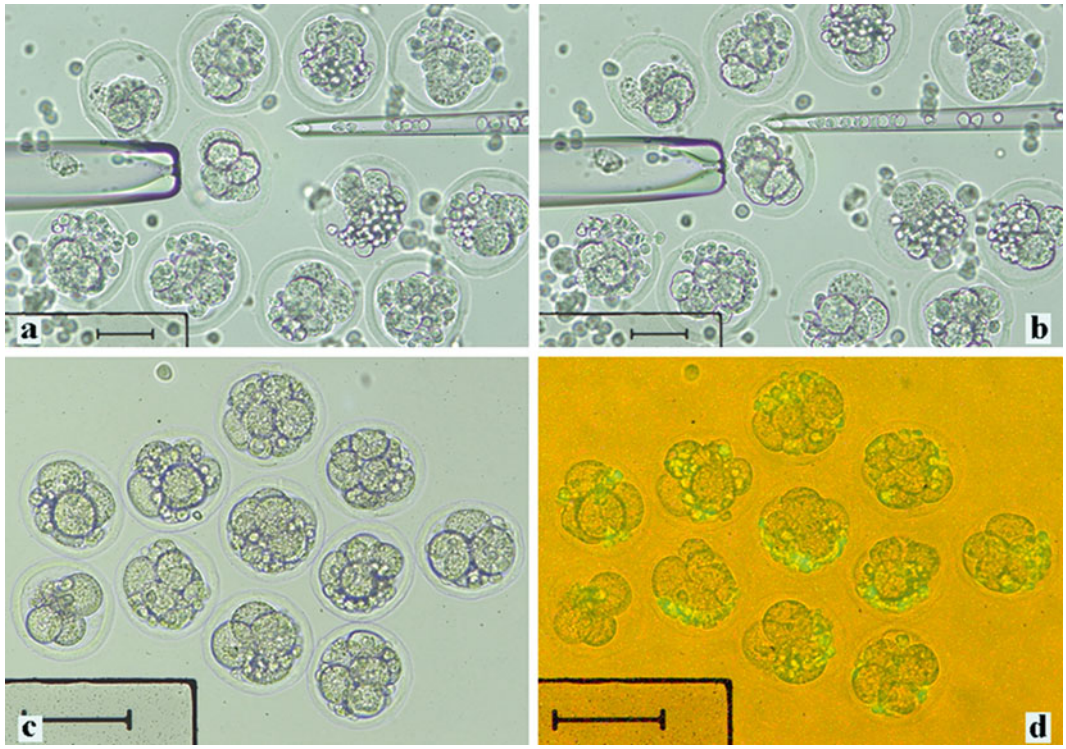

Fig. 5 The generation of mouse chimeric embryos via the hypertonic microinjecting enriched ESCs into a subzonal space of ICR × ICR 2.5-dpc embryos. (**a**, **b**) Injected and injecting embryos and enriched ESCs (ESC 26GJ9012-8-2, P14) in 0.2 M sucrose EHK (37.5 % K ESC medium and 62.5 % HK) hypertonic injection medium (500 ± 10 mOsm/kg H_2O) show the large subzonal space. (**c**, **d**) After injected ~25 ESCs into embryos, which were washed to isotonic EK (37.5 % K ESC medium and 62.5 % KSOM-AA; 285 ± 10 mOsm). The bright (**c**) and bright plus green fluorescent (**d**) images show the same view of 11 injected embryos. Modified from Lee [1]

1. Set up the microinjection devices for microinjecting enriched ESCs into the subzonal space of 2.5-dpc embryos. In my laboratory, a homemade injection chamber (Fig. 3) has been used for decades. Two clean injection chambers (one is for work, and another is a spare) are ethanol flame-sterilized. Each chamber is placing a ~6 μL of 12 % PVP (optional) on the left and a ~100 μL of injection medium on the center of the glass (Fig. 3). They are then covered with lightweight mineral oil until use. Alternatively, depression slides, a Petri dish lid [50], and other commercial or homemade injection chambers [21, 49] can also be used.
2. To introduce cells into a subzonal space of embryos or the cavity of blastocysts, a 200× magnification is the best and suggested for most experiments. In some situations, opening an anti-vibration table is necessary; otherwise, microinjection is impossible due to a magnified vibration. A cooling stage (~10 °C) is sometimes adopted to increase the rigidity of embryos and cells and make them less sticky (optional).

3. Put the prepared spare injection chamber on the stage of the inverted microscope.
4. Insert a holding pipette to the pipette holder on the left side of the micromanipulator while heavyweight mineral oil is flowing out slowly under a positive pressure to avoid air bubbles in the tubing and pipette as this may cause uncontrollable movement of contents. Cross the tubing behind the microscope. The tubing connects to a 500-μL screw-drive (or micrometer-drive) gastight glass syringe, which should be controlled by the right hand. Position the pipette holder with holding pipette in the micromanipulator. Insert the holding pipette into the lightweight mineral oil of the spare injection chamber and maintain a positive pressure.
5. Place a microinjection pipette on the right side of the micromanipulator but air bubbles avoided via a positive pressure from the lightweight mineral oil reservoir syringe. Cross the tubing filled with the mineral oil behind the microscope. The tubing connects to a 50-μL micrometer-drive gastight glass syringe, which should be controlled by the left hand. Position the microinjection pipette in the micromanipulator and then insert the pipette into the lightweight mineral oil of the spare injection chamber and maintain a positive pressure.
6. Both pipettes are adjusted to be parallel with each other under a ~50× magnification. The holding pipette is washed inside several times in the lightweight mineral oil (optional). Move the microinjection pipette into the 12 % PVP droplet, wash ~1 cm tip via suck in, push out two to three times, and keep at RT until use (optional).
7. Use a dissection microscope with a ~20× magnification. Transfer the enriched ESCs (in HKKF containing 0.2 M sucrose) from the centrifuge tube or vial into the upper left corner of the injection medium in the work injection chamber, but leave cell-free at other areas (*see* **Note 15**).
8. The recovered 2.5-dpc embryos in HK are washed using injection medium two to three times and then transferred to the left-middle side of the work injection medium, which has been loaded with enriched cells and kept at RT. If more than 60 embryos are recovered, they may be divided into two groups. The first group comprises embryos with ≦8 blastomeres. These embryos will be injected in the first round. Another group comprises compacting morulae, which are washed into KSOM-AA and put back into a CO_2 incubator until the next round of microinjection (*see* **Note 16**).
9. Remove the microinjection pipette from the PVP to the injection medium and wash one to two times. Both the microinjection pipette and holding pipette are lifted and the spare

injection chamber is replaced by the prepared work injection chamber. Reposition both pipettes in the injection medium.

10. The microinjection pipette is washed once using injection medium and then under a 200× magnification, hundreds (enough for the entire microinjection) of small, round, smooth-looking enriched cells (mainly ESCs) are sucked in within ~5 min. The holding pipette sucks in a little injection medium and waits for holding embryos.
11. After the holding pipette is sucked in an eight-cell embryo that adjusts to touch the surface, the microinjection pipette is moved into the same focal plane. Then move the spike to the high midpoint of the ZP, which is penetrated by mechanical force via the fine micromanipulator. The cells are slowly expelled into the subzonal space by a gentle positive pressure (Fig. 5). Approximately 6–8, 10–15, and 15–20 enriched cells are introduced into the subzonal space of four-cell, eight-cell, and morulae embryos, respectively (*see* **Note 17**).
12. The step is optional. After expel cells to the subzonal space, suck in a little medium to release the possible buildup of overpressure that might push out the injected cells while withdrawing the microinjection pipette. Alternatively, when the tip of the microinjection pipette reaches the entry point to a ZP, overpressure is allowed to release by waiting for a few seconds. One then moves the injected embryos aside and holds the next embryo.
13. The microinjection procedure is repeated until all embryos are injected. Using this protocol, more than 60 embryos can be injected routinely within ~1 h. One pair of micropipettes usually can finish the entire microinjection without any replacement due to clog of sticky cells or materials in tips of pipettes.
14. On microscopic stage, transfer injected embryos into KKF drops in dish (Fig. 1) and wash embryos two to three times. Put the dish back into the CO_2 incubator.
15. Embryos in KSOM-AA drops in dish are washed with injection medium twice and transferred to the injection medium in work injection chamber on stage. Repeat **steps 11–14** for the second or the third round of microinjection (optional).
16. Approximately 1.5 h after finishing the microinjection, injected embryos are washed again with KKF or KSOM-AA and cultured overnight until embryos are transferred.

3.8 Uterine Transfer

Chimeric embryos, via either vial coculturing or hypertonic injection, are transferred on the next morning into the uterus lumen of 2.5-dpc pseudopregnant recipients (*see* **Note 18**). A conventional embryo transfer protocol is followed [16, 23, 51]. Approximately 10–16 injected embryos (with ZP) are transferred bilaterally or ≦8

embryos to unilaterally into one recipient (*see* **Note 19**). However, the success rate for transferred sticky ZP-free embryos is somewhat lower than embryos with ZP. Therefore, 16–20 embryos could be transferred to uterine horns bilaterally.

1. Homemade transfer pipettes are prepared via hand-drawn clean and sterilized glass Pasteur pipette on the flame of an alcohol lamp. The pulled-out narrow tip should be ~2 cm. One breaks the pipette using fine forceps at the position of ~250 μm in diameter. The opening is polished and sterilized by quick passes through an alcohol flame. The internal diameter now is ~150 μm and ready for embryo transfer. Pipettes are placed on the acrylic rack or secured on other devices until loading embryos. Using one pipette for one embryo transfer is recommended.
2. One 2.5-dpc pseudopregnant recipient is anesthetized via avertin ip injection, and placed on a paper towel.
3. The embryo is loaded to transfer pipettes via a mouthpiece as described elsewhere [16, 23] but HK replaces M2 or PB1. Draw in lightweight mineral oil until it reaches the section where the pipette widens (to avoid capillary suction)—HK medium—air bubble—HK—air bubble—5–10 closely stacked embryos—air bubble—HK. Put the embryos in loaded transfer pipettes on the acrylic rack or fix on other devices next to the microscope until transfer. One may load 2–4 pipettes at once. Ensure that the pipette does not touch anything and is not disturbed.
4. After checking the reflex response of anesthetization (*see* **Note 20**), the recipient is placed on the stage of a dissection microscope.
5. The back of the recipient is cleaned with 75 % ethanol; grasp and lift skin with curved, blunt, and serrated tip forceps and make a ~1 cm transverse incision with fine dissection scissors in the skin just below the rib cage at the level of the first lumber vertebra. Wipe the hair away with 75 % ethanol-soaked cotton.
6. The skin incision is lateral until the right or the left ovarian or fat pad is visible through the peritoneal wall, which is grasped and lifted with the watchmaker's forceps and cut open (~5 mm) via the scissors while avoiding blood vessels.
7. The uterine horn, oviduct, and ovary are gently pulled out by the fat pad attached to the ovary; attach a small Kimwipes or serrefine clamp to the fat pad and position the clamp over the recipient's flank in such a manner that visualizes the uterine horn.
8. A sterile 26–32G needle is carefully inserted into the uterine lumen through the uterine wall, ~2 mm from the utero-tubal junction under ~10× magnifications. The insertion direction

should be parallel to the uterine horn to avoid puncturing the uterus. Remove the needle and without taking your eyes off the hole, carefully insert the tip of the transfer pipette connected to a mouthpiece into the uterine lumen. The embryos are gently expelled into the lumen by blowing (the last air bubble indicates success) and the pipette is pulled out gently. The uterus is replaced in the abdominal cavity using blunt forceps.

9. Proceed to another side for a bilateral uterine transfer and close the body wall with a small wound clip or suture. One stitch and a tight knot may be used to close the skin.
10. When transfer is complete, ear-mark the recipient [52], and return the animal to a clean cage. Keep the recipient warm until it wakes up.
11. Repeat these steps for other recipients.
12. One may expect the pups to be born ~17.5 days after embryos are transferred.

4 Notes

1. Breeding records of all stud and vasectomized males should be kept at all times. Successful mating over 50 % is required to maintain efficiency; otherwise, the male should be replaced.
2. The skill to control a mouth pipette is critical for handling embryos smoothly. One should have the ability to transfer ~100 embryos at one time in any situation and lose none. For this criterion, the opening of the pipette should be ~150 μm.
3. An FBS is an expensive product with a varying biopotency. Therefore, new FBS batches are tested to select those providing the most undifferentiated ESC cultures that maintain pluripotency. Briefly, 200–250 ES cells (~150 cells/mL ESC media) are plated onto a 35-mm gelatinized cell culture dish without feeder cells. At least two dishes (duplicates) are plated for each condition. The ESCs are cultured in ESC medium containing 10, 20, and 30 % non-inactivated and/or inactivated (56 °C for 30 min) FBS, and the medium is replaced every 2 days. After 4–5 days, serum quality is assessed by screening the morphology of ESC colonies (relative 3D vs. flat colony shape reveals undifferentiated vs. differentiated cells) under a phase microscope. Thereafter, dishes are stained with methylene blue to determine plating efficiency. The biopotency of a new FBS batch should be the same or better than the batch used currently.
4. For an FBS ESC medium, FBS can be replaced completely by SR (e.g., KSR or N2B27); otherwise, 5–15 % FBS can be replaced by SR. When changing FBS to a new tested batch,

ESCs sometimes have to adapt to a new batch. For instance, one can mix 50 % old FBS with 50 % new FBS and allow the ESCs to acclimatize to the new medium. Generally, ESCs could be changed from SR to FBS ESC media by sequential adaptation with two to three passages. Reversely, acclimatizing ESCs from FBS to SR ESC medium is occasionally difficult and can fail. When one is switching to a serum-free cell culture, sequential adaptation with three to four passages is required.

5. Avertin and ketamine/xylazine mixtures [44, 53, 54] are two reliable different anesthetics that are the most commonly used for mouse surgery. Avertin provides rapid and deep anesthesia in mice, followed by fast and full postoperative recovery [55]. Induction requires only minutes and a mouse will remain anesthetized for 30–60 min and recover within 1–1.5 h. A disadvantage is that tribromoethanol degrades rapidly at high temperatures (>40 °C) or under light to produce toxic by-products (dibromoacetaldehyde and hydrobromic acid).
6. For uterine transfers, use transfer pipettes with an internal diameter slightly greater than that of embryos (diameter: ~85 μm). Controlling the movement and moving of contents into uterine lumens with a transfer pipette that is too wide is difficult. My laboratory typically uses flame-polished pipettes with an external diameter of 200–300 μm and internal diameter of 150–200 μm. A hemocytometer or micrometer can be applied to estimate the bore size of transfer pipettes.
7. In general, which breed/strain is used as an embryo donor largely depends on the ESCs used [5, 16, 21, 31]. Practically, coat color, green fluorescence protein (GFP), enzymes, and DNA markers are useful criteria.
8. The female recipients should be good mothers as they must carry transferred embryos to term and nurse newborns to wean at 3–4 weeks of age. They should also allow the inspection of newborn pups and accept pups from another mother. Typically, mating females, at least 2 months old, with vasectomized males [16, 51] will generate pseudopregnant females required for transferred of chimeric embryos. To increase the chance of mating a female in natural estrus, 1–2 females are placed with one resident vasectomized male. The vasectomized male can be used twice weekly for mating. The surplus of plugged, pseudopregnant fosters and superovulated females can be reused after 2 weeks.
9. The light:dark cycle is extremely important in regulating circadian rhythms and synchronizing the estrus cycles of mice. The mating, ovulation, fertilization, and subsequent development of mice zygotes follow a preset time course, mainly based on the light:dark cycle. Therefore, most animal facilities have automated light during 06:00–18:00 or 05:00–19:00 in

temperate/frigid or subtropical/tropical zones all year round, respectively. The light:dark cycle may be adjusted for convenience. However, interrupting the dark period, even with short light pulses, can markedly disrupt and alter the circadian clock of animals. Females maintained on a constant light:dark cycle tend to ovulate once every 4–5 days, 3–5 h after the onset of the dark period [49].

10. The ESCs tend to lose the normal number of chromosomes when doubling [56] and differentiate while culture conditions are unsuitable. Therefore, my laboratory always grows ESCs, with an early passage number, in quantity and enough frozen cells (e.g., 10 confluent 100-mm dishes frozen ~100 vials within 1 week) for further experiments. Generally, after a vial is thawed, cells should be re-plated into two 35-mm dishes for 2–3 days to reach 60–90 % confluence. For convenience, thawed and then enriched ESCs can be used directly for either microinjection or coculturing [26]. In this way, variation in the highly spontaneous differentiation of ESCs is fixed.
11. For maintenance and propagation, most ESCs culture on fibroblast feeder layers. Therefore, for convenience, my laboratory always freezes many mitomycin C-inactivated STO and mEFs [1, 25, 26]. In practice, a fresh feeder cells are mitomycin C treated, washed, and prepared or frozen. Sometimes, inactivated cells remained in fibroblast medium for culturing overnight or at least hours before they are prepared or frozen [21]. I usually begin to re-plate ESCs on feeders that are thawed and monolayer prepared 1–3 days earlier. However, in an emergency, inactivated feeder cells and ESCs can be thawed and placed together on gelatin-coated (even wet) dishes when FBS ESC medium is used. Otherwise, feeders should be prepared 1 day earlier because STO and mEF do not attach to the dish surface well when SR ESC medium is used.
12. In summary, when using the double-plating enrichment method, ~96 % of harvested cells expressed bright-green fluorescence, ~92 % of these cells were <12 μm in diameter, and the recovery rate of cells was ~25 % (Fig. 4) [26]. This method is implemented easily, and is selective, effective, and reproducible in removing debris, dead cells, and feeder cells from the prepared ES single-cell suspension. Further, the first 80 min of incubation is the appropriate time to recover embryos for generation of chimeric embryo. Previous study indicated that activated ESCs (culture in fresh ESC medium for 2 h before trypsinization) before microinjecting to eight-cell embryos and blastocysts will increase the efficiency to generate fertile (F0) chimeras effectively [57].
13. The STO or mEF cells are attached to the surface of a dish faster than ESCs; too much shaking would lift them again.

This is used to eliminate most feeders prior to injection without having to culture ESCs in feeder-free conditions for one passage. During this incubation period, only viable cells will attach, leaving dead cells and debris in the medium.

14. Notably, an over-exposure in acidic Tyrode's solution will compromise the viability of embryos. Denuded embryos are sticky using media supplemented with ≦5 % FBS in air. To avoid touching them directly with a glass pipette, manipulation by blowing the medium is suggested.
15. To avoid residual feeder cells (their size can be quite large), differentiated cells or ESCs sticking to the tip of the holding pipette while catching the embryos, a cell-free injection area for microinjection, or an isolated ESCs droplet is recommended.
16. I usually inject eight-cell embryos and then four-cell embryos. The morulae are injected (could be directly into an embryo proper) at the last round or simply used as the sham control (do everything the same as microinjection without expelling cells).
17. The optimal number of ESCs needed to generate high percentage of fertile chimeric mice depends largely on the characteristics of the ESC line and the number of passages used. Even under optimal culture conditions, the ESCs accumulate chromosome errors and DNA mutations, reducing the generation of chimeric mice [56, 58]. Generally, as the passage number (usually no more than 15–20) increases the number of ESCs the need to be injected increases.
18. When the number of 2.5-dpc pseudopregnant recipients is lacking, the cultured overnight denuded chimeric embryos can be transferred into the uterine horns of 3.5-dpc recipients. The hypertonic microinjected chimeric embryos with ZP can be transferred into either 3.5-dpc recipients or the infundibulum of 0.5-dpc recipients [57].
19. The technique used to transfer embryos is very important for the generation of chimeric mice. Generally, if normal and untreated embryos are transferred to four recipients, three births will be of the accepted standard. Furthermore, the number of pups should be ≧65 % of the embryos transferred to a recipient.
20. An indicator of full sedation is the absence of a corneal (blinking) reflex when one softly blows or touches on an animal's eyes.

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Chapter 6

Genetic Inducible Fate Mapping in Adult Mice Using Tamoxifen-Dependent Cre Recombinases

Susanne Feil, Jana Krauss, Martin Thunemann, and Robert Feil

Abstract

The Cre/lox site-specific recombination system allows the control of gene activity in space and time in almost any tissue of the mouse. A major technical advance was the development of tamoxifen-dependent Cre recombinases, such as CreER^{T2}, that can be activated by administration of tamoxifen to the animal. This powerful tool greatly facilitates the study of gene functions and the generation of more realistic animal models of sporadic human diseases. Another important application of tamoxifen-dependent Cre recombinases is genetic inducible fate mapping (GIFM). In GIFM studies, the inducible Cre/lox system is used to genetically label a defined cell population at a selected time by irreversible activation of the expression of a Cre-responsive reporter transgene. Then, marked cells are detected at later time points to determine how the originally labeled progenitors contribute to specific structures and cell types during pre- and postnatal development. GIFM was initially applied during mouse embryogenesis, but is now increasingly used for cell lineage tracing in adult mice under physiological and pathophysiological conditions. Here we describe the design of GIFM experiments in adult mice as exemplified by CreER^{T2}-assisted tracing of vascular smooth muscle cells during the development of atherosclerotic lesions. First, we give an overview of reporter transgenes available for genetic cell marking that are expressed from the *Rosa26* locus, such as β-galactosidase and fluorescent proteins. Then we present detailed protocols for the generation of experimental mice for GIFM studies, the induction of cell labeling by tamoxifen treatment, and the detection of marked cells in fixed and live tissues. Each section also provides a discussion of limitations and common pitfalls of GIFM experiments. Most of the protocols can be easily adapted to other developmental stages, cell types, Cre recombinases, and reporter transgenes and, thus, can be used as general guidelines for GIFM studies in mice.

Key words Atherosclerosis, Cardiovascular cell tracking, Fluorescence detection, GFP, Inducible Cre, LacZ, R26R reporter, RFP, SM22, Transgenic mice, VSMC, Whole mount, X-Gal staining

1 Introduction

In the last two decades, the site-specific recombinase Cre (*c*yclization *re*combination) has emerged as an indispensable tool for the precise in vivo manipulation of the mouse genome [1–3]. Cre catalyzes recombination between two 34-bp DNA recognition sites named loxP (*lo*cus of crossing [*x*-ing]-over of bacteriophage *P*1).

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The basic strategy for Cre/lox-directed gene *inactivation* is to flank ("flox") an essential exon of the gene of interest with two loxP sites (by homologous recombination in embryonic stem cells) followed by "delivery" of Cre to excise the intervening DNA including the exon from the chromosome, thus generating a null allele in all cells where Cre is active. Delivery of Cre can be achieved by crossing mice carrying the floxed target gene with transgenic Cre-expressing mice. By use of a tissue-specific promoter to drive Cre expression, the gene is inactivated only in specific cell types, so that a tissue-specific knockout mouse is generated. A similar strategy can also be applied for Cre/lox-directed gene *activation*, for example, to switch on the expression of a reporter protein in a selected cell type. The most popular Cre reporter mouse is the so-called Rosa26 lacZ reporter line (R26R or R26-lacZ) [4]. This line produces β-galactosidase after Cre-mediated excision of a floxed STOP cassette from a reporter transgene that has been integrated into the broadly expressed *Rosa26* locus (*see* also Fig. 1a and Subheading 1.1).

To add inducibility to the Cre/lox system, ligand-dependent chimeric Cre recombinases, the so-called CreER recombinases, have been developed [5–8]. They consist of Cre fused to mutated hormone-binding domains of the estrogen receptor that bind the synthetic drug, 4-hydroxytamoxifen (OHT), but not the endogenous estrogen receptor ligand, estradiol. In the absence of OHT, the recombinase is retained in the cytosol. Binding of OHT induces the translocation of the recombinase into the nucleus, where it can recombine its loxP substrates (Fig. 1a, b). Thus, tamoxifen-dependent CreER recombinases allow for external temporal control of Cre activity. Their properties were continuously improved to decrease background activity in the absence of inducer and to increase the sensitivity and efficiency of tamoxifen-induced recombination in vivo. The $CreER^{T2}$ recombinase, which contains the human estrogen receptor ligand-binding domain with a G400V/M543A/L544A triple mutation, is currently the sharpest tool in the CreER box and its use is highly recommended for inducible site-specific recombination in the mouse [6, 9, 10]. To induce recombination in $CreER^{T2}$-expressing mice, animals are usually treated with tamoxifen, which is metabolized to OHT in the liver [11, 12]. Location, duration, and efficiency of $CreER^{T2}$-mediated recombination depend on the promoter driving recombinase expression as well as the dose, frequency, and route of tamoxifen administration. By using $CreER^{T2}$ or similar recombinases it is now feasible to control the onset of defined mutations in almost any tissue of the mouse, thus greatly facilitating the generation of sophisticated animal models for sporadic human diseases and drug action [13–15].

Another powerful application of site-specific recombination technology is the genetic labeling and mapping of specific cell

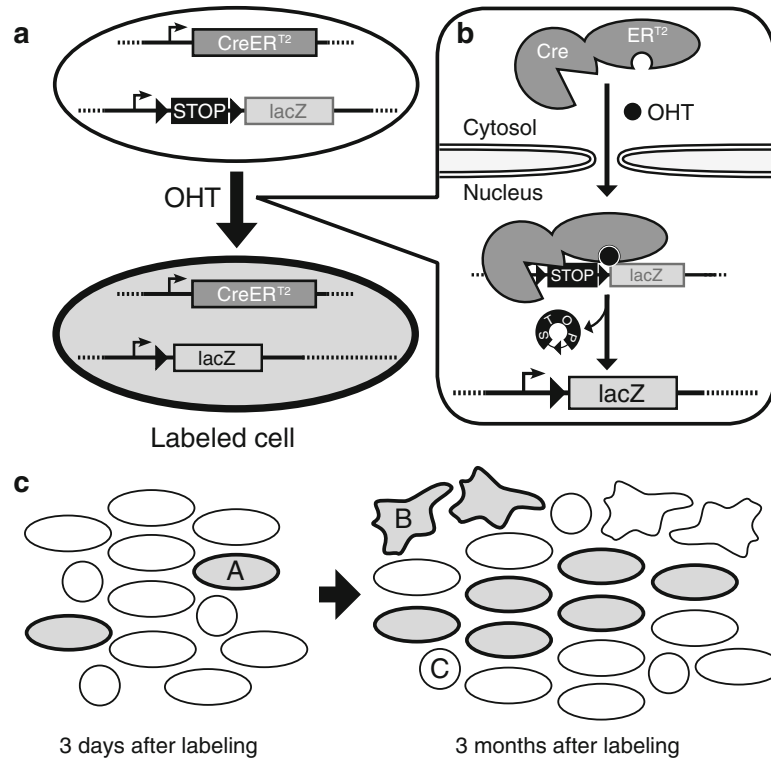

Fig. 1 Basic concept of Cre/lox-assisted GIFM experiments. (**a**) Experimental mice carry a cell type-specific CreER^{T2} transgene as well as a conditional *lacZ* reporter construct that expresses β-galactosidase after Cre-mediated excision of a loxP (*triangle*)-flanked STOP cassette. *Arrows* indicate promoter regions and transcriptional start points. Individual cells are genetically marked upon administration of tamoxifen to the animal. Note that tamoxifen is metabolized to OHT, which is presumably the ligand that activates CreER^{T2} in vivo. By choosing the appropriate time window, dose, and frequency of tamoxifen treatment, cells of a particular type are irreversibly labeled. Once recombined, reporter expression is independent of the presence of Cre activity. The activated reporter transgene is stably inherited to all progeny of the initially marked cell. Its gene product, bacterial β-galactosidase, can be detected any time after labeling by staining cells blue with X-Gal, a chromogenic substrate of β-galactosidase. (**b**) Mechanistically, binding of OHT (*black circle*) causes the translocation of CreER^{T2} from the cytosol into the nucleus, where it has access to its loxP substrates (*triangles*) and activates the *lacZ* reporter. (**c**) Illustration of a hypothetical GIFM experiment. Labeled cells (*grey*) were detected by X-Gal staining at two time points after tamoxifen treatment. Analysis of the tissue of interest at 3 days after tamoxifen induction revealed that only cell type "A" was initially marked (*left side*, *grey ovals*). Note that usually not *all* cells of a particular cell type are labeled in a given tissue (*left side*, *white ovals*). This is most likely due to transient CreER^{T2} activity combined with its mosaic expression in many tissue-specific CreER^{T2}-transgenic mouse lines. X-Gal staining of the tissue of interest at 3 months after labeling (*right side*) indicates that cell type "A" is apparently able to proliferate and maintain its population and that type-"A" cells may also give rise to cell type "B" but not to cell type "C"

lineages in vivo on a wild-type or a mutant genetic background [16]. Because site-specific excision of chromosomal DNA generates an essentially irreversible genomic alteration, which is stably inherited to all cells derived from the original recombined population, it is ideal for genetic labeling of a cell lineage. Cre-directed cell fate mapping is based on the intercrossing of a cell type-specific Cre mouse and a Cre reporter mouse (e.g., R26R) resulting in expression of the reporter gene (e.g., β-galactosidase) in all initially recombined cells and their progeny. Detection of the marked cells over time (e.g., by staining cells blue with X-Gal) reveals their contribution to embryonic and adult tissues. Ideally, Cre expression should be under the control of an endogenous gene locus specifying the cell lineage of interest, whereas the reporter transgene should be linked to a widely active promoter capable of driving its expression in all cell types and at all stages of pre- and postnatal development [17]. Genetic fate mapping in mice was first applied by developmental biologists to characterize cell lineages during embryogenesis [18, 19]. It is important to note that cell labeling with conventional Cre recombinase lacks external temporal control. It occurs in all cells that have ever expressed Cre, even transiently, until the time point of analysis. Therefore, fate maps that were generated with conventional Cre-transgenic mouse lines are cumulative and often difficult to interpret.

A significant advance was the introduction of genetic inducible fate mapping (GIFM) in mice [20, 21]. This method is based on the use of inducible recombinases like CreER^{T2} allowing the investigators to control *when* the cells are marked and, thus, to label relevant lineages at defined developmental stages. The general design of a GIFM experiment is illustrated in Fig. 1a, c. GIFM has been applied to study organogenesis during embryonic development [22, 23] as well as various biological processes in adult mice, for instance, the contribution of bone marrow-derived cells to tumor endothelium [24], the existence of native cardiac progenitor cells in the postnatal heart [25], and stem cell-driven tissue homeostasis [26]. Furthermore, as demonstrated by two of the first reports of GIFM in adult mice [27, 28], it is feasible to combine tamoxifen-induced cell marking and gene inactivation to monitor the fate of wild-type versus mutant cells during disease development, such as atherosclerosis. Clearly, GIFM in adult mice is a powerful approach to study biology in vivo and to provide new answers to old questions in human (patho-)physiology. The following sections describe some of the most useful reporter transgenes for Cre-directed cell labeling followed by protocols for CreER^{T2}-assisted GIFM in adult mice as exemplified by tracing of vascular smooth muscle cell (VSMC)-derived cells in a mouse model of atherosclerosis. Most of the protocols can be used as general guidelines for GIFM experiments in mice. Additional protocols for GIFM in embryonic and adult mice can be found elsewhere [29, 30].

1.1 Cre Reporter Transgenes for GIFM Studies

The selection of appropriate reporters to label a defined cell population is a critical factor in fate-mapping studies. As described above, the prototype of a Cre-inducible reporter construct consists of the reporter-encoding sequence preceded by a floxed STOP cassette. The latter contains at least polyadenylation signals and/or stop codons to block reporter gene transcription and/or translation in the absence of Cre. Cre-mediated excision of the STOP cassette is de facto irreversible and, thus, allows for permanent expression of the previously "silenced" reporter. Once activated, reporter expression is independent of subsequent Cre expression/activity. The stable inheritance of the activated reporter transgene from the originally labeled cell to its progeny is the underlying principle of all GIFM studies. Importantly, in order to generate a complete fate map, the activated reporter transgene should be expressed in *all* genetically marked cells. Therefore, the promoter driving transcription of the reporter transgene should have a broad, cell type-*independent* activity.

Reliable expression in virtually any tissue of pre- and postnatal mice can be achieved by targeted integration of reporter gene constructs into the *Rosa26* gene locus. This gene locus was identified in an embryonic stem cell-based gene trap screen for genes involved in embryogenesis [31, 32]. The name "Rosa" stands for "*r*everse *o*rientation *s*plice *a*cceptor" and was derived from the promoterless gene trap construct that contained a splice acceptor (SA) site and a β-galactosidase/neo (*βgeo*) fusion gene flanked by retroviral integration sites in reverse orientation. The gene trap construct had integrated into the first intron of a gene, which is now called *Rosa26*; it is located on mouse chromosome 6 at 113.07–113.08 Mbp. The *Rosa26* promoter directed ubiquitous *βgeo* expression in embryos as well as in newborn and adult mice [32]. Endogenous *Rosa26* transcripts do not encode proteins. Their biological function is still not understood. Mice with two gene trap- or transgene-containing *Rosa26* alleles do not show obvious developmental defects and are viable and fertile ([4, 32], and M. Thunemann, S. Feil, and R. Feil, unpublished data).

Because of its transcriptional accessibility in most embryonic and adult cells, the *Rosa26* gene locus has been frequently used for the targeted insertion of transgenes including numerous Cre-switchable reporter constructs. Table 1 lists examples of *Rosa26*-based Cre reporter mouse lines for GIFM studies. The first such line, R26-lacZ or R26R, was generated by Philippe Soriano, who introduced a SA site followed by a conditional *lacZ* gene construct into the first intron of the *Rosa26* gene locus, in close vicinity to the position where the original gene trap was integrated [4]. After Cre-mediated excision of a STOP cassette, which contains a neomycin resistance gene, *lacZ* expression is driven by the endogenous *Rosa26* promoter (Fig. 1a and Table 1). Recombined/labeled cells express bacterial β-galactosidase in their cytosol and

Table 1
Examples of *Rosa26*-based reporter constructs for GIFM studies

Name	Reporter expression after recombination[a]	Transgene structure[a,b]	Detection in adult mice	JAX stock[c]	Reference
Rosa26 promoter-driven constructs[d]					
R26-lacZ (R26R)	β-Galactosidase		X-Gal staining	003309 003310 003474	[4]
R26-EGFP	GFP		Antibody staining[e]	004077	[47]
R26-EYFP	YFP		Antibody staining[e]	006148	[48]
Rosa26/CAG promoter-driven constructs[d]					
R26-mT/mG	Membrane-targeted GFP		Fluorescence	007576 007676	[36]
R26-NZG	Nuclear β-galactosidase or GFP[f]		X-Gal staining or fluorescence[f]	012429	[49]
R26-Ai14	RFP		Fluorescence	007914	[37]
R26-Confetti	nGFP or YFP or RFP or mCFP[g]		Fluorescence[g]	013731 017492	[38]

Abbreviations: *CD* cytosine deaminase, *CFP* cyan fluorescent protein, *EGFP* enhanced GFP, *EYFP* enhanced YFP, *GFP* green fluorescent protein, *lacZ* gene encoding bacterial β-galactosidase, *mCFP* membrane-targeted CFP, *mG* membrane-targeted GFP, *mT* membrane-targeted tandem-dimer tomato protein, *neo*^{R} neomycin resistance gene, *nGFP* nuclear-targeted GFP, *nlacZ* nuclear-targeted β-galactosidase, P_{CAG} cytomegalovirus enhancer/chicken β-actin hybrid promoter, *Puro* puromycin resistance gene, *RFP* red fluorescent protein, *SA* splice acceptor site, *WPRE* woodchuck hepatitis virus posttranscriptional regulatory element, *YFP* yellow fluorescent protein

[a]For fluorescent proteins, only principal colors (e.g., CFP, GFP, RFP, YFP) and subcellular targeting (e.g., membrane- or nuclear-targeted) are denoted; for details on the respective fluorescent protein(s) used in each construct (e.g., enhanced GFP), the reader is referred to the original publication

[b]Symbols in constructs: Black triangles, loxP sites; white triangles, FRT sites; small black rectangle, attL site; black rectangle with white hexagonal "STOP sign," stop codons, and/or polyadenylation signals

[c]Mouse lines can be obtained from The Jackson Laboratories see indicated stock numbers (http://jaxmice.jax.org)

[d]In the first group of reporter constructs, reporter expression is driven by the endogenous *Rosa26* promoter (not shown); these constructs are integrated into the first intron of the *Rosa26* gene and contain a splice acceptor site (SA), so that mRNA appropriate for reporter gene expression is generated from the recombined transgene. The second group of reporter constructs contains the CAG promoter that drives reporter gene transcription presumably independent of the *Rosa26* promoter

[e]Reporter fluorescence in hematopoietic cells of adult mice might be sufficient for fluorescence-activated cell sorting

[f]In the R26-NZG reporter construct, the nlacZ gene is flanked by FRT recognition sites (white triangles) for Flp recombinase. In the basal reporter configuration (see table), Cre-mediated recombination of loxP sites (black triangles) activates nlacZ expression. After Flp-mediated excision of the nlacZ gene, the transgene is converted into a Cre reporter that expresses enhanced GFP upon Cre-mediated recombination

[g]In the R26-Confetti reporter construct, the arrangement and relative orientation of the loxP sites combined with transient Cre recombinase activity result in stochastic activation of the expression of one out of four fluorescent proteins

can be detected by histochemical X-Gal staining of fixed tissues. Note that the fixation step prior to X-Gal staining precludes the analysis and isolation of viable cells. Alternatively, β-galactosidase can be detected by antibody staining of fixed tissues. All in all, the R26R line has proven to be a very useful reporter for GIFM studies, because (a) *lacZ* can principally be expressed in a wide range of embryonic and adult cell types of this reporter line without interfering with their physiological functions and (b) the enzymatic nature of X-Gal staining mediated by bacterial β-galactosidase combined with the low background from endogenous β-galactosidases in most mouse tissues allows the detection of labeled cells with high sensitivity.

Meanwhile, *Rosa26*-based Cre reporter mouse lines have been generated that express a variety of reporter proteins with potential advantages over cytoplasmic β-galactosidase, such as nuclear β-galactosidase or fluorescent proteins derived from green fluorescent protein (GFP) (Table 1). For instance, nuclear-localized β-galactosidase can provide a higher sensitivity and resolution of cell detection than cytoplasmic β-galactosidase. An advantage of fluorescent proteins over β-galactosidase is the possibility that labeled cells can be detected in situ in unfixed tissues, thus principally enabling the isolation of viable cells. However, a review of the relevant literature and our own experience (M. Thunemann and R. Feil, unpublished data) indicate that in many cell types of adult mice the level of fluorescent protein expression driven by the endogenous *Rosa26* promoter is too low for direct fluorescence monitoring. Therefore, many studies could not utilize the full potential of fluorescent reporters as they had to be detected by antibody staining of fixed tissues.

An elegant strategy to increase the expression of transgenes integrated into the *Rosa26* locus is the co-insertion of the strong and broadly active CAG promoter, which consists of a cytomegalovirus enhancer and a modified chicken β-actin promoter [33]. The expression level of *Rosa26*/CAG-driven reporter constructs is usually ten times higher as compared to expression from the endogenous *Rosa26* promoter alone and superior to other strong promoters that have been integrated into this locus [34, 35]. Presently, several *Rosa26*/CAG-based Cre reporter lines are available that show sufficient signal intensities for reliable detection of fluorescent proteins in cells of adult mice (Table 1). For instance, in the R26-mT/mG line, Cre induces a switch from expression of a membrane-targeted red fluorescent protein to a membrane-targeted GFP [36]; in the R26-Ai14 line, Cre activates expression of a red fluorescent protein [37]; and in the R26-Confetti line, Cre-mediated recombination results in the stochastic activation of one out of four different fluorescent proteins, which simplifies the clonal analysis of cell fate [38].

2 Materials

2.1 Generation of Experimental Mice

2.1.1 Mouse Breeding

1. In general, two to three mouse lines are required for GIFM studies: (a) an inducible Cre line expressing, for instance, the CreER^{T2} recombinase in the cell type of interest for initial labeling (examples of available CreER^{T2} lines are given in [14, 15]); (b) a Cre reporter line that, after Cre-mediated recombination, has the potential to express the reporter protein in a wide range of cell types and tissues (for examples, *see* Table 1 and [39, 15]); and, optionally, (c) a third line that carries a gene mutation or a disease-promoting genetic modification. Here, we describe GIFM of VSMC-derived cells in atherosclerotic plaques by using the following three mouse lines:
   (a) The SM-CreER^{T2} line [10] expresses CreER^{T2} under the control of the endogenous SM22α promoter selectively in smooth muscle cells of adult mice (transgene integration site: chromosome 9).
   (b) The R26R Cre reporter line [4] shows broad expression of cytoplasmic β-galactosidase from the recombined reporter allele in adult mice (transgene integration site: chromosome 6).
   (c) The ApoE knockout line [40] carries a null allele of the gene encoding apolipoprotein E and is commonly used as a mouse model of atherosclerosis (gene location: chromosome 7).

   At least five male and five to ten female mice of each mouse line should be available, preferably on the same genetic background, e.g., C57BL/6.
2. Mouse facility with standard equipment and room capacity for approx. 30 type III and 50 type II cages, in which approx. eight and four mice can be housed, respectively.

2.1.2 PCR Genotyping of Mice Using Ear Punch Tissue

1. Ear punch tool.
2. Thermocycler.
3. Standard equipment for agarose gel electrophoresis.
4. 50 mg/mL Proteinase K (in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0); store aliquots at −20 °C.
5. 10× PCR buffer: 500 mM KCl, 100 mM Tris–HCl, pH 8.0, 15 mM $MgCl_2$; store aliquots at −20 °C.
6. 10× PCR buffer with dNTPs: 2 mM of each dNTP (dGTP, dATP, dTTP, dCTP) in 10× PCR buffer; store aliquots at −20 °C.
7. Taq polymerase; store at −20 °C.
8. Oligonucleotide primers: Each 25 pmol/μL (in H_2O), store at −20 °C. The primer sequences for detection of the SM-CreER^{T2}, R26R, and mutated ApoE alleles and the corresponding wild-type alleles are as follows:

(a) SM-CreER^{T2}:
RF67: 5′-CTC AGA GTG GAA GGC CTG CTT-3′.
RF90: 5′-CAC ACC ATT CTT CAG CCA CA-3′.
SC135: 5′-GGC GAT CCC TGA ACA TGT CC-3′.

(b) R26R:
BB01: 5′-CTC TGC TGC CTC CTG GCT TCT-3′.
BB02: 5′-CGA GGC GGA TCA CAA GCA ATA-3′.
RF127: 5′-GCG AAG AGT TTG TCC TCA ACC-3′.

(c) ApoE:
RF115: 5′-GCC TAG CCG AGG GAG AGC CG-3′.
RF117: 5′-GCC GCC CCG ACT GCA TCT-3′.
RF151: 5′-AGT TCT TGT GTG ACT TGG GAG-3′.

2.2 Induction of Cell Labeling with Tamoxifen

1. Tamoxifen (Sigma T5648); protect from light.
2. Ethanol.
3. Sunflower oil.
4. Syringes (1 mL, graded in 100-μL intervals) and 22-gauge needles.

2.3 Induction of Atherosclerosis

1. Atherogenic diet (20 % fat, 1.5 % cholesterol by weight).

2.4 Detection of Labeled Cells

2.4.1 Isolation of Mouse Tissues of Interest

1. CO_2 for euthanasia.
2. Syringe pump or peristaltic pump for perfusion fixation.
3. 27-Gauge needles.
4. Dissecting tools (forceps, scissors, etc.).
5. Collecting tray for fixative solution.

2.4.2 X-Gal Staining of Whole Mounts and Preparation of Paraffin Sections

For X-Gal Staining of Whole Mounts

1. Phosphate-buffered saline (PBS): 135 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4. Prepare at least 2 L and sterilize by autoclaving; store at room temperature (RT).
2. Fixative solution for X-Gal staining (prepare under a fume hood): 2 % Formaldehyde, 0.2 % glutaraldehyde in PBS (~50 mL per mouse); store at 4 °C for a maximum of 4 weeks.
3. X-Gal stock solution: 40 mg/mL 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) in dimethylsulfoxide. Prepare 10 mL and store 1-mL aliquots at −20 °C.
4. X-Gal staining solution: 2 mM $MgCl_2$, 2.5 mM $K_3Fe(CN)_6$, 2.5 mM $K_4Fe(CN)_6$ in PBS; prepare 500 mL and store in the dark at RT. Before use, add X-Gal stock solution (40 mg/mL) to a final concentration of 1 mg/mL of X-Gal. Depending on

the amount of tissues to be stained, prepare 2–20 mL of X-Gal staining solution per mouse.

5. Stereomicroscope.

For Preparation of Paraffin Sections of X-Gal-Stained Whole Mounts

1. Ethanol.
2. Toluene.
3. Paraffin.
4. Microtome.
5. Water bath.
6. Polylysine-coated microscope slides.
7. Aqueous mounting medium.
8. Light microscope.

2.4.3 X-Gal Staining of Cryosections

1. 30 % Sucrose (in PBS).
2. OCT embedding medium.
3. Cryostat.
4. Superfrost Plus microscope slides.
5. Glass coplin jars.
6. Solutions for fixation and X-Gal staining (*see* Subheading 2.4.2).
7. Aqueous mounting medium.
8. Light microscope.

2.4.4 Fluorescence Detection in Whole Mounts and Cryosections

For Fluorescence Detection in Whole Mounts

1. PBS (*see* Subheading 2.4.2).
2. Fixative solution for fluorescence detection (prepare under a fume hood): 4 % Paraformaldehyde (PFA) in PBS (~50 mL per mouse):
   - Add 4 g PFA per 100 mL PBS.
   - Heat to 60 °C and add few drops of 1 M NaOH to dissolve PFA.
   - Allow to cool to RT and filtrate through a paper filter.
   - Store at 4 °C for a maximum of 2 weeks.
3. Fluorescence stereomicroscope with appropriate filter sets (*see*, for example, legend to Fig. 2c).

For Fluorescence Detection in Cryosections, in Addition

1. 30 % Sucrose (in PBS).
2. OCT embedding medium.
3. Cryostat.
4. Superfrost Plus microscope slides.
5. Mounting medium: 80 % Glycerol; store at RT.
6. Fluorescence microscope with appropriate filter sets (*see*, for example, legend to Fig. 2c).

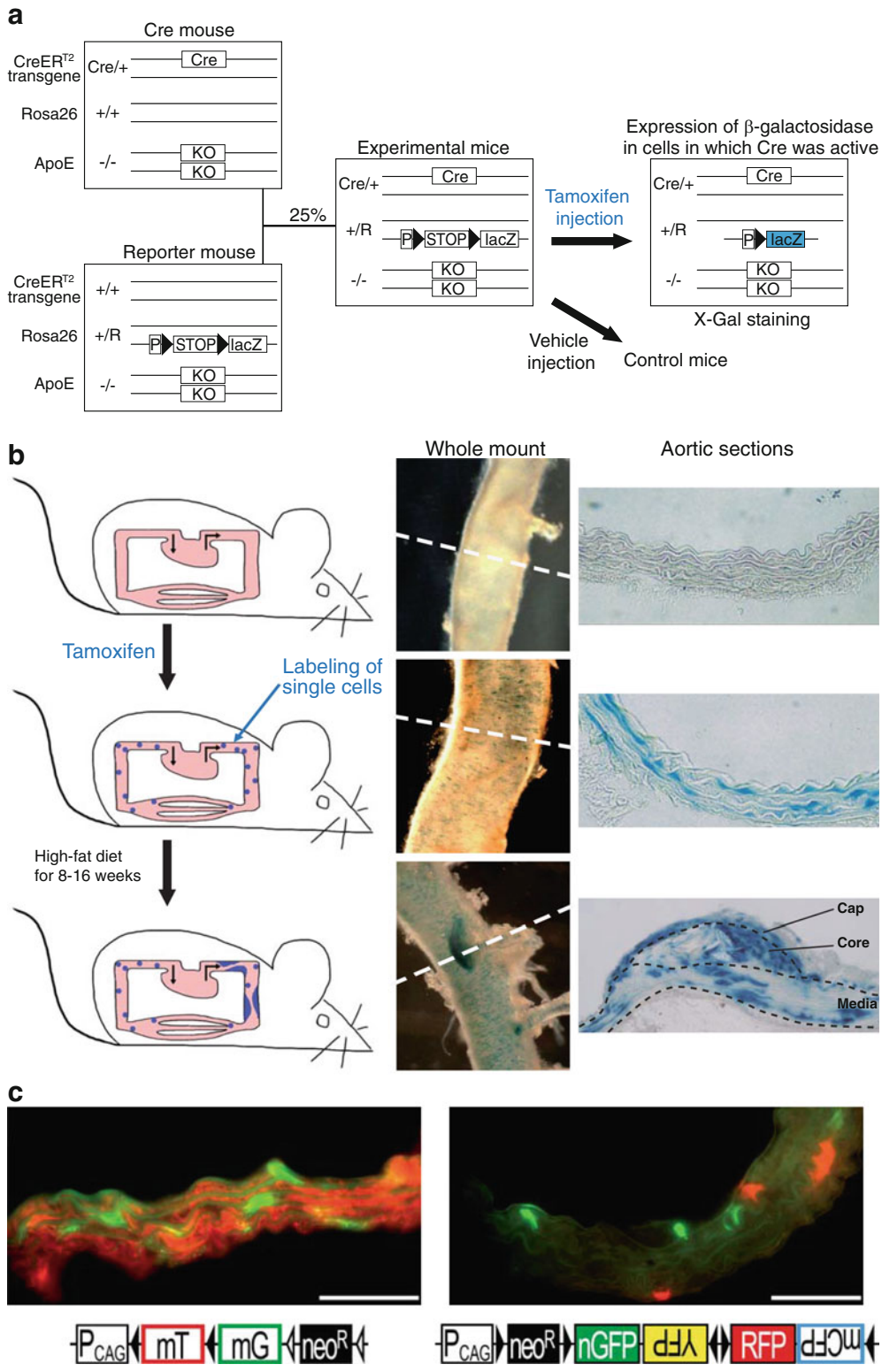

Fig. 2 GIFM of VSMCs in a mouse model of atherosclerosis. (**a**) Breeding scheme for the generation of experimental animals. ApoE(–/–) mice that are also heterozygous for the smooth muscle-specific SM-CreER^{T2} transgene (*upper box*) are bred to ApoE(–/–) mice that are also heterozygous for the R26R *lacZ* reporter transgene

3 Methods

This section outlines protocols for GIFM studies to elucidate the fate of medial VSMCs during the development of atherosclerotic plaques in the ApoE-deficient mouse model of atherosclerosis. After experimental mice with the genotype SM-CreER^{T2}(Cre/+); R26R(+/R);ApoE(−/−) have been generated (Subheading 3.1 and Fig. 2a), the GIFM experiment is performed as follows:

1. First, VSMCs in the medial layer of the aorta are labeled by treating the non-atherosclerotic animals for a short time with tamoxifen, i.e., labeling of medial VSMCs is induced by a "tamoxifen pulse" (Subheading 3.2) (*see* **Note 1**).

Fig. 2 (continued) integrated into the *Rosa26* locus (*lower box*). According to Mendel's rule, 25 % of offspring are expected to be experimental mice carrying both transgenes on an ApoE null background (*middle box*). Most of these mice are injected with tamoxifen to induce CreER^{T2} activity and, thereby, reporter gene recombination and expression of β-galactosidase (*right box*). Genetically labeled cells can be detected by staining them *blue* with X-Gal at various time points after tamoxifen induction. Some experimental mice are injected with vehicle to serve as controls for background Cre and reporter activity. Note that the activated *lacZ* reporter (*right box*) is present only in those cells, in which the CreER^{T2} recombinase was active; all other cells still carry the non-recombined *lacZ* reporter transgene (*middle box*). (**b**) GIFM of VSMCs in atherosclerotic plaques. Four-week-old experimental mice (see panel **a**) were injected intraperitoneally with tamoxifen for five consecutive days (1 mg tamoxifen per day) to induce labeling of individual VSMCs in the medial layer of the non-atherosclerotic aorta (*blue spots* in the *middle mouse*). Most of the "labeled" mice were then fed a high-fat diet to promote the development of atherosclerotic lesions potentially containing marked cells (*blue plaques* in the *lower mouse*). Aortae were isolated from three experimental groups (represented by the mice in the *left column*), stained with X-Gal (whole mounts in the *middle column*), and subsequently sectioned in paraffin (*right column*). Firstly, X-Gal staining of tissues from vehicle-treated mice showed that there was no background staining in the aorta in the absence of tamoxifen (*upper row*). Secondly, the analysis of tamoxifen-treated mice shortly after tamoxifen induction confirmed that the initially labeled cells were indeed medial VSMCs (*middle row*) and that other cell types, such as bone marrow cells, were not marked (data not shown). Note that recombination is mosaic, so that only a subset of the VSMCs is labeled. Thirdly, older mice that were on the atherogenic diet for 12 weeks had atherosclerotic plaques containing X-Gal-stained cells (*lower row*). Marked cells were present in the media and in the fibrous cap, two plaque regions that are well known to contain VSMCs. Interestingly, X-Gal-positive cells were also detected in the plaque core indicating a previously unknown VSMC-derived population of plaque cells. (**c**) Representative fluorescence images of cryosectioned aortae from tamoxifen-treated SM-CreER^{T2} mice carrying either the R26-mT/mG reporter (*left*) or the R26-Confetti reporter (*right*). The structure of the respective reporter constructs is shown in the non-recombined state. Scale bars: 50 μm. Filter set for CFP: excitation BP445/20, emission LP480; filter set for YFP: excitation BP497/16, emission BP535/22; filter set for RFPs: excitation BP543/22, emission BP610/75. Sections in **b** and **c** are orientated with the vessel lumen towards the top. Symbols and abbreviations: triangles, loxP sites; +, wild-type; Cre or CreER^{T2}, SM-CreER^{T2} transgene that is driven by the endogenous smooth muscle-specific SM22α promoter; KO, knockout; P, endogenous *Rosa26* promoter; STOP, sequence that prevents expression of the downstream *lacZ* gene encoding β-galactosidase; for abbreviations of elements of the reporter constructs shown in panel **c**, *see* Table 1. For further explanations, see text. The X-Gal-stained aortic sections shown in panel **b**, *upper right* and *middle right*, were taken from Wolfsgruber et al. (2003) Proc Natl Acad Sci U S A 100(23):13519–13524 (© 2003 National Academy of Sciences, U.S.A.)

2. Subsequent to genetic labeling of VSMCs, atherosclerosis is induced by feeding the mice a high-fat diet for several months (Subheading 3.3).
3. The fate of the initially labeled medial VSMCs is analyzed at various time points during the development of atherosclerotic lesions (Subheading 3.4).

The experimental design and representative results are shown in Fig. 2b. For correct interpretation of the fate map, it is of paramount importance that the labeled cells in the atherosclerotic plaques were indeed derived from medial VSMCs that have been genetically marked during the defined tamoxifen pulse. It must be excluded that other cell types (e.g., circulating blood cells or bone marrow-derived cells) have also been marked and, therefore, could also contribute to labeled plaque cells (*see* **Note 2**).

3.1 Generation of Experimental Mice

3.1.1 Mouse Breeding

Some guidelines for efficient mouse breeding are given in **Note 3**. Here, we describe the crossing of three mouse lines, the SM-CreER^{T2} line (also referred to as "Cre mouse"), the R26R Cre reporter line (also referred to as "reporter mouse"), and the ApoE knockout line, to generate experimental animals for GIFM of VSMC-derived cells in atherosclerotic plaques (*see* also Subheading 2.1.1). The respective breeding scheme is shown in Fig. 2a. Note that the parental mice are heterozygous for the SM-CreER^{T2} allele (genotype: Cre/+) or for the R26R reporter allele (genotype: +/R), respectively, and both of them are homozygous for the ApoE null allele (genotype: −/−). These parental mice have to be generated by at least two successive rounds of breeding Cre mice as well as reporter mice with homozygous ApoE(−/−) animals (not shown in Fig. 2a). According to Mendel's rule, crossing of SM-CreER^{T2} (Cre/+);ApoE(−/−) mice with R26R(+/R);ApoE(−/−) mice should yield 25 % SM-CreER^{T2}(Cre/+);R26R(+/R);ApoE(−/−) progeny that can be used for GIFM experiments (*see* **Note 4**). Most of the experimental animals are injected with tamoxifen to induce cell labeling (Subheading 3.2). In particular, at the beginning of a new GIFM study, some of the experimental mice should be injected with vehicle and analyzed in parallel to the tamoxifen-treated animals to control for background cell labeling. Additional important controls should be performed to verify the cell-type specificity of the Cre mouse as well as the widespread expression profile of the recombined reporter allele (*see* **Note 2**)

The breeding strategy can easily be adapted to label other cell types by exchanging the Cre mouse, or if other reporters or disease/knockout models are being used. For instance, the combination of a CreER^{T2} mouse, reporter mouse, and conditional knockout mouse carrying a floxed target gene should result in reporter gene activation and target gene inactivation simultaneously in the

same cells after tamoxifen treatment, so that the gene's effect on cell fate can be directly analyzed [28] (*see* **Note 5**). An overview of published Cre lines and Cre reporter lines and their activity profiles can be found in the Cre-X-database (http://nagy.mshri.on.ca/cre) and The Jackson Cre Repository (http://cre.jax.org/index.html). Many of these mouse lines can be obtained from The Jackson Laboratory (http://jaxmice.jax.org).

3.1.2 PCR Genotyping of Mice Using Ear Punch Tissue

Mice used in GIFM studies carry at least two, but sometimes also three, four, or more, transgenes or other genetic modifications in a specific combination (Fig. 2a). The generation of such compound mutants requires multiple rounds of breeding and genotyping. It is associated with considerable expenditure of time and money. The efficient generation of experimental animals depends on reliable genotyping. Incorrect genotypes can lead to time-consuming additional breeding steps, loss of mouse lines, or, if undetected, incorrect interpretation of GIFM results. Below we describe a simple, fast, and reliable PCR genotyping protocol based on the isolation of DNA from small ear punch tissue that is obtained during numbering of mice (*see* **Note 6**).

1. Place ear punch tissue (ca. 1–2 mm in diameter) in a microcentrifuge tube.
2. Add 50 μL freshly prepared DNA digestion buffer (0.5 mg/mL proteinase K in 1× PCR buffer) and incubate overnight at 55 °C.
3. Mix and centrifuge for 3 min at 10,000 × *g*.
4. Incubate the supernatant for 15 min at 95 °C. Centrifuge for 3 min at 10,000 × *g*. It is recommended to use 2 μL of the supernatant of this DNA preparation immediately for PCR genotyping. Alternatively, it can be stored at −20 °C.
5. The following PCR conditions are used to genotype the below-named mouse lines. For each mouse line, a "three-primer" PCR is performed that detects the modified and respective wild-type allele in the same sample. Similar PCR conditions can be applied for the detection of other Cre transgenes (*see* **Note 7**).
   - The standard reaction mix (25 μL) contains:

DNA	2 μL
Primer A (25 pmol/μL)	0.3 μL
Primer B (25 pmol/μL)	0.3 μL
Primer C (25 pmol/μL)	0.3 μL
10× PCR buffer	2.5 μL
H_2O	19.3 μL
Taq polymerase (5 U/μL)	0.3 μL

- Standard PCR program: Initial denaturation step for 5 min at 95 °C; then 35 cycles of denaturation (10 s at 95 °C), annealing (30 s at 65 °C), and polymerization (30 s at 72 °C); and a final polymerization step for 5 min at 72 °C; store at 4 °C.
- The expected product sizes are as follows:

Mouse line/allele	Detected by primer pair	Product size
(a) SM-CreER^{T2} line		
Wild-type allele (+)	RF67 + RF90	290 bp
Cre allele (Cre)	RF67 + SC135	220 bp
(b) R26R line		
Wild-type allele (+)	BB01 + BB02	322 bp
Reporter allele (R)	BB01 + RF127	~210 bp
(c) ApoE line		
Wild-type allele (+)	RF115 + RF151	163 bp
Knockout allele (−)	RF115 + RF117	245 bp

6. Load 12 μL of the PCR reaction on a 2 % agarose gel and analyze products by agarose gel electrophoresis.

3.2 Induction of Cell Labeling with Tamoxifen

This section describes our standard protocol for the induction of cell labeling in adult SM-CreER^{T2}(Cre/+);R26R(+/R);ApoE(−/−) mice. Five consecutive daily injections of 1 mg tamoxifen result in mosaic labeling of ~10–30 % of the VSMCs in the media of the non-atherosclerotic aorta (Fig. 2b, middle row). Variations of this labeling scheme might be required, if other CreER^{T2} mice are used or if cells are to be marked during embryogenesis, in other tissues, or with other efficiencies (*see* **Note 8**). The injection of mice with tamoxifen is an animal experiment and has to be performed in accordance with the local guidelines for animal welfare.

1. Suspend 100 mg of tamoxifen (Sigma T5648) in 0.5 mL of ethanol in a 15-mL tube and add 9.5 mL of sunflower oil (do not autoclave). Mix the suspension thoroughly using a vortexer and sonicate in an ultrasonic bath for 5 min at 37 °C (*see* **Note 9**). In addition, prepare 1.0 mL of sunflower oil containing 5 % ethanol for vehicle injection. Protect tamoxifen and tamoxifen solutions from light. The tamoxifen solution (10 mg/mL) can be stored at −20 °C for up to 4 weeks.
2. Before use, thaw and sonicate the tamoxifen solution in an ultrasonic bath at 37 °C for 5 min.

3. Experimental mice including controls (*see* **Note 2**, Subheading 3.1.1, and Fig. 2a) are injected with tamoxifen or vehicle at an age of 4–5 weeks. Inject each mouse intraperitoneally (i.p.) with 100 μL of tamoxifen solution (corresponds to 1 mg of tamoxifen) or with vehicle for five consecutive days. Do not house tamoxifen-treated mice together with vehicle-treated or untreated animals in the same cage (*see* **Note 10**).
4. Sacrifice some of the animals 3 days after the last injection to characterize the initially labeled cell population (*see* **Note 2**).

3.3 Induction of Atherosclerosis

1. Start feeding the vehicle- and tamoxifen-treated mice, which are on an ApoE-deficient genetic background, with the atherogenic diet at the age of 8 weeks.
2. Monitor labeled cells in atherosclerotic plaques after 8–16 weeks atherogenic diet (*see* **Note 11**).

3.4 Detection of Labeled Cells

Labeled cells of the isolated tissues (Subheading 3.4.1) can be detected in several ways:

If a *lacZ* reporter is used, the options are the following:

- X-Gal staining of whole mounts and preparation of paraffin sections from stained tissues (Subheading 3.4.2): Representative results of X-Gal-stained normal and atherosclerotic aorta are shown in Fig. 2b.
- X-Gal staining of cryosections (Subheading 3.4.3).

If a fluorescence reporter is used, the options are the following:

- Fluorescence detection in whole mounts and/or cryosections (Subheading 3.4.4): Fluorescent cells may be observed in fixed or unfixed tissue. The latter option allows, in principle, the isolation of viable marked cells for further analyses (*see* **Note 12**). Examples of aortic sections with fluorescently labeled cells are shown in Fig. 2c.

3.4.1 Isolation of Mouse Tissues of Interest

1. Sacrifice the mouse with CO_2 (*see* **Note 13**).
2. Perfusion fixation:
   The goal of this procedure is to clear the vascular system of blood, to achieve uniform fixation, especially in bigger organs, and to obtain tissue preparations that are appropriate for sectioning (*see* **Note 14**).
   - Set up the perfusion system.
   - Place the mouse in a collecting tray and arrange the whole setup in a fume hood (*see* **Note 15**).
   - Wet the fur of the animal with 70 % ethanol.
   - Open abdominal and thoracic cavity.

- Insert a 27-gauge needle about 2 mm into the left ventricle of the heart oriented towards the ascending aorta. Be careful to hold the needle in place throughout the perfusion procedure. Make an incision to the right atrium to create an outlet without damaging the descending aorta. Perfuse the animal at 60 mL/h with ice-cold PBS for 5 min followed by ice-cold fixative solution for 10 min (*see* **Note 16**).

3. The composition of the fixative solution depends on further processing:
   - For X-Gal staining of whole mounts (Subheading 3.4.2) or cryosections (Subheading 3.4.3), the fixative contains 2 % formaldehyde and 0.2 % glutaraldehyde (in PBS).
   - For fluorescence detection in whole mounts or cryosections (Subheading 3.4.4), tissues are fixed with 4 % PFA (in PBS). Alternatively, fluorescent cells can be observed in unfixed tissue (*see* **Note 12**).
4. Dissect aorta and other tissues of interest. The aorta is isolated as follows:
   - Remove internal organs and place the body with the tail to the left and the head to the right (for right-handed persons). The alignment helps to keep orientation during dissection of the aorta, especially when remaining blood, fat, and/or muscle may prevent a clear view on the aorta. Cut ascending vessels, trachea, and esophagus below the pharynx; grab the heart with forceps; and lift it carefully to set the aorta under some tension. Cut along the spine towards the tail. Avoid rupture of the aorta during dissection; it is very difficult to find its loose end in the body.
   - Transfer the excised aorta (still attached to the heart) into a 10-cm Petri dish with PBS and remove the heart by carefully cutting the left ventricle in 5 mm distance around the aortic valve.
5. Transfer aorta into a 1.5-mL microcentrifuge tube with 1 mL of fixative solution and other tissues into a 15-mL tube with 10 mL of fixative solution (*see* **step 3**). Tissues of the same mouse can be pooled into the same tube. Postfix tissues for 30 min at RT with gentle shaking (*see* **Note 16**).
6. Wash fixed tissues three times for 5 min with PBS at RT with gentle shaking and process immediately for further analysis.

3.4.2 X-Gal Staining of Whole Mounts and Preparation of Paraffin Sections

1. Incubate fixed aorta (Subheading 3.4.1) in 1.5 mL and other tissues in 10 mL of X-Gal staining solution overnight in the dark at RT with gentle shaking (*see* **Note 17**).
2. Wash tissues three times for 5 min with PBS at RT with gentle shaking.

3. Analyze X-Gal-stained whole mounts in a Petri dish using a stereomicroscope. For photo-documentation, various lighting conditions should be tested. Reflections can be avoided by taking pictures from samples totally covered with PBS.
4. Store X-Gal-stained tissues in PBS at 4 °C until further processing. For long-time storage (>1 week) at 4 °C, transfer tissues into 70 % ethanol (*see* **Note 18**). For detailed analysis of labeled cells, paraffin sections can be prepared (*see* **Note 19**).

Preparation of paraffin sections from X-Gal-stained whole mounts (perform **steps 5**–7 and **12** in a fume hood):

5. Dehydrate the tissue in 70, 80, and 95 % ethanol, 45 min each, followed by three changes of absolute ethanol, 1 h each.
6. Clear tissue in toluene for 30 min. Exchange toluene and incubate tissue for another 15 min (*see* **Note 20**).
7. Isolate the region of interest for sectioning. Use a scalpel and trim tissue to the appropriate size for embedding in paraffin.
8. Immerse the tissue in paraffin, 3× for 1 h to overnight.
9. Embed the tissue in paraffin. Place aorta in a small mold in appropriate orientation for cross-sectioning. Paraffin tissue blocks can be stored at RT for years.
10. Section the paraffin block at 6–10 μm thickness on a microtome and let sections flatten by floating in a 40 °C water bath containing distilled water.
11. Transfer sections onto glass slides suitable for immunohistochemistry (e.g., polylysine-coated slides). Allow slides to dry overnight at 37 °C and store them at RT.
12. Deparaffinize and rehydrate sections. Place slides in a rack and perform the following washes:
    Toluene, 2× 3 min (*see* **Note 20**); toluene/ethanol (1:1), 1× 3 min; ethanol, 2× 3 min; 95 % ethanol, 1× 3 min; 70 % ethanol, 1× 3 min; 50 % ethanol, 1× 3 min; rinse slides with cold tap water.
13. If required, X-Gal-stained paraffin sections can be co-stained by conventional histochemistry (e.g., eosin, nuclear fast red) and/or immunohistochemistry (*see* **Note 21**).
14. Add aqueous mounting medium and a glass cover slip, and allow the mounting medium to solidify.
15. Observe sections with a light microscope under bright-field illumination.

3.4.3 X-Gal Staining of Cryosections

1. Embed fixed aorta or other tissues (Subheading 3.4.1) for cryosectioning (*see* **Note 22**). To preserve tissue architecture during freezing, soak tissues in 30 % Sucrose solution (aorta in

1.5 mL and other tissues pooled in 10 mL) until tissues sink. This can take approx. 1–3 days.

2. Transfer tissues into embedding molds (e.g., self-made from aluminium foil) containing OCT embedding medium. Carefully remove air bubbles with forceps or needles and freeze on dry ice.
3. Remove mold from dry ice, wrap in labeled aluminium foil, and store at −80 °C until sectioning.
4. Prepare 10-µm sections using a cryostat and mount them on Superfrost Plus slides. Air-dry slides for 1 h at RT and continue with X-Gal staining. Cryosections can be stored at −80 °C.
5. Transfer slides into a glass coplin jar and incubate them for 5 min in PBS at RT.
6. Incubate slides in X-Gal staining solution at 37 °C in the dark until color develops (~2–24 h).
7. Wash slides carefully three times with PBS at RT.
8. If required, X-Gal-stained cryosections can be co-stained by conventional histochemistry (e.g., eosin, nuclear fast red) and/or immunohistochemistry (*see* **Note 21**).
9. Add aqueous mounting medium and a glass cover slip, and allow the mounting medium to solidify.
10. Observe sections with a light microscope under bright-field illumination.

3.4.4 Fluorescence Detection in Whole Mounts and Cryosections

Fluorescence Detection in Whole Mounts

1. Place PFA-fixed or unfixed tissue (Subheading 3.4.1) in a Petri dish with PBS (*see* **Note 12**).
2. Observe whole mount under a fluorescence stereomicroscope (*see* **Note 23**) and acquire images using appropriate software.

Fluorescence Detection in Cryosections

1. Use PFA-fixed or unfixed tissue (Subheading 3.4.1) for cryosectioning (Subheading 3.4.3, **steps 1–3**). For rapid sectioning of unfixed tissue, soaking in 30 % Sucrose is omitted. Protect tissues from light.
2. Prepare 10-µm sections using a cryostat and mount them on Superfrost Plus slides. Air-dry slides for 1 h at RT in the dark. Cryosections can be stored at −80 °C.
3. Mount sections in 80 % glycerol and analyze them by fluorescence microscopy (*see* **Note 23**). Acquire images using appropriate software.
4. Cryosections can be co-stained with fluorescent dyes (e.g., 1 µg/mL Hoechst 33258 in PBS to stain cell nuclei) and/or by immunofluorescence. The chosen fluorophores should be compatible with the simultaneous detection of the respective fluorescent reporter protein(s).

4 Notes

1. In the GIFM strategy described here, cell labeling is induced by a tamoxifen pulse given to the animal. Tamoxifen is metabolized to OHT, which is presumably the actual ligand that binds and activates the CreER^{T2} recombinase. The window of cell marking and, thus, the temporal resolution that can be provided for the GIFM study are determined by the pharmacokinetics of tamoxifen in the mouse or, more precisely, by the rise and fall of OHT [11, 12]. After a single dose of tamoxifen, recombination events that activate reporter gene expression appear to begin within ~6–12 h and continue for ~24–36 h, thus defining a minimal labeling window of ~24 h [20, 21]. Considering the lag phase between activation of the reporter transgene by recombination at the DNA level and its phenotypic expression as protein, the analysis of initially marked cells is usually performed not earlier than 48 h after tamoxifen treatment.
2. In GIFM experiments several controls are essential. First, it is very important to analyze which cell types have been labeled *initially* during the pulsed tamoxifen treatment. Ideally, only one cell type should be marked to allow firm conclusions about the origin of labeled cells visualized at any later time point. Thus, some animals are analyzed for marked cells shortly (e.g., 2–3 days) after tamoxifen treatment. Many tissues should be included to get a complete picture of the tissue- and cell-type specificity of the CreER^{T2} mouse line. Second, it is recommended to control for unwanted recombinase activity and cell labeling in the absence of tamoxifen. Therefore, tissues of interest are obtained from vehicle-treated mice at the same time points as from tamoxifen-treated animals and analyzed for marked cells. If background recombinase activity is detected, then consider that this could be due to the presence of estrogenic compounds in the chow, such as phytoestrogens. Third, tissues of interest should also be isolated from wild-type mice and incubated with X-Gal to control for endogenous β-galactosidase activity, which typically generates a greenish-blue stain. Some tissues show a relatively high β-galactosidase background, for instance, some regions of the gut, the intestinal mucosa, ovaries, and the testes. This might impede unequivocal detection of labeled cells. Similar controls should be performed, if fluorescence reporter mice are used. Some tissues show relatively strong endogenous fluorescence, especially fatty and collagen-rich tissues like the atherosclerotic aorta. Fourth, to verify that the recombined reporter allele can indeed be expressed in a wide range of cell types, at least in the tissues of interest, germ line-recombined reporter mice can be analyzed.

Such mice are generated by crossing the reporter line to a "deleter" Cre line that expresses Cre in germ cells, such as the EIIa-Cre mouse [41].

3. In general, breeding cages are set up by placing two female mice (>6 weeks old) to one male mouse (>6 weeks old, if possible use an experienced breeder). The average litter size is five to ten pups. Each breeding step requires 2–3 months, resulting from ~3 weeks of pregnancy and 6–9 weeks until the offspring reaches sexual maturity. Each of the individual mouse lines (i.e., Cre line, reporter line, and ApoE line) that are crossbred to obtain the experimental animals should also be maintained separately by breeding with wild-type mice of the same genetic background.
4. If the breeding scheme does not yield experimental animals for tamoxifen injection, then this might be related to the fact that both the Cre transgene and the ApoE gene are located on the same chromosome, in this case mouse chromosome 7. If so, then another Cre mouse line that carries the transgene on a different chromosome should be used. Similarly, if one of the other transgenes is integrated on the same chromosome as the *Rosa26*-based reporter (chromosome 6), then one could use an alternative Cre reporter mouse, such as the CAG-CAT-EGFP line [42]. The yield of double-heterozygous experimental animals (genotype: Cre/+;+/R) can be increased by using parental mice homozygous for Cre (genotype: Cre/Cre) and/or the reporter (genotype: R/R). Furthermore, the labeling efficiency can be increased, if the experimental animals are homozygous for Cre and/or the reporter (genotype: Cre/Cre;+/R, Cre/+;R/R, or Cre/Cre;R/R). Such mice can be generated by interbreeding double-heterozygous mice (genotype: Cre/+;+/R).
5. Investigators should be aware of the potential pitfalls, if Cre recombinase, in particular an inducible Cre, has to recombine two floxed sequences in the same cell, for instance, a floxed reporter and a floxed target gene. For several reasons, a labeled cell expressing the reporter protein must not necessarily be deficient for the target protein. First, the sensitivity of the Cre reporter to recombination might be higher than that of the floxed target gene. Second, the *onset* of reporter protein expression is usually detectable within 1 day after tamoxifen treatment, but the *elimination* of the target protein might take much longer. The half-life of individual mRNAs and proteins can vary significantly, so that it can take several days to weeks after tamoxifen treatment until the target protein is lost from a cell. Therefore, it is strongly recommended to monitor the expression of the target protein at the cellular level [14].

6. In general, ear punches can be collected without anesthesia. It is not recommended to store the biopsies at −20 °C. Best genotyping results are obtained, if freshly obtained ear tissue is processed immediately. In principle, the DNA from other tissues like the tail can also be used for PCR genotyping, but the conditions (e.g., DNA and primer concentrations) might require some modifications to get reliable results.
7. The following primers amplify a 403-bp fragment of the Cre-encoding sequence and, hence, can be used to genotype most Cre-transgenic mouse lines: Cre800 (5′-GCT GCC ACG ACC AAG TGA CAG CAA TG-3′) and Cre1200 (5′-GTA GTT ATT CGG ATC ATC AGC TAC AC-3′).
8. Examples of induction protocols that have been used with $CreER^{T2}$ transgenic mice are given in [14]. In general, tamoxifen-induced recombination can be achieved in any pre- and postnatal tissue, but in certain organs, such as brain, efficient cell labeling might require a higher dose of tamoxifen and/or injection frequency [43]. To mark cells during embryogenesis in utero, tamoxifen or OHT can be given to pregnant mice [14]. However, this treatment can impair normal delivery of the pups. To obtain live offspring, it is recommended to perform caesarean section. By decreasing dose and frequency of tamoxifen injections, the labeling efficiency can be "titrated" to a few single cells, which facilitates clonal analysis of cell fate. Moreover, recombination can also be controlled locally in a light-directed manner by using photoactivatable ("caged") tamoxifen analogs, thus allowing for high-precision labeling of preselected cells [44].
9. Tamoxifen is not soluble in water. Oil should be added after suspending tamoxifen in a small volume of ethanol. Note that after sonication the tamoxifen solution might still be slightly turbid. Variations of the standard protocol include the use of peanut oil or Miglyol instead of sunflower oil, or the use of OHT (Sigma H6278) instead of tamoxifen. OHT is presumably the actual ligand that binds with high affinity to the estrogen receptor ligand-binding domain of CreER recombinases. However, because tamoxifen is metabolized to OHT [11, 12] and is less costly than OHT, it is the drug of choice for most induction protocols that are based on systemic drug administration. OHT should be used directly whenever limited conversion of tamoxifen to OHT is anticipated, for instance, when recombination is to be induced by locally restricted drug application or in cultured cells.
10. Cross-contamination with tamoxifen can take place if treated and untreated animals are housed in the same cage. Licking of oily tamoxifen suspension, grooming, or coprophagous behavior can already cause recombination [45].

11. Beginning at ~8 weeks after initiation of the atherogenic diet, small plaques can be detected in ApoE-deficient mice. Atherosclerotic lesions do also develop in ApoE null mice on a normal chow. These plaques can be observed in ~6-month-old animals, but their cellular composition might differ from plaques that have developed in mice on a high-fat diet (S. Feil and R. Feil, unpublished data).
12. Many GIFM experiments would be more informative, if the labeled cells could be isolated and characterized in detail, for instance, by gene expression profiling or growth assays. In principle, cells marked by *lacZ* expression can be isolated from frozen sections by navigated laser capture microdissection, and intact mRNA can be recovered from these cells for transcriptome analysis [46]. However, the fixation step required for visualization of the labeled cells by X-Gal staining precludes the isolation of viable cells. In contrast, using fluorescent reporter proteins for cell labeling allows the isolation of viable cells from unfixed tissues, e.g., by fluorescence-activated cell sorting, thereby opening additional avenues for their characterization.
13. Alternatively, other methods can be used to sacrifice mice, if they are compatible with downstream processing of the tissue samples. Cervical dislocation prior to perfusion fixation is not recommended, because vessels can be destroyed and the fixative solution will not reach every organ.
14. For analysis of smaller organs and tissues like aorta, perfusion fixation of the whole animal is not absolutely required. These tissues can be directly isolated and immersion-fixed prior to further processing such as X-Gal staining. If a fluorescent reporter is used, cells can be directly observed in situ in unfixed tissue.
15. Formaldehyde is a carcinogenic gas. Therefore, experiments should be performed under a fume hood and the mouse placed in a dish to collect the fixative solution that drips from the animal.
16. Since over-fixation can impair β-galactosidase activity, the optimal fixation time should be determined empirically.
17. X-Gal should be added freshly to the staining solution before use. The exact staining time depends on the size of the samples and the level of β-galactosidase activity. Check regularly for the development of the blue X-Gal stain. The recommended time for staining of tissues is about 12–24 h. Background staining might increase with extended incubation time. Alternatively, β-galactosidase can be detected by antibody staining.
18. The blue X-Gal stain is stable in ethanol. Moreover, ethanol clears the tissue for better contrast in whole-mount preparations.

19. X-Gal staining solution penetrates only the outer layer of bigger organs, e.g., 2–3 mm of the heart. For uniform staining of deeper regions of organs, tissues should be sliced with a razor blade or scalpel before X-Gal staining is performed. Thinner slices with defined thickness (e.g., 0.1–1 mm) are prepared with a vibratome. X-Gal-stained tissue slices can be directly mounted on Superfrost Plus slides or further processed for sectioning.
20. It is not recommended to incubate tissues in organic solvents, such as toluene or xylene, longer than required, because the blue precipitate may leach out.
21. In general, co-staining of X-Gal-stained sections with antibodies is possible, but some restrictions apply. First, the fixation with glutaraldehyde-containing solution prior to X-Gal staining results in relatively strong tissue autofluorescence, which impedes the staining of sections with fluorescent antibodies. Therefore, it is recommended to use immunohistochemistry, e.g., the ABC method, for co-staining of X-Gal-stained sections. In addition, if the R26R reporter mouse is used, the blue X-Gal precipitate is diffusely distributed in the cytoplasm of labeled cells and might interfere with histochemical antibody staining. The abovementioned problems can be avoided by several strategies: (a) by using a *lacZ* reporter that expresses *nuclear* β-galactosidase (e.g., R26-NZG, Table 1); (b) by detection of β-galactosidase with a fluorescently labeled antibody on conventional cryosections; and (c) by using a fluorescent reporter mouse (Table 1).
22. As an alternative to X-Gal staining of whole mounts and subsequent sectioning, it is feasible to prepare frozen sections first and stain them with X-Gal subsequently. However, an atherosclerotic aorta contains only few plaques and not all of them contain marked cells. If cryosections were to be prepared, then the whole aorta needs to be sectioned (e.g., in 10 μm thick sections) and many of these sections must be stained with X-Gal (e.g., every tenth section) to screen for labeled plaques. In contrast, if the whole aorta is first stained with X-Gal, then one can preselect the appropriate regions containing blue plaques for subsequent sectioning (*see* also Fig. 2b, lower row).
23. For unequivocal detection of fluorescently labeled cells, mice with and without the recombined reporter must be carefully compared. Be aware of the high autofluorescence of fatty, collagen-rich regions in the vessels, particularly in aortic plaques. If a fluorescence stereomicroscope is not available, then thin layers of tissue, e.g., the medial layer of the aorta, can be prepared with forceps, mounted on a glass slide and observed with the fluorescence microscope.

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Chapter 7

Intracytoplasmic Sperm Injection (ICSI)-Mediated Transgenesis in Mice

Pedro N. Moreira and Lluís Montoliu

Abstract

Over the years many well-described techniques for the introduction of transgene DNA into host organisms have been used, including pronuclear injection, in vitro fertilization-mediated transgenesis, transfection of ES and spermatogenic cells, nuclear transfer of somatic cell nuclei, and lentiviral vectors. The application of these techniques has been limited however either by the time and effort to be executed or by their narrow efficiency with large transgenes. The greatest advantage of intracytoplasmic sperm injection (ICSI)-mediated transgenesis is precisely its ability to stably introduce large DNA molecules into the genome of host organisms with relatively high efficiency, as compared to alternative procedures. In mice, this procedure has been shown to be a reproducible method to generate transgenic offspring with a high efficiency. Recently, it proved also to be a viable method to generate transgenic rats and pigs, and as such, it is foreseen with great interest for the production of transgenic farm animals, where it would constitute an important tool for the production of recombinant proteins and livestock improvement.

Key words Intracytoplasmic sperm injection (ICSI), Transgenesis, DNA injection, Sperm-mediated gene transfer (SMGT), Piezo-actuated micromanipulation, Embryo micromanipulation

1 Introduction

The production of transgenic animals has been an important tool for the understanding of molecular mechanisms underlying normal biological processes as well as for the assembly of animal models of human disease. The introduction of foreign DNA in the genome of a host animal has been used to study the characteristics of exogenous molecules and modified genes carrying insertions or deletions.

Over the years, many well-described techniques for the introduction of transgene DNA into host organisms have been used, such as pronuclear injection [1], in vitro fertilization-mediated transgenesis [2], transfection of ES [3, 4] and spermatogenic cells [5–7], nuclear transfer of somatic cell nuclei [8–10], and the use of lentiviral vectors [11].

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The microinjection of foreign DNA into the male pronuclei of fertilized oocytes is routinely used in mice and has been proved efficient in several other animal species as well [12]. Although widely used across species, this procedure has limited efficiency, in delivering molecules larger than plasmid size [13], and has been shown to generate high levels of chromatin damage in host embryos capable of compromising embryo development [14, 15]. The technical simplicity of producing genetically modified animals by heterologous DNA interaction with mammalian spermatozoa and subsequent delivery into the oocyte by in vitro fertilization could open the possibility of large-scale production of transgenic animals for basic research and biotechnology purposes, but the results obtained so far, not only in mice but also in domestic species, have been inconsistent [16, 17]. The poor efficiency of cloning in erasing epigenetic marks of donor cell nuclei, which across organisms does not allow for live offspring rates grater that 1–10 %, depending on the species [18], limits the broader use of somatic cell nuclear transfer for the production of transgenic animals. The low efficiency and difficulty involved in ES and germ cell-mediated transgenesis also suggest the need for the development of alternative approaches for the insertion of DNA fragments into the genome of host organisms. This need is even greater when trying to understand the biological significance with large transgenes of wide genomic regions, which have been identified as fundamental for phenotypic expression in certain diseases.

In mice, many of the ES cell lines widely used were derived from the 129Sv mouse inbred strain, which is associated with abnormal brain morphology and behavioral and developmental difficulties [19, 20].

The most effective procedure of generating transgenic founders described up to date involves the use of disarmed retroviral vectors, usually lentiviral, to insert transgenes of interest at seemingly random locations into the genome of host organisms [11]. However, the use of lentiviral vectors is restricted in many laboratories. They generate high embryo mortality rates and the size of the RNA viral genome limits the length of the exogenous DNA molecule that can be transmitted to the host organism to 7–8 kb, limiting the application of this technology in a broader way.

The greatest advantage of intracytoplasmic sperm injection (ICSI)-mediated transgenesis, also called “metaphase II-mediated transgenesis” is precisely its ability to stably introduce large transgenes of submegabase dimensions such as BACs [15, 16] and YACs [13, 21] with relatively high efficiency when compared with alternative procedures into the genome of host organisms. ICSI-mediated transgenesis involves the use of DNA-coated spermatozoa, where the sperm cells are first exposed to freeze-thaw cycles, detergent, or alkaline treatments [13, 21–24], to remove the cellular membrane and expose their nuclei to exogenous DNA molecules.

After incubation and interaction with the transgene of interest, demembranated sperm cells are used for assisted reproduction and individually injected into the cytoplasm of unfertilized metaphase II oocytes. In certain species, such as mice, this procedure has been shown to be a reproducible method to generate transgenic offspring at high efficiency [13, 21–24]. Recently it proved to be a viable method to generate transgenic rats [25] and pigs [26] and it is foreseen with great interest for the production of transgenic farm animals, where it would constitute an important tool for the production of recombinant proteins and livestock improvement [26–29]. The use of ICSI as a vehicle for animal transgenesis has several advantages. It only requires intact DNA, DNA-coated spermatozoa can be delivered anywhere in the cytoplasm of unfertilized oocytes (unlike pronuclear injection of DNA which requires proper visualization of these structures inside the embryo), and the time window for oocyte manipulation is quite wide. Few reports have been published describing the molecular mechanisms responsible for ICSI-mediated transgenesis. Sperm cell binding of exogenous DNA molecules seems to be mediated by DNA-binding proteins of 30–35 kDa present in the postacrosomal region of spermatozoa [30, 31] with the subsequent uptake by the sperm nucleus, involving the collaboration of CD4 and other major histocompatibility complex class II proteins [32]. Delivery of large DNA constructs by pronuclear injection frequently generates DNA molecule fragmentation, likely to be caused by the small pore pipette used for embryo delivery, and hence compromising proper expression. During ICSI-mediated transgenesis the sperm cell structure offers a matrix for attachment for the DNA molecules helping reducing DNA sharing. Freeze-thawing and detergent treatment of spermatozoa have been showed to generate chromosomal damage and, therefore, it is possible that DNA repair mechanisms in the oocyte are involved in promoting transgenesis using exogenous DNA molecules as templates for DNA structure repair. The extensive reorganization of the sperm cell nucleus, involving chromatin decondensation and exchange of many structural associated proteins, may also promote and facilitate the incorporation of exogenous DNA molecules into the host genome.

2 Materials

2.1 Reagents

1. Mice: Preferably of the hybrid strain B6D2F1 (*see* **Note 1**) (suppliers include Charles River Laboratories and Harlan).
2. Hormones for superovulation: Pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet, Boxmeer) and human chorionic gonadotropin (hCG, Intervet, Boxmeer).
3. M2 medium (Sigma, cat. no. M7167).

4. BSA (Sigma, cat. no. A-3311).
5. Potassium simplex optimized medium (KSOM; Zenith Biotech, cat. no. ZEKS-100).
6. Embryo-tested bovine testis hyaluronidase type IV (Sigma, cat. no. H4272).
7. Polyvinyl-pyrrolidone (PVP; Sigma, cat. no. PVP-360).
8. Mineral oil (Sigma, cat. no. M8410A).
9. Mercury (Sigma, cat. no. 83359-100G): Careful handling and disposal are required (*see* **Note 2**).
10. Spermidine (Sigma, cat. no. S-2501).
11. Spermine (Sigma cat. no. S-1141).
12. Fluorinert (Sigma, cat. no. FC-770).

2.2 Equipment

1. Borosilicate glass capillaries 1.0 mm o.d., 0.75 mm i.d (Kwik-Fil, Sarasota, FL, cat. no. TW100-4) (*see* **Note 3**).
2. Scissors for fine dissection such as Iris straight tip (FST, cat. no. 14040-10).
3. Forceps such as Dumont #5 (FST, cat. no. 11251-30).
4. Aspirator tube assemblies for mouth pipetting (Sigma, cat. no. A5177-5EA).
5. Falcon dishes of 100 × 20 mm, 60 × 15 mm, and 35 × 10 mm (Becton Dickinson cat. nos. 353003, 3551007 and 351008 respectively).
6. Disposable 1 ml syringes with 26-gauge needle (BD Plastipak, cat. no. 300015).
7. Cryogenic vials of 1.8 ml (Sigma, cat. no. V7884).
8. Stereomicroscope, such as the SMZ 1500 (Nikon, Nakagawa, Japan).
9. Pipette Puller, such as the P87 (Sutter Instrument Co., Novato, CA).
10. Precision pipettes, such as P20, P200, and P1000 Pipetman (Gilson, Inc.).
11. Microforge for glass capillaries, such as the MF-900 (Narishige Scientific Instruments Lab, Tokyo, Japan).
12. Workstation comprising inverted microscope, such as the TE Eclipse 2000S (Nikon, Nakagawa, Japan), equipped with MMO-202ND micromanipulators, Cell Tram Vario microinjectors (Eppendorf, Hamburg, Germany), and Hoffman modulation contrast lenses or equivalent (4×, 20×, and 40× objectives).
13. Piezo impact unit PMM150FU (Prime Tech, Ibaraki, Japan).
14. Humidified CO_2 (5 % volume per volume in air) incubator (suppliers include Sanyo and Heraeus).

15. Anti-vibration table or platform (suppliers include Leica and Technical Manufacturing Corporation).

2.3 Reagent Setup

1. Mice: For all experiments use mice with ad libitum feeding of a standard diet and maintain in a temperature- and light-controlled room (23 °C, 14-h light, 10-h dark). Carry out all animal experimentation according to national and international regulations.
2. PMSG and hCG for superovulation: Both hormones are usually distributed in a lyophilized form, in ampullae of 500–2,000 IU. Dissolve them in saline physiological serum (0.9 % NaCl) or phosphate buffer solution (PBS) to a final concentration of 5 IU per 0.1 ml, which is a reasonable volume to be injected into the peritoneal cavity of oocyte donors (*see* **Note 4**). Store aliquots of PMSG at −80 °C for up to 3 months and those of hCG for up to 1 month. Thaw aliquots at room temperature before use and do not refreeze (*see* **Note 5**).
3. KSOM: This mouse embryo culture medium from Zenith Biotech already contains amino acids, d-glucose, and phenol red. Just supplement with 1 mg/ml BSA (referenced in the above Reagents list).
4. Hyaluronidase stock: Dilute the 30 mg of the bovine testis hyaluronidase type IV supplied by Sigma in 100 ml of M2 medium to obtain a final working concentration of 300 μg/ml. Store in aliquots of 500 μl at −20 °C until use. Repeated freezing and thawing are not advisable (*see* **Note 6**).
5. 10 % PVP solution: In a Petri dish of 35 mm Ø, overlay 2 ml of M2 medium with 200 mg of PVP_{360}. Without agitation, seal the dish with Parafilm to avoid evaporation and salt concentration and place it at 4 °C. In 48 h total PVP_{360} dissolution has been accomplished. Sterility should be a concern throughout the procedure. This solution can be used for up to 2 weeks if stored at 4 °C.

2.4 Equipment Setup

2.4.1 Preparation of Microinjection Needles

1. For the preparation of holding and injection pipettes, borosilicate glass capillaries are extended on a pipette puller equipped with a 4.5 mm box filament. As initial settings we suggest 830, 75, 130, and 100 for the parameters heat, pull, velocity, and time, respectively (*see* **Note 7**).
2. Using a microforge, the tip of the pulled capillaries is tailed to generate holding and injection pipettes. At the lowest amplification place pulled capillary horizontally on the microforge and slide it down close to the glass bead. At the highest magnification the edge of the glass bead on the platinum filament of the microforge should be in the center of the optical field and in sharp focus.

3. Place the tip of the pulled capillary and the edge of the glass bead on the same plane of focus. The diameter of the micromanipulation pipette is defined by moving the capillary left or right relatively to the edge of the glass bead.
4. For holding pipettes, an external diameter of 80–90 μm is appropriate. Injection pipettes should have an external diameter at the tip around 6–8 μm. Once the external diameter of the pipette tool is defined bring the capillary in contact with the glass bead and apply a brief heat pulse, just enough to fuse the external wall of the capillary to the expanding glass bead. Fusion point defines breakage point. Either breakage occurs during retraction and culling down of the glass bead or it can be intentionally generated by vertically pulling apart the capillary fused to the glass bead. A good injection pipette should have a sharp blunt-ended tip (*see* **Note 8**). On the contrary, holding pipettes need to be heat-polished. These are obtained by vertically flush-ending the 80–90 μm o.d. broken capillaries to a pore size of ~10 μm.
5. Before assembly on the piezo impact unit, load injection needles with 5 mm of Hg through its wider end with a disposable 1 ml syringe and a 26-gauge needle.

2.4.2 Preparation of Microinjection Workstation

1. Embryo micromanipulation requires a firm and stable platform. During microinjection, glass needle vibration will result in embryo lysis. For best results, microinjection workstations should be assembled on air-cushioned tables, in dedicated low-traffic environments, far from any known source of vibration. Traffic, elevators, motorized electrical instruments, and room ventilation frequently interfere with the steadiness of the microinjection pipettes. Holding and injection pipette should be firmly mounted on the micromanipulation arms. The micromanipulation arm in which the injection pipette is assembled is the one surrounded by the ceramic core of the piezo impact unit (*see* **Note 9**). Microinjectors allow for placement of meniscus, and control over fluid and cells inside the capillaries. After assembly of the microinjection tools the oil inside the holding pipette and the mercury front of the injection needle should remain static (*see* **Note 10**).

3 Methods

3.1 Preparation of Plasmid and BAC DNA for Microinjection

1. The preparation of transgene DNA with a quality compatible for ICSI can be achieved using any of the standard methods currently available. Reproducible protocols for the routine preparation of plasmid DNA [33], BAC DNA [34], or YAC DNA [35] for microinjection purposes have been reported

elsewhere and can be directly adopted for ICSI. In particular, for the preparation of YAC-DNA meant for ICSI a detailed protocol is already available [21].

2. Plasmid DNA is best obtained in standard microinjection buffer (10 mM Tris–HCl buffer, 0.1 mM EDTA, pH = 7.5) [33] whereas BAC or YAC DNA should be prepared in polyamine-containing microinjection buffer (standard microinjection buffer with 100 mM NaCl, 70 μM (micromolar) spermidine, and 30 μM (micromolar) spermine for optimal results [21, 34, 35]).
3. BAC DNAs can be prepared through a variety of protocols [34, 36] but simple reproducible methods, based on commercially available reagents, provide optimal results. In this regard, it is highly recommended the Qiagen Large-Construct Kit, used according to the indications provided by the supplier.
4. Irrespective of the protocol used for preparing plasmid, BAC, or YAC DNA (as described above) it is always strongly advisable to perform a last dialysis step on floating filters (Millipore dialysis filter 0.05 μm pore size), strictly as reported [21, 35], prior to using these DNA samples for ICSI purposes. This dialysis step will effectively remove any traces of contaminants that might have been copurified with the DNA and, thus, will produce the cleanest transgene DNA available for microinjection or ICSI purposes.

3.2 Collection of Oocytes

1. In order to collect a large number of oocytes, superovulate female donors by intraperitoneal injection of 5 IU of PMSG followed 48 h later by 5 IU of hCG. Administer the corresponding hormone volumes of 0.1 ml (if stored at 5 IU per 0.1 ml) to oocyte donors with a disposable 1 ml syringe and a 26-gauge needle. Optimal hormonal response is age dependent and mouse strain specific but for most strains, 6–8-week-old female mice respond well to this superovulation protocol.
2. Collect the oocytes soon after ovulation, 13–14 h post-hCG administration (*see* **Note 11**). Sacrifice female mice by cervical distention, remove the oviducts, and place the organs into a 35-mm petri dish containing 2 ml of M2 medium pre-warmed to room temperature. Gentle swirl allows segregation of fur, blood, and adipose tissue. Repeat this procedure as much as necessary. Rupture of the oviductal ampulla and release of cumulus masses are done under the stereomicroscope at 15–20× magnification in clean M2 medium with the help of sterile forceps. Discard the oviductal remnant before processing the following.
3. Transfer the cumulus masses with a glass pipette, in the minimum volume possible (*see* **Note 12**), into a 300 μl dispersion drop of M2 medium containing 300 μg/ml of hyaluronidase.

For oocyte dispersion, place the dish for 3–5 min on a heated stage at 37 °C.

4. In order to eliminate traces of hyaluronidase activity (*see* **Note 13**) and scattered cumulus cells, pass sequentially the oocyte through ten drops of pre-warmed M2 medium. During this washing process, perform oocyte selection. The final drop of M2 medium should only contain the best-looking oocytes totally striped of cumulus cells. Wash the selected denuded oocytes two to three times in equilibrated KSOM (*see* **Note 14**) to dilute any traces of M2 medium before placing them in KSOM droplets in the incubator (droplets of 50 μl, covered with mineral oil to prevent evaporation, incubated overnight at 37 °C in a humidified 5 % (v/v) CO_2/air atmosphere). Oocytes can remain in KSOM droplets for several hours until use.

3.3 Sperm Collection and Freezing

1. Spermatozoa are collected from cauda epididymides of reproductively mature males (*see* **Note 15**). Males 3–6 months old ensure a good yielding of spermatozoa. The cauda epididymis is a yellow pouch of convoluted tubules surrounded by fat and muscle situated along the posterior margin of each testis. Upon excision, place cauda epididymides in a 35 mm petri dish with M2 medium and remove the excess of fat and muscle as much as possible. Repeat the transfer into fresh M2 medium until all traces of blood are eliminated. Force the sperm out of the cauda epididymides in clean M2 medium by gentle squeeze with forceps.
2. Before dispersion, transfer the released mass of sperm cells in a minimal volume with a sterile pipette tip into a cryogenic vial, and extend with fresh M2 medium to a concentration of 2×10^6 cells/ml. Throughout the procedure, asepsis should be maintained as much as possible. Generate a homogenous sperm cell suspension by gentle pipetting and transfer aliquots of 50–60 μl into cryogenic vials of 1.8 ml. Tightly cap the cryogenic vials and place them in liquid nitrogen without complete immersion. Direct contact between the liquid nitrogen and the sperm sample should be avoided (*see* **Note 16**). Store the sperm samples at −75 °C, which in these conditions can be used for at least 1 month. The use of sperm samples stored for longer periods is not advisable (*see* **Note 17**).

3.4 Sperm/DNA Mixing

1. Thaw an aliquot of frozen sperm at room temperature and mix 5–10 μl of the sperm sample with an equivalent volume of DNA. Best results are obtained with DNA concentrations in the mixture of 3–5 ng/μl for small transgenes such as plasmids, and 1–2 ng/μl for large constructs such as BACs or YACs. Handling of large constructs requires constant incubation on ice, gentle mixing, and the use of truncated pipette tips in order to minimize molecule fragmentation.

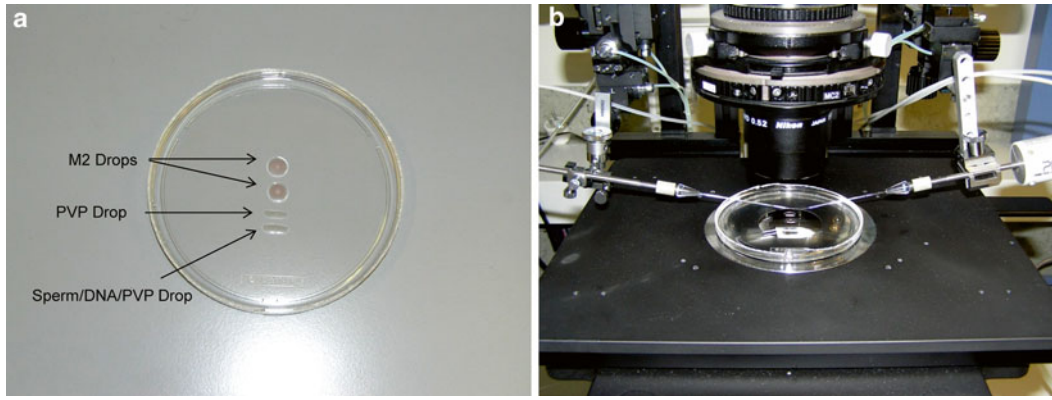

Fig. 1 Microinjection dish setup. The microinjection dish for intracytoplasmic sperm injection should contain under mineral oil two manipulation drops of 50–60 μl of M2 medium, one needle cleaning drop of 20–30 μl of 10 % PVP_{360} solution, and one drop of 20–30 μl of the sperm/DNA/PVP_{360} mixture. A convenient disposable support is the plastic lid of a 100 × 20 mm Falcon dish (**a**). By cutting the sides of the lid, oocyte manipulation can be done with straight glass needles using a minimal working angle (**b**)

2. After 2–3-min incubation on ice (*see* **Note 18**), mix 5 μl of the sperm/DNA solution with 25 μl of 10 % PVP_{360} solution. The sperm drop in the microinjection dish will be done from this final blend.

3.5 Piezo-Actuated Intracytoplasmic Sperm Injection

1. The microinjection dish for intracytoplasmic sperm injection should contain under mineral oil two manipulation drops of 50–60 μl of M2 medium (*see* **Note 19**), one needle cleaning drop of 20–30 μl of 10 % PVP_{360} solution, and one drop of 20–30 μl of the sperm/DNA/PVP_{360} mixture prepared as previously described (*see* Fig. 1). The 10 % PVP_{360} needle-cleaning drop is used to lubricate the injection pipette before and during the injection session (*see* **Note 20**). In preparation for oocyte manipulation, push the mercury placed inside the injection needle close to the tip by applying positive pressure with the microinjector. Immerse the injection needle into the PVP_{360} needle-cleaning drop, release some drops of mercury, and fill the tip with PVP_{360} solution while maintaining the mercury meniscus visible at 100×, and under microinjector control (*see* **Note 21**). The holding pipette does not require previous lubrication in the needle-cleaning drop before being lowered into the manipulation drop. M2 medium will rush inside by capillarity until it stabilizes. Before oocyte manipulation, align holding and injection needles with each other in the center of the manipulation drop, on the same plane of focus, close to the bottom of the microinjection dish. Aligned microinjection tools should not drag the surface of the dish.

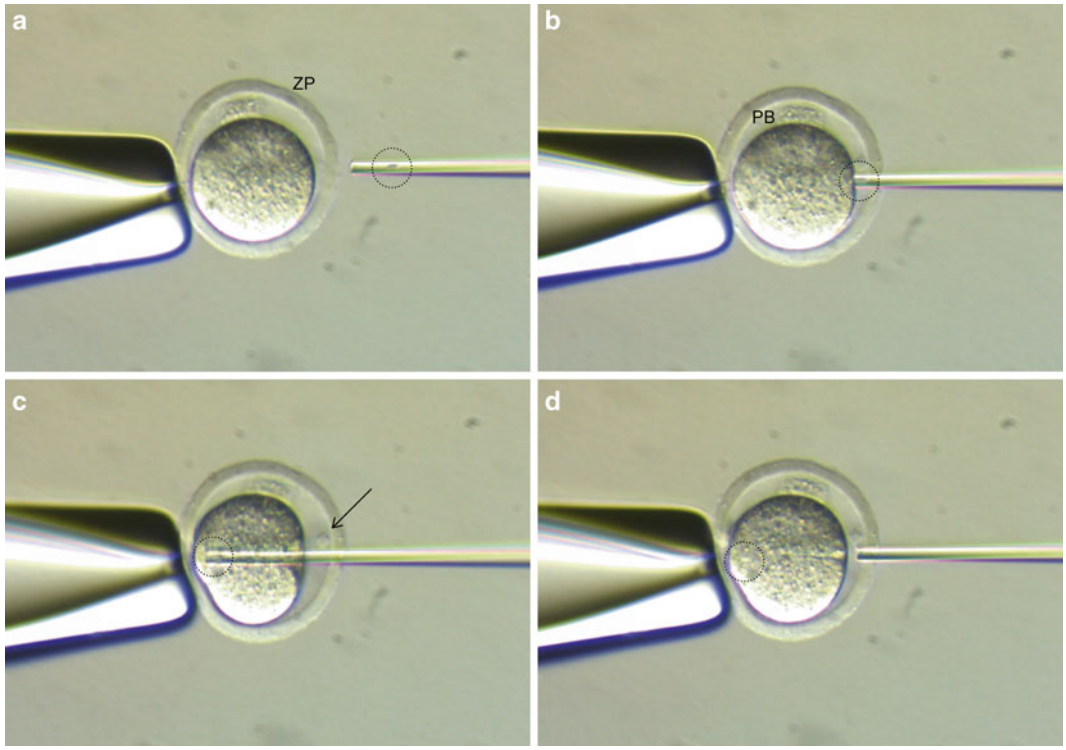

Fig. 2 Mouse oocyte injection of DNA-coated sperm heads. Sperm heads are drained within injection pipette intervals that ensure single delivery per oocyte. The oocyte is rotated on the holding pipette so that its MII plate or the polar body (when the first is not visible) becomes located at the 12 or 6 o'clock position and the largest perivitelline space for zona drilling is selected (**a**). At 400× magnification, the plasma membrane of the oocyte is placed in sharp focus. Zona drilling is accomplished by applying gentle negative pressure within the injection needle placed in contact with the zona pellucida at the 3 o'clock position, and piezo pulses of strong intensity. Upon release of the cored fragment in the perivitelline space a single sperm head is pushed to the tip of the injection needle (**b**). The needle is then advanced into the oocyte (about 90 % of total diameter), and the oolemma penetrated with a single piezo pulse of low intensity (**c**). The sperm head is released in the ooplasm in the minimum volume of extraneous medium and the pipette immediately removed (**d**). *ZP* zona pellucida, *PB* polar body. *Dashed circle* indicates location of DNA-coated sperm head. *Arrow* indicates zona fragment in perivitelline space

2. Sperm head injection (*see* Fig. 2) is performed at room temperature and should not last more than 2 h (*see* **Notes 22** and **23**). In order to reduce the extent of suboptimal conditioning outside of the incubator, inject the oocytes in small groups of 10–20. With the help of a mouth pipette assembly and a common embryo transfer glass capillary transfer the oocytes into the manipulation drop in a minimal volume of KSOM (*see* **Note 24**). From the sperm/DNA/PVP drop, at 400× magnification, aspirate individual sperm heads separated by the freeze/thaw procedure into the injection needle. With experience, five or more sperm heads can be drained within

pipette intervals that ensure single sperm head delivery per oocyte, considerably reducing the time necessary to inject a group of oocytes. If necessary, mechanical separation of sperm heads and tails can also be accomplished with the piezo unit. Bring the sperm cell in sharp focus at 400× magnification, draw its tail first into the injection pipette in a way that the head-midpiece junction stays in close contact with the sharp edge of the injection needle, and apply several pulses of strong intensity and low frequency with the piezo impact unit (piezo setup will depend on the pipette sharpness; start with intensity 13 and frequency 4, and reduce to the lowest working settings). Release the sperm cell tails in the drop before aspiration of the separated sperm heads.

3. Under low 40× magnification realign the injection needle loaded with DNA-coated sperm heads with the holding pipette in the oocyte manipulation drop. This can be simply done by moving the microscope stage, but avoid as much as possible the carryover of sperm cells and PVP solution into the manipulation drop.
4. Successful ICSI requires correct oocyte positioning. Use the microinjection needle to rotate the oocyte loosely secured on the holding pipette so that its MII plate (*see* **Note 25**) becomes located at the 12 or the 6 o'clock position and the largest perivitelline space for zona drilling is selected. Damage to the metaphase plate of the oocyte during sperm injection will affect normal embryo development. In addition, membrane penetration close to the MII plate frequently results in oocyte lysis. In order to prevent against dislodgements during microinjection secure tightly the correctly positioned oocyte by increasing suction in the holding pipette. At 400× magnification, place the plasma membrane of the oocyte in sharp focus. Perform zona drilling prior to ICSI by applying gentle negative pressure within the injection needle placed in contact with the zona pellucida at the 3 o'clock position, and piezo pulses of strong intensity (intensity 13 or higher, frequency of 6–8). During this process it is important to avoid aspiration and needle contact with the oolemma, which would result in rapid cell lysis. The fragment of the zona pellucida generated during zona drilling can be expelled in the perivitelline space without exiting the oocyte with the pipette, which avoids having to reenter through the zona core, difficult to see without experience. By applying positive pressure with the microinjector force a single sperm head to the tip of the injection needle. Advance the needle into the oocyte (about 90 % of total diameter), and penetrate the oolemma by applying a single piezo pulse of low intensity (frequency 1, intensity 2–3). Relaxation of the oocyte plasma membrane stretched along the injection needle indicates membrane puncture. Release the sperm head

in the ooplasm in the minimum volume of extraneous medium and remove the pipette immediately but smoothly from the oocyte (*see* **Note 26**). Gently release the injected oocyte in the manipulation drop far apart from the non-injected population. Microinjected oocytes should rest in the manipulation drop of M2 medium for at least 10–15 min (*see* **Note 27**). Those that survive should be washed two to three times in equilibrated KSOM to dilute any traces of M2 medium before being placed in mineral oil-covered KSOM culture droplets in the incubator. Twenty-four hours later transfer the embryos that have progressed to two-cell stage into the oviducts of surrogate mothers (*see* **Note 28**).

5. Details on the oviductal embryo transfer procedure are described elsewhere [20]. CD1 females selected for estrus and mated with vasectomized CD1 males are normally used as foster mothers. They are characterized by allowing large litters, good nursing behavior, and rare cannibalism of newborn. Detection of transgenic DNA in F_o mice can be investigated by polymerase chain reaction (PCR), Southern blot, and slot analysis. All animal experimentation described here should be performed in accordance with Institutional Animal Care and USE Committee (IACUC) guidelines, and in adherence with the established Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

4 Notes

1. Although ICSI-mediated transgenesis can be done with several stains of mice, for novices the use of oocytes, which resist well the manipulation procedure such as those of the hybrid strain B6D2F1 is advisable (female C57Bl6 × male DBA/2 F1 hybrid).
2. Mercury is a hazardous reagent. Although it is used in very small amounts in this method, elemental mercury vaporizes, has neurotoxic properties, and can be absorbed by the skin for which handling and disposal should carefully follow local institutional guidelines for hazardous reagents. In some institutions this use of mercury is not allowed, in which case it can be substituted with Fluorinert.
3. The efficiency of the microinjection procedure is highly dependent on the assembly of good microinjection pipettes. Hardness and flexibility of the TW100-4 capillaries from Kwik-Fil assure the assembly of efficient microinjection tools.
4. Intraperitoneal injection of volumes grater than 0.2 ml can lead to animal injury.

5. Loss of hormone activity and poor superovulation responses are frequently associated with hormone thawing above 37 °C and repeated freezing.
6. Repeated freezing and thawing of the hyaluronidase solution lead to loss of enzymatic activity and inefficient disaggregation of cumulus masses.
7. Optimal pipette puller settings vary considerably depending on the unit, laboratory temperature, and humidity. Suggested settings are just to be considered as an initial reference.
8. The drilling efficiency of the piezo-impact unit greatly depends on the sharpness of the microinjection needle. Mistakenly, frustrating results are frequently attributed to the instrument when in reality they are just a consequence of a poorly assembled injection pipette with sooth edges.
9. The ceramic core of the piezo impact unit is very delicate. Extreme care during handling is necessary as breakage of this core will interfere or even cancel the impact function of the unit.
10. Autonomous front recession on the microinjection tools independently of microinjector control indicates the existence of a leak in the hydraulic line, which needs to be solved.
11. Oocyte fertilization should be concluded before 20 h post-hCG. Delay in oocyte collection reduces the time window for oocyte manipulation. In addition, with aging, oocytes become more prone to parthenogenic activation and less resistant to the microinjection procedure.
12. Transfer of too much M2 medium and dilution of the hyaluronidase concentration in the dispersion drop may result in incomplete removal of cumulus cells. In order to avoid oocyte damage, cumulus masses should not be forced through the opening of the transfer pipette.
13. Long exposure to hyaluronidase solution can induce parthenogenic oocyte activation.
14. In order to avoid pH alteration in KSOM droplets it is very important that oocytes and embryos are transferred into these without any traces of M2 medium.
15. Sperm from outbred CD1 mice and some hybrid mouse strains (i.e., B6D2F1) generate better results than those obtained from inbred mouse strains (i.e., C57Bl/6J) and other hybrid mouse strains (C57CBAF1).
16. Direct sperm sample contact with liquid nitrogen has been shown to compromise embryo development [20]. This is possibly a consequence of extensive sperm DNA fragmentation.
17. A gradual decrease in embryo development should be expected as result of the gradual deterioration of the sperm samples stored at −75 °C.

18. An incubation period of at least 2–3 min on ice is important for proper interaction and adherence between DNA molecules and spermatozoa.
19. The assembly of at least one backup manipulation drop is recommended in case for some reason (contamination with mercury, PVP_{360}, sperm cells, and so on) work in the initial M2 drop becomes difficult.
20. The 10 % PVP_{360} solution of needle cleaning drop helps decreasing the adhesiveness of DNA-coated sperm cells to the glass pipette, which is one of the main technical difficulties associated with ICSI-mediated transgenesis.
21. Fragmentation of the mercury column inside the injection pipette decreases the efficiency of the piezo impact unit and microinjector. The same occurs if there is discontinuity in the hydraulic fluid inside the injection pipette or in the tubing connecting to the microinjector.
22. Oocyte membrane healing can be enhanced by performing the microinjection on a cooling stage preset to 19 °C; however, embryo exposure to low temperature decreases subsequent development. On the other hand, manipulation on a heated stage at 37 °C may result in increased parthenogenic activation and oocyte lysis.
23. Because sperm cell freezing releases nucleases that degrade endogenous and transgenic DNA, the injection session should not last for more than 2 h.
24. The transfer of elevated amounts of KSOM into the manipulation drop interferes with oocyte membrane healing resulting in increased lysis. If preferable wash oocytes in M2 medium before placement in the manipulation drop.
25. The metaphase II plate in mouse oocytes can be identified by a translucid, peripheral area that frequently protrudes causing oocyte deformation.
26. The intensity of the piezo pulse to penetrate the oolemma and the volume of injected fluid should be the smallest possible in order to reduce oocyte injury and increase the possibility of oocyte survival and development after microinjection.
27. Immediate transfer of manipulated oocytes into KSOM decreases oocyte survival. Recovery in a medium with lower osmolarity at higher temperature interferes with membrane healing.
28. Microinjection of DNA-coated sperm heads occasionally results in slower transition through first mitosis influenced by DNA purity and concentration.

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Chapter 8

Generation of Bacterial Artificial Chromosome (BAC) Transgenic Mice

Jane Beil and Thorsten Buch

Abstract

Transgenic mice are among the most helpful tools to study the role of genes in physiological conditions. In this protocol, we describe the generation of bacterial artificial chromosome (BACs) constructs, which are used to express a gene of interest under a particular promoter. BACs as driver of transgenes have the advantage that a characterization of transcriptional control elements is unnecessary and the construct's size usually reduces position effects from random integration. In the following, we firstly explain in detail the amplification of the BAC, the generation of the targeting construct as well as the recombination by ET-cloning, and the analysis of the recombined clones by Southern blot analysis. Finally, we also describe the preparation of the BACs for oocyte injection. In total, the construction of such BAC transgenes needs around 6–8 weeks.

Key words Bacterial artificial chromosome, BAC, Transgene, Recombineering, Oocyte injection

1 Introduction

Model organisms like *Caenorhabditis*, *Drosophila*, zebrafish, and mouse have proven to be invaluable for biological research. As model organisms evolutionarily closest to the human the mouse has always played a special role in biomedical research. Fortunately, for the investigation of gene function in the mouse, modification of the mouse genome has been possible for now almost 20 years. To achieve this, two main methods are used. The first one is used to generate knockout and knock-in mice. It is specifically designed to modify a particular gene of interest within its physiological location in the genome. The older and more established, but time-consuming, technique to achieve this is the generation of gene-modified mice by gene targeting in embryonic stem (ES) cells. Currently this method may be superseded by new techniques like zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) or the Cas9/CRISPR system [1–3].

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The second method is transgenesis by random integration of an expression vector. Such expression vector-driven transgenes usually consist of transcriptional control elements (promoter, enhancer, and silencer), an open reading frame (ORF), and a polyA element. While these simple transgenes have proven to be valuable in many research projects they suffer from the fact that they are extremely sensitive to position effects due to the random integration. Further, exact knowledge of the transcriptional control elements is required before such a construct can be generated. Both disadvantages can be overcome by the use of bacterial artificial chromosomes (BACs) for generation of specific transgenes. In BACs the actual transgene is placed into the 100–300 kb of genomic sequence of the BAC which contains the specific promoter and in all likelihood the other transcriptional control elements. Overcoming the two described disadvantages of conventional transgenes leads to BAC transgenes mimicking the natural expression of the gene of interest more faithfully than conventional transgenes [4, 5]. Thus, in many cases also the screening of a large number of founder lines is not necessary. Similar to conventional transgenes BACs are in most cases inserted into the mouse genome via oocyte injection. Admittedly, BAC transgenesis still leads to multiple insertions, but low-copy-number transgenic founders can be easily selected through Southern blot analysis. Until now, many useful applications have been developed that make the BAC approach very interesting to work with. It is possible to transgenically mark cells by the use of fluorescent proteins or generate cell-specific or inducible mutations by using Cre or CreER^{t2}. Furthermore, the transgenic expression of herpes simplex thymidine kinase [6, 7] or diphtheria toxin receptor [8, 9] enables induced cell death by the injection of ganciclovir or diphtheria toxin, respectively. Two methods for effective BAC transgenesis through recombineering are frequently used. One system is usually referred to as ET cloning and in this protocol, we describe this technique based on the publication of Johansson et al. [10]. Further information about the in silico analysis and also about the other recombineering system can be found in Johansson et al. [10].

2 Materials

2.1 Reagents

1. Luria Broth Base (Thermo Fisher Scientific).
2. LB Agar, Powder (Lennox L Agar) (Thermo Fisher Scientific).
3. Chloramphenicol (Sigma-Aldrich).
4. Ampicillin (Sigma-Aldrich).
5. Kanamycin (Sigma-Aldrich).
6. Tetracycline (Sigma-Aldrich).

7. Glycerol (Sigma-Aldrich).
8. Spermine (Sigma-Aldrich, tetrahydrochloride).
9. Spermidine (Sigma-Aldrich, trihydrochloride).
10. Dextran sulfate (Sigma-Aldrich).
11. DNA, MB-grade from fish sperm (Roche Applied Science).
12. Phusion High-Fidelity PCR Kit (Thermo Scientific).
13. GoTaq Green Master Mix (Promega).
14. L-(1)-Arabinose (Sigma-Aldrich).
15. GeneRuler 1 kb Plus (Thermo Scientific).
16. Plasmid Maxi Kit (Qiagen).
17. Gel Extraction Kit (Qiagen).
18. PhasePrep BAC DNA Kit (Sigma-Aldrich).
19. Ladderman Labeling Kit (Takara Bio).
20. EasyTides Deoxycytidine 50-triphosphate [a-32P] (PerkinElmer).
21. Illustra Micro-Spin S-200 HR Columns (GE Healthcare).
22. Sepharose CL-4B (GE Healthcare).
23. MidRange PFG Marker II (New England Biolabs).

2.2 Equipment

1. Standard molecular biology equipment.
2. Centrifugation bottle (250 ml).
3. 50-ml Conical tubes.
4. Electroporator (Eppendorf electroporator 2510 or similar).
5. Gene Pulser electroporation cuvette 0.1 cm (BioRad).
6. 10-ml Sterile syringe.
7. Infusion tube.
8. Syringe filter Acrodisc 0.1 μm (PALL).
9. 3MM Chr Paper (Whatman).
10. Paper towels.
11. Isotope lab for P-32 work, with hybridization oven (bottle roller), bag sealer, heat plate, thermoblock, and 1.5-ml tube centrifuge.
12. Southern blot membrane: Biodyne B 0.45 μm (PALL).
13. Degassing setup: Vacuum flask (Büchner flask, Kitasato flask), plug, tubing and pump.
14. BioMax film (Sigma Aldrich, size depends on your gel and autoradiography setup).
15. Pulse Field Gel Electrophoresis System (e.g., BioRad CHEF-DR II System).

2.3 Plasmids and Bacteria

1. Plasmids:
   - pSC101-BAD-gbaA-tetra (GeneBridges).
   - 706-FLP (GeneBridges).
   - A targeting cassette (*see* Subheading 3.2).
2. Bacteria:
   - Top 10 (Thermo Fisher Scientific) or DH5a (Thermo Fisher Scientific).

2.4 Reagent Preparation

1. 1,000× Polyamine stock: Dissolve 30 mM spermine and 70 mM spermidine in dH_2O. Autoclave the H_2O before using. Sterilize your solution through 0.2 μm filters. Store at −20 °C (*see* **Note 1**).
2. Microinjection buffer (50 ml): 10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, pH 8.0, 100 mM NaCl, 1× polyamines, fill up to 50 ml with dH_2O, filter sterilize (0.1 μm filters), and use the degassing device to degas the buffer (*see* **Note 2**).
3. Antibiotics: Ampicillin, dissolve 100 mg/ml ampicillin in 50 % ethanol; chloramphenicol, dissolve 30 mg/ml chloramphenicol in 100 % ethanol; kanamycin, dissolve 100 mg/ml kanamycin in H_2O; tetracycline, dissolve 3 mg/ml tetracycline in 100 % methanol.
4. Hybridization buffer (200 ml): Prepare in 180 ml dH_2O 1 M NaCl (11.8 g NaCl), 50 mM Tris–HCl pH 7.5, 10 % dextran sulfate (20 g), 1 % SDS, 250 μg/ml fish sperm DNA. Dissolve at 65 °C, fill up to 200 ml, and store 30 ml aliquots at −80 °C.
5. 10 M NaOH: Dissolve 200 g NaOH in 500 ml dH_2O.
6. 5 M NaCl: Dissolve 292 g NaCl in 1 l dH_2O.
7. 0.4 M NaOH: 40 ml 10 M NaOH in 960 ml dH_2O.
8. Transfer buffer: Measure 40 ml 10 M NaOH and 120 ml 5 M NaCl, and fill up to 1 l with dH_2O (0.4 M NaOH, 0.6 M NaCl end concentration).
9. Neutralization buffer: Weigh 35.1 g NaCl and 60.1 g Tris, fill up to 1 l with dH_2O, and equilibrate to pH 7.0 with HCl (0.5 M Tris–HCl, 0.6 M NaCl, pH 7.0).

2.5 Preparation of the Separation Column

1. Twist the two cotton filaments from a 5-ml serological pipette around each other and place them again into the pipette, keeping the end. Place your finger on top of the pipette, apply a vacuum to the tip, and remove your finger. The cotton will be sucked down to the tip. This creates a liquid-permeable plug.
2. Fix the pipette in a holder and provide a 50-ml beaker glass under the tip.
3. Invert the Sepharose multiple times to homogenize it, degas approximately 10 ml Sepharose and 5 ml injection buffer per column, and store on ice (*see* **Note 3**).

4. A 10-ml syringe is connected together with a sterile perfusion tube. Then cut the tube to a similar length as the pipette and mix the Sepharose with the injection buffer. Suck the mixture through the tube to fill the syringe.
5. The tube is inserted into the pipette right down to the plug at the bottom. Slowly press the mixture into the pipette. Keeping the end 1–2 cm below the liquid surface by pulling the tube slowly out of the pipette. During the procedure the liquid will start to drop out of the pipette but the Sepharose stays in the column. Repeat the whole process until the Sepharose surface is about 3–4 cm below the top of the pipette (*see* **Note 4**).
6. Insert a 50-ml syringe without plunger into the top of the pipette and seal the connection between syringe and pipette with Parafilm. 30 ml of degassed microinjection buffer is filled into the syringe. The Sepharose column is ready to use as soon as all buffer has passed through the column.

3 Methods

3.1 Amplification of the BAC

1. At www.ensemble.org you can find your gene of interest within its genomic environment. You can choose to have BACs displayed and then choose the corresponding BACs. You should take one in which your start of the gene is located in the middle of the insert sequence. You can export the BAC sequence for in silico analysis and order it from resources like www.chori.org (BACPAC Resource Center). For a detailed description, please check Johansson et al. [10].
2. Upon arrival inoculate some of the BAC glycerol stock into 3 ml fresh LB media supplemented with the appropriate antibiotics and culture it overnight at 37 °C and 250 × *g*. Because most BACs contain a chloramphenicol resistance we use chloramphenicol throughout this protocol. You may have to use another antibiotic.
3. Streak out some of the culture on an LB plate (containing 15 µg/ml chloramphenicol) and place it overnight at 37 °C.
4. Pick one colony on the next day and grow it in 3 ml LB media (with 15 µg/ml chloramphenicol) throughout the day at 37 °C, shaking at 250 × *g*.
5. Mix 300 µl 50 % glycerol with 700 µl of the bacteria culture and freeze it at −80 °C (glycerol stock).
6. Inoculate 1 l LB media (with 15 µg/ml chloramphenicol) with the rest of your suspension and culture it overnight at 37 °C, shaking at 250 × *g*.
7. Use the PhasePrep BAC DNA Kit to extract the DNA following the manufacturer's instructions.

3.2 Generation of the Amplified Targeting Cassette

The targeting cassette (Tg-Kan(R) Cassette) consists of a transgene (e.g., fluorescence protein, cre, or diphtheria toxin receptor) and a kanamycin resistance cassette flanked by FRT sites. Many such cassettes have been published (see table in reference 10) and can be used directly. Dependent on your aim you may have to clone a different transgenic insert yourself. The targeting cassette serves as template while performing a PCR with primers complementary to the homology arms of your gene of interest (*see* Fig. 1).

1. Design the primers (P1 and P2, *see* Fig. 1) for your PCR so that they contain around 50 bp of the homology arm (*see* **Note 5**).
2. Test the PCR while using a proofreading polymerase (e.g., Phusion High-Fidelity PCR Kit). Although your primer conditions can vary, you can start with the following conditions (*see* **Note 6**):

5× HF buffer	10 µl
Flanked primer for	5 µl of 5 µM
Flanked primer rev	5 µl of 5 µM
dNTP	2 µl 10 mM (each)
$MgCl_2$ (50 mM)	2.5 µl (titrate 2.0, 2.2, 2.5)
DMSO	3 µl (titrate 0, 1.5, 3, 4.5 µl)
Pfu polymerase	0.1 U
Template	50 ng
H_2O	Fill up to a total volume of 50 µl

95 °C	5 min	35 cycles
95 °C	30 s	
58 °C	1 min	
72 °C	4 min	
72 °C	10 min	

3.3 Recombination of Mini Targeting Cassette into the BAC by ET Cloning

During these steps you electroporate the plasmid for ET cloning (pSC101-BAD-gbaA-tetra) into your bacteria containing the BAC. Adding L-arabinose to those bacteria leads to the expression of necessary genes triggering recombination with the later transfected, amplified Tg-Kan(R) cassette.

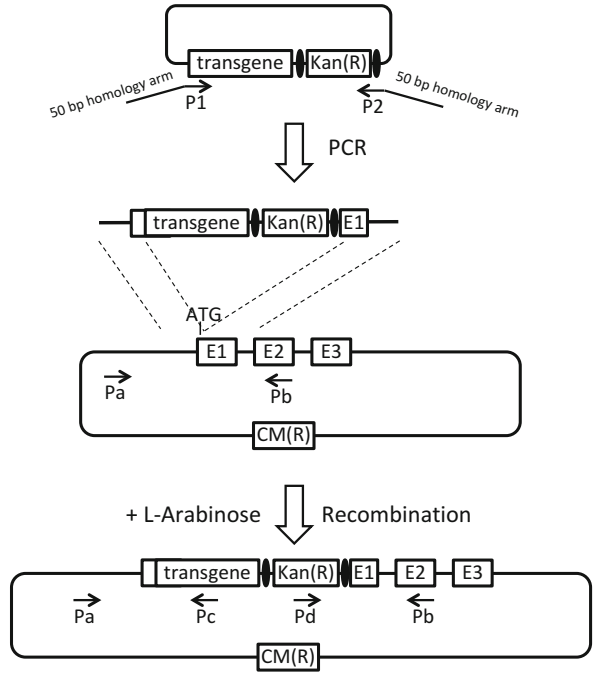

Fig. 1 Scheme of BAC modification by ET cloning. First a vector containing the targeting cassette is constructed. It consists of the transgene with pA followed by a FRT-flanked kanamycin resistance gene. Transgene and resistance gene are amplified by PCR with primers including 50 bp homology sequence from the insertion of the BAC, frequently 5′ and 3′ of the ATG. The PCR product is integrated into the BAC by recombination mediated through recombinases expressed after induction through arabinose in the BAC containing bacteria. Recombinants are found by selection and initially confirmed by PCR using the primers Pa–Pd

1. First the bacteria containing the BAC have to be made electrocompetent:
   (a) Punctuate ten 1.5 ml Eppendorf tubes for ventilation.
   (b) Add 1 ml LB media with corresponding antibiotics to the Eppendorf tubes.
   (c) Pick ten colonies of the bacteria carrying the BAC and place one into each Eppendorf tube.
   (d) Grow the bacteria overnight at 37 °C, shaking at 1,000 × *g*.
   (e) Take a new punctured tube, fill it with 1.4 ml LB media (containing 15 μg/ml chloramphenicol), and add 30 μl of the overnight culture.
   (f) Incubate the bacteria for 3 h at 37 °C, shaking at 1,000 × *g*.
   (g) Centrifuge the bacteria for 30 s (4 °C, 11,000 × *g*) and remove the supernatant.
   (h) Resuspend the pellet in 1 ml ice-cold dH_2O.

(i) Repeat **steps f** and **g**.

(j) Add 20–30 µl dH_2O (*see* **Note 7**).

2. Add 1 µl of the pSC101-BAD-gbaA-tetra plasmid to the bacteria and slowly pipette it into a cooled 0.1 cm electroporation cuvette. Electroporate the cells using Eppendorf electroporator 2510: 1,350 V, 10 µF, 600 Ω.
3. Resuspend the cells in 1 ml LB media and incubate them for 60–80 min (30 °C, 1,000 × *g*).
4. Take 100 µl of the bacteria and plate them on an LB agar plate with tetracycline (3 µg/ml) and chloramphenicol (15 µg/ml). The plate should be incubated for at least 24 h at RT (<28 °C).
5. Pick again ten colonies but this time inoculate them together in one punctured tube. Add LB media containing tetracycline and chloramphenicol and incubate the bacteria overnight at 30 °C, 1,000 × *g*.
6. Prepare a tube with 1.4 ml LB media containing 15 µg/ml chloramphenicol and add 30 µl of the overnight culture. Incubate them at 30 °C and 1,000 × *g* until a density of OD_{600} 0.2 (it will take about 2 h).
7. By adding of 20 µl of 10 % (w/v) L-arabinose you will induce the expression of recombinase. Let the bacteria grow at 37 °C and 1,000 × *g* for 40 min, but not more than OD_{600} 0.4.
8. Repeat **steps 2a–2i** to make the bacteria electrocompetent.
9. Use 1 µl (200 ng) of the amplified Tg-Kan(R) cassette to electroporate into the bacteria (**step 3**).
10. Resuspend the cells in 1 ml LB media and incubate them for 70 min at 37 °C.
11. The bacteria are then plated on LB agar plates containing 15 µg/ml chloramphenicol and 15 µg/ml kanamycin and incubated overnight at 37 °C.

3.4 Analysis of Recombined Clones

1. After 18–24 h you can pick around ten colonies (*see* **Note 8**). Inoculate each colony separately in 3–5 ml LB media containing 15 µg/ml chloramphenicol and 15 µg/ml kanamycin.
2. Screen your clones by setting up a PCR (e.g., Promega GoTaq Green) with 1 µl bacteria culture. Set up a multiplex PCR by using primers Pa, Pb, Pc, and Pd to verify that you inserted your transgene of interest (*see* Fig. 1). Continue only with clones that have your insertion but lack the wild-type product.
3. Use two to four positive clones and inoculate each colony in 250 ml LB media containing 15 µg/ml chloramphenicol and 15 µg/ml kanamycin.
4. Perform a Maxi-Prep.
5. Repeat the screening PCR of your plasmid.

6. Perform a restriction length analysis of your candidate BAC to confirm correct modification.
   (a) Start with an in silico digestion and choose an enzyme with around 20–30 cutting sites.
   (b) Digest 1–5 μg of your candidate BAC as well as wild-type BAC.
   (c) Cast a 0.7 % agarose gel of more than 20 cm. Load your digested DNAs and a DNA ladder (1 kb Plus). Run the gel 15–24 h at 30 V and take several pictures.
   (d) Compare your results carefully to your in silico analysis.
7. Perform a Southern blot analysis of your modified BAC.
   (a) Amplify an external and internal probe by PCR using a proofreading polymerase (*see* Fig. 1). Run it on a gel and purify it. Dilute the DNA to 30 ng/μl.
   (b) Digest 1 μg of your BAC DNA with a restriction enzyme that cuts 5′ and 3′ of both probes.
   (c) Cast a 0.7 % agarose gel and load your digested BAC DNA and a 1 kb Plus marker. Run it overnight at 30 V and take a picture on the following day.
   (d) Place the gel in 0.4 M NaOH and shake it for 15 min.
   (e) Cut eleven 3MM Chr paper in pieces slightly bigger than the membrane.
   (f) Set up the transfer solution containing 0.4 M NaOH and 0.6 M NaCl.
   (g) Two paper towel stacks of 5 cm height are placed next to each other.
   (h) Take three 3MM Chr papers, soak them in transfer solution, and place them on top of the paper towels.
   (i) Wet the membrane first in dH_2O by letting it slip in without bubbles. Use forceps! Then place it in transfer buffer.
   (j) Place the wet membrane on top of the paper stack.
   (k) Now the gel can be placed on the membrane.
   (l) Take another three 3MM Chr papers, soak them in transfer solution, and place them on top of the gel.
   (m) Use two further 3MM Chr papers to build a bridge to a tank filled with transfer buffer. Place this tank at least 5 cm higher than the top of the stack.
   (n) Transfer for at least 4 h (better overnight).
   (o) The membrane is then disassembled and incubated in neutralization buffer for 10 min.
   (p) Put the membrane between 3MM Chr papers and bake it in a hybridization oven for 1 h at 65–70 °C.

(q) The membrane is prewetted with 2× SSC and afterwards incubated with 15–20 ml prehybridization/hybridization solution in the hybridization oven overnight at 65 °C. Make sure that it is rotating.

(r) Label your probes with P-32 by using the Ladderman labeling kit according to the manufacturer's instructions. Follow the guidelines and safety instruction for working with radioactivity.

(s) Vortex the S-200 HR columns, remove the bottom seal, and centrifuge the liquid into a tube (1 min, 3,000 × *g*). Discard the liquid. Put the column back into the tube, add 100 µl H_2O to your probe, and transfer it to the column. Centrifuge again. Measure the counts of column and probe and discard the column. In an optimal labeling 50 % of the radioactivity is in the probe.

(t) Close the tube with your sample properly and place it for 5 min in boiling water (*see* **Note 9**). Place the tube on ice for 5 min.

(u) Pipette the probe into the prehybridization solution. Use a hybridization tube and do no pipette directly onto the membrane. Hybridize overnight at 65 °C.

(v) Wash the membrane twice with preheated (65 °C) 2× SSC at 65 °C for 5 min and measure the counts. The counts should be around 200 (*see* **Note 10**).

(w) Place the membrane in a plastic bag, seal it, and put it in a BioMax cassette film for 2 h. If the bands are too week you can leave it overnight.

3.5 Excision of the Resistance Cassette Kan(R)

Opinions about the excision of the resistance cassette are contradictory. Some people published that it may result in unpredicted phenotypes of the transgenic mice if the cassette is not cut out prior to injection, and others have observed that the Cre activity was higher in the presence of the resistance cassette [11, 12]. You need to decide what is best for your approach. If you keep the resistance cassette, you can still cross the mice with Flp deleter mice which leads to a removal because of the flanked FRT sites. However, beware that you then also resolve concatemers of the BAC at the insertion site. In the following, the excision of Kan(R) with the help of a transformation with the FLP-expressing plasmid 706-FLP is described in detail.

1. Make your bacteria that contain the modified BAC electrocompetent (*see* Subheading 3.3, **step 2**). Use LB media containing 15 µg/ml chloramphenicol and 15 µg/ml kanamycin.
2. Perform the electroporation according to Subheading 3.3, **step 3**, with 1 µl of 706-FLP (10–100 ng).

3. Incubate your cells after electroporation and resuspend in 1 ml LB media and incubate for 60–80 min (30 °C, 1,000 × *g*).
4. Plate 100 μl on LB plates (3 μg/ml tetracycline and 15 μg/ml chloramphenicol supplemented) and incubate them for at least 24 h at room temperature.
5. Pick ten colonies and inoculate them in 3 ml LB media containing 15 μg/ml chloramphenicol. Culture them individually and use ventilated tubes, which are incubated for 2–3 h at 30 °C and 250 × *g*. Finally, change the temperature to 37 °C and incubate the culture overnight.

3.6 Preparation of Your BAC Before Oocyte Injection

1. Choose a restriction enzyme that cuts the backbone out of your gene of interest or at least linearizes the vector. Set up a digestion solution with a total volume of 300–400 μl. Use 50 μg of the BAC and 100 U restriction enzyme (*see* **Note 11**). Incubate overnight.
2. Prepare the injection buffer.
3. After overnight incubation, the enzyme must be heat-inactivated.
4. 200 μl Injection buffer and 10 μl 6× loading dye are added to the digestion solution.
5. Follow Subheading 2.5 to prepare the separation column.
6. As soon as all the liquid is added to the syringe, remove the syringe. Add the digested BAC once the liquid reaches the level of the Sepharose.
7. Wait until the liquid reaches the surface of the Sepharose and add 1 ml of the injection buffer.
8. Collect volumes of 500 μl while repeating the addition of 1 ml injection buffer until 10 ml has been run through the column (*see* **Note 12**).
9. Use 20 μl from each fraction to run a pulse-field gel (120 V, 20 h). Run a MidRanger PFG Marker II, as well.
10. The transgene is expected to be in fractions 6–10. Follow on with three fractions that show no/less smear and measure the DNA concentration.
11. Inject your DNA into the pronuclei of the oocytes (*see* **Note 13**).

4 Notes

1. Order and use only small quantities (1 g) at once because of the hygroscopicity of the polyamines.
2. The buffer should be freshly prepared. Unused buffer should be discarded. The injection buffer may be different in your facility.

3. Prepare fresh solutions for each day of separation. Degassing the Sepharose/injection buffer prohibits bubble formation during the procedure.
4. It is very important to avoid bubbles in the matrix of the column.
5. Use PAGE-purified oligonucleotides to ensure best quality of the primers.
6. If your PCR is not working the primer length is probably a reason. To avoid long primers you may want to try a two-step PCR: For the first PCR use the same primers as mentioned above for a short PCR (i.e., 16 cycles). This PCR product can be used as a template for the second PCR where primers with normal length are used which are complementary to the end of each homology arm.
7. By adding 10 % glycerol electrocompetent bacteria can be stored at −80 °C.
8. Do not pick colonies after only 24 h of incubation.
9. The tube must be sealed tightly to make sure that no radioactivity can escape.
10. If the counts are too high, you should wash as follows, 2× SSC 0.1 % SDS, 1× SSC 0.1 % SDS, 0.5× SSC 0.1 % SDS for 10 min, and measure the counts between each washing step.
11. Use a rare cutter like Not I to excise the vector backbone or at least linearize the vector (e.g., Asc I).
12. This method is the same as running a gel electrophoresis but without the gel extraction step. Your backbone will run through the column earlier than the insert containing the used genomic region because smaller fragments are faster than bigger fragments. Be careful not to let the column run dry.
13. The most efficient injections can be performed using CBF1 or BDF1 hybrids as well as FVB/N inbred strain. C57BL/6 and BALB7c are more challenging.

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Chapter 9

Generation of Genetically Engineered Mice by the *piggyBac* Transposon System

Sheng Ding, Tian Xu, and Xiaohui Wu

Abstract

Genetically engineered mice (GEM) are invaluable tools not only for understanding mammalian biology but also for modeling human diseases. Here we present protocols to generate GEM with the *piggyBac* (*PB*) transposon system. In the first part, we describe a transgenic procedure that co-injects the transgene carried by a *PB* donor plasmid and a *PB* transposase (*PBase*)-expressing helper plasmid into the pronuclei of fertilized eggs. In the second part, we provide a large-scale, cost-effective insertional mutagenesis strategy that remobilizes single-copy *PB* transposons in the male germ line. Given that *PB* can transpose in a broad spectrum of eukaryotic hosts, the protocols described here could be adapted for other species in the future.

Key words *piggyBac*, Transposon, Genetically engineered mice, Transgenesis, Insertional mutagenesis

1 Introduction

Genetically engineered mice (GEM) are mice that stably carry transgenes or induced mutations in their genomes. GEM has played a pivotal role in improving our understanding of the underlying mechanisms of mammalian biology and diseases. In addition, they have served as human disease models that are extensively used to develop therapeutic reagents and strategies.

Pronuclear injection has become the prevailing method to make transgenic mice for more than two decades [1, 2]. Normally, linearized transgenic DNA is injected into one of the two pronuclei of the fertilized eggs. The zygotes are then transplanted into a pseudopregnant recipient female so that 10–20 % of the offspring will be transgene positive. This technique is also widely used in the transgenesis of rats, rabbits, sheep, goats, cows, and pigs with varied efficiencies [3]. Pronuclear injection can introduce large DNA fragments of megabase scale into the mouse genome [4]. However, transgenes resulted from this method randomly integrate into the genome with at least 5–15 % hitting endogenous genes [5]. It is technically difficult to map the integrations because transgenes

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frequently form head-to-tail concatemers with copy numbers varying from one to several hundred. Functional studies of the transgenes, especially the dosage-sensitive ones, are also complicated by the abnormal transgenic expression resulted from high copy numbers. Thus, it is usually recommended to characterize multiple transgenic lines to get a reliable conclusion.

Retroviral vectors usually bring single-copy transgenes into the genome. The prototypical retroviruses can only infect cells that are actively proliferating. Moreover, promoter and enhancer sequences in the retroviral long terminal repeats (LTRs) cause de novo methylation and silence expression of the transgene [6]. Lentiviral vectors could overcome these limitations [7]. In addition, lentiviral particles can be injected into the perivitelline space of single-cell embryos to get transgenic mice, which was claimed to bring less damage to the embryos than pronuclear injection [8]. However, both retroviral and lentiviral vectors have limited cargo capacity, which usually does not exceed ~8.5 kb [7].

Artificial mutations can be introduced into the mouse genome either on target or at random. Gene targeting is the most popular method for the former purpose [9]. Mutated allele of the target gene is introduced into the embryonic stem (ES) cells to replace the wild-type allele through homologous recombination (targeted integration) [10]. Targeted ES cells are then injected into the blastocyst cavity to develop chimeras and then mutant animals in successive generations. Limitations of this method include the requirement of a well-defined structure of the targeted genomic region, as well as the technical and financial challenges of ES cell culture. Thus, although gene targeting can be performed in any region of interest, most studies are focused on the sequences surrounding known exons [11].

Random mutagenesis induces mutations genome-widely. It is essential for unbiased genetic screens that may result in unique discoveries [12, 13]. Chemical mutagens such as the *N*-ethyl-*N*-*nitrosourea* (ENU) can generate random mutations throughout the genome with a high frequency [14]. However, ENU usually causes single-nucleotide changes, leaving mutation identification a laborious task. In contrast, mutations generated by the insertions of mobile genetic elements (retroviruses or transposons) are tagged with the inserted sequence for easy identification. Several retroviral based large-scale mutagenesis projects have been performed in mouse ES cells [15, 16]. It is estimated that 40 % of the annotated mouse genes have been hit by this method (http://www.igtc.org).

Meanwhile, several transposon systems have been developed to work in mammals, which make them attractive tools for mouse genetics [17–21]. Among all the transposon systems established in mammals, *piggyBac* (*PB*) is considered to be the most effective one [22]. *PB* was originally isolated from the cabbage looper moth *Trichoplusia ni* [23]. It transposes with a cut-and-paste manner in

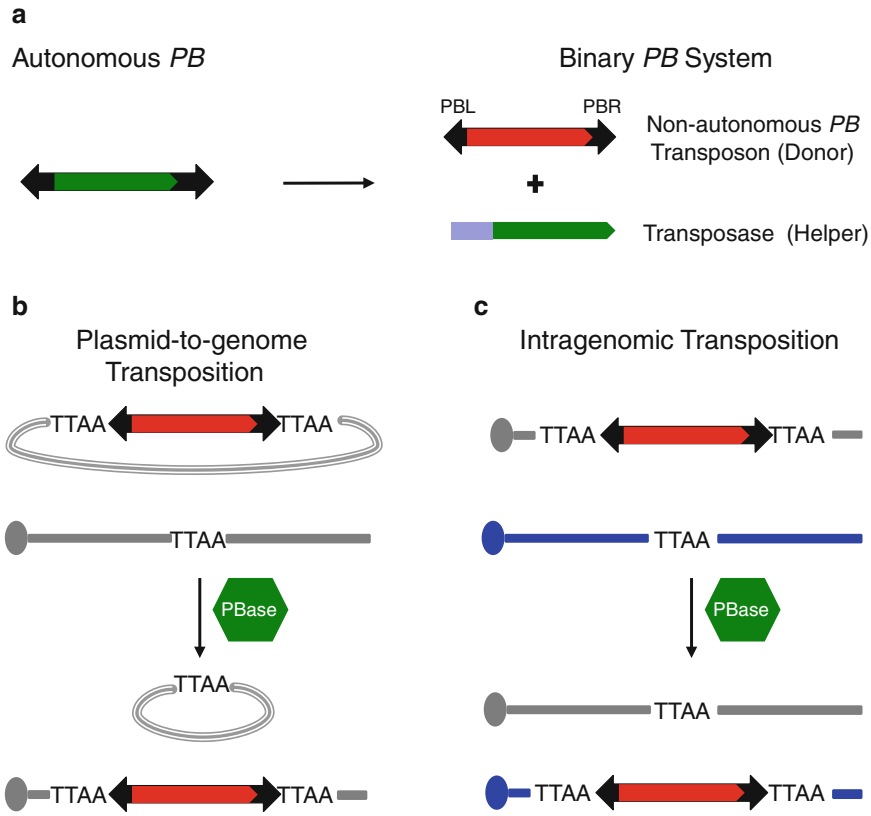

Fig. 1 Principle of *PB* transgenesis and mutagenesis. (**a**) Composition of a binary *PB* system for mice. (**b**) As a transgenic vector, *PB* can carry a transgene (*red*) and transpose from an epigenomic plasmid into the mouse genome at the presence of PBase (*green*). (**c**) As an insertional mutagen, *PB* can be precisely excised from the donor site and reintegrate into a new genomic location at the presence of PBase

the genome to hit the TTAA tetranucleotide under the catalysis of the *PB* transposase (PBase) (Fig. 1a). We have developed a binary *PB* system in mice, consisting of a donor and a helper element [17]. The donor element contained a non-autonomous *PB* transposon in which the *PBase* sequence was replaced by a drug selection marker or other transgene cassette. The helper element carried the *PBase* fragment but lacked the terminal sequences of *PB* (PBL and PBR) that are required for transposition. By adding or removing the helper element, the donor element could be mobilized or stabilized in the mouse genome, respectively (Fig. 1a).

PB has been successfully used for transgenesis and insertional mutagenesis in mice [17, 24]. As a transgenic vector, *PB* has a cargo capacity much bigger than that of retroviruses and lentiviruses. It efficiently produces single-copy integrations that mimic the situation of endogenous genes. As an insertional mutagen, *PB* effectively inserts into the transcription units and interferes

with gene expression [25]. In both cases, *PB* integrations can be easily mapped, so that desired animals could be selected before further breeding.

In Subheading 3.1, we describe a protocol for *PB*-mediated transgenesis that has been successfully applied for transgenes up to 16.3 kb. In our hands, this protocol resulted in more than 30 % transgene-positive founders. In Subheading 3.2, we provide a mutagenesis protocol by mobilizing *PB* in vivo. Combined the mating scheme with different visible markers, this protocol provides a cost-effective method for mutagenesis and helped us to generate a collection of more than 5,000 mutants [26] (Wu, X., Ding, S., and Xu, T., unpublished). Although the protocols described here use *PB* elements carrying a ubiquitously expressed red fluorescent protein (RFP) marker and the albino inbred *FVB/N* strain, they can be applied to other *PB* vectors and mouse strains with minor modifications.

PB transposon has been shown to be able to transpose in yeast [27], planarian [28], insects [29], chicken [30], rats [31], rabbits [32], and various vertebrate cells (Ding, S. et al., unpublished). Recently, we and other groups have shown that *PB* can transpose with bacterial artificial chromosomes (BACs) [33–35]. Given the broad host spectrum and the huge cargo capacity, *PB* possesses a great potential to serve as a universal genetic manipulation tool in eukaryotes.

2 Materials

2.1 Reagents

1. *PB* donor plasmid: The transgene cassette cloned into donor plasmid such as *PB*[*Act-RFP*]*DS* [17].
2. *PB* helper plasmid: *Act-PBase*, in which *PBase* expression is driven by the actin promoter [17].
3. Plasmid *PB*[*K14-Tyr*], which expresses the tyrosinase gene under the control of a skin-specific *K14* promoter. It provides a visible gray color in the albino mice such as *FVB/N*.
4. pGEM-T vector system (Promega).
5. *E. coli* strain: XL1-Blue, XL10-Gold, or DH5α.
6. XA medium for *E. coli* culture:

Yeast extract	5 g/L
Tryptone	20 g/L
$MgSO_4 \cdot 7H_2O$	5 g/L
KCl	0.76 g/L

Adjust pH to 7.6 with 1 M KOH.

7. Injection buffer (*see* **Note 1**):

Tris–HCl	10 mM
EDTA	0.25 mM
H_2O	

   Adjust pH to 7.4 with 1 M HCl or 1 M NaOH.
8. Restriction enzymes and buffers (New England Biolabs).
9. T4 ligase (Takara).
10. Taq polymerase (Promega).
11. 10× Genotyping buffer:

$(NH_4)_2SO_4$	166 mM
Tris–HCl, pH 8.8	670 mM
$MgCl_2$	67 mM
Beta-mercaptoethanol	50 mM
EDTA	67 μM

12. PCR primers:

    RF1: 5′-CCT CGA TAT ACA GAC CGA TAA AAC ACA TGC-3′.

    RR1: 5′-AGT CAG TCA GAA ACA ACT TTG GCA CAT ATC-3′.

    RV: 5′-GAT TAA CGT GTC TCT GCT CAA GTC-3′.

    BacEN-F: 5′-GCC ACC ATG GGA TGT TCT TTA G-3′.

    BacEN-B: 5′-GTA CTC AGA AAC AAC TTT GGC-3′.

2.2 Equipment

1. QIAGEN plasmid midi kit (QIAGEN).
2. QIAquick PCR purification kit (QIAGEN).
3. QIAquick gel extraction kit (QIAGEN).
4. Horizontal agarose electrophoresis apparatus (e.g., Owl separation systems).
5. Gel documentation system (e.g., UVP BioDoc-It).
6. Spectrophotometer (e.g., Eppendorf Biophotometer).
7. Pipetman (e.g., Gilson P-20, P-200, and P-1000).
8. Centrifuge (e.g., Heraeus Biofuge Stratos).
9. Dissecting microscope (e.g., Leica MZ7.5).
10. Micropipette puller (e.g., Sutter P-97).
11. Microforge (e.g., Narishige MF-900).
12. Microinjection setup (e.g., Leica DM IRB).

13. Microinjector (e.g., Narishige IM-300).
14. Tissue culture incubator (e.g., Thermo HEPA Class 100).
15. Water bath (e.g., Fisher Isotemp 210).
16. Mini incubator (e.g., Merck Cultura).
17. #5 Watchmaker forceps (e.g., Dumont Dumoxel).
18. Handheld long-wave UV lamp (e.g., UVP UVL-56).
19. Thermocycler (e.g., MJ PTC-100).
20. Bacteria incubator (e.g., Heraeus Function Line).
21. Personal computer with an Internet connection (e.g., Apple iMac).

2.3 Animal

FVB/N mice (e.g., Jackson laboratory).

3 Methods

3.1 PB-Mediated Transgenesis (Fig. 2a)

1. Design transgene as described [5]. Clone the transgene cassette into the *Asc I*, *Swa I*, *BamH I*, *Mfe I*, or *Hind III* site of the donor plasmid *PB*[*Act-RFP*]*DS*.
2. Prepare donor and helper plasmid DNA from the bacteria culture with QIAGEN plasmid midi kit. Purified DNA can be left overnight at 4 °C or for years at −80 °C.
3. Mix donor and helper plasmid DNA at a molar ratio of 2:1 with the total concentration at 2 ng/μL in the injection buffer, and spin briefly at maximal speed to pellet any debris immediately before injection to avoid clogging the needle (*see* **Note 2**).
4. Harvest fertilized eggs and perform nuclear injection as described [5].
5. Transfer injected eggs to the pseudopregnant recipient mice as described [5]. Check for progeny 3 weeks later.
6. Identify transgene-positive founders according to the red skin color resulting from RFP expression. RFP has a maximum excitation wavelength of 584 nm and a maximum emission wavelength of 607 nm. Its signal can be detected under daylight. If necessary, use a handheld UV lamp to help highlight the color (Fig. 2b). Alternatively, identify positive founders with PCR-based genotyping in **steps 7–9**.
7. Isolate genomic DNA with protease K and phenol from mouse tail tips as described [5]. Measure the DNA concentration by the spectrophotometer at the wavelength of 260 nm.

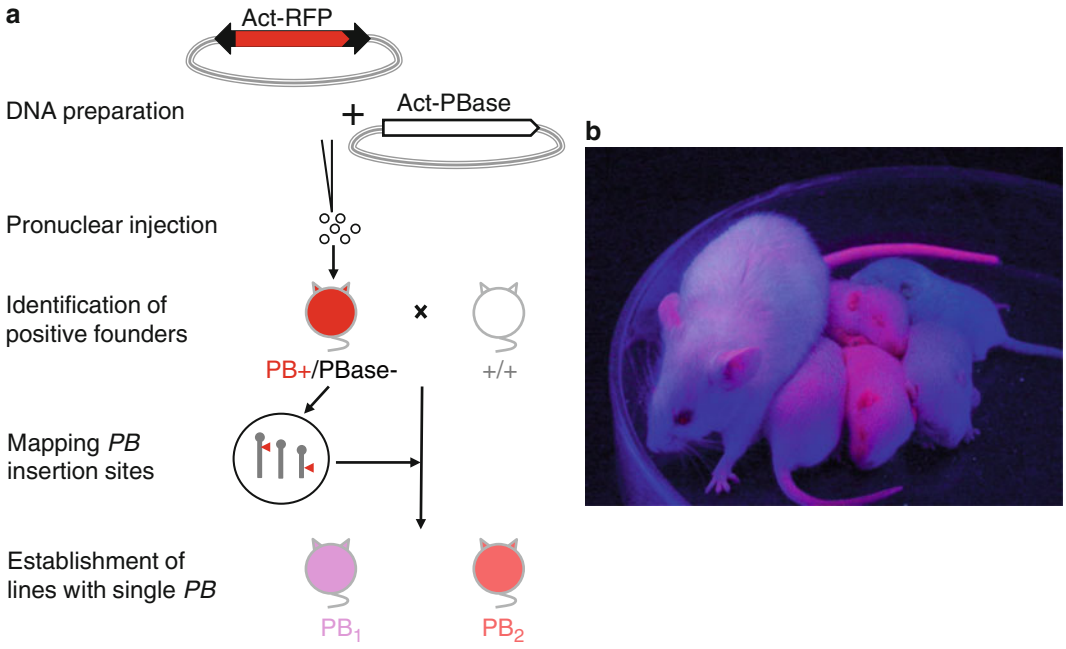

Fig. 2 Generation of transgenic mice by the *PB* transposon. (**a**) Step-by-step illustration of *PB*-mediated transgenesis in mice. PB_1 or PB_2 stands for different *PB* insertions. (**b**) A founder female mouse carrying multiple *PB*[*Act-RFP*] transposons which segregated in her progeny

8. Use 100 ng genomic DNA as template to perform genotyping PCR with transgene-specific primers. PCR products can be left at 4 °C overnight.

   PCR mixture:

DNA sample	100 ng
10× Genotyping PCR buffer	2 μL
DMSO	2 μL
25 mM dNTP mix	0.8 μL
20 μM of primer 1	0.12 μL
20 μM of primer 2	0.12 μL
Taq polymerase (5 U/μL)	0.2 μL
H_2O	To 20 μL

   Thermofile:

   1) 93 °C: 90 s.
   2) 93 °C: 30 s.
   3) 57 °C: 30 s.
   4) 65 °C: 3 min.

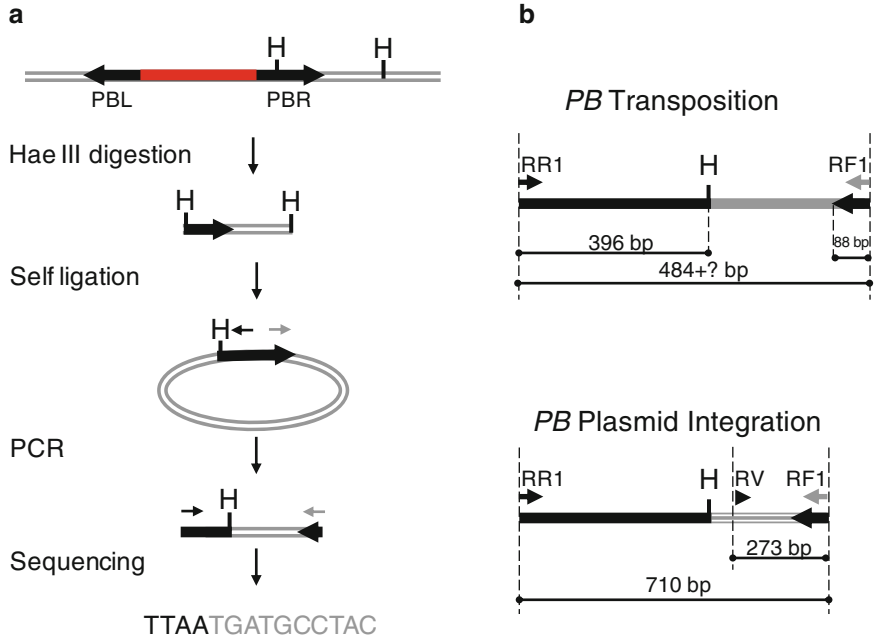

Fig. 3 Identification of *PB* insertion sites by inverse PCR. (**a**) Principle of inverse PCR. **H**: *Hae III sites*. *Arrows* stand for the PCR primers. (**b**) Anticipated results of inverse PCR with *Hae III* digestion

5) Go to step 2) for 39 more cycles.
6) 65 °C: 10 min.
7) End.

9. Run an aliquot of 5 μL of the PCR products in a 1 % agarose gel.
10. Perform genotyping PCR for transgene-positive founders with *PBase*-specific primers BacEN-F and BacEN-B with the same procedure as **steps 8** and **9**. Discard *PBase-positive* mice giving a PCR product of 1,795 bp (*see* **Note 3**).
11. **Steps 11–28** describe procedures to map *PB* integration sites by inverse PCR (Fig. 3a). Digest 2 μg genomic DNA of the transgene single-positive founders with *Hae III* as shown below (*see* **Note 4**). Incubate at 37 °C for 6 h or overnight.

10× NEB CutSmart Buffer	4 μL
Genomic DNA	2 μg
Hae III	2 μL
H_2O	To 40 μL

12. Run a 5 μL aliquot of the digests in a 0.7 % agarose gel to confirm a complete digestion. A smear with one or two intense pseudobands should be seen.

13. Inactivate the restriction enzyme by incubating the digests at 80 °C for 20 min.
14. Set up self-ligation reactions as follows. Incubate the samples overnight at 16 °C.

10× Ligase buffer	40 μL
Digested genomic DNA	35 μL
T4 DNA ligase	2 μL
H_2O	To 400 μL

15. Add 40 μL of 3 M sodium acetate and 1 mL 100 % ethanol. Mix well and incubate the tubes at –20 °C for 2 h. Samples can be left at –20 °C overnight.
16. Centrifuge the samples at 4 °C for 15 min at 16,000 × *g* Discard the supernatant.
17. Wash the DNA pellet with 200 μL of 70 % ethanol and discard the supernatant.
18. Air-dry the samples for about 10 min.
19. Add 60 μL H_2O to the sample and incubate at 55 °C for 1 h to help the DNA dissolve. Store DNA samples at –20 °C.
20. Prepare the following mixture for inverse PCR:

DNA sample	5 μL
10× Genotyping PCR buffer	2.5 μL
DMSO	2.5 μL
25 mM dNTP mix	1 μL
20 μM of primer RF1	0.16 μL
20 μM of primer RR1	0.16 μL
Taq polymerase (5 U/μL)	0.3 μL
H_2O	To 25 μL

21. Perform PCR with the thermofile in **step 8**. PCR products can be left at 4 °C overnight (*see* **Note 5**).
22. Run an aliquot of 5 μL of the PCR products in a 1 % agarose gel.
23. If PCR products show a single band other than 710 bp in electrophoresis, then purify the rest of the PCR products with QIAquick PCR purification kit. Send the purified sample for sequencing and go to **step 28** (*see* **Note 6**).
24. Otherwise, purify the rest of the PCR products with QIAquick PCR purification kit, clone the purified sample with pGEM-T vector system, and transform into *E. coli* (*see* **Note 7**).

25. Pick several transformants for each inverse PCR reaction. Let them grow at 37 °C overnight in test tubes with 3 mL XA.
26. Prepare the following mixture for 3-primer PCR:

E. coli culture	1 μL
10× PCR buffer	1.5 μL
25 mM $MgCl_2$	0.9 μL
25 mM of each dNTP	0.12 μL
20 μM of primer RF1	0.2 μL
20 μM of primer RR1	0.2 μL
20 μM of primer RV	0.1 μL
Taq polymerase (5 U/μL)	0.15 μL
H_2O	To 15 μL

Perform PCR with the thermofile in **step 8**. PCR products can be left at 4 °C overnight.

27. Run an aliquot of 5 μL of the PCR products in a 1 % agarose gel. Purify plasmid DNA from the colonies giving single bands bigger than 484 bp for sequencing (*see* **Notes 6** and 7).
28. Use BLAST searches (www.ncbi.nlm.nih.gov) or Ensembl mouse genome database (www.ensembl.org) to compare the sequencing results from **step 23** or **27** with the mouse genome sequence to map the integration sites of *PB*.
29. Breed desired transgene-positive founders to wild-type mice to generate lines carrying a single *PB* transposon (*see* **Note 8**).

3.2 Generation of Mouse Mutants by PB-Mediated Insertional Mutagenesis (Fig. 4a)

1. Generation of mutator lines: Follow Subheading 3.1 to generate mouse lines carrying a single *PB* transposon (*see* **Note 9**). Maintain these mutator lines by back-crossing with wild-type mice. Mutator mice carrying the *PB* transposon can be visually identified by the red fluorescence of RFP.
2. **Steps 2**–7 describe procedures to generate jumpstarter lines. Linearize the helper plasmid *Act-PBase* with restriction enzymes *Sal I* and *Hind III*. Purify the 4,095-bp *PBase* expression cassette with agarose gel electrophoresis and the QIAquick gel extraction kit.
3. Linearize plasmid *K14-Tyr* with *Asc I* and *Sma I*. Purify the 4,824-bp tyrosinase expression cassette with agarose gel electrophoresis and the QIAquick gel extraction kit.
4. Mix linearized *Act-PBase* and *K14-Tyr* DNA at a molar ratio of 2:1 with the total concentration at 2 ng/μL in the injection buffer, and spin briefly at maximal speed to pellet any debris immediately before injection to avoid clogging the needle.

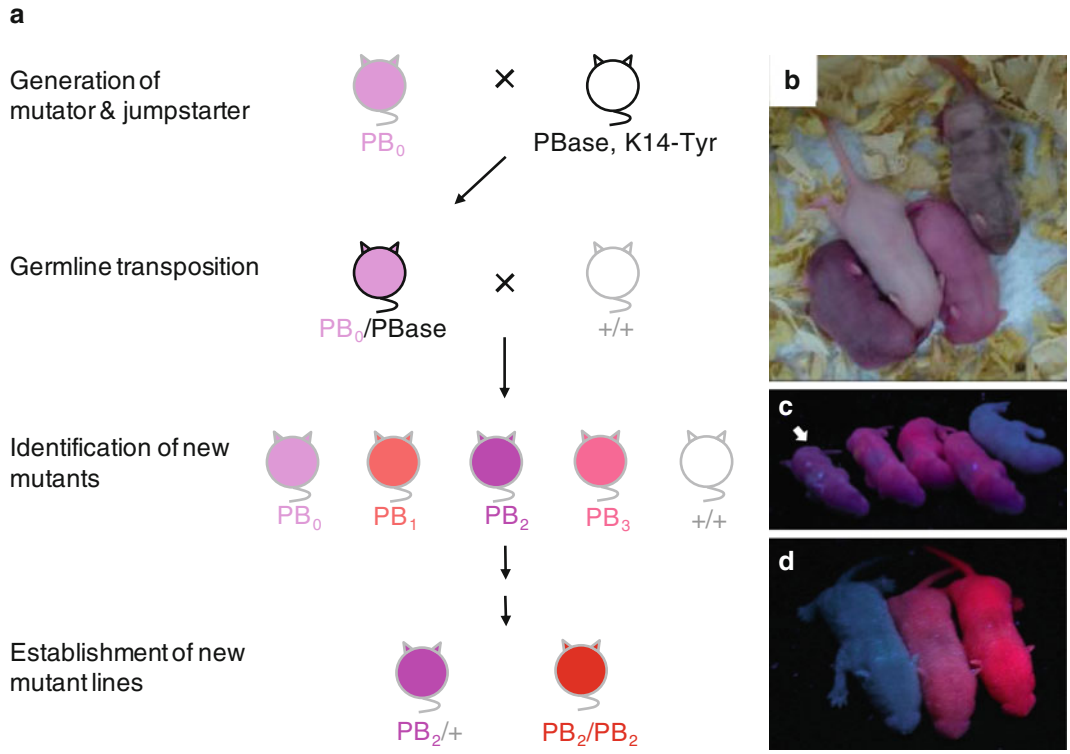

Fig. 4 Generation of mouse mutants by germline remobilization of a single-copy *PB* transposon. (**a**) Breeding strategy for producing new mutants. PB_0 stands for the original *PB* insertion, while PB_1, PB_2, or PB_3 stands for newly derived *PB* insertion. (**b**) An example of visible marker expression. The photo was taken under daylight. These four pups are progeny of a mating between a *PB*/*PBase* double-positive male and a wild-type female. Genotypes from the *left* to the *right*: *PB*/*PBase* double positive, wild type, *PB* positive, and *PBase* positive. (**c**) Identification of mice carrying new *PB* insertions by RFP intensity. The photo shows five 4-day-old littermates from a mating between a *PB*/*PBase* double-positive male and a wild-type female. *Arrow* shows the pup carrying the original *PB* insertion, while the other pink mice carry different new *PB* insertions. (**d**) Utilization of RFP to distinguish heterozygotes and homozygotes. The photo shows three 5-day-old littermates. Genotypes from the *left* to the *right*: wild type, heterozygote, and homozygote

5. Harvest fertilized eggs and perform pronuclear injection to generate transgenic founders as described [5].
6. Identify jumpstarter founders both by the 1,795-bp *PBase* band after genotyping PCR with primers BacEN-F/BacEN-B and by gray coat color resulted from the expression of *K14-Tyr*.
7. Breed jumpstarter founders with wild-type mice to confirm that both transgenes are co-segregated in the next generation, and then generate jumpstarter lines with desired founders.
8. Cross a mutator mouse with a jumpstarter mouse. Select *PB*/*PBase* double-positive progeny by red fluorescence and

gray coat color resulted from the *Act-RFP* in *PB* and *K14-Tyr* co-segregated with *PBase*, respectively (*see* Fig. 4b and **Note 10**).

9. Breed the *PB/PBase* double-positive animal with wild-type mice. Keep the progeny with red fluorescence (*PB* positive) and albino coat color (*PBase* negative).
10. Identify mice carrying new *PB* insertions by the different RFP intensity they have than that of their parental mutator line (*see* Fig. 4c and **Note 11**).
11. Map the new insertion by inverse PCR, as described in **steps 11–28** of Subheading 3.1.
12. Breed the mouse carrying a new *PB* insertion with wild-type mice to establish a new mutant line. Breed heterozygous siblings of the next generation to produce homozygous mutants. Homozygotes can be identified from heterozygotes as they have stronger RFP signal (Fig. 4d).

4 Notes

1. The quality of injection buffer affects the survival rate of injected embryos significantly. Use embryo tested-grade reagents whenever possible.
2. To reduce random integration events of donor and helper plasmid, it is not recommended to linearize these vectors before injection. In our hands, *PB* transposes quite efficiently in a wide range of donor-helper ratios (from 10:1 to 1:10) in tissue culture cells (Ding, S., Wu, X., and Xu, T., unpublished). The ratio of 2:1 is optional.
3. Among the progeny born in **step 5** of Subheading 3.1, more than 30 % are expected to be *PB* transgene single-positive founders, less than 3 % are *PBase* positive as well [17]. The latter group should be discarded, since the expression of *PBase* can remobilize the *PB* element and make the genome unstable. If the *PB* vector carries an *Act-RFP* cassette, positive founders can be easily identified according to the red fluorescence (Fig. 2b).
4. Any restriction enzyme cuts both the *PB* element and the flanking genomic sequences can be chosen for inverse PCR. We choose 4-nucleotide cutters so that the flanking genomic sequence after digestion will not be too long to be amplified by PCR. In our hands, *Msp I* is also successfully used for inverse PCR. If *MspI* is used, random integration of *PB* plasmids will generate a band of 814 bp as in **step 23** of Subheading 3.1.
5. Troubleshooting for inverse PCR.

Problem	Possible Reason	Solution
Inverse PCR get no products	Self-ligation failed	Check the quality of ligase used in **step 14** of Subheading 3.1
	Template DNA too few	Adjust DNA amount used in **step 20** of Subheading 3.1
	Flanking genomic *Hae III* too far from *PB* transposon	Try other restriction enzymes (e.g., *Msp I*) in **step 11** of Subheading 3.1
Inverse PCR only get products with the sizes similar as 484 bp	Flanking genomic Hae III too close to *PB* transposon	Try other restriction enzymes (e.g., *Msp I*) in **step 11** of Subheading 3.1

6. It needs to be noted that up to 40 % of the positive founders also have random integrations of the *PB* plasmid, which may exist as concatemers. *PB* plasmid integrations produce a characteristic 710-bp (Fig. 3b) fragment in inverse PCR with *Hae III*. If this fragment is cloned into the *pGEM-T* vector, it can produce two bands with the size of 710 and 273 bp in PCR with three primers RR1, RF1, and RV (**step 27** of Subheading 3.1). *PB* plasmid integrations can be segregated from *PB* transpositions during the successive breeding process, which will not significantly interfere with functional studies of the transgene. During the breeding process, primers RF1 and RV can also be used to identify *PB* plasmid integrations.
7. A *PB*-positive founder may have experienced multiple *PB* transposition events, resulted in single-copy insertions in different genomic loci. Different insertion sites result in bands with various sizes no less than 484 bp (Fig. 3b) in **step 22** of Subheading 3.1. To avoid sequencing failure of the inverse PCR product from a founder that carries multiple *PB* insertions, subcloning the PCR products with *pGEM-T* vector is recommended. This additional step is extremely useful if bands of similar sizes are generated in inverse PCR.
8. Single-copy *PB* insertions in different loci can be readily segregated during the successive breeding process. Individual *PB* insertions can be followed by genotyping PCR with RF1 and a locus-specific genomic primer.
9. To increase the efficiency of insertional mutagenesis, gene trap strategy can be adopted in the design of the *PB* donor vector [26, 36].

10. *PB* mobilizes following a "cut-and-paste" manner. Most of the mutants generated by remobilizing a single-copy *PB* insertion will not carry the original *PB* insertion. Due to different effects of surrounding genomic sequences, different *PB* insertions express the RFP at different levels. We recommend to select a mutator line with weak fluorescence at this step, so that most of the mice carrying new *PB* insertions can be easily identified in **step 10** of Subheading 3.2 by stronger RFP expression (Fig. 4c).
11. In our hands, more than 10 % of the visually identified *PB+/PBase*-progeny carry new insertions. Genotyping PCR can be performed in order to identify all the new *PB* insertions.

Acknowledgement

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Chapter 10

Generation and Applications of MADM-Based Mouse Genetic Mosaic System

Hui Zong

Abstract

Genetic mosaics describe organisms that contain cells with distinct genotypes related to somatic transposition, mitotic recombination, or genomic aberrations. Most, if not all, human cancers are genetic mosaics because cancer cells bear mutations that are absent in normal cells within the same body. While naturally occurring mutant cells in genetic mosaic animals are difficult to track down, a genetically engineered mosaic mouse model termed MADM (Mosaic Analysis with Double Markers) enables one to perform phenotypic analysis of mutant cells at single-cell resolution in vivo. While cancer modeling is the most obvious application, MADM is also highly suitable for studying developmental biology, neuroscience, and regenerative biology problems to investigate clonal contributions. Here we describe the construction of the MADM model on a specific chromosome through ES cell-based targeting of MADM cassettes into a pair of homologous chromosomes. We also detail procedures to verify the labeling efficiency of the newly established MADM model. Finally, we explain the breeding schemes and analytical principles that enable using MADM for in vivo phenotypic analysis at single-cell resolution.

Key words Mouse genetic mosaics, MADM, ES targeting, Gene knockout, Cell labeling, Phenotypic analysis, Single-cell resolution, Internal control, Cancer modeling

1 Introduction

Mouse gene knockout (KO) technique has been widely used to decipher the role of a particular gene in normal development and diseases. Conventional gene KO disrupts the gene function in zygotes and thus affects every cell in a mouse. Due to pleiotropic functions of many genes, embryonic lethality often precludes the analysis of gene functions in adult animals. Conditional KO, in which a loxP-flanked gene is deleted only in tissues or cell types where Cre recombinase is expressed, mostly resolves this issue [1]. However, the population of mutant cells generated by conditional KO is still quite large, making it difficult to differentiate cell-autonomous from cell-non-autonomous gene functions. To circumvent this problem and enhance the cellular resolution of

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phenotypic analysis in vivo, we established the MADM system (Mosaic Analysis with Double Markers) that uses Cre/loxP-mediated interchromosomal recombination to generate fluorescent protein labeled, sparsely distributed mutant cells within an otherwise heterozygous, colorless mouse [2]. Specifically, MADM generates a pair of GFP and RFP labeled daughter cells from a colorless progenitor cell (Fig. 1). If a mutant gene resides at the telomeric side of one of the MADM cassettes, the green cell will be homozygous

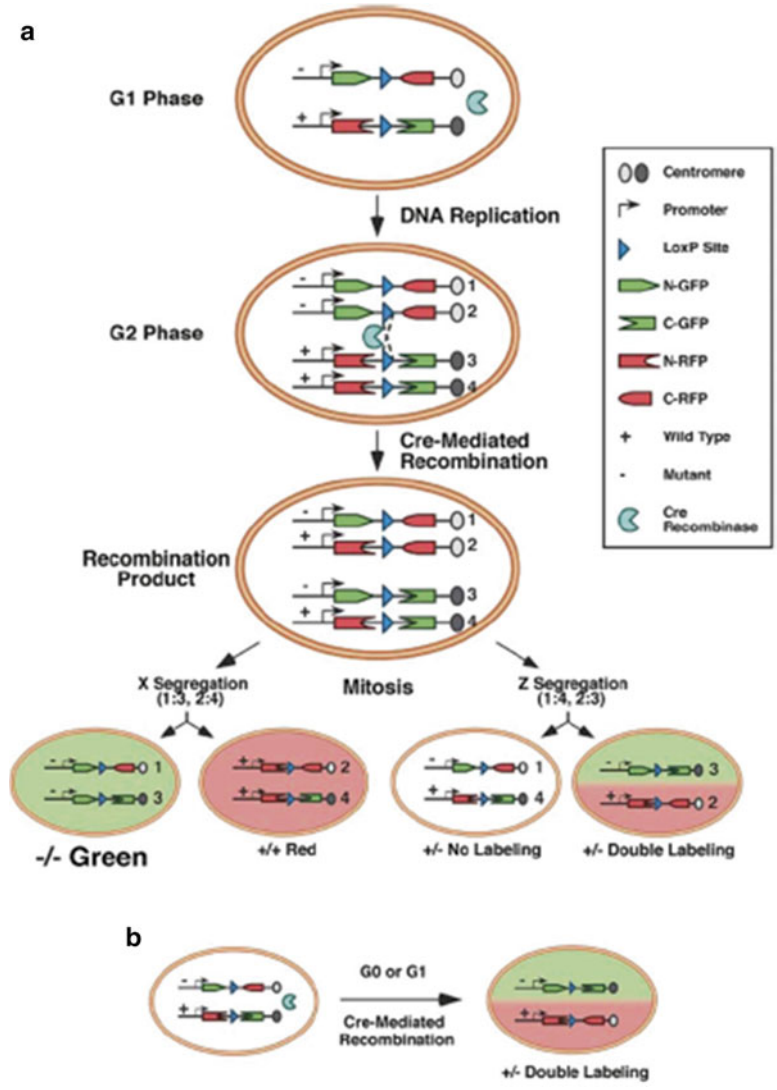

Fig. 1 Scheme of the MADM system. (**a**) From a colorless heterozygous mother cell, interchromosomal recombination after DNA replication restores GFP and RFP coding sequences and exchanges the telomeric side of homologous chromosomes. Subsequent X segregation produces one *green* mutant and one *red* WT daughter cell, while Z segregation produces two heterozygous cells, one still colorless and the other *yellow*. (**b**) Post-mitotic recombination produces heterozygous *yellow* cells

mutant while the red cell will be WT (an ideal internal control for in vivo phenotypic analysis). Since progeny from labeled cells will always express the same fluorescent protein, one could trace the lineage of both GFP- and RFP-labeled cells through their final fate. With these unique features, MADM has been applied to study various important biological questions, including the correlation between lineage relationship and axonal wiring of cerebellar granule cells [2], cell division patterns in the granule neuron lineage [3], neuronal morphogenesis [4] and migration [5], cell cycle regulation [6], the identification of the cell of origin for glioma [7], and the effects of loss of imprinting (LOI) in somatic cells [8].

While powerful, genes amendable to MADM-based phenotypic analysis are limited to the availability of MADM-bearing chromosomes. Currently published ones are chromosomes 6, 7, 11, and 12 [2, 5, 7–10]. If one's gene of interest is not located on these chromosomes, it would be recommended to contact Drs. Liqun Luo (Stanford), Hui Zong (University of Virginia), and Simon Hippenmeyer (IST Austria) for new MADM chromosomes that are currently under construction. If an MADM-bearing chromosome is not available, one could then follow the protocol in this chapter to construct an MADM chromosome. Since the targeting backbone and MADM cassettes are all available at Addgene (see below for details), the main effort is to follow Subheading 3.1 to identify an intergenic targeting locus with open chromatin structure that supports bi-allelic, ubiquitous expression of marker genes. After successful germ line transmission of MADM mice, one should then follow Subheadings 3.2–3.4 to verify ubiquitous cell labeling, bi-allelic expression of GFP and RFP, and possible imprinting effects. Finally, after building up stock mice (Subheadings 3.5 and 3.6), one can analyze phenotypes caused by the loss of gene of interest in all tissues at single-cell resolution (Subheading 3.7).

It should be noted that while all can label cells for the lineage tracing purpose, MADM and Brainbow or confetti systems have distinct features [11, 12] (http://jaxmice.jax.org/findmice/brainbow.html). Brainbow/confetti systems are Cre reporter mice that stochastically express different reporter genes, which are particularly suitable for analyzing multiple lineages at the same time but not designed for gene KO studies. MADM, on the other hand, achieves gene KO and cell labeling in one recombination event that enables single-cell resolution analysis of phenotypes, including lineage aberration of GFP + mutant cells in comparison to RFP + WT cells [2, 6]. One should choose carefully which system to use according to one's research questions.

While at the first glance MADM seems to require a lot of mouse breeding, in fact the number of mouse cages required is quite limited, and the overall time from stock breeding to data collection is short compared to conditional KO system. Significant savings are achieved because: (1) unlike conditional KO that requires the presence of floxed allele in both parents, MADM only

need a heterozygous allele in one of the MADM stocks; (2) the internal control provided by red WT cells greatly reduces the number of mice needed to generate statistically significant data to pinpoint any phenotypes. From our experiences, MADM-based models have consistently outpaced and outperformed conditional KO models to reach unequivocal conclusions [6, 7]. Therefore, building an MADM-bearing chromosome should be a great investment for the lab in the short term, and for the entire field of mouse genetics in the long run.

2 Materials

1. MADM-GT plasmid (http://www.addgene.org/40020/).
2. MADM-TG plasmid (http://www.addgene.org/40021/).
3. Targeting backbone vector pROSA26PA (http://www.addgene.org/21271/).
4. Phusion high-fidelity DNA polymerase (New England Labs).
5. Plasmid miniprep and maxiprep kits (QIAGEN).
6. LA Taq Polymerase (TaKaRa).
7. Fluorescent dissection scope.
8. Confocal laser-scanning microscope.
9. Chicken anti-GFP antibody (Aves Labs).
10. Goat anti-myc antibody (Novus Biologicals).
11. Donkey anti-chicken Alexa Fluor 488 secondary antibody (Invitrogen).
12. Donkey anti-goat Alexa 555 secondary antibody (Invitrogen).

3 Methods

3.1 Targeting of the MADM Cassettes onto a Given Chromosome

1. Identify the chromosome location of your gene of interest. Go to Mouse Genome Informatics Web site http://www.informatics.jax.org/. Enter the gene name in the search box. Click the proper entry, and copy down chromosome number and recombination distance (unit in cM as centi-Morgan) in the "Genetic Map" box (Fig. 2a).
2. Go to Mouse Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway?org=mouse), and look for a region between centromere and your gene of interest to insert MADM cassettes (Fig. 2b) (*see* **Note 1**).
3. Zoom in within the region to identify a few intergenic sequences as candidate targeting sites (*see* **Note 2**).
4. To identify a locus within open chromatin structure among choices from **step 3**, look up the expression data of both flanking

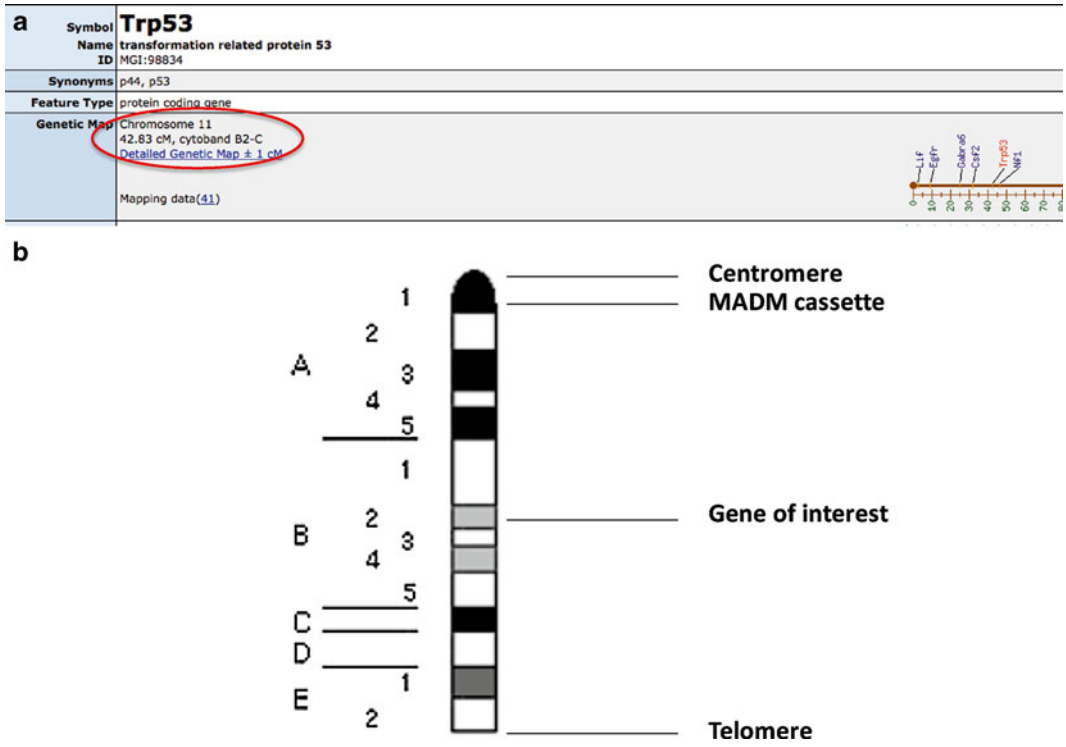

Fig. 2 Position of the MADM cassette on the target chromosome. (**a**) Screenshot of gene information at the Mouse Genome Informatics Web site http://www.informatics.jax.org/, showing the genomic location of the gene of interest (*red circle*). (**b**) MADM cassette should be inserted between centromere and the gene of interest. Whenever possible, it should be positioned as close as to the centromere so that as many genes as possible on that chromosome can be studied with the MADM system

genes by going to http://www.ncbi.nlm.nih.gov/, selecting UniGene as the search option, typing in the gene name into the search box, clicking the entry of mouse gene (*Mus musculus*), and then clicking "EST profile" (*see* **Note 3**).

5. To avoid potential disruption of regulatory elements around the locus selected in **step 4**, go to http://genome.lbl.gov/vista/index.shtml, compare the mouse sequence against equivalent sequences from human, and choose non-conserved areas (*see* **Note 4**).
6. Among areas chosen from **step 5**, pinpoint an MADM cassette insertion site that is surrounded by balanced Base composition and devoid of long stretch of mono- or bi-nucleotide repeats. Decide on 5′ and 3′ targeting arms that flank the insertion site (*see* **Note 5**).
7. Request MADM-GT (http://www.addgene.org/40020/) and MADM-TG (http://www.addgene.org/40021/) cassettes, and targeting backbone vector pROSA26PA (http://www.addgene.org/21271/) from Addgene.

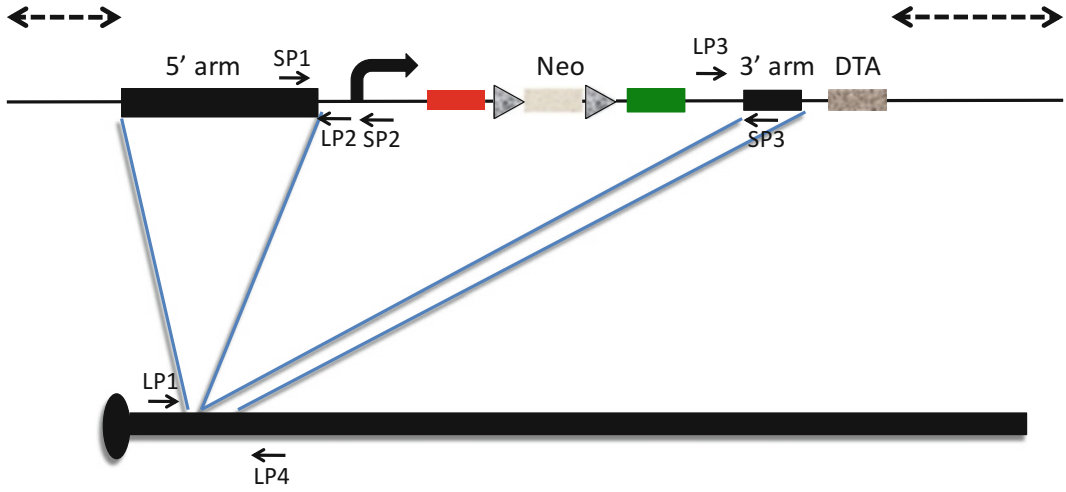

Fig. 3 Configuration of the MADM-GT targeting construct and primers for genotyping. MADM-TG is almost identical except for the coding sequence of the fluorescent proteins

8. Analyze DNA sequences of all the components to design a cloning scheme to replace the ROSA26 targeting arms pROSA26PA with "[5′ targeting arm] - [MADM GT] - [3′ targeting arm]" or "[5′ targeting arm] - [MADM TG] - [3′ targeting arm]", respectively. Order primers for amplify targeting arms with restriction enzyme sites at both ends according to the design (Fig. 3) (*see* **Notes 6** and **7**).
9. Purify genomic DNA from ES cells that will be used for gene targeting as the PCR template to amplify targeting arms (*see* **Note 8**).
10. PCR amplification of targeting arms from genomic DNA purified in **step 9** with high fidelity DNA polymerase, such as Phusion.
11. Clone amplified targeting arms from three independent PCR reactions and send one clone each for sequencing. Align the sequencing data with mouse genome data at NCBI Web site (*see* **Note 9**).
12. Based on the design in **step 8**, use restriction enzymes to digest pROSA26PA, targeting arms, and the MADM cassettes. Then assemble the targeting construct by ligating 5′ arm, MADM-GT or TG, 3′ arm together into pROSA26PA backbone (*see* **Note 10**).
13. Spread the ligation products on LB plates with 50 mg/L ampicillin and 5 mg/L kanamycin (*see* **Note 11**).
14. Next day, pick colonies and grow up overnight culture.

15. Miniprep plasmids and identify potential constructs based on restriction enzyme digestion maps.
16. Sequence the entire targeting construct to verify its accuracy.
17. Purify targeting constructs using QIAgen maxiprep kit.
18. Linearize the targeting constructs with a restriction enzyme that cuts outside, not within, the targeting arms-DTA region (Fig. 3). Use phenol–chloroform extraction to purify linearized DNA.
19. Submit the linearized constructs to the core facility for standard ES targeting.
20. In 2–3 week after ES cell electroporation, expect to receive 96-well plates containing neomycin/G418 resistant ES cells clones.
21. Purify genomic DNA from 96-well plates according to the protocol 11 in Chapter 3 of the book titled *Gene Targeting*: *A Practical Approach* [13].
22. Order primer sets, LP1, LP2, LP3, and LP4, for the screening of correctly targeted ES clones for both 5′ and 3′ arms (Fig. 3) (*see* **Note 12**).
23. Use TaKaRa LA Taq DNA polymerase to perform PCR screening of candidate ES clones with genomic DNA and primer sets from steps above and select clones that are properly targeted at both 5′ and 3′ sides (*see* **Note 13**).
24. After identifying all positive clones, ask ES facility to thaw at least three ES clones for MADM-GT and TG targeting, respectively (*see* **Notes 14** and **15**).
25. After thawing and expansion, ask ES facility to seed some ES cells in a 6-well plate. Purify genomic DNA from each clone and use PCR-based method to verify the proper targeting again as described above.
26. Choose one positive clone each (MADM-GT and TG) that is free of trisomy based on karyotyping results and ask ES facility to inject into WT C57bl/6 blastocysts.
27. At ~30-day post injection (~12 days after birth), check the coat color of pups for ES cell contribution to the chimeric pups. Keep the pups that have predominantly agouti coat color but not the black ones (*see* **Note 16**).
28. Genotype the agouti chimeric pups for the presence of targeted allele.
29. 7–8 weeks after birth, breed a few male chimeric mice that have mostly agouti coat with black female mice (*see* **Note 17**).
30. Rotate each male with two new black females each week to generate multiple litters.

31. ~12 days after the litters are born, check the coat color of pups and keep the agouti ones but not the black ones. Genotype agouti pups to look for germ line transmission of the targeted alleles (*see* **Note 18**).
32. Design SP1, SP2, and SP3 primers for a short, internal PCR that can detect both the GT and TG alleles for future use (Fig. 3) (*see* **Note 19**).
33. 7 weeks after birth, incross each strain (GT or TG) to make homozygous stock (*see* **Note 20**).

3.2 Verify Cell Labeling in the Newly Established MADM System

1. Breed GT line with ubiquitously expressed Hprt-Cre to get GT; Hprt-Cre.
2. When GT; Hprt-Cre mice are sexually mature, breed them with TG mice. Use the short internal PCR that can detect both GT and TG alleles and a primer set for Cre to genotype pups. Keep pups that have the MADM-ready genotype (GT/TG; Hprt-Cre) and control genotype (GT/TG).
3. At the weaning age, dissect the mice with both genotypes and collect multiple tissues to examine the cell labeling efficiency by the MADM system. Tissues from mice with the control genotype should have no color at all [2].

3.3 Verify Bi-allelic, Ubiquitous Expression of MADM Cassettes (See Note 21)

1. Breed GT/TG; Hprt-Cre mice generated in Subheading 3.2 with WT mice. Use fluorescent dissection scope to look for green and red pups (*see* **Note 22**).
2. In ~7 weeks, breed green and red mice to get double positive pups.
3. At the weaning age, dissect these pups and collect multiple tissues to examine the color of cells under a confocal laser scanning microscope. If all cells are yellow and no cells are green or red, then bi-allelic expression is confirmed (*see* **Note 23**).

3.4 Examine Potential Imprinting Effect (See Note 24)

1. Breed GT; Hprt-Cre male with TG female mice. Collect tissues from GT/TG; Hprt-Cre pups (*see* **Note 25**).
2. Breed GT; Hprt-Cre female with TG male mice. Collect tissues from GT/TG; Hprt-Cre pups (*see* **Note 26**).
3. Calculate the ratio of cell numbers and compare general properties between green and red cells. If the ratio remains at 1 in both cases and there is no obvious difference between green and red cells, then imprinting effect does not manifest in the examined tissue (*see* **Note 27**).

3.5 Recombine Mutant Allele with the TG Allele for Phenotypic Analysis

1. Breed mutant mice with TG mice. Genotype and keep TG/mutant trans-heterozygous pups.
2. In 7–8 weeks, breed TG/mutant mice with WT mice. Genotype for both alleles and keep TG, mutant recombinant

pups (*see* **Note 28** for the number of pups needed for getting recombinant pups).

3. In 7–8 weeks, breed TG,mutant mice with TG/TG mice to get TG,mutant/TG mice as the final stock (*see* **Note 29**).
4. Maintain the stock by breeding TG,mutant/TG with TG/TG mice.

3.6 Introduce Desired Cre Transgene into the GT Line

1. Breed Cre mice with GT mice. Genotype for both alleles and keep GT/+; Cre/+pups.
2. In 7–8 weeks, breed GT/+; Cre/+with GT/GT mice. Genotype for both GT and Cre and keep GT/GT; Cre/+pups. If homozygous Cre is viable and fertile, one should incross GT/+; Cre/+mice to get GT/GT; Cre/Cre stock to double the breeding efficiency.
3. Maintain the stock by crossing GT/GT; Cre/+with GT/GT, or incrossing GT/GT; Cre/Cre stock mice.

3.7 Perform In Vivo Phenotypic Analysis of Mutant Cells with MADM

1. Breed TG,mutant/TG with GT/GT; Cre mice.
2. Genotype for MADM alleles, mutant allele and Cre. Keep TG,mutant/GT; Cre pups.
3. At desired age, dissect these mice, collect tissues of interest, and section them onto slides.
4. Stain slides with anti-GFP (Alexa 488), anti-myc (epitope tag for tdTomato, Alexa 555), and other relevant antibodies (Fig. 4) (*see* **Note 30**). If antibody recognizing the gene of interest is available, it is advised to confirm the loss of gene product in all GFP + cells [6].

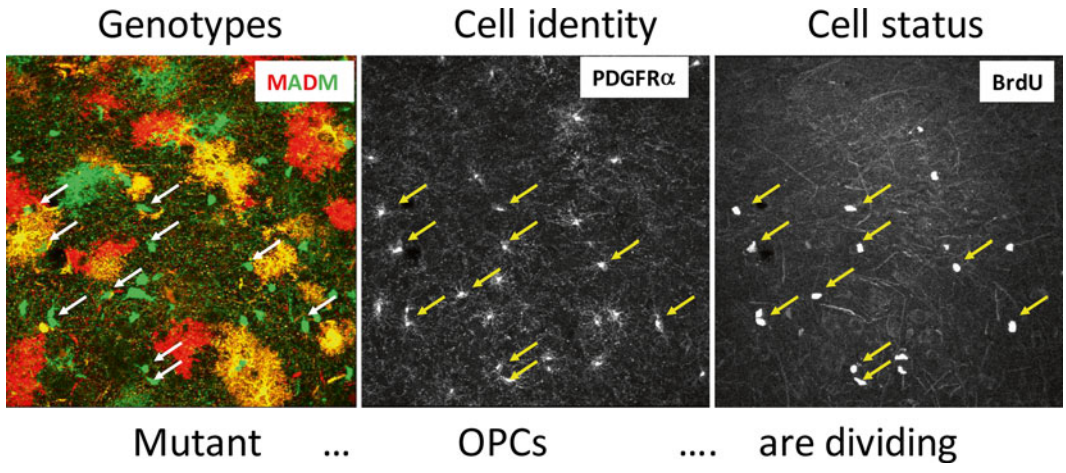

Fig. 4 MADM allows phenotypic analysis at the single-cell resolution. Use a 4-channel staining scheme, *green* and *red* channels define mutant vs. WT genotypes; *far-red* channel can be used to pinpoint cell identity (in this case PDGFRα stains for oligodendrocyte precusor cells); while *blue* channel reveals cellular state (in this case BrdU stains for DNA synthesis during cell division)

5. Acquire images with confocal laser scanning microscope. Focus on the differences between green mutant and red WT cells as phenotypes caused by mutation(s). A few examples of basic phenotypic analysis:
   (a) Compare morphologies between green and red cells [4].
   (b) Compare cell number between green and red cells [6, 7].
   (c) Compare migration pattern between green and red cells [5].
   (d) Compare proliferation, differentiation, and apoptosis rate between green and red cells [7].

4 Notes

1. Based on the design principle of MADM, only mutant genes located between MADM cassette and telomere can be turned into homozygous mutations upon Cre/loxP-mediated interchromosomal mitotic recombination. Therefore, the insertion locus of MADM must reside between centromere and your gene of interest. To ensure most genes on this chromosome suitable for MADM analysis, it would be ideal to insert MADM cassettes as close as possible to the centromeric region, as long as the insertion site satisfies considerations detailed below.
2. Since MADM consists of the knock-in of two complementary cassettes into the equivalent loci on a pair of homologous chromosomes, the insertion site will be homozygously disrupted in MADM mice. Therefore, the insertion site must locate in intergenic sequences to avoid disrupting coding sequence of endogenous genes.
3. Since MADM relies on GFP and RFP marker expression to identify mutant cells, it is critical to insert MADM cassettes into open chromatin structure where housekeeping genes reside to avoid silencing of marker expression. Ideally, EST profiles of both flanking genes should show high expression value in multiple tissues and at multiple developmental stages. As references, both Gt(rosa)26sor and eif4enif1 loci are proven open chromatin structure with relatively high EST value, and thus can be used as references. Since it is unlikely for any gene to express in all tissues at all developmental stages, special attention should be paid to EST values in tissues for intended studies, such as brain for neuroscience research.
4. Since disrupting regulatory regions by MADM cassettes could lead to alteration of the expression of neighboring genes, best effort should be made with VISTA comparison to avoid these elements, along with repetitive elements such as LINE, SINE, LTR, etc.

5. As a rule of thumb, one of the targeting arms should be ≥5 kb whenever possible to ensure high targeting efficiency, and the other arm could be 1–3 kb long. While in theory one could use even longer targeting arms, there is limitation of Taq polymerase to faithfully amplify long chromosome fragments. Long targeting arms also pose difficulties for the verification of proper targeting in ES cells, using PCR with primers that flank targeting arms.
6. pROSA26PA contains three parts: pSK plasmid backbone with ampicillin resistant gene; targeting arms for ROSA26 locus, and a DTA negative selection marker against random integration of targeting constructs into the genome after ES cell electroporation [14]. One could either replace the ROSA26 targeting arms with the ones for current targeting, or just cut out the full DTA cassette (promoter, coding region, and polyA sequence) and reconstruct with the current targeting arms into a "clean" pSK plasmid.
7. The MADM cassettes can be cut out from MADM-GT and MADM-TG plasmids with restriction enzymes that recognize 8 bp sequences: PacI and PmeI on the 5′-end, SawI and AscI on the 3′-end. When designing primers to amplify targeting arms, it is important to design compatible (do not have to be exactly the same) restriction enzyme sites to these 8-cutters in between 5′ and 3′ targeting arms, which should be unique and do not cut elsewhere in the targeting arms and the plasmid backbone. Finally, a restriction site in the plasmid backbone (outside of targeting arms-DTA region) needs to be identified for construct linearization during the ES cell electroporation step. If there are none, one should add a unique restriction site at the 5′ end of the 5′-arm during primer design.
8. Different ES cell lines are derived from different mouse strains, thus have some degrees of polymorphism. Because even small mismatch between targeting arms and genomic DNA could drastically reduce the efficiency of homologous recombination, it is critical to amplify targeting arms from genomic DNA of ES cells that will be used for targeting.
9. It is expected that there will be discrepancies between cloned targeting arms and the NCBI mouse genome data, because (1) most ES cell lines were derived from 129-based mouse strains, while the NCBI data were based on C57bl/6 strain; (2) NCBI data set still contains some errors; (3) PCR amplification could introduce some mutations into the targeting arm. By sequencing three independent PCR clones, one would not need to be concerned if the differences between targeting arms and NCBI data set are exactly the same in all three clones (issue #1 and #2) since targeting efficiency will not be affected. However, any unique differences in one clone should be attributed to

issue #3, alerting the researcher not to use that clone as the targeting arm. If necessary, more clones should be sequenced to find a correct targeting arm.

10. There are two ways to perform the ligation experiment. In a stepwise fashion, one could first replace the ROSA26 targeting arms with the current targeting arms, then use restriction enzymes to cut in between 5′ and 3′ arms to insert the MADM cassettes. Or one could perform a 4-piece ligation to assemble [pROSA26PA without original arms] - [5′ targeting arm] - [MADM cassette] - [3′ target arm] together, as long as the restriction enzyme sites at all four junctions are distinct.
11. The neomycin/kanamycin gene in the MADM cassettes has both mammalian and bacteria promoters and thus can be used to facilitate the selection of correctly ligated clones. Both ampicillin and kanamycin are used at lower concentration because large-sized targeting constructs tend to have lower copy numbers thus reduced resistance.
12. It would be wise to test LP1 and LP4 primers with genomic DNA of non-targeted ES cells.
13. Since the primer sets that can verify proper targeting must flank the targeting arms, the fragment size of these PCRs could be more than 5 kb. Long fragment PCR with crudely purified, small amount of genomic DNA tends to be very challenging. Based on the recommendation by Dr. Hong Zeng at Stanford Transgenic Facility, we found that Takara LA Taq polymerase works the best for such PCR reactions. It is recommended to follow product instructions and analyze the GC content in the targeting arms to determine the optimal PCR conditions.
14. It would be wise to thaw multiple clones at this stage since some clones don't grow very well while some clones could have a trisomy problem.
15. Whether or not to use neomycin during the expansion stage is worth considering. If neomycin is not used, WT ES cells attached to properly targeted ES cells could take over the clone. However, prolonged culture with neomycin will retard ES cell growth and increase the chance of trisomy. It is recommended to use moderate dose of neomycin in the first a few days of the expansion phase but not to its entirety.
16. It should be noted that agouti coat color is an exaggerated representation of chimeracy because the gene product of agouti is secreted and affects larger than the actual ES cell contributed areas.
17. Unless C57bl/6 genetic background is critical for experiments, it is recommended to use other mouse strains with black coat color that have better fecundity.

18. It is possible that some males never sire any litters, due to either developmental defects of reproductive organ or trisomy in ES cells. If all males fail to breed or all agouti pups contain no MADM targeting due to WT ES cell contamination, it would be necessary to thaw another positive ES clone, perform karyotyping test for trisomy, and repeat the blastocyst injection.
19. Make sure that fragment size from PCR with SP1-SP2 primer set (MADM allele) is different from that with SP1-SP3 primer set (WT allele), so that three primers could be mixed together for genotyping purpose. It is recommended to use long PCR for mouse genotyping up to this step just in case the injected ES clone contains cells with both properly targeted and randomly inserted MADM cassette, which would derail the entire experiment. At this step, it would be a good idea to perform both long and short PCR reactions side by side. If all positive clones match with each other in both reactions, then it will be certain a properly targeted mouse has been obtained.
20. With great care during the locus selection process, the insertion of MADM cassettes should not affect endogenous gene expression, making homozygous stock mice viable.
21. Since GFP and RFP expression will be used to determine genotypes of cells in an MADM mouse (green as −/−, red as +/+, yellow and colorless as +/− for the gene of interest), mono-allelic or silenced expression of fluorescent proteins will invalid the scheme entirely. Therefore, bi-allelic expression from the locus is absolutely essential for the MADM system to work according to the design.
22. Because Hprt-Cre is ubiquitously expressed and can catalyze recombination in the germ line, some germ cells will have recombined, full-length GFP or RFP. Pups containing these alleles will be green or red, respectively.
23. If the targeting locus allows bi-allelic expression, cells in all tissues should appear yellow. If cells in some tissues appear green or red rather than yellow, it would suggest that the targeting locus expresses marker genes in a mono-allelic fashion. If colorless cells are found, then MADM cassette must be silenced in them. If your tissue of interest contain significant amount of colorless, green, or red cells, the MADM system would not work properly. A different locus should be chosen to repeat the entire experiment.
24. When homozygous mutant and WT cells are generated from heterozygous mother cells through mitotic recombination, these cells will be either bi-paternal or bi-maternal [8]. If there are imprinted genes on the newly targeted chromosome, bi-paternal and bi-maternal cells could behave differently without

any additional mutations, which could confound the phenotypic analysis of the gene of interest.

25. Red cells will be bi-paternal and green ones will be bi-maternal in the pups.
26. Red cells will be bi-maternal and green ones will be bi-paternal in the pups.
27. Imprinting effect could be variable among tissues. One could proceed as long as his/her tissue of interest is free of such effect. Even if there is imprinting effect, one could still perform the experiments as long as some pups get mutant allele from father while others get it from mother. Any phenotypes that manifest consistently in both cases are due to gene mutations rather than imprinting effects.
28. Calculate the genetic distance between TG and mutant gene loci (cM) based on information at the MGI Web site. For 95 % confidence (less than 5 % probability of failure) to get a recombined TG, mutant mouse, the equation $(1-\text{cM}\%/2)^n < 5\ \%$ should be used. The total pups needed will be $n > \ln 0.05/\ln(1-\text{cM}\%/2)$. For example, to recombine a mutated gene that is 10 cM away from MADM cassette, it will take $\ln 0.05/\ln 0.95 = 58$ pups to get 95 % confidence. When gene of interest and MADM alleles are tightly linked (small cM value), many more pups will be needed to obtain recombination. In extremely difficult case, one could consider using CRISPR strategy to directly mutate the gene of interest in zygotes containing the MADM cassette [15–17]. It is also possible to recombine multiple mutated genes on the same chromosome with the MADM cassette [7].
29. Even after recombining TG and mutated gene, they will segregate in germ line during further breeding, especially if they are far apart on the chromosome. Keeping the stock as TG,mutant/TG ensures 50 % TG,mutant transmission rate in all circumstances and greatly increases the efficiency in producing pups with desired genotype.
30. If Cre labels multiple cell types, additional marker can be used to identify the cell type of interest. Markers for cell proliferation, differentiation, and apoptosis can also be stained [7]. Depending on the availability of secondary antibodies with distinct excitation/emission wavelength and spectrum-separation capability of your confocal laser-scanning microscope, you can use more markers for you to analyze phenotype of specific cell types in vivo. Alternatively, fluorescent conversion of LacZ reaction can be used to image gene expression [18].

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Chapter 11

Generation of Mouse Lines Conditionally Over-expressing MicroRNA Using the Rosa26-Lox-Stop-Lox System

Claudia Piovan, Foued Amari, Francesca Lovat, Qun Chen, and Vincenzo Coppola

Abstract

MicroRNAs are currently the object of intensive investigation due to their role in a myriad of physiological processes and pathological conditions, such as gene regulation and tumorigenesis. To better understand microRNA function, numerous laboratories have already taken advantage of the available techniques of genome editing in mouse. Here, we describe how to generate genetically engineered mouse lines using the popular Rosa-26 Lox-Stop-Lox Knock-In (Rosa-LSL-KI) targeting. This strategy allows for the selective overexpression of microRNAs of interest when coupled to a tissue-specific Cre-expressing line. The present protocol illustrates in detail both the engineering of the targeting vector and the generation of mutated ES clones ready for injection into mouse blastocysts.

Key words MicroRNA, Gene targeting, Knock-in mice, Transgenic mice, Rosa26 Locus

1 Introduction

With improved methods to explore the transcriptome, there has been great attention to the world of the noncoding RNAs, such as microRNAs. MicroRNAs are small (19–22 nucleotides) noncoding RNAs, highly conserved across different species, which play crucial functions in important processes as key regulators of gene expression [1]. Although novel unconventional actions have recently been described [2, 3], the main function of microRNAs is to negatively regulate protein coding gene expression [4]. In many cases the overall effect of microRNAs appears to be context specific [5]. Consequently, mouse models conditionally overexpressing specific microRNAs are invaluable tools to elucidate their role in vivo.

The ROSA26 locus on mouse chromosome 6 has proven to be an ideal genomic location to insert genes for regulated overexpression purposes. The use of this locus was pioneered by the Soriano's laboratory to engineer a reporter line that is still widely used to investigate Cre-expression in vivo [6]. It is now established that: (1)

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the disruption of the Rosa26 gene does not cause any obvious phenotype; (2) the locus is relatively easy to target; (3) the inserted transgenes are not subject to silencing. Thus, numerous groups have used the same LSL (LoxP-Stop-LoxP) strategy and now many Rosa26-LSL-KI murine lines are available. The first reports of a successful microRNA overexpression exploiting the Rosa-LSL-KI strategy in the mouse were published in 2007 [7, 8].

Over the past few years, we have worked to improve the system. We engineered different vectors resulting in alleles with various levels of expression. The present protocol describes how to generate Rosa26-LSL-microRNA-KI mouse lines, but it can be applied to any other Rosa26-LSL-gene-KI strain generation, from the targeting vector construction to the expansion of targeted ES clones ready to be injected into blastocysts, a service that can be routinely performed by institutional mouse transgenic core facilities.

The Rosa-LSL-KI system is only one of the possible strategies to engineer mouse lines in order to obtain specific microRNA overexpression. However, it is beyond the scope of this chapter to discuss the advantages and disadvantages of all the different approaches available for the establishment of microRNA overexpressing mouse lines.

2 Materials

2.1 Engineer Rosa-LSL-microRNA Targeting Vector and Genotype mES Rosa26-Targeted Clones

1. Refrigerator/Freezer (4 °C/−20 °C) to store reagents and solutions.
2. Thermocycler (Veriti 96-well thermal cycler, AB Applied Biosystems).
3. Incubator at 37 °C.
4. H_2O bath (37 °C and 42 °C).
5. Microcentrifuge (model 5424, Eppendorf).
6. Centrifuge (Sorvall Legend XFR, Thermo Scientific).
7. Large gel electrophoresis apparatus.
8. NanoDrop Spectrophotometer (ND-1000, Thermo Scientific).
9. Gel-imaging device with UV-transillumination cabinet (AlphaImager HP, Alpha Innotech).
10. UV crosslinker (Stratalinker 2400, Stratagene).
11. Film developer (model M35A, Ti-Ba Enterprises).

2.2 Culture Rosa26 Targeted mES Clones

It is strongly recommended dedicating a tissue culture room to mouse ES (mES) cell culture in order to limit access and exposure to the rest of the laboratory environment. In the absence of a dedicated room, we recommend taking special care to assure that the

equipment is decontaminated. It is very important to avoid at all times mycoplasma contamination: persistent infections of cells can result in genetic and phenotypic changes and, most importantly, it can affect postnatal development and germ line transmission of the resulting chimeras.

1. Tissue culture incubators. Set at 5 % CO_2, 20 % O_2, with a saturated aqueous atmosphere. Routinely monitor CO_2 levels and ensure that the recovery time after openings is not too extended. Primary mouse embryonic fibroblasts (MEFs) grow particularly well in a low-oxygen condition due to reduced oxidative stress, replicative senescence, and DNA damage. For this reason, it is also recommend using an O_2/CO_2 incubator connected to a nitrogen tank to maintain the percentage of O_2 at 3 % (MCO-18 M, Sanyo).
2. Inverted Microscope with 5×, 10×, and 20× objectives (Olympus GX41).
3. Electroporator capable of reaching the capacitance of 500 mFarads (Gene Pulser, Biorad).
4. Tissue culture hood (biological safety cabinet; BSC) with UV light. Make sure that it has been properly decontaminated before use. Routinely wipe BSC with 70 % ethanol before and after use. Turn on the UV light when not in use.
5. Tabletop centrifuge (Hermle Z300, Labnet).
6. H_2O bath at 37 °C for regular use (Polyscience).
7. Magnetic stirrer (Fisher Scientific™).
8. Orbit shaker with temperature monitor (Environ-Shaker, Lab-Line).
9. Cell counter (Cellometer Auto T4, Nexcelom Bioscience).
10. Refrigerator/Freezer (4 °C/−20 °C) to store tissue culture reagents and solutions.
11. Deep freezer (−80 °C) for short-term storage of mES clones, MEFs and ES certified Fetal Bovine Serum (FBS).
12. Liquid Nitrogen Cryotank (Cryoplus 3, Thermo Scientific) for long-term storage of mES clones and primary MEFs.

2.3 Rosa26-LSL-KI Plasmids

We use three different versions of the Rosa26-LSL targeting vectors.

1. *pCAG-STOP-eGFP-RosaTV*: basic targeting vector containing 4.3 kb 5′- and ~1 kb 3′- homology arms separated by a compound cassette that includes (a) CAG promoter; (b) STOP cassette (loxP-neo resistance gene-3X polyadenylation sites-loxP); (c) *Asc*I unique cloning site where the microRNA is cloned; (d) frt-IRES-eGFP-frt cassette; (e) polyadenylation site [7].

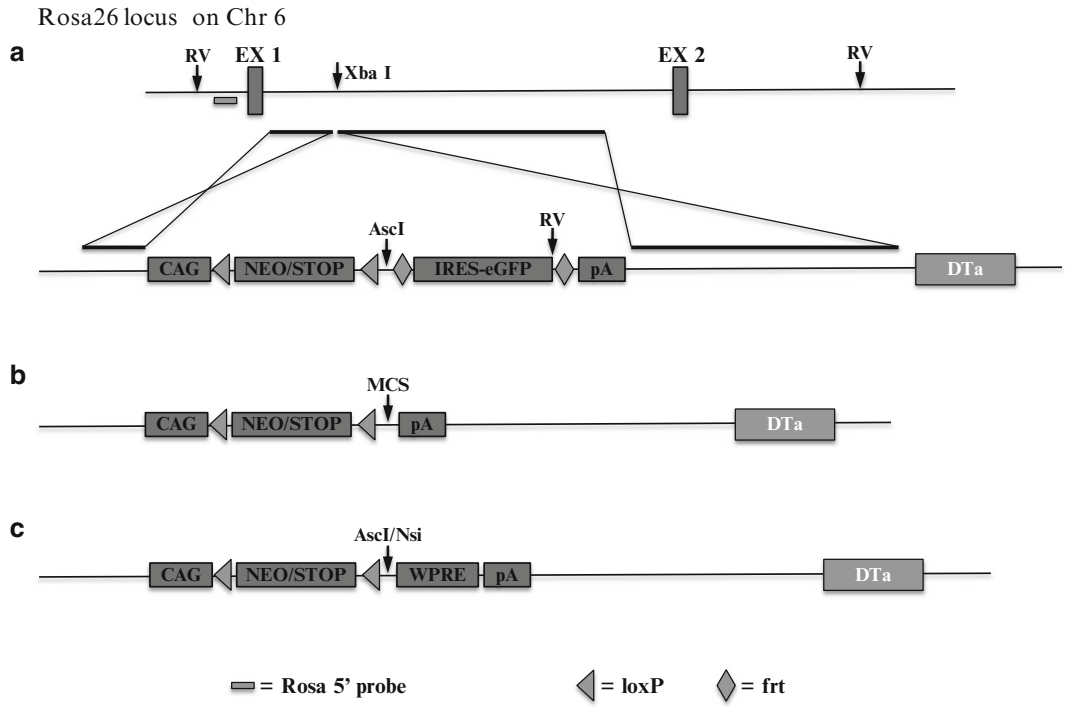

Fig. 1 Rosa26-LSL-microRNA targeting strategies. To obtain the expression in vivo of a microRNA of interest from the Rosa26 locus we use three different versions of the Rosa26-LSL targeting vector: (**a**) pCAG-STOP-eGFP-RosaTV; (**b**) pRosa-LSL-MCS; (**c**) pRosa-LSL-MCS-WPRE. All three targeting vectors have a neo resistance gene for positive selections and a DTa minigene for negative selection of targeted mES clones in culture. The basic pCAG-STOP-eGFP-RosaTV contains about 5 kb of homology sequence with the Rosa26 locus on mouse chromosome 6. It also includes a CAG promoter and a STOP cassette (loxP-neo resistance gene-3X polyadenylation sites-loxP) preceding the *Asc*I unique cloning site where the microRNA is cloned (the loxP site is indicated by a *triangle*). Following Cre excision of the STOP cassette, the CAG promoter drives ubiquitous expression of the microRNA of interest. The frt-IRES-eGFP-frt cassette is useful for tracking the expression of the microRNA and it can be removed by Flpe recombination (Rodriguez et al., 2000) (frt site is indicated by a rhombus). The pRosa-LSL-MCS targeting vector is a derivative of pCAG-STOP-eGFP-RosaTV replacing the fragment from the *Asc*I site to the 3′ frt site with a Multiple Cloning Site (MCS) that includes *Asc*I, *Nsi*I, *Fse*I, *Sma*I/*Xma*I sites for directional cloning of the microRNA of interest. The pRosa-LSL-MCS-WPRE version of the targeting vector was derived from pRosa-LSL-MCS inserting a Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) sequence flanked by two *Kpn*I sites into the *Fse*I site. In this configuration, the WPRE sequence enhances the expression of the microRNA by creating a tertiary structure (Donello et al., 1998). When using the pCAG-STOP-eGFP-RosaTV, the screening for correctly targeted clones by Southern analysis can be done using the indicated 5′ external probe (indicated by a *small rectangle* before Ex 1) and on genomic DNA digested by EcoRV (*see* Subheading 3.5.2)

The microRNA of interest is conditionally expressed from the CAG promoter upon Cre-mediated excision of the STOP cassette and the frt-IRES-eGFP-frt cassette allows tracking its expression in vivo. This GFP reporter can be deleted in vivo by crossing to a FLP deleter strain [9]. This vector also carries the Diphtheria Toxin subunit-a minigene (*DTa*) as negative selection marker (Fig. 1).

2. *pRosa-LSL-MCS*: targeting vector derived from pCAG-STOP-eGFP-RosaTV in which the fragment from the *Asc*I site to the 3′ frt site has been replaced by a Multiple Cloning Site (MCS) that includes *Asc*I, *Nsi*I, *Fse*I, *Sma*I/*Xma*I sites for directional cloning of the microRNA of interest (Fig. 1).
3. *pRosa-LSL-MCS-WPRE*: targeting vector derived from pRosa-LSL-MCS in which a Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) sequence flanked by two *Kpn*I sites has been inserted into the *Fse*I site. In this configuration, the WPRE sequence enhances the expression of the microRNA by creating a tertiary structure [10] (Fig. 1).

2.4 Generate a Rosa26-LSL-microRNA Targeting Vector

1. TBE 10×. Mix 108 g of Tris base, 55 g of Boric acid, and 40 mL of 0.5 M EDTA (pH 8.0) in 1 L final volume of H_2O.
2. Gel loading buffer: 1 % SDS (w/v), 50 mM EDTA, 0.02 % bromophenol blue (w/v), 50 % glycerol (v/v), adjust pH to 7.5.
3. Luria Broth (LB) media. Mix 10 g of Bacto-Tryptone, 5 g of Bacto-Yeast extract, and 10 g of NaCl in 1 L of H_2O. For LB agar plates, add 15 g Agar powder to 1 L of LB media. Autoclave before use.
4. 10× Carlos buffer: 330 mM Tris-Ac pH 7.9, 660 mM KAc, 100 mM MgAc, 30 mM Spermidine, 1 mg/mL Bovine Serum Albumin (BSA, Sigma).
5. Ampicillin 100 mg/mL (1,000×, Sigma).
6. Ethidium Bromide (EtBr) 10 mg/mL (100,000×, Bio-Rad).
7. Advantage 2 PCR kit (Clontech,).
8. UltraPure™ Agarose (Life Technologies).
9. QIAquick PCR Purification Kit (Qiagen).
10. *Asc*I restriction endonuclease (New England Biolabs).
11. CIP (Calf Intestinal Alkaline Phosphatase, New England Biolabs).
12. UltraPure™ Phenol–Chloroform–Isoamyl Alcohol (25:24:1, v/v; Phe:Chl, Life Technologies).
13. Glycogen, molecular biology grade (Sigma).
14. T4 DNA Ligase (Roche).
15. MAX Efficiency DH5α™ Competent Cells (Life Technologies).
16. SOC media (Sigma).
17. QIAprep Spin Miniprep Kit (Qiagen).
18. EndoFree Plasmid Maxi Kit (Qiagen).

2.5 Plasticware and Other Materials

1. Tissue culture quality culture plates: 10-cm polystyrene dishes (fill with 10–12 mL media), 6-cm polystyrene dishes (fill with 4–5 mL media), 12-well plates (fill with 2–3 mL media/well),

24-well plates (fill with 1–2 mL media/well), round-bottom and flat-bottom 96-well plates (fill with 100–250 μL media/well).

2. Falcon 50-mL and 15-mL tubes.
3. Cell strainers (100-μm pore size, Falcon)
4. 1.5-mL freezing vials.
5. 1.5-mL eppendorf tubes.
6. 25-mL, 10-mL, 5-mL, and 1-mL plastic pipettes.
7. 200-μL aerosol barrier tips.
8. 125-mL and 1-L filter units (Nalgene).
9. 12-well multichannel pipettor.
10. Repeat pipetman.
11. 0.4 cm electrode gap electroporation cuvettes (Bio-Rad).
12. Equipment for dissection: razor blades, microdissecting scissors, straight and curved forceps, tweezers.
13. PCR tubes (0.2 mL or 0.1 mL).
14. Sterile glass beads, 5-mm diameter, autoclaved.
15. X-ray films (Amersham Hyperfilm HP).

2.6 Culture mES Cells

All reagents used for the culturing ES cells should be at least "ES qualified". Alternatively, use "tissue culture grade" or "tissue culture tested" reagents. Execute all procedures under sterile conditions in BSC using sterile instruments and detergent-free glassware.

2.6.1 ES Cell Culture Media

1. Dulbecco's Modified Eagle Media (D-MEM): use D-MEM (Life Technologies) as base for culturing MEF and STO and Knockout-D-MEM (Life Technologies) containing 4.5 g/L of D-glucose, with sodium pyruvate as base for mES cultures (*see* **Note 1**).
2. Fetal Bovine Serum (FBS). Test every new lot of FBS purchased to assure that it is optimal for the ES cell line in use in the laboratory. We routinely test for (a) plating efficiency and (b) toxicity. Briefly, 10^3 cells are plated onto a 6-cm plate with feeder cells. Media is prepared with 10 % or 30 % of the new batch of heat-inactivated (30 min at 56 °C) FBS. Colonies are counted after 5–7 days. The number of colonies is compared between different lots. The presence of a greater number of colonies in the 10 % FBS media compared to the 30 % is usually suggestive of serum toxicity. We store FBS at −80 °C for up to 3 years.
3. Penicillin-Streptomycin (Pen/Strep, 100×, Life Technologies).
4. GlutaMAX (100×, Life Technologies).
5. 2-Mercaptoethanol (1,000×, Life Technologies).

Table 1
MEF and ES Cell media composition (Use 500-mL bottle of D-MEM or KO D-MEM, respectively)

	MEF Media with D-MEM (1×)	ES media with knockout D-MEM (1×)
FBS	74 mL	88 mL
Pen/Strep (100×)	6 mL	6 mL
GlutaMAX (100×)	–	6 mL
2-Mercaptoethanol (1,000×)	29 µL[a]	60 µL
LIF (10^7 Units/mL)	–	60 µL

[a]Use MEF media with added 2-mercaptoethanol for primary MEFs and STO

6. MEM Non-Essential Amino Acids (100×, Life Technologies).
7. Leukemia Inhibitory Factor (LIF, available from Millipore as ESGRO® Murine Leukemia Inhibitory Factor 10^7 Units/mL).

The above supplements (2–7) should be added to fresh D-MEM to prepare two different media needed for the protocol (Table 1).

2.6.2 Other Solutions to Culture mES Cells

1. 2× freezing media: 80 % FBS–20 % Dimethyl Sulfoxide (DMSO, Sigma).
2. D-PBS without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$, Life Technologies).
3. 0.1 % Gelatin (Millipore).
4. Trypsin–EDTA. Available from Life Technologies in two different concentrations 0.05 % used mainly for mES cells, and the 0.25 % used for MEFs.
5. Mitomycin C (MMC) at 10 µg/mL (Sigma).
6. 100× Salt I stock solution. Dissolve 3.6 g of KCl, 1.6 g of KH_2PO_4, and 1.42 g of $MgSO_4$ in 20 mL of H_2O and make up to 100 mL final volume. Filter the solution with a 125-mL unit. Store at 4 °C.
7. 100× Salt II stock solution. On a magnetic stirrer, mix 2.5 g of $CaCl_2.2H_2O$ in 20 mL of H_2O and then adjust to 100 mL final volume. Sterilize the solution with a 125-mL filter unit and store at 4 °C.
8. 1,000× Salt III stock solution. Dissolve 0.36 g of EDTA-Na (sodium salt) in 10 mL of H_2O. Store at 4 °C.
9. 1,000× Gentamicin Sulfate (Sigma) stock solution. Dissolve 0.5 g in 10 mL in H_2O. Store at 4 °C.

Table 2
Formulation of CZB-HEPES media for mouse cloning [14]

	CZB-H Embryo handling	
	mM	mg/100 mL
NaCl	82.0	478.9
KCl	4.9	36.3
KH_2PO_4	1.2	15.9
$MgSO_4.7H_2O$	1.2	29.1
$NaHCO_3$	15.0	126.0
Glucose	5.6	100.0
Sodium pyruvate	0.3	2.9
$CaCl_2.2H_2O$[a]	1.7	25.1
HEPES	10	238.0
Glutamine[a]	1.0	14.6
Sodium lactate (60 % syrup)	20.0	0.37
EDTA	0.1	3.8
Polyvinyl alcohol[b]		10.0

[a]$CaCl_2$ and glutamine can be added also from 100× stock solutions
[b]Polyvinyl alcohol (cold H_2O soluble) is first dissolved in ultrapure H_2O at 80 °C for 1 h

10. CZB-H (Chatot–Ziomek–Bavister HEPES culture media, pH 7.2–7.4, Table 2). To prepare 1 L, proceed as follows (*see* **Note 2**):
    (a) Pour into a beaker 100 mL of ddH_2O
    (b) Add 10 mL of 100× Salt I, 10 mL of 100× Salt II, 1 mL of 1,000× Salt III, 1 mL of 1,000× Gentamicin Sulfate stock, and finally 1 mL of phenol red solution (Sigma).
    (c) Individually weigh the following and add to the stirring solution: 0.029 g of Na Pyruvate (Sigma) (kept at 4 °C) and 0.146 g of L-Glutamine (Sigma), 5.847 g of Na Lactate (60 % syrup, kept at 4 °C), 1 g of D-glucose, 5 g of BSA, 5 g of NaCl, 0.292 g of $NaHCO_3$, and 4.776 g of HEPES (free acid).
    (d) Adjust the pH to 7.2–7.4 by adding NaOH pellets, one at a time. If the pH is still <7.2, dissolve one pellet in 1 mL of H_2O and add drop by drop to adjust pH to 7.3.
    (e) Adjust to 1 L final volume and then filter the solution with 1 L unit working in a BSC.

2.7 mES Cell DNA Extraction and Analysis

1. Lysis buffer: 100 mM Tris–HCl pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, 0.2 mg/mL proteinase K (*see* **Note 3**).
2. 0.1 M spermidine trihydrochloride (Sigma).
3. 3 M NaOAc, pH 5.2.
4. 70 % and 100 % ethanol (EtOH).
5. Depurination solution: 0.25 M HCl.
6. Denaturation solution: 1.5 M NaCl, 0.5 M NaOH.
7. 20× SSC. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H_2O, adjust pH to 7.0 with 10 M NaOH, and bring the volume up to 1 L.
8. Hybridization solution. Mix 5× SSC, 10 % Polyethyleneglycol MW 8,000 (Sigma), 5 % Polysodium 4-styrenesulfonate (Sigma), and 0.2 % Cetylpyridinium chloride monohydrate (Sigma, *see* **Note 4**).
9. 0.5 M EDTA, pH 8.0. Dissolve 18.6 g EDTA, 2.2 g NaOH in 80 mL of dH_2O then adjust the volume to 100 mL.
10. 10× TBE. See Subheading 2.4.
11. DNA dye and loading buffer 6× (Azvision, Amresco).
12. Biodyne B Nylon membrane, 0.45 μm (PALL Life Sciences).
13. Prime-It Random Primer Labeling Kit (Agilent Technologies).
14. pRosa26-5′ (plasmid #: 21715, Addgene).
15. EcoRV-HF restriction enzyme (NEB).

3 Methods

3.1 Engineering of Rosa-26-LSL-microRNA-KI Targeting Vector

The following procedure illustrates how to construct a Rosa26-LSL-microRNA-KI targeting vector cloning a selected microRNA at the *Asc*I site (*Asc*I: GGCGCGCC) of the basic pCAG-STOP-eGFP-RosaTV. Once the sequence of the microRNA of interest has been identified (http://genome.ucsc.edu/), design forward and reverse primers both containing *Asc*I sites. Although *Asc*I is a "rare cutter" of the mammalian genome, it is fundamental to rule out the presence of the sequence recognized by this endonuclease within the microRNA of choice. Carry out all procedures at room temperature unless otherwise specified.

1. PCR amplification of the microRNA of interest flanked by *Asc*I sites (microRNA insert) from a murine genomic DNA. Mix 5 μL of 10× Advantage 2 PCR buffer, 1 μL of Forward Primer (10 μM), 1 μL of Reverse Primer (10 μM), 1 μL 50× dNTP Mix (10 mM each), 1 μL 50× Advantage 2 Polymerase, and 1 μL of mouse genomic DNA (100 ng/μL) and add H_2O up to a total volume of 50 μL. For the amplification of fragments of less

than 1 kb in length, use the following cycling parameters: denaturation at 95 °C for 2 min, 25–35 cycles at 95 °C for 30 s, at 55–68 °C for 30 s (based on the primer design), at 68 °C for 1 min. For larger fragments it is suggested to increase the elongation time up to 12 min (generally 1 min/kb).

2. Analyze 5 μL of PCR product, along with suitable DNA size markers, on an agarose gel containing 0.1 μg/mL of EtBr. Keep the remaining PCR product at 4 °C. Use a different agarose percentage depending on the expected range of the amplified product: for 0.3–1.5 kb the recommended percentage is 1.5, for 0.5–10 kb is 1.2 and for > 5 kb is 0.8. For a fragment of about 500 bp, dissolve 1.5 g UltraPure™ Agarose in 100 mL of 0.5× TBE buffer. Microwave until the agarose dissolves. Add EtBr at the final concentration of 0.1 μg/mL. Pour the gel, wait until it solidifies, then mix 1 μL of gel loading buffer with 5 μL of PCR reaction and load sample. Run the gel at 100 V for approximately 30 min or until the molecular weight ladder is well separated. Visualize microRNA insert with a UV light box.
3. Sequence the microRNA insert using the same primers used for PCR amplification to make sure the sequence is correct.
4. Digestion of the microRNA insert. Purify the microRNA insert using QIAquick PCR Purification Kit. Elute it in 30 μL of H_2O and add 4 μL of 10× NEB CutSmart™ buffer, 1 μL of *Asc*I restriction endonuclease, and 5 μL H_2O (40 μL final volume). Incubate for 2 h at 37 °C. Purify the *Asc*I-digested microRNA insert using QIAquick PCR Purification Kit and elute the digested PCR product in 30 μL of H_2O. Purified fragments can be stored at −20 °C until ready to proceed with the cloning.
5. pCAG-STOP-eGFP-RosaTV digestion and dephosphorylation with CIP. Mix 3 μg of vector DNA, 3 μL of 10× Carlos buffer, 3 μL of 25 mM DTT, 1 μL of *Asc*I restriction endonuclease and add H_2O up to 30 μL final volume. Incubate for 2 h at 37 °C. Then add 2 μL of 10× NEB CutSmart™ buffer, 0.5 units of CIP/μ g vector DNA and add H_2O up to 50 μL final volume. Incubate for 60 min at 37 °C. Purify digested and dephosphorylated DNA by phenol–chloroform extraction. Briefly, add H_2O to the digested and dephosphorylated DNA up to 200 μL, add one volume of UltraPure™ Phenol–Chloroform–Isoamyl Alcohol to the sample, and vortex for approximately 20 s. Centrifuge at room temperature for 10 min at 16,000 × *g*. Carefully transfer the upper aqueous phase to a fresh tube. Be sure not to carry over any phenol during pipetting. Add one volume of chloroform to the aqueous phase; shake by hand thoroughly for approximately 20 s and

centrifuge at room temperature for 10 min at 16,000 × *g*. Carefully transfer the upper aqueous phase to a fresh tube. Add to the aqueous phase: 1/10× volumes of 3 M NaOAc solution, 2× volumes of 100 % EtOH, and 1 μL of glycogen (20 μg/μL) to precipitate DNA. Centrifuge for 10 min at 16,000 × *g*. Then discard the supernatant and rinse the pellet with 70 % cold EtOH. Air-dry the pellet and dissolve in 30 μL of H_2O.

6. Cloning of the microRNA insert into digested and dephosphorylated pCAG-STOP-eGFP-RosaTV. Determine the concentration of both the *Asc*I-digested vector and microRNA insert after they have been prepared for ligation. Suggested vector–insert DNA ratio is 1:5. The following formula is used to calculate the necessary amount of insert in nanograms.

$$\text{insert mass in ng} = \left(\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \right) \times \text{molar ratio of} \left(\frac{\text{insert}}{\text{vector}} \right)$$

Remember to include negative controls. (1) The digested vector alone without ligase (control for uncut vector). (2) The digested and dephosphorylated vector with ligase without the insert (control for insufficient dephosphorylation). The ligation reaction (3) will include: digested and dephosphorylated vector, fragment, and ligase. Add digested and dephosphorylated vector and digested fragment in H_2O to a final volume of 10 μL in a sterile reaction vial. Add 10 μL of 2× T4 DNA Ligation Buffer to the reaction vial. Mix thoroughly and add 1 μL of T4 DNA Ligase. Mix thoroughly and incubate for 5 min at 15–25 °C.

7. Bacterial transformation. Pre-warm agar plates (containing ampicillin at 100 μg/mL) at 37 °C. Thaw MAX Efficiency DH5α Competent Cells on ice; add the ligation reaction to 50 μL of competent cells. Mix gently by flicking the bottom of the tube and leave it on ice for 30 min. Heat-shock the competent cell–DNA mixture by placing the tube into 42 °C water bath for 45 s and then put the tubes back on ice for 2 min. Add 500 μL SOC media (without antibiotic) and grow the culture in a shaking incubator for 45 min a 37 °C. Plate all of the transformation reaction onto pre-warmed LB agar plates using sterile glass beads. Incubate plates overnight at 37 °C.
8. The following day, check agar plates. If there are colonies on control 1 (digested vector alone without ligase) it means that restriction digestion didn't work. If there are colonies on 2 (digested and dephosphorylated vector with ligase) but not 1, it means that the CIP treatment didn't work. The presence of colonies only in the reaction 3 indicates a correct ligation reaction. Pick colonies with a sterile tip and grow bacteria in

LB media with ampicillin 100 μg/mL, overnight in an orbital shaker at 37 °C at 180–200 x g. Pick at least 20 colonies.

9. The next day extract the DNA using QIAprep Spin Miniprep Kit according the included protocol. Elute DNA in 50 μL of H_2O.
10. Check by restriction digestion the recombinant colonies. Mix 6 μL of miniprep (usually 50 ng/μL), 1 μL 10× NEB CutSmart™ buffer, 1 μL *Asc*I endonuclease. Make up to 10 μL with H_2O. Incubate for 1 h at 37 °C. Add 1 μL of loading dye and load onto a 1.5 % agarose gel.
11. Sequence positive clones to verify the correct orientation of the fragment and appropriate PCR amplification. Design the forward and reverse primer for sequencing just upstream and downstream of the *Asc*I restriction site.
12. Once identified the recombinant clones, proceed to the bacterial transformation (point 7) with 2 μL of miniprep DNA. The following day, set up a pre-culture of 3 mL LB media with ampicillin at 100 μg/mL by picking a single colony from a fresh agar plate. After 3 h when the pre-culture is slightly turbid, inoculate 1 mL of it into 250 mL of pre-warmed LB media (with ampicillin) and grow overnight 37 °C. The day after, proceed to maxiprep DNA purification using EndoFree Plasmid Maxi Kit. Determine DNA yield and purity by measuring absorbance at 260 nm at the spectrophotometer.
13. Linearize 110 μg of the final KI vector in 2× 400 μL digests with 55 μg of DNA each, at 37 °C overnight (*see* notes below).
14. Run 1 μL of the digest on a minigel to confirm complete digestion.
15. After linearization, perform Phe:Chl extraction of the linearized final targeting vector. Extract the vector DNA as follows: for each tube with 400 μL of digestion add 400 μL of phenol; mix well by hand and spin down at 16,000 × *g* for 3 min; transfer supernatant to a new tube and add 400 μL of chloroform; mix well and spin down at 16,000 × *g* for 3 min. Repeat the last two steps then precipitate the DNA with 1/10× volume 3 M NaOAc, pH 5.2 (~20 μL) and 2.5× volumes (~1 mL) EtOH at −80 °C for 1 h or −20 °C O/N. Pellet the DNA by centrifuging at 4 °C, 16,000 × *g* for 15 min. Then wash with 0.3 mL of 70 % EtOH, air-dry, and resuspend the DNA in the suitable volume of sterile ddH_2O to obtain a final concentration of 1 μg/μL (*see* **Note 5**). Store at −20 °C.

3.2 Preparation of Feeder Layer for mES Cells

Murine ES cells need to be maintained in a totipotent state until they are injected into blastocysts. For this purpose they are grown on a feeder layer of mitotically inactivated primary MEFs or STO fibroblasts [11].

In our lab, we use a 1:1 ratio (MEFs:STOs) feeder mix. MEFs and STO cells are cultured in MEF media + 2-mercaptoethanol (MEF+ media, Table 1).

3.2.1 Isolation of Primary MEFs

We derive MEFs from 12 to 14 days post coitum embryos of the Tg(DR4)1Jae/J mouse strain (The Jackson Laboratory), resistant to concentrations of the drugs neomycin/G418, puromycin, 6-thioguanine, and hygromycin well above those used normally for the selection of ES clones subsequently to the electroporation [12].

1. Euthanize a 12–14-day pregnant female by CO_2 asphyxiation.
2. In a BSC, aseptically remove the uterus and place it in a dish containing sterile D-PBS$^{-/-}$.
3. Carefully dissect out embryos and transfer them in a new 10-cm tissue culture dish with sterile D-PBS$^{-/-}$.
4. Decapitate embryos with the single-edged razor blades and hold bodies in order to eviscerate them using fine forceps.
5. Place the carcasses in a clean 10-cm dish and quickly mince the tissue with a sterile razor blade until a gelatinous suspension is created.
6. Filter the viscous suspension through cell strainers (100 μm pore size) into 50-mL tubes and bring the total volume up to 50 mL by adding MEF+ media.
7. Spin down for 10 min at 1,000 × *g* and discard supernatant.
8. Carefully resuspend cell suspension using 15 mL of MEF+ media per dissected embryo.
9. Distribute 15 mL onto 1× 15-cm tissue culture dish (e.g., for ten embryos, resuspend in 150 mL of media and seed onto ten dishes).
10. Grow MEFs at low O_2 (3 %) until they are about 80 % confluent, freeze them (6–8 vials/dish), and keep at −150 °C in liquid nitrogen cryotank for future use.

3.2.2 Culture of STO Cells

The STO cells we use are the SNL 76/7 (available from Cell Biolabs). They were established in Dr. Allan Bradley laboratory [13] and they are transfected with *LIF* and *neoR* genes. These cells are grown and mitotically inactivated similarly to primary MEF. In our culture system, they represent an extra source of LIF to keep mES cells undifferentiated.

3.2.3 Mitotic Inactivation of Primary MEFs and STO Cells

1. Thaw one vial of primary MEFs/STO cells onto a 15-cm dish with MEF+ media.
2. Grow cells at 37 °C, 3 % O_2, and 5 % CO_2 to 80 % confluency and then split 1:5. When these four plates are again confluent, split one more time 1:6 (total 30× 15-cm dishes).

Table 3
Densities of Feeder cells (mix 1:1 MEFs:STO) onto different gelatin coated dishes and plates

Dish/plate size	Total feeders (mix MEFs: STOs/1:1)
10 cm	10×10^6 cells (5×10^6 MEFs + 5×10^6 STOs) per dish
6 cm	1.5×10^6 cells per dish
6 well plate	1.25×10^6 cells per well
24 well plate	4.16×10^5 cells per well
96 well plate	10^5 cells per well

3. Reconstitute MMC at 10 μg/mL final concentration (e.g., 4 mg dissolved in 4 mL of D-PBS$^{-/-}$, filtered and added to 396 mL of pre-warmed MEF+ media, *see* **Note 6**).
4. When MEFs have almost reached 80 % confluency, remove the media and add 13 mL/dish of MEF+ media containing MMC.
5. Incubate for 3.0 h at 37 °C to mitotically arrest cells.
6. After treatment, remove media and wash at least twice with D-PBS$^{-/-}$ (~30 mL each time), to ensure complete removal of MMC.
7. Add 3 mL of trypsin–EDTA 0.25 %/dish and incubate for 5 min at 37 °C.
8. Neutralize Trypsin by adding 15 mL of MEF+ media and collect cells in a 50-mL tube.
9. Spin down for 10 min at 1,000 × *g* and remove the supernatant. Resuspend in 1 mL of MEF+ media and gently pipette up and down in order and obtain a single cell suspension.
10. Bring up the volume to 50 mL with MEF+ media. Use cell counter to determine the total number of cells, centrifuge at 1,000 × *g* for 10 min, and resuspend cells in a convenient volume of MEF+ media for immediate plating or freezing.

3.2.4 Preparation of Feeder Plates

1. Prepare gelatin-coated plates by covering the dish surface with 0.1 % gelatin (*see* **Note 7**). After at least 30 min in BSC remove the remaining gelatin from dishes and let them completely dry for at least 30 additional minutes.
2. Plate the 1:1 mix of feeder cells onto gelatin-coated dishes in order to obtain a monolayer (Table 3).
3. Change media daily and use seeded feeder within 7–10 days.

3.3 Culturing mES Cells

If maintained in optimal conditions, murine ES cells will continue to grow indefinitely in an undifferentiated diploid state. They are sensitive to pH changes, overcrowding, and temperature changes. It is imperative to take care of mES cells daily to avoid spontaneous differentiation.

3.4 Rosa26-LSL-microRNA KI Targeting of mES Cells

The following section describes how to prepare and execute the electroporation (EP) of five different targeting constructs in the hybrid C57Bl/6-129Sv mES cell line S1B6a at early passages.

3.4.1 Targeting mES Cells: Electroporation, Selection, and Picking Colonies

1. Day -8 and -7: gelatinize and seed feeder cells as described in Subheading 3.2.4 and Table 3 onto 1× 6-cm dish and 18× 10-cm dishes.
2. Day -6: change media and allow cells to completely settle on dishes.
3. Day -5: thaw one vial of S1B6 ES cells (1×10^6) and resuspend them in 5 mL of ES media (Table 1). Remove MEF medium from the 6-cm dish with feeder and seed S1B6a cells. Change medium to the other 10-cm dishes (MEF media).
4. Day -4: change media to ES and to feeder dishes.
5. Day -3: S1B6a cells should be about 80 % confluent. Remove the medium and rinse twice with D-PBS$^{-/-}$. Add 0.5 mL of Trypsin–EDTA 0.05 % and incubate at 37 °C for ~5 min. Add 1 mL of ES media and gently pipette to obtain a single cell suspension. Bring the volume up to 10 mL with ES medium and seed all onto 1× 10-cm feeder dish.
6. Day -2: change the medium to ES and to feeder dishes.
7. Day -1: S1B6a cells should be again about 80 % confluent. Remove media and rinse twice. Add 1.5 mL of Trypsin–EDTA 0.05 % and leave in incubator for ~5 min and seed all onto 2× 10-cm feeder dishes (20 mL total volume).
8. The day of the electroporation (Day 0, *see* **Note 8**) trypsinize ES cells as above, collect in 50 mL total volume. Use the cell counter to count cells and take the volume equivalent to 50×10^6 (10×10^6 cells/construct).
9. Spin them at 1,000 × *g* for 10 min at room temperature. Wash once in 20 mL of D-PBS$^{-/-}$ and gently resuspend pellet in 4.0 mL total of D-PBS$^{-/-}$ (800 μL/construct).
10. Using pre-labeled EP cuvettes in BSC, first pipette in the DNA targeting vector (25 μg) on the bottom and then add the resuspended mES cells (800 μL).
11. Electroporate S1B6a cells at a 230 V and 500 μFarads of capacitance. Immediately transfer the cells from each EP cuvette to a 30 mL of ES media. Plate onto 3× pre-labeled 10-cm dishes with feeders making sure to well distribute the electroporated ES cells and then return dishes to incubator.

12. Day 1: change media to the electroporated ES cells with 10 mL of fresh ES media.
13. On Day 2 after the electroporation, start selection by using 250 μg/mL of G418 for 5–6 days and change media with antibiotic daily (*see* **Note 9**).
14. On Day 4–5 start preparing 96-well feeder plates as described in Subheading 3.2.4 and Table 3.
15. By Day 7 after EP, distinct G418/DTa double-selected colonies should be clearly visible on the dishes. To start picking clones one dish at the time, remove the media from the dish and add 10 mL of D-PBS$^{-/-}$. Pick colonies under inverted microscope by using a P20 filter tip (new for each clone) mounted on a micropipette set at 10 μL. Transfer each clone to a well of a round bottom 96-well plate containing 40 μL of trypsin–EDTA 0.05 % and incubate the plate for 5 min at 37 °C. Then, using a multichannel P200 pipettor, add 60 μL/well of ES media and gently dissociate colonies by pipetting 15–20 times. Finally, transfer picked colonies onto prepared flat bottom 96-well plates ("master plates") seeded with mix feeder in 100 μL/well of the ES media (*see* **Note 10**).

3.4.2 Freezing mES neo/DTa Selected Clones and Generating Duplicate Plates for DNA Analysis

1. Prepare 2× flat-bottom 96-well gelatin coated plates marked "A" and "B" (*see* Subheading 3.2.4, **step 1**) for each "master" plate. The day of the procedure, add ES media (100 μL/well) in both plates and put them in the incubator.
2. Grow selected clones on mother plates for 3–4 days changing ES media (150–200 μL/well) daily.
3. When wells of the "master" plate are about 80 % confluent, remove media, wash twice with D-PBS$^{-/-}$ (200 μL/well), and add 50 μL/well of Trypsin–EDTA 0.05 %.
4. Incubate the plate at 37 °C for 5–10 min.
5. With a multichannel, add 100 μL/well of ES media and pipette 15–20 times. Transfer 50 μL of the suspension to each prelabeled "DNA" plate A and B, respectively.
6. When the whole master plate is completed, add 50 μL/well of freezing media (FBS-20 % DMSO) to the 50 μL/well of cell suspension.
7. Seal the sides of the mother plate with Parafilm and then wrap it in Saran™ plastic.
8. Freeze down the mother plate by sitting it on a plastic rack on dry ice in a styrofoam box for at least 2 h before transferring to −80 °C.
9. Return "DNA" duplicate plates to the incubator and change ES media as needed.

10. When clones are fully confluent (usually after 3–5 days), remove media and wash twice with D-PBS$^{-/-}$. Wrap the plates in Saran™ plastic, snap-freeze them on dry ice, and store at −80 °C or proceed to DNA extraction (*see* **Note 11**).

3.5 Analysis of DNA from ES Cells Clones

3.5.1 Lysis of ES Cell Clones and DNA Extraction

1. Add 200 μL/well of lysis buffer (*see* Subheading 2.7, **item 1**) and incubate at 56 °C for 15–20 min and then harvest the lysates into 1.5 mL eppendorf tubes.
2. Incubate overnight at 56 °C in an orbital shaker (200–250 × *g*) (*see* **Note 12**).
3. Add 500 μL of 100 % EtOH and 5 μL of 3 M NaOAc (pH 5.2), and then mix thoroughly by hand (DNA precipitate should already be visible).
4. Spin at 16,000 × *g* for 20 min and remove carefully the supernatant away from the pellet.
5. Add 100 μL of 70 % EtOH and spin at 16,000 × *g* for 8 min.
6. Carefully remove the supernatant and air-dry for 5 min.
7. Resuspend pellet in 20 μL of dH_2O and determine the DNA amount by spectrophotometric measurement (*see* **Note 13**).

3.5.2 Southern Blot Screening of Rosa26-LSL-microRNA KI Targeted Clones

To screen for correctly targeted clones by Southern, we routinely digest the DNA by EcoRV and use a 5′ Rosa26 genomic-specific probe. This probe can be excised from the pRosa26-5′ plasmid available from Addgene [6]. When mouse genomic DNA is digested with EcoRV and probed with this 5′ probe, the wild type Rosa26 locus shows a fragment of ~11.5 kb. On the other end, the Rosa26 targeted allele shows a fragment of ~8.5 kb plus the size of the inserted microRNA that is usually less than 1 kb. In fact, EcoRV cuts at the end of the polyA signal following the eGFP gene (Fig. 1).

1. Set a 30 μL reaction as follows and incubate overnight at 37 °C:

   mES Clone DNA—19 μL

   10× NEBuffer 4—3 μL

   Spermidine (0.1 M)—2 μL

   EcoRV-HF—2 μL

   ddH_2O—4 μL
2. The following day, add loading buffer to samples and load onto a 0.8 % agarose gel in 1× TBE buffer.
3. Run gel overnight at 30 V.
4. Take picture of agarose gel to be blotted with phoshorescent ruler lined up along side it, such that the ruler is lined up with the top of the wells, so that you can later estimate the size of your band on your film/blot.

5. Immerge gel in 0.25 M HCl solution to depurinate DNA for 20 min gently shaking (50–75 × *g*).
6. Denature DNA in 0.5 M NaOH, 1.5 M NaCl for 45 min gently shaking.
7. Alkali transfer of DNA to the Biodyne B nylon membrane overnight:
   - Place the gel on a Plexiglas support. Remove all air bubbles between the gel and the glass plate by rolling a serological pipette on the gel.
   - Place a piece of the pre-wetted membrane (with denaturing solution) on top of the gel. Roll pipette over the membrane to remove all air bubbles between the gel and the membrane.
   - Place 2 sheets of pre-wetted 3-mm Wattman paper on membrane and roll pipette again.
   - Cover with a stack of paper towel and place a Plexiglas plate on the paper towel.
   - Place on the top at center of the Plexiglas plate a weight like for example a 1 L bottle containing about 750 mL of liquid.
8. The following day, remove paper towels and 3-mm paper, mark the membrane and wells by using a pencil, and then allow to air-dry on 3-mm paper for 10 min.
9. UV-crosslink the membrane (1,200 uJoules/cm^2).
10. Immerse the membrane in 2× SSC and transfer to the hybridization bottle.
11. Add 10–15 mL of hybridization solution (see Subheading 2.7, **item 8**) and incubate for 45 min in a rotating hybridization oven at 65 °C.
12. In the meantime, start the probe labeling using the Prime-It Random Primer Labeling Kit.
    - In 1.5 mL screw cap tube, mix 50 ng of Rosa26 5′ probe with 10 μL of random oligonucleotide primers and H_2O to bring the volume up to 34 μL. Spin briefly and then keep on ice.
    - Boil for 5 min and put back on ice for 2 min.
    - Add 10 μL of 5× dCTP buffer, mix and briefly spin.
    - Go to the radiation area and, from now on, use all the safety measures required when working with radioactive material.
    - Add 5 μL of ^{32}P-α-dCTP and 1 μL of NEB Exo(−) Klenow (5 U/μL).

- Mix by flicking, spin briefly, and incubate at 37 °C for 15 min (always spinning down prior to open).
- Add 2 μL of stop mix and 250 μL of pre-boiled salmon sperm DNA (10 μg/mL).
- Boil for 10 min and briefly spin before chilling on ice for 2 min.

13. Add the probe to the hybridization bottle avoiding direct touch of the membrane with the tip and hybridize overnight at 65 °C.
14. The following day, discard the hybridization solution in a labeled ^{32}P liquid container. Rinse the membrane with 2× SSC at room temperature and discard again the solution into the ^{32}P liquid waste container.
15. Take out membrane and wash in 2× SSC–0.5 % SDS (1st) for 10 min, 1× SSC–0.5 % SDS (2nd) for 10 min, and 0.1× SSC–0.1 %-SDS 0.5 % (3rd) for 10 min at 65 °C.
16. At the end, air-dry the membrane on 3-mm paper for 5–10 min.
17. Expose overnight the membrane to X-ray film at −80 °C and then develop to obtain an autoradiographic image.
18. Label the film and look for specific bands at the expected size of both the wild type and mutant clones (*see* **Notes 14** and **15**).

3.6 Preparation of Rosa26-LSL-microRNA KI ES Clones for Injection

1. Two days prior thawing the selected ES clones, seed a 24-well plate with mix feeder (*see* Subheading 3.2 and Table 3).
2. Take out 96-well master plate from the −80 °C and spray it with 70 % EtOH before putting it in BSC.
3. Quickly add 150 μL of pre-warmed ES media to the wells of selected Rosa26-LSL-microRNA-KI positive primary clones and transfer them immediately to the prepared 24-well plate containing fresh ES media (1.5 mL/well).
4. Allow primary clones to grow for 3–4 days changing ES media daily. In the meantime, prepare a 6-well plate with mix feeder as in Subheading 3.2.4 and Table 3. Then split as follows:
   - Wash well twice with D-PBS$^{-/-}$, add 0.3 mL of Trypsin–EDTA 0.05 %, and leave plate in incubator for ~5 min.
   - Add 0.9 mL of ES media and gently pipette to obtain single cell suspension.
   - Transfer 0.9 mL of suspension onto a 1 well of the 6-well plate with 4.5 ml of fresh ES media and distribute carefully.
   - Seed the remaining 0.3 mL onto two gelatinized wells of a 24-well plate (no feeder) to be used for DNA extraction to reconfirm the positivity of the selected clones.

5. Let selected clones grow to about 80 % confluency and split 1:8 two days before injection. Every passage freeze at least 2× vials with 1×10^6/each.
6. The day of the injection trypsinize 1× well of the selected clones as above (Subheading 3.6, **step 4**) and transfer immediately to 2.5 mL of ES media in a 15 mL pre-labeled tube.
7. Centrifuge for 3 min at 1,000 × *g*.
8. Remove supernatant and gently resuspend pellet in 1.0 mL of CZB-H media. Keep tubes on ice until injection.

4 Notes

1. Always pre-warm MEF and ES cell media before use. In order to keep the media as fresh as possible, aliquot only the required amount. Use ES Cell medium older than 3 weeks only for expanding mES cells needed for DNA analysis.
2. Store CZB-H media at 4 °C for short periods of times. Make it fresh every 2 weeks. Use glassware (cylinders and beakers) dedicated only to CZB-H preparation. Use disposable transfer pipet to withdraw CZB-H media during pH measurement.
3. Proteinase K is prepared as a stock solution at 20 mg/mL in H_2O and stored at −20 °C. It should be added fresh to the other components of the lysis buffer just before lysing the cells.
4. Cetylpyridinium chloride monohydrate is highly toxic; it should be handled with extreme care.
5. The final targeting vector quality and concentration should be verified by running a 0.8 % agarose minigel with 1 μL of the final linearized vector (=1 μg) and 1 μL of a 1:10 dilution of the same. Include 1 μL of the non-linearized final vector. Verification of the functionality of the integrity of the LSL system by transfecting the final vector into mammalian cells of choice (HeLa or HEK293, for example) with and without a Cre expressing plasmid is also recommended in order to measure overexpression of the microRNA of interest when the STOP cassette is removed. The purity of the DNA and the absence of any contamination derived from salts or proteins are required for an efficient targeting.
6. Make sure to fully dissolve MMC in D-PBS$^{-/-}$ mixing thoroughly. MMC is light sensitive and the MMC containing media should be protected from light before use.
7. Never leave gelatin-coated plates inside the BSC if the UV light is on: UV radiation damages the gelatin layer on the plates, which will result in a reduced adhesion efficiency of feeder cells.

8. The day of the electroporation (Day 0), feed the mES cells with fresh ES media 4–5 h prior to electroporation (12 mL/dish) to stimulate their proliferation. We routinely perform the EP in late afternoon (5:00–6:00 p.m.). Due to the shock, many cells die after EP. The cell suspension in the EP cuvette appears somehow viscous because of the DNA released by the disrupted cells. It is important to record the voltage and the time constant of the electroporated samples to document the success of the procedure.
9. Between day 2–4 post electroporation, massive cell death will appear in the dishes. When changing media, swirl dish to remove debris.
10. Pick 12–24 colonies at the time in order not to leave colonies in trypsin for more than 10–15 min. For microRNA that are less than 1 kb of length, it is usually enough to pick only 48 clones because the efficiency of targeting is usually well above 50 %.
11. The "master" and "DNA" plates should be labeled in a way that will be easy to be traced for later use. In general, the following information should be found on the plates: date, EP #, construct/project name, plate #. "Master" plates with clones to be used for injections can be kept at −80 °C only for few months. The sooner they are expanded and used the better. There is a significant loss of efficiency in chimera generation and germline competency if clones are stored at −80 °C for longer than 6 months. In order to maximize DNA recovery, wells should be grown and harvested at maximum confluency.
12. Lysed samples can be stored at −20 °C until processing for extraction. However, it is very important to completely thaw and pre-mix samples (shaking for 15–20 min at 37 °C) before adding EtOH.
13. One confluent well from a 96-well plate provides approximately 2–6 μg of DNA that is sufficient at least for 1× Southern analysis.
14. Mark the potential mutant clones in a way to be easily identified in the ES mother plate (96 well plate) for expansion and microinjection.
15. In general, when the ES cells injection is planned, a second Southern screening for the selected clones is performed (DNA isolated from the expanded cells) and multiple freezing vials for a specific mutant clone are stored at −80 °C. For long-term storage, transfer the cryo-vials of frozen ES cells into the −150 °C Cryotank.

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Chapter 12

In Situ Hybridization (Both Radioactive and Nonradioactive) and Spatiotemporal Gene Expression Analysis

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Abstract

Section in situ hybridization using either radioactive or nonradioactive labeled cDNA probes is an invaluable technique that enables the investigator to detect and localize mRNA expression within tissue sections and cells. Here, we describe the labeling of ^{35}S-UTP radioactive and nonradioactive digoxigenin probes, preparation of tissue sections, hybridization, and washing of non-hybridized probes, followed by the detection of radioactive signals via dipping in nuclear emulsion and the immunohistochemical and subsequent colorimetric detection of nonradioactive signals.

Key words mRNA localization, Radioactive and nonradioactive in situ hybridization, Tissue sections, cDNA probes, ^{35}S-UTP, Digoxigenin, Immunohistochemistry

1 Introduction

Detecting messenger RNAs (*mRNA*) within either fixed individual cells, tissue sections or whole mount preparations has become one of the fundamental molecular biology techniques that is routinely used in a broad range of research fields from developmental genetics to mouse modeling of human diseases and from neuroscience to pathology. In situ hybridization provides information of abundant to moderate mRNA expression levels (threshold levels of detection are in the region of 10–20 copies of mRNA per cell) within heterogeneous tissues and most significantly, it provides a spatiotemporal representation of gene expression without disturbing the cell's form or location. The technique started as early as 1969, when Gall and Pardue amplified the signal of ribosomal genes in oocytes of *Xenopus* [1] and was then modified via Angerer and Angerer for use in sections [2]. It is a technique sensitive enough to provide information of mRNA at a single cell level, whereas other techniques such as northern blot or polymerase

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chain reaction (PCR) would not allow for this level of detail [3]. The in situ hybridization method utilizes a labeled complementary DNA or RNA strand, utilizing a radioactive or nonradioactive labeled nucleotide, to specifically localize the sequence within a cell and/or in a particular tissue. In situ hybridization presents a unique set of complications as the mRNA to be detected will be at a lower concentration, may be masked because of concomitant proteins, or protected within a cell or cellular structure. Thus, the key variables for efficient hybridization include probe design and concentration, appropriate pretreatment (none, proteinase K, or antigen retrieval) of tissue to permit probe access and stringency of hybridization reaction without destroying the structural integrity of the cell or tissue. Following washing to remove unbound probe, the key variables for the subsequent probe detection steps (be they nuclear emulsion or immunohistochemical) are the dipping and exposure methodology or the concentration of the primary antibody and use of a highly sensitive detection system.

Although there are a wide variety of methods available [4], the use of radioactively labeled probes and detection via autoradiography still offers the greatest sensitivity [4–7]. In this chapter, we describe the use of two of the most frequently used protocols to effectively accomplish a radioactive and nonradioactive in situ hybridization and demonstrate the critical parameters of these methods for mRNA expression detection in comparison to other methods. The methods used will vary depending upon the tissues that are being probed; upon the nature of the sequence being detected (i.e., detection of precursor vs. mature forms of micro RNAs [8]); whether both mRNA and proteins are being simultaneously detected [9, 10]; as well as the type of questions being asked, facilities available, and how one intends to assess the outcome (i.e., qualitatively or quantitatively). Thus, in this chapter we also highlight the formulae and the rationale for their use, therefore enabling the reader to understand the key parts with in the process and their purpose, so that they may be modified and tailored for individual use to give the best level of resolution with the highest level of stringency.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) that has been made RNAse-free via treatment with diethylpyrocarbonate (DEPC) and autoclaving and use of analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Preparation of Sections

1. Frozen tissue is embedded in Tissue-Tek OCT embedding cryo-support medium.
2. Fixed serial wax sections are mounted on Superfrost Plus slides.
3. 4 % paraformaldehyde (PFA), 4 g PFA dissolved in 100 ml DEPC-treated PBS.

2.2 Nonradioactive Probe Preparation

1. Sterile distilled water treated with diethylpyrocarbonate (DEPC), add 1 mL of DEPC to 1 L of sterile distilled water.
2. 10× Transcription buffer: 400 mM Tris–HCl, pH 8.0, 60 mM $MgCl_2$, 100 mM DTT, 20 mM spermidine.
3. DIG RNA labeling mix, 10×: Roche, 10 mM ATP, 10 mM GTP, 10 mM CTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP, pH 7.5.
4. 4 M ammonium acetate, dissolve 308 g of ammonium acetate in 800 mL of water. Adjust the volume to 1 L and filter. Store at room temperature.

2.3 Radioactive Probe Preparation

1. TE buffer is composed of 10 mM Tris–Cl pH 7.5 and 1 mM EDTA, and is made up from 1 M stock of Tris–Cl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).
2. Carbonate hydrolysis buffer, is made of 80 mM $NaHCO_3$ and 120 mM Na_2CO_3 in DEPC-H_2O (pH 10.2)
3. TD buffer is composed of 10 mM Tris–Cl pH 7.5 and 20 mM DTT (*see* **Note 1**).
4. Purify the ^{35}S-labeled RNA using the Quick Spin Columns (Boehringer/Roche).

2.4 Pre-hybridization

1. A 10μg/mL Proteinase K stock (1,000× concentration) solution can be made from which aliquots are thawed once or twice and then discarded. Stored stock at −20 °C and make up fresh working concentration by adding 1 μL Proteinase K Stock to 1 mL PBST
2. 2 mg/mL glycine in PBST, 0.2 g glycine (Tissue Culture Grade) is dissolved in 100 ml PBST and chilled.
3. Ultrapure formamide, an organic solvent, which reduces the thermal stability of the bonds allowing hybridization to be carried out at a lower temperature.
4. 20× saline sodium citrate buffer (SSC), dissolve 175.2 g sodium chloride in 700 mL of distilled H_2O, then add 88.2 g sodium citrate dehydrate then adjust the pH to 4.5 with 16–20 mL of concentrated HCl. Adjust the volume to 1 L and filter and treat with DEPC.
5. 10 % sodium dodecyl sulfate (SDS), dissolve 100 g SDS in 800 mL of DEPC-H_2O and adjust the volume to 1 L.
6. 0.1 M TEA–HCl Buffer, is 0.1 M Triethanolamine–HCl and 0.9 % NaCl in DEPC-H_2O, pH 8.0

2.5 Post-hybridization

1. To make up 40 ml of post-hybridization wash # 1, add 20 ml Formamide, 10 ml 20× SCC pH 4.5, 4 ml 10 % SDS to 6 ml distilled H_2O.
2. To make up 40 ml of post-hybridization wash # 2, add 20 ml Formamide, 4 ml 20× SCC pH 4.5, to 16 ml distilled H_2O.
3. 10× stock TBS solution (80 g sodium chloride, 2 g potassium chloride, 250 ml 1 M Tris–HCl pH 7.5, and make upto 1 l with distilled H_2O).
4. To make 1 l of TBST working solution #1, add 100 ml 10× TBS, 10 ml 10 % Tween 20 and make up to 1 l with distilled H_2O.

2.6 Radioactive Post-hybridization

1. 20× saline sodium citrate (SSC): 175.3 g NaCl and 88.2 g $Na_3C_6H_5O_7$ in 900 mL water, pH 7 adjusted by NaOH or HCl, sterilized by autoclaving.
2. To make 1 l of washing solution #1, add 233.8 g NaCl, 100 ml 1 M Tris–HCl pH 7.5, 0.5 M EDTA and make up to 1 l with distilled H_2O.

2.7 Detection of Hybridization Signal

1. To make 1 l of TBST working solution #2, add 0.48 g levamisole to 1 l of TBST working solution #1.
2. To prepare 100 ml of NTMT solution, add 2 ml 5 M NaCl, 5 ml 2 M Tris–HCl pH 9.5, 2.5 ml 2 M $MgCl_2$, 1 ml 10 % Tween 20, 0.048 g Levamisol and make up to 100 ml with distilled H_2O.
3. To prepare 10 ml NTMT color solution, 45 µl 4 Nitro blue tetrazolium chloride (NBT, Boehringer-Mannheim) and 35 µl X-Phosphate (BCIP, Boehringer-Mannheim) to 10 ml of NTMT solution.
4. Use photographic nuclear emulsion Ilford K-5 or Kodak NTB-2 and developer (D19 Kodak) with fixer (Ilford Hypam).

3 Methods

The most common tissues used with in situ hybridization are:

1. Frozen sections: Fresh tissue is snap-frozen (either in liquid nitrogen or rapidly put into a –80 °C freezer) and then when frozen, tissue is embedded in embedding cryo-support medium for thin cryosectioning (usually at 10 µm). The sections are lightly and rapidly fixed in fresh 4 % PFA just prior to processing for hybridization.
2. Paraffin embedded sections: Formalin-fixed autopsy tissues or fresh tissues fixed in 4 % PFA are then embedded in wax (paraffin sections) before being serially sectioned at 10 µm and mounted on charged slides. Wax embedded tissues can be stored at 4 °C indefinitely.

3. Whole embryos or organs: Whole mouse embryos (from embryonic (E) day 6 until E14) and isolated mouse fetal organs, as well as both Zebrafish and *Drosophila* can be used a whole mount preparations following 4 % PFA fixation and permeabilization, as long as the labeled probe can reach its target sequence and both the unbound probe and detection antibodies can be washed away. This method provides a powerful 3D view of gene expression.
4. Cells in suspension: Cells can be cytospun onto charged glass slides and fixed with methanol.

3.1 Preparation of Material

1. Dissect collected tissue in ice-cold phosphate buffered saline pH 7.5 (PBS).

3.1.1 Frozen Sections

2. Place tissue in fresh ice-cold 4 % PFA (4 g PFA dissolved in 100 ml DEPC-treated PBS), fix it for 30 min–1 h depending upon age at 4 °C, then rinse twice with cold PBS.
3. Place fixed or unfixed tissue in 10 %, 20 %, 30 % sucrose solutions (made up with DEPC-H_2O) respectively at 4 °C for 1 h or until the tissue sinks (can take each step up to overnight).
4. Place tissue in OCT–30 % sucrose mixture (1:1) for 10–15 min at room temperature to infiltrate (mix OCT and 30 % sucrose at room temperature ahead of time and rock gently to avoid bubbles).
5. Embed embryo in OCT under the microscope and freeze the mold in liquid nitrogen. Blocks can be stored at −80 °C until ready to section.
6. Cut blocks in cryostat into 5–12 μ sections at −20 °C. Place sections onto a slide and store slides at −80 °C.

3.1.2 Wax Sections

1. Dissect collected tissue in ice-cold PBS.
2. Place tissue in fresh ice-cold 4 % PFA and leave rocking at 4 °C overnight
3. Place tissue in 70 % ethanol (made up with DEPC-H_2O) and leave rocking at 4 °C overnight (note: tissue can be stored in 70 % ethanol indefinitely).
4. Run tissue through 95 % and 100 % ethanol at room temperature, twice in each ethanol concentration for at least 30 min (time can be increased for larger fetal and adult samples ~ upto 2 h). Run tissue through Pro-Par clearant twice for at least 30 min at room temperature. Replace solution with Pro-Par–wax mixture (1:1) for at least 30 min in vacuum oven at 55–60 °C, followed by three changes of wax, each for 30 min (*see* **Note 2**).
5. Orient tissue with warmed needle under the microscope in a mold and allow wax to set overnight at room temperature.

Cut serial section ribbons of 5–12 μm sections, float them in 42–44 °C water bath (with DEPC-H_2O) until creases disappear, and place sections onto a slide. Dry slides overnight and store them at room temperature in a slide box.

3.1.3 Whole Embryos or Organs

1. Dissect out embryos and/or organs in ice-cold PBS.
2. Fix samples in fresh ice-cold 4 % PFA at 4 °C for various times depending on age (note: for E8.5 mouse embryos leave 1 h, for E11.5 and older samples fix overnight and open cavities).
3. Wash samples thoroughly in PBST (PBS with 0.1 % Tween 20 detergent) three times for 10 min each. Place samples in 70 % methanol (made up with DEPC-H_2O) and leave rocking at 4 °C overnight.
4. Run tissue through 85 % and 100 % methanol at 4 °C room for at least an hour each and then store samples in 100 % methanol at −20 °C until use. Embryos and organs can be stored for several months in 100 % methanol prior to use.

3.2 Nonradioactive In Situ Hybridization

3.2.1 Nonradioactive Probe Preparation

As there are several type of probes, the investigator will have to consider the type to use and how best to label it, to give the best level of resolution with the highest level of stringency. There are essentially three major forms of probe that can be used: namely, oligonucleotide probes that can be chemically synthesized, are resistant to RNases and are small (~40–50 bp); single-stranded DNA probes that are larger (~200–500 bp) and are produced by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer; and RNA probes (RNA probes or riboprobes). As RNA probes are the most widely used and versatile, and as they have the advantages that RNA–RNA hybrids are very thermostable and resistant to digestion by RNases (allowing for post-hybridization digestion with RNase to remove non-hybridized RNA and thus minimizing any possible nonspecific background staining), we will describe their preparation. Although there are two methods of preparing RNA probes, either via RNA polymerase-catalyzed transcription of mRNA in the 3′ to 5′ prime direction or in vitro transcription of linearized plasmid DNA with RNA polymerase (wherein plasmid vectors contain polymerase from bacteriophages T3, T7, or SP6).

In order to generate nonradioactive in situ RNA probes, a non-isotopic method of labeling via in vitro transcription using either labeled dUTP biotin-16-dUTP, or digoxigenin-11-dUTP, enables visualization of signal at the cellular level in tissues. Digoxigenin (DIG) is a steroid isolated from the digitalis plant and as the blossoms and leaves are the only known source of DIG, the anti-DIG antibodies are not likely to bind to other biological material. In general, it is thought that the DIG label is more sensitive

than the biotin label and that the DIG label allows comparable sensitivity to ^{35}S-radiolabeled probes (*see* Subheading 3.3). Since these nonradioactive labels have no inherent "decay" kinetics, once a probe has been labeled it can either be used immediately or be divided into aliquots, lyophilized and stored at −20 °C for later use for as long as 1–2 years with careful storage. All solutions during probe preparation are made with autoclaved DEPC-treated sterile distilled water, keep eppendorf tubes on ice or at 4 °C at all times, aerosol barrier filter RNAse-free pipette tips are used and gloves are always worn.

Preparation of Linearized Plasmid Template

1. Digest 50 μg of plasmid DNA in a total reaction volume of 200 μl with an appropriate restriction enzyme. Before the addition of the enzyme, save 1 μl of undigested DNA mixture to run on diagnostic gel (*see* **step 3** below).

   Plasmid DNA (if stock at 1 μg/μl) : 50 μl

   10× Restriction Enzyme Buffer : 20 μl

   DEPC-treated H_2O: 125 μl

   Restriction Enzyme (typically, 5–20 Units/μl): 5 μl
2. Incubate for 1 h at appropriate temperature (usually 37 °C) until linearization is complete.
3. Run 1 μl of undigested plasmid DNA (from **step 1** above) and 1 μl of digested linear DNA (from **step 2** above) side by side, within a 1 % agarose gel, to confirm complete digestion. Once digestion is verified, extract DNA with phenol, phenol–$CHCl_3$, $CHCl_3$–isoamyl alcohol, and then ethanol-precipitate.
4. Rinse precipitate with 70 % ethanol (made up with DEPC-H2O), then dry the pellet and dissolve into 40 μl of DEPC-H2O. This will give a final DNA concentration of ~1 μg/μl, and this can then be stored store at −20 °C until probe generation.

In Vitro Transcription of Digoxigenin UTP-Labeled RNA Probe

1. Add the following to a siliconized microfuge tube on ice:

   DEPC-H_2O: 11 μl

   10× Transcription Buffer: 3 μl

   DIG RNA Labeling Mix: 2 μl

   RNase inhibitor (RNase OUT): 1 μl

   Template DNA (1 μg/μl): 1 μl

   Enzyme (T3, T7, or SP6; typically, 10–20 Units/μl): 2 μl
2. In vitro transcription for 3 h at 30–37 °C.
3. Add 1 μl of DNaseI (RNase-free) to remove DNA template to minimize DNA–RNA annealing, and incubate at 37 °C for further 15–30 min (optional step).

4. Add 2 µl of 200 mM EDTA (made up in DEPC-H_2O) to stop reaction, add DEPC-H_2O to make up to the final volume of 100 µl. Save 1 µl of the mixture to run on diagnostic gel (*see* **step 10** below).
5. Add 10 µl of 10 mg/ml yeast transfer carrier RNA (RNAse-free), mix by hand and then add equal volume (100–110 µl) of 4 M ammonium acetate. Mix by hand again, and add 2 volumes of ice cold 100 % ethanol (400–450 µl).
6. Leave at room temperature for 15 min and then centrifuge at 12,000–15,000 × *g* for 10–30 min at 4 °C.
7. Resuspend in 100 µl DEPC-H_2O and repeat steps 5 and 6.
8. Rinse the precipitate with 500 µl 70 % ethanol (made up with DEPC-H_2O) and spin again and then dry briefly, as over drying is not recommended, since the precipitate will be hard to dissolve if totally dry.
9. Dissolve the pellet in 100 µl 10 mM dithiothreitol (DTT) (made up in DEPC-H_2O), and add 1 µl of RNase inhibitor. Save 1 µl of the final solution to run on diagnostic gel (*see* **step 10** below), and store the rest in −80 °C (*see* **Note 3**) to prevent degradation.
10. Prepare a fresh 1 % agarose gel, and run sample (pre addition of tRNA, from **step 4**) and final RNA probe (following addition of DTT from **step 9**) side by side in cold buffer at 60 V for 30 min. Then compare the intensity with DNA markers to estimate the concentration (*see* **Note 4**).

Pre-hybridization

The major function of pretreatment is to reduce background staining and permeabilize the tissue. Many of the nonradioactive detection methods utilize enzymes such as peroxidases or alkaline phosphatases to visualize the label, and thus endogenous tissue enzymes, which could result in nonspecific background staining, are neutralized. This is usually accomplished by treating the tissues with 1 % H_2O_2 in methanol for 30 min (to inhibit with peroxidases) or levamisole (to inhibit alkaline phosphatases). Another commonly observed pretreatment is acetylation with acetic anhydride (0.25 %) in triethanolamine, as this is thought to decreasing background. Pre-hybridization involves incubating the tissue/sections with a solution that is composed of all the elements of the hybridization solution, minus the probe.

For Whole Mount In Situ Hybridization

1. Embryos are received in 100 % Methanol.
2. Rehydrate through 75 %, 50 %, and 25 % methanol made up with PBST (PBS with 0.1 % Tween 20) at 4 °C on rocker for 30 min each change or until embryos sink.
3. Wash in PBST at 4oC on rocker for 15 min two times.

4. Bleach samples in 6 % H_2O_2 in PBST at 4 °C for 60 min, embryos will turn whitish and make-up 6 % solution just prior to use.
5. Wash in PBST at 4 °C on rocker three times for 10 min. During this time, prepare the 2 mg/mL Glycine in PBT.
6. Treat samples with 10 μg/mL Proteinase K in PBST at room temperature for 5–15 min, may shake gently from time to time and take care embryos are fragile at this point). Digestion times vary depending upon developmental stage and size of samples E8.5 embryos are Proteinase K digested for 5 min, E10.5 for 10 min, and E12.5 and older/larger samples are digested for 15 min). Alternatively, smaller embryos can be permeabilized with RIPA solution (*see* **Note 5**).
7. Stop digestion via washing in 2 mg/mL Glycine in PBST solution on ice (NO ROCKING) for 20 min. The wash twice in PBST for 5 min at 4 °C.
8. Re-fix samples to preserve histology in freshly made up ice cold 0.2 % glutaraldehyde in 4 % PFA at 4 °C on rocker for 20 min. Re-wash three times in large volumes of PBST for 5 min at 4 °C to remove all fixative.
9. Remove as much PBST as possible and place samples in heated pre-hybridization buffer (*see* below). Samples are now ready for hybridization. To prepare 40 ml pre-hybridization buffer, add following in order and heat to 65 °C (initially solution will appear opaque but will clear upon heating):

   Formamide: 20 mL

   20× saline sodium citrate buffer pH 4.5: 10 mL

   Yeast RNA (tRNA): 40 μL

   50 μg/mL Heparin stock: 40 μL

   DEPC H_2O: 36 mL

   10 % SDS: 4 mL

For Section In Situ Hybridization

1. Prepare RNase-free Coplin jars by spraying with RNase Zap, then washing three times in DEPC-H_2O.
   (a) For Paraffin Sections: De-wax slides in Xylene for 10 min two times. Rehydrate slides with two changes of 100 % ethanol, followed by 90 %, 80 %, 70 %, 50 %, and 30 % ethanol, then two washes in DEPC-H_2O (note: each change should be 30 s).
   (b) For frozen sections: Fix in 4% PFA + 0.25 % glutaraldehyde for 15 min on ice. Wash twice in PBS for 5 min each (first wash on ice, the next at room temperature).
2. Prepare sections

3. Digest paraffin sections in Proteinase K working solution (10 μg/mL) at 37 °C for 10 min depending on the age of the embryo, but digest frozen sections for 5 min only.
4. Rinse twice in DEPC-H_2O for 5 min, and place slides in 0.1 M TEA–HCl for 10 min and shake on shaker.
5. Transfer slides to 0.1 M TeHCl + 0.25 % acetic anhydride for 10 min and shake at room temperature. Wash slides in 2× SSC twice for 10 min.
6. Dehydrate slides through graded ethanol (50 %, 70 %, 80 % and 90 % once, then twice in 100 % ethanol) for 30 s each change and air-dry for at least 2 h. Sections are now ready for hybridization

Hybridization

The composition of the hybridization solution is critical in controlling the efficiency of the hybridization reaction, with maximum sensitivity achieved when probe concentrations are just high enough to saturate the target sequence. Hybridization depends on the ability of the labeled probe to anneal to a complementary mRNA strand just below its melting point (Tm). The factors that influence the hybridization to the target mRNA are temperature, pH, monovalent cation concentration, and the presence of organic solvents. Other components are added to decrease the chance of nonspecific binding of the probe, including tRNA, which acts as a carrier RNA.

For Whole Mount In Situ Hybridization

1. Replace preheated pre-hybridization after 1 h with fresh preheated hybridization buffer containing labeled RNA probe (50 % formamide, 5× saline sodium citrate buffer pH 4.5, 1 % SDS, 50 μg/mL heparin, 50 μg/mL yeast RNA, 4 μL/mL digoxigenin labeled probe made up in DEPC-H_2O), so that final concentration of probe is about 200 ng/ml and make sure hybridization solution completely covers samples.
2. Incubate samples at 70 °C overnight in water bath (*see* **Note 6**).

For Section In Situ Hybridization

1. Heat probe to 80 °C for 2 min to denature any possible secondary structure which would inhibit hybridization, and then place on ice for 1 min. To a new aliquot of pre-warmed prehybridization buffer (50 % formamide, 5× SSC buffer pH 4.5, 1 % SDS, 50 μg/mL heparin, 50 μg/mL yeast RNA made up in DEPC-H_2O), add 4 μL/mL digoxigenin labeled probe (so that final concentration of probe is about 200 ng/ml).
2. Place 50–100 μl of prepared probe (as above) on each slide and coverslip (note: coverslips were initially sprayed with RNase Zap, then washed three times in DEPC-H_2O prior to use). Place in a humidified chamber (chamber consists of slides elevated from paper towels), with chamber buffer (5× SSC buffer pH 4.5, 1 % SDS) soaked paper towels to prevent drying out and incubate overnight at 55 °C.

Post-hybridization

Following hybridization, the unbound probe and probe that has bound loosely to imperfectly matched sequences is washed away. Two key steps ensure low background, which ultimately governs the techniques sensitivity. Importantly, washing is carried out at or close to the stringency condition at which the hybridization takes place with a final low stringency wash. This is followed via RNAse treatment to remove any unbound single-stranded probe in high salts. All solutions are made with sterile distilled water, as DEPC-H2O is no longer necessary for the preparation of the solutions in the following steps. It is important to keep the tissues moist.

For Whole Mount In Situ Hybridization

1. Remove probe solution and save if required (note: store at −20 °C and can reuse up to 3 times) and wash samples twice for 30 min in post-hybridization wash # 1 (*see* Subheading 2) in water bath at same temperature used for hybridization.
2. Wash in post-hybridization wash # 2 (*see* Subheading 2) at 50–65 °C (temperature should be 5 °C less than hybridization temperature) in water bath twice for 30 min.
3. Wash samples in TBST working solution #1 (*see* Subheading 2) twice for 10 min each at 37 °C. Samples are now ready to be blocked prior to antibody detection of labeled probe.

For Section In Situ Hybridization

1. Remove probe solution and save if required (note: store at −20 °C and can reuse up to 3 times) and wash slides in a Coplin jar in post-hybridization wash # 2 (*see* Subheading 2) for 15 min at 65 °C.
2. Wash slides in post-hybridization wash # 2 (*see* Subheading 2) at 60 °C (temperature should be 5 °C less than hybridization temperature) twice for 30 min.
3. Wash slides in TBST working solution #1 (*see* Subheading 2) twice for 10 min each at 37 °C.
4. Incubate slides in RNase (100 mg/ml) diluted 1:5,000 in TBST working solution #1 for 30 min at 37 °C.
5. Rinse with distilled H_2O and then wash slides twice in TBST working solution #1 for 10 min each at 37 °C. Slides are now ready to be blocked prior to antibody detection of labeled probe.

Detection of Labeled Probe and Hybridization Signal

For Whole Mount In Situ Hybridization

1. Replace TBST working solution with 10 % heat-inactivated sheep serum (or goat depending upon which animal anti-DIG antibody was raised in) in TBST for 90 min at 4 °C on rocker. To heat inactivate sheep/goat serum prior to use, incubate at 50 °C in water bath for 4 h and then cool to room temperature.
2. Remove blocking solution and incubate samples in 1 % heat-inactivated sheep serum (or goat) in TBST with1:2,000 dilution of alkaline phosphatase labeled anti-Digoxigenin antibody at 4 °C on rocker overnight.

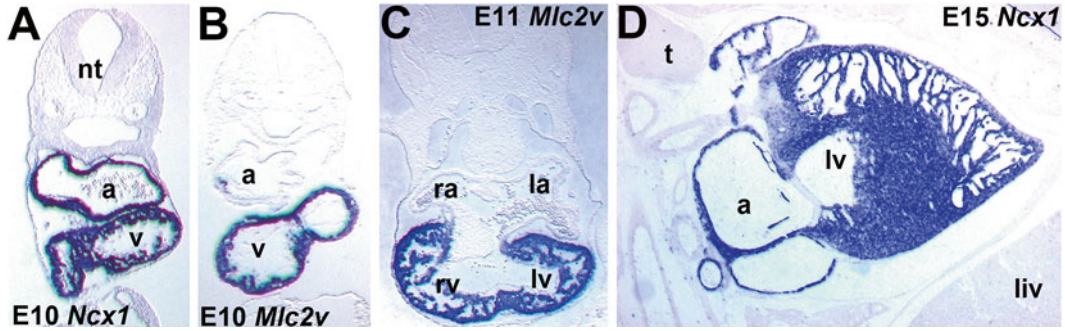

Fig. 1 Nonradioactive in situ hybridization. Antisense mouse cDNA probes against *Sodium-calcium exchanger* (*Ncx1*) and *Myosin light chain-2 ventricular restricted* (*Mlc2v*) were labeled with DIG and used to probe both embryonic and fetal mouse heart 6 μm thick wax sections. (**a**) Transverse E10 mouse embryo sections were probed with DIG-labeled *Ncx1* cDNA [11] to detect cardiomyocytes-restricted *Ncx1* mRNA expression within both the ventricle (v) and atria (a). Following detection of the DIG-labeled probe (30 min in dark) via staining with NBT-X-phosphate, the specific signal appears as a blue-purple precipitate within only the cardiomyocytes. (**b**) Similarly, when sections through the same E10 embryo were probed with DIG-labeled *Mlc2v* cDNA, only the ventricular cardiomyocytes were positive for *Mlc2v* mRNA expression. (**c**) Mlc2v ventricular expression remains restricted to the left and right ventricles (lv, rv) of the E11 heart, and there is no nonspecific expression within either the left or right atria (la, ra), nor there is blood trapped within the atria. (**d**) Equally, when older fetal E15 sagittal mouse sections were probed with DIG-labeled *Ncx1* cDNA, robust signal is detectable within the ventricular and atrial cardiomyocytes, without any nonspecific signal within the liver (liv) or thymus (t)

3. Transfer embryos to larger 50 mL tubes and wash in TBST working solution # 2 (*see* Subheading 2) at 4 °C on rocker for 60 min, at least five times. Leave in last wash at 4 °C on rocker overnight.
4. Wash samples in NTMT solution (*see* Subheading 2) three times for 10 min at room temperature. Replace NTMT with fresh NTMT containing NBT-BCIP substrate (*see* Subheading 2) and place vials containing samples/NTMT/NBT-BCIP substrate in dark drawer (as light sensitive) and wait for color development. Signal may take anywhere from 1 h with strong probes to overnight depending upon level of mRNA expression and efficiency of probe hybridization; can place samples at 4 °C overnight to several days for weaker probes. *See* Fig. 1 for an example.
5. Following appropriate color development (usually judged via observing similar staining pattern in duplicate/triplicate samples and an absence of staining in negative control embryos; *see* **Note 11** for discussion of inclusion of suitable controls), samples are washed in PBS for 5 min twice at room temperature and post-fixed in 4 % PFA overnight at 4 °C to preserve histology.
6. To clear and store samples, rinse in PBS for 5 min twice and dehydrate samples though graded clearing glycerol solutions (10 %,

20 %, 50 % glycerol in PBS). Embryos may be left in any step in 4 °C refrigerator overnight or until embryos sink. Store completely cleared embryos, wrapped in foil in the 4 °C refrigerator.

For Section In Situ Hybridization

1. Remove TBST working solution #1 and incubate slides in blocking solution (10 % heat-inactivated sheep serum (or goat) in TBST for 2 h at 4 °C, rocking.
2. Remove blocking solution and incubate slides overnight in fresh 1 % heat-inactivated sheep serum (or goat) in TBST with 1:2,000 dilution of alkaline phosphatase labeled anti-Digoxigenin antibody added at 4 °C.
3. Wash slides in TBST working solution # 2 (*see* Subheading 2) at least five times at room temperature for 30 min each.
4. Wash slides in NTMT solution (*see* Subheading 2) three times for 10 min at room temperature. Replace NTMT with fresh NTMT containing NBT-BCIP substrate (*see* Subheading 2) and place slides in dark drawer (as light sensitive) and wait for color development (may take anywhere from 20 min to overnight depending upon level of mRNA expression and efficiency of probe hybridization).
5. Wash slides in PBS for 5 min twice at room temperature and post-fix in 4 % PFA at 4 °C for 20 min. Rewash in PBS and dehydrate slides through 50 %, 70 %, 90 %, and 100 % ethanol series for 2 min each. Run tissue through Pro-Par clearant twice for at least 10 min each and coverslip slides.

3.3 Radioactive In Situ Hybridization

Despite its long exposure times, radiolabeled probes are still the choice for many investigators due to its unique sensitivity. ^{35}Sulfur (^{35}S) is the most commonly used radioisotope because its high activity enables the detection of transcripts present in low amounts. Radiolabeled probes are visualized by exposure of the tissue section or cells (to which the labeled RNA probe has been hybridized) against photographic film which is then developed. When using radioactivity, strict waste disposal and containment measures will need to be adhered to, and it must be remembered that the useful shelf life of the labeled probe is inherently dependent on the half-life of the radionucleotide. ^{35}S has an experimental life time of ~80 days, so probes will need to be remade if in situ hybridizations are to be repeated several months apart.

3.3.1 Radioactive Probe Preparation

Although there are several type of probes, here we describe the in vitro synthesis of radioactive in situ RNA probes using ^{35}S-labeled dUTP. All solutions during probe preparation are made with autoclaved DEPC-treated sterile distilled water, keep eppendorf tubes on ice or at 4 °C at all times, aerosol barrier filter RNAse-free pipette tips are used and gloves are always worn. Solutions are stored at room temperature unless otherwise indicated.

1. Add the following to a siliconized microfuge tube on ice:
   DEPC-H_2O: 2 µl
   10× Transcription Buffer: 2 µl
   Alpha-^{35}S-UTP 200 µci: 10 µl
   ATP, CTP, GTP (10 µm) each: 1 µl
   100 mM DTT: 1 µl
   RNase inhibitor (RNase OUT): 1 µl
   Template DNA (1 µg/µl): 1 µl
   Enzyme (T3, T7, or SP6; typically, 10–20 Units/µl): 2 µl
2. In vitro transcription for 3 h at 30–37 °C.
3. Add 1 µl of DNaseI (RNase-free) to remove DNA template to minimize DNA–RNA annealing, and incubate at 37 °C for further 10 min (optional step).
4. Add 3 µl of 200 mM EDTA to stop reaction, add DEPC-H_2O to make up to the final volume of 50 µl. Save 1 µl of the mixture for scintillation counter determination of labeling efficiency.
5. Purify the ^{35}S-labeled RNA using the columns to remove free rNTPs according to the manufacturer's instructions.
6. Add 10 µl of 10 mg/ml yeast carrier tRNA, 10 µl 5 M NaCl, and 50 µl DEPC-H_2O to the tube and mix by hand. Next, add 500 µl cold 100 % ethanol and precipitate probe in a –20 °C freezer overnight. Or, if continuing on the same day, place in –70 °C for 2 h.
7. Centrifuge precipitate for 15 min at 14,000 g at 4 °C.
8. Wash pellet in 500 µl of 70–75 % ETOH made with DEPC-H2O and spin at 14,000 g for 10 min. Then remove and discard supernatant.
9. Resuspend pellet in 50 µl DEPC-H_2O containing 20 mM DTT plus 50 µl carbonate Buffer-hydrolysis Buffer (*see* Subheading 2) and incubate at 60 °C for the appropriate time depending upon the insert/probe size. In order to generate desired ~100 base pair labeled probe fragments, hydrolysis is required. Hydrolysis incubation time can be calculated using the following equation (time in minutes = [starting kb - desired kb]/0.11 [starting kb] [desired kb]).
10. Neutralize reaction with 3 µl 3 M sodium acetate (pH 6) and 5 µl 10 % glacial acetic acid. Add 300 µl 100 % ETOH. Store at –70 °C for 2 h or overnight.
11. Precipitate by centrifuging at 14,000 × *g* for 20 min. Rinse the pellet in 500 µl of 70–75 % ethanol for 10 min by centrifuging at 14,000 g.

12. Dry briefly (*see* **Note 7**) and resuspend the pellet in 50 µl TD buffer (*see* Subheading 2). Save 1 µl for scintillation counting and store rest at −20 °C until required. Radioactive ^{35}S RNA probes should be used within 1 month.
13. Spot samples from **steps 3** and **11** on filter paper, place filters in Scinitiverse vials and count the radioactivity using a scintillation counter. Usually, the count of unpurified labeling mix is ~4×10^6 cpm/µl, which is the total count of transcribed products and free, unincorporated nucleotides), whereas that of purified labeled probe (theoretically, consisting of only transcribed labeled riboprobes) should be more than 3×10^6 cpm/µl.

3.3.2 Pre-hybridization and Hybridization

Pre-hybridization and hybridization for radioactive in situ on either frozen or wax embedded sections is the same as for nonradioactive section in situ (*see* above). Pre-hybridization is carried out in RNase-free Coplin jars rinse with DEPC-H_2O and then dried in an oven at 70 °C for 1 h. It is recommended that hybridization occur the same day that the slides are prepared. It is important to keep the sections moist, thus transfer the slides from solutions into racks already immersed in solutions whenever possible. Additionally, as DTT is labile it should thus be the last item added to solutions just prior to incubation. Prepare the probe (1×10^6 counts per minute per 100µl hybridization buffer per slide) and add to the hybridization buffer (50 % formamide, 5× saline sodium citrate buffer pH 4.5, 1 % SDS, 50 µg/mL heparin, 50 µg/mL yeast RNA, and ^{35}S-labeled probe made up in DEPC-H_2O). Vortex probes mixture briefly, then centrifuge to get rid of any bubbles and add ~100 µl probe solution to each slide and coverslip. Hybridization is then carried out overnight at 65 °C within a Parafilm-sealed humidified chamber. Chamber consists of slides elevated from chamber buffer (5× SSC buffer pH 4.5, 1 % SDS) wetted paper towels in a radiation safe hybridization oven.

Post hybridization Washing

1. Remove probe solution and discard, wash slides in a Coplin jar in post-hybridization wash # 1 (*see* Subheading 2) for 30 min at 65 °C.
2. Wash slides in post-hybridization wash # 2 (*see* Subheading 2) at 60 °C (temperature should be 5 °C less than hybridization temperature) twice for 30 min.
3. Wash slides in washing solution #1 (*see* Subheading 2) twice for 10 min each at 37 °C.
4. Incubate slides in RNase (100 mg/ml) diluted 1:5,000 in washing solution #1 for 30 min at 37 °C.
5. Rinse briefly with distilled H_2O and then wash slides twice in washing solution #1 for 5 min each at 37 °C.
6. Wash in 2× SSC, then 0.1× SSC for 15 min each at 37 °C.

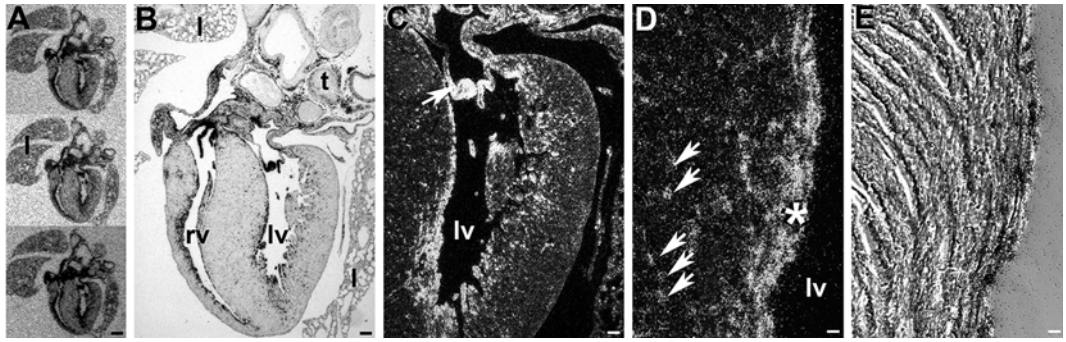

Fig. 2 Radioactive in situ hybridization. Antisense mouse *Periostin* cDNA [12] probe was synthesize using [35S] UTP to a specific activity of 2×10^5 dpm/μg and used for in situ hybridization to detect *Periostin* mRNA within mouse postnatal day 14 heart and lung 10 μm thick wax sections. (**a**) Light field image of 3 consecutive heart and lung (l) sections following 3 day exposure of radioactive slides via preliminary auto radiographic film detection of signal. Note consistent and reproducible signal (*black*) patterns within all three sections. (**b**) Higher power light field view of heart, following emulsion dipping and exposure after 10 days in the dark. Note that signals detected upon the X-ray film are consistent with signal within image in B, and that silver grains (appear *black* in light field) are present within the cardiac valves and particularly the cardiac fibroblasts lining the left and right ventricles (lv, rv). Also note that *Periostin* is expressed within the capsule surrounding the neonatal thymus (t) but is absent from the thymus itself. (**c**) Enlarger dark field view of the left ventricle from panel B, illustrating the robust signal (silver grains appear white in dark field) present within the vales (*arrow*) and the punctate signal within cardiac fibroblasts lining the ventricular lumen and intermingled within the cardiomyocytes. (**d** and **e**) High power dark field (**d**) and phase (**e**) images of *Periostin*-positive cardiomyocytes within the ventricles (*arrows* in D) and those lining the left ventricular lumen (indicated via *). Bar magnifications: (**a**) = 1 mm, (**b**) = 100 μm, (**c**) = 50 μm, (**d**, **e**) = 10 μm

7. Dehydrate slides quickly by taking them through 30 %, 50 %, 70 %, and 90 % ethanol series (including 0.3 M ammonium acetate), followed by dehydration in 100 % ethanol twice. Air-dry for 1 h, and then slides are ready for a preliminary autoradiographic verification of signal.

Preliminary Autoradiographic Check

As a preliminary verification that a radioactive signal is present and prior to the expensive and time consuming process of dipping the slides in nuclear emulsion, that dried slides can be placed against an autoradiographic film to produce a low-resolution image (*see* Fig. 2a). Using a rough relationship that one day exposure on film equates to three days of exposure of dipped slides, it is possible to estimate how long exposure is likely to be for each of the probes used. This step can also be used to gauge whether the experiment worked and if it is worthwhile to proceed to dipping (*see* below).

Chemical Emulsion and Autoradiographic Signal Detection

1. Following preliminary X-ray verification of signal and assessment of exposure time, air-dried slides are dipped in photographic nuclear emulsion. Dipping takes place within the darkroom (note: a light tight darkroom is essential and we prearrange slides and solution in a linear fashion and dip in total darkness, however, some investigators use a dark red light

whilst dipping). Emulsion within slide mailers is re-warmed to 40 °C in total darkness, then each slide is sequentially dipped for 20 s and then placed within a wooden rack to air-dry for 1 h in total dark.

2. Place dry slides in lightproof box and expose for 10–16 days at 4 °C. Several Drierite pouches are placed within the slide boxes to prevent humidity and slide boxes are wrapped with 3 separate sheets of foil. Wrap with lab-diaper to ensure dryness and store at 4 °C room away for radioactivity stocks.
3. In dark room (no red light), develop (dissolve at 18 g/100 ml distilled H_2O) slides in developer for 5 min and rinse in water for 5 s.
4. In dark room (no red light), place slides in fixer for 3 changes of 5 min each. Dilute 1 part liquid fix with 4 parts distilled H_2O.
5. In dark room (no red light), wash in gentle running H_2O for at least 10 min, as this wash is essential for low background).
6. Can then turn light on, repeat wash in H_2O then counter stain in 0.02 % Toluidine blue for 5–30 s. Rinse in H_2O, dehydrate through graded ethanol series (70 %, 90 %, and twice in 100 %) and clear in xylene. Coverslip with synthetic mounting media and allow slides to dry before assessing signal. After the mounting medium has dried, there may be some residual medium on the back of the slide that can be removed with a razor blade. Viewed under dark field, the location of the radionucleotide beneath the emulsion will appear as a sliver grain (Fig. 2c, d) but under light field and phase contrast will appear as black grains (Fig. 2b, e).
7. There are various glitches which can sometimes be encountered together (for example; low hybridization (*see* **Note 8**), high background (*see* **Note 9**) and patchy background (*see* **Note 10**), but with appropriate remedies these problems can be circumvented.

3.4 Necessary Controls

Of course the most important part of any experimental procedure is the inclusion of controls. If the quality of your tissue is poor and/or your RNA is degraded it will be very hard to get good results with in situ hybridization. There are, however, a number of controls (*see* **Note 11**) you can add to your experiment to verify the status of your tissue and mRNA within the tissue. If you are using fresh tissue and these controls are negative, then this suggests a problem with your technique or protocol.

4 Notes

1. Do not exceed 60 °C in the vacuum oven as temperatures higher than 60 °C can damage tissue histology.
2. 10 mM DTT is necessary to minimize loss of probe due to nonspecific sticking to the eppendorf tubes.

3. Labeled probes can be stored for several months at −20 °C, especially if stored in hybridization solution (*see* Subheading 2).
4. Typically, the reaction should yield ~100 ng/μl probe and if the optional DNase1 step was omitted, then the smaller labeled RNA probe band should also be ~10× as intense as the larger plasmid DNA band.
5. RIPA, a solution with a very high concentration of detergents, is gentler than proteinase K and can be used to permeablize very young embryos. It results in better retention of morphology and higher penetration of probes but only in younger E6-9 embryos. After washing three times for 30 min in RIPA buffer (150 mM NaCl, 1 % NP40, 0.5 % deoxycholate, 0.1 % SDS, 1 mM EDTA, 50 mM Tris, pH 7.5), embryos are post-fixed as usual in 4 % PFA and then washed thoroughly with PBST.
6. We use RNAase-free screw cap eppendorf vials with conical bottoms for hybridization and probes may be reused several times.
7. Note over drying is not recommended, since a totally dry RNA precipitate is difficult to dissolve.
8. Low hybridization: Low probe concentration, which can be corrected by increasing the probe concentration (and amount of probe should be checked via gel after purification). Very weak signal may also be due to your tissue RNA being degraded, if old samples are used or were not stored appropriately. Probe degradation, always keep probe reduced by adding 10 mM DTT and ensure solutions and equipment are sterilized and RNase-free. Wear gloves at all times. Use truncated templates to synthesize RNA probes to eliminate vector sequences (which tend to cross react with ribosomal RNA). Low specific activity, make sure use fresh radioactive label prior to half-life decay. When using large probes and ^{35}S labeling, alkaline hydrolysis to reduce probe size is critical and you may need to optimize hydrolysis times for each RNA probe. When using DIG labeling, label incorporation can be checked via dot-blot against a stand and antibody detection, to ensure efficient incorporation of label during transcription reactions.
9. High background: Use a long first wash to remove most of the unhybridized probe. Use high stringency washing. If high background is consistently observed, then use RT-PCR and/or sense control to determine if gene being analyzed is constituently expressed. Longer RNA probes can exhibit poor tissue penetration and while hydrolysis enhances tissue penetration it also increases the likelihood of non-selective binding to other non-targeted gene sequences. Can use shorter inserts within plasmid vectors. Essentially, the solutions should be prepared fresh before use, since many of the components of

such solutions are extremely labile. Nuclear emulsion exposed to light/radiation. It is recommended for the emulsion be prepared freshly and utilized in complete darkness; however a reasonable distance from safelight should render the same effect. Avoid storing the slides during exposure time in a cold room were other radionucleotides, such as ^{32}P, is usually stored. Excessively high probe concentration, make sure you check probe levels after purification.

10. Patchy background: Incomplete removal of wax during pretreatment, make sure that clearant is fresh and not saturated with dissolved wax. It is very important that after slides are submerged in the nuclear emulsion, they are allowed to dry completely before starting exposure. During the emulsion step avoid formation of bubbles on the slides; this can prevent slides from getting coated evenly, and later interfering with autoradiography. Use new slide mailers for dipping in nuclear emulsion and to avoid old emulsion from dipping chamber.
11. Necessary controls: Always use a sense strand RNA probe as a negative control and to measure nonspecific background in situ. If your sense probe detects nothing, then you can be sure that any signal detected by your antisense probe is due to sequence-specific binding to mRNA and not due to binding to other targets within the cell. Use a positive control on fresh, positive control tissue known to have the sequence of interest (not always possible). If you detect no signal then this suggests the problem exists within your technique or protocol. Perform specificity control and determine that your probe is only binding to RNA. An absence of binding after RNase treatment indicates that binding was indeed to RNA within the tissue. Competition studies with labeled and excess unlabeled probes can also help distinguish between specific versus nonspecific binding. The best way to ensure that your probe is binding to the correct target sequence is by choosing a correct probe sequence from the start and having high stringency hybridization and wash conditions in your experiment. If your probe is binding to the tissue in a sequence-specific fashion and this pattern is repeated in at least three (or more) serial sections or stage matched embryo samples, then your in situ will be a success.

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Part II

Mouse Genetics in Stem Cells

Chapter 13

Isolation and Handling of Mouse Embryonic Fibroblasts

Kanika Jain, Paul J. Verma, and Jun Liu

Abstract

Primary mouse embryonic fibroblasts (MEFs) are the most commonly used feeder layers that help to support growth and maintain pluripotency of embryonic stem cells (ESC) in long-term culture. Feeders provide substrates/nutrients that are essential to maintain pluripotency and prevent spontaneous differentiation of ESC. Since embryonic fibroblasts stop dividing after a few passages, care must be taken to isolate them freshly. Here, we provide a protocol to derive MEFs and describe the method to inactivate the cells using mitomycin C treatment. The protocol also describes freezing, thawing, and passaging of MEFs. This basic protocol works well in our laboratory. However, it can be modified and adapted according to any user's particular requirement.

Key words Mouse embryonic fibroblasts, Feeder layers, Mitomycin C, Cell culture

1 Introduction

MEFs are the primary cell lines that are generally derived from 12.5- to 13.5-day fetuses from pregnant female mice [1]. MEF isolation involves dissecting the uterus and embryonic sac to release the fetuses, discarding the head and visceral tissue, mincing the remaining tissue, and culturing the minced tissue in MEF medium. All newly isolated MEF cultures should be tested for mycoplasma before serving as feeder layers for ES cell culture [2].

The use of MEFs as feeder layers for the culture of pluripotent stem cells (PSC) has been well established since the first mouse ESC were derived in 1981 [3, 4]. Many ES cell lines have been established on MEFs and it is recommended to maintain each of these cell lines on the feeder type on which it was originally established. Induced pluripotent stem cells (iPSC), like ESC, should be grown on monolayers of MEF feeders for long-term culture and maintenance [5, 6]. To generate the feeder cells, MEFs are mitotically inactivated by treatment with mitomycin C or γ irradiation, which inhibits their cell division but keeps the cells metabolically active [7, 8]. Feeders are known to provide a complex but somewhat

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unknown mixture of nutrients and factors that helps to maintain the pluripotency of PSC during their long-term growth and proliferation [9]. Both, MEFs and inactivated feeder MEFs can be frozen and stored in liquid nitrogen indefinitely.

Care must be taken while culturing and maintaining MEFs, since being a primary cell line, they have a limited life-span in culture [10]. If these cells begin to elongate or increase the doubling time, they are going beyond their normal and useful state. Our recommendation is passaging cells no more than five times, which necessitates new stocks being made on a regular basis. The most important factor while culturing MEFs is to keep them in a healthy and proliferative state.

Appropriate institutional animal ethics approvals must be obtained before any work is commenced.

2 Materials

All culture medium must be filtered with a 0.22 μm filter unit. Store all media at 2–6 °C for up to 2 weeks.

2.1 Mice

1. Naturally mated or superovulated day-12.5–13.5 post-coitus (*dpc*) pregnant CD1 mice (*see* **Note 1**).

2.2 Media

1. MEF culture medium: Add 10 % fetal bovine serum (FBS) to 90 % Dulbecco's minimal essential medium (DMEM) with high glucose, supplemented with penicillin/streptomycin (0.5 mL/100 mL). Store at 4 °C.
2. 2× Freezing medium: 20 % FBS, 20 % DMSO, and 60 % DMEM. Pour all components, with DMSO last, into a 0.22 μm filter container and filter (*see* **Note 2**).
3. Mitomycin C solution: 8 μg/mL Mitomycin C. Weigh 2 mg mitomycin C (Sigma) and add to 250 mL DMEM. Filter through a 0.22 μm filter unit and aliquot 7 or 14 mL per Falcon tube. Store at –20 °C.

3 Methods

3.1 MEF Stocks

1. Sacrifice the pregnant mice when embryos are about 12.5–13.5 *dpc* by exposure to CO_2 or cervical dislocation. Sterilize the abdomen with 70 % ethanol and dissect the abdominal cavity to expose the uterine horns.
2. Transfer the uterine horns into 100 mm Petri dishes containing PBS. Use two pairs of watchmakers' forceps to dissect the embryos away from the uterus and all the membranes. Transfer the embryos into a new dish containing PBS. Count the number of the embryos.

3. Wash retrieved embryos three times with PBS. Remove heads and visceral organs (liver, heart, kidney, lung, and intestine) with the same instruments.
4. Wash three times with PBS to remove as much blood and cell debris as possible.
5. Mince the embryos using sharp scissors.
6. Add 10 mL trypsin-EDTA (0.5 %, 10×), transfer into a 50 mL Falcon tube, and incubate for at least 20 min at 37 °C with occasional shaking.
7. Add 10 mL MEF medium to neutralize trypsin (to stop trypsin activity), pipetting the cell suspension up and down thoroughly.
8. Distribute the cell suspension evenly into T75 culture flasks (two embryos per flask), and add additional MEF medium in flasks to a total volume of 20 mL.
9. Culture the cells in a 5 % CO_2 incubator until confluent, and change medium when it is yellowish (do not vacuum the lumps).
10. After 2 to 3 days of culture, the MEF should form a confluent monolayer. Trypsinize each flask and re-plate further into five T75 flasks.
11. When the flasks are confluent (usually after 2 or 3 days), freeze the cells.

3.2 MEF Freezing

Check flasks to see if the cells cover the whole surface of the flask. If there are still some spaces between cells, wait for another day before freezing. If confluent, then freeze cells.

1. Remove medium and all lumps possible from the flasks.
2. Add 3 mL trypsin-EDTA (0.25 %, 10×) to cover the entire culture-flask surface. Incubate for at least 10 min at 37 °C.
3. Tap the side of the flask to loosen the cells, add 6 mL MEF medium to neutralize the trypsin, and wash the flask wall with the medium.
4. Collect cell suspension into a 50 mL Falcon tube; cell suspensions in other flasks from the same mouse could be collected together in the same 50 mL tube (*see* **Note 3**).
5. Centrifuge for 5 min at 200 × *g*.
6. Remove supernatant, resuspend in desired volume of MEF medium, and pipette in order to fracture the pellet.
7. Add equivalent volume of freezing medium drop by drop and mix gently (*see* **Note 4**).
8. Distribute to each freezing vial with 1 mL of cell suspension (*see* **Note 5**).
9. Freeze vials overnight at –70 °C in Nalgene freezing boxes (containing Iso-Propanol) and transfer freezing vials to liquid nitrogen container.

3.3 MEF Thawing

1. Remove vials from liquid nitrogen and thaw briefly in a 37 °C water bath (*see* **Note 6**).
2. When a small ice pellet of frozen cell remains, clean the vial using 70 % ethanol.
3. Pipette the contents of the vial once, and transfer cells into a 15 mL Falcon tube.
4. Drop by drop add 2 ml MEF medium, and mix gently (*see* **Note 7**).
5. Centrifuge for 5 min at 200 × *g*.
6. Resuspend the pellet in 5 ml MEF medium, pipetting up and down twice.
7. Transfer the cell suspension into a TC75 flask and add additional 10 ml MEF medium (*see* **Note 8**).

3.4 MEF Splitting

1. Remove the conditioned medium from the flask and add 3 mL trypsin-EDTA (0.25 %) to cover the entire culture-flask surface.
2. Incubate for at least 10 min at 37 °C.
3. Tap the side of the flask to loosen the wells, and add 6 mL MEF medium.
4. Wash the flask wall to remove cells completely and then transfer the cell suspension to a 50 mL Falcon tube. Cell suspension from the same batch can be collected in the same Falcon tube.
5. Centrifuge for 5 min at 200 × *g*.
6. Remove supernatant and resuspend the cell pellet in desired volume of MEF medium and pipetting up and down to fracture the pellet.
7. Distribute cell suspension to desired number of culture flasks (*see* **Note 9**).
8. Add MEF medium to final volume of 10–15 ml per flask.

3.5 Preparation of MEF Feeder Layers

1. Remove conditioned medium from the confluent T75 flask.
2. Add 7 mL mitomycin C solution (8 μg/mL) per flask and incubate cells at 37 °C, 5 % CO_2, for 2–3 h.
3. Remove mitomycin C solution and wash twice with 10 mL PBS.
4. Add 3 mL trypsin-EDTA (0.25 %) for 10 min at 37 °C.
5. Add 6 mL MEF medium to neutralize the trypsin and break any cell aggregates by pipetting.
6. Transfer cell suspension into a conical tube.
7. Centrifuge for 5 min at 200 × *g*.
8. Remove supernatant and resuspend the cell pellet in 10 mL MEF medium.

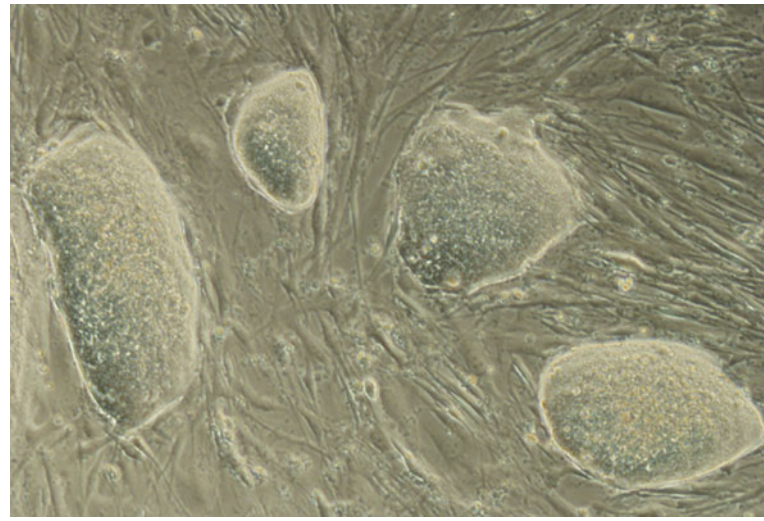

Fig. 1 Morphology of MEFs supporting growth of mouse iPSC colonies. Image of mitomycin C-inactivated MEFs displaying typical morphology and seeding density, supporting mouse iPSC colony growth

9. Titrate the pellet up and down to dissociate.
10. Count the cells and resuspend in desired medium volume.
11. Plate the cells immediately onto tissue culture plates containing MEF medium. For 6-well plate, 400,000 cells/well and 2 ml per well, and for 4-well plate, 100,000 cells/well and 0.5 ml per well (*see* **Note 10**).
12. Allow feeders to attach at least 2 h, but preferably overnight, before seeding ESC or iPSC.
13. Change the medium to ESC medium immediately before adding ESC or iPSC. Mitomycin C-treated MEF feeders can be used for up to 5 days with medium changes every 3 days (Fig. 1).

4 Notes

1. We have not found obvious difference between MEF made from CD1 or Swiss outbred, C57BL/6j inbred, or MTKneo transgenic mice expressing the bacterial neomycin phosphotransferase gene (neo). Superovulated or natural estrus female mice can be used for mating with fertile stud males to generate pregnancies. The superovulation regime entails administration of 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet) per prepubertal (~3–4 weeks old) female mouse followed by 5 IU human chorionic gonadotrophin (hCG: Chorulon, Intervet) 48 h later. The females are placed with stud male mice immediately following the hCG injection.

2. We find that it is best to prepare this fresh each time.
3. Collect all flasks from the same mouse and mark them with a batch number (e.g., the date of freezing), due to possible variations between different mice.
4. Adding the freezing medium drop by drop is crucial for cell recovery.
5. It is recommended to freeze four vials from one confluent T75 flask.
6. Normally two different batches of MEF are thawed and cultured parallelly, in case one is contaminated or of bad quality.
7. Adding the medium drop by drop is crucial for cell recovery.
8. It is recommended to thaw one or two vials into one T75 flask.
9. Normally one confluent flask cell is split to three or four flasks.
10. Cell number can also be calculated as $3–4 \times 10^4$ cells per cm^2.

Acknowledgement

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Chapter 14

Generation of Induced Pluripotent Stem Cells from Mouse Adipose Tissue

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Abstract

The discovery that embryonic stem (ES) cell-like cells can be generated by simply over-expressing four key genes in adult somatic cells has changed the face of regenerative medicine. These induced pluripotent stem (iPS) cells have a wide range of potential uses from drug testing and in vitro disease modeling to personalized cell therapies for patients. However, prior to the realization of their potential, many issues need to be considered. One of these is the low-efficiency formation of iPSC. It has been extensively demonstrated that the somatic cell type can greatly influence reprogramming outcomes. We have shown that adipose tissue-derived cells (ADCs) can be easily isolated from adult animals and can be reprogrammed to a pluripotent state with high efficiency. Here, we describe a protocol for the high-efficiency derivation of ADCs and their subsequent use to generate mouse iPSC using Oct4, Sox2, Klf4, and cMyc retroviral vectors.

Key words Embryonic stem cell, Induced pluripotent stem cells, Adipose tissue-derived cells, Adipose tissue

1 Introduction

The discovery that mouse embryonic stem cells (ESC) could be propagated indefinitely in culture while maintaining the potential to be differentiated into any cell type [1] generated great excitement for the field of regenerative medicine. Subsequent isolation of human ESC and findings of their pluripotency [2] further fuelled interest in the use of ESC as a potentially unlimited source of replacement cells for patient therapies. Unfortunately, the major hurdle for using ESC clinically is the possibility of immune rejection in patients, given that ESC can only be isolated from the developing blastocyst. However, the birth of the cloned sheep, Dolly, was a breakthrough study showing that adult mammalian cells could be "reprogrammed" to a state of totipotency by somatic cell nuclear transfer (SCNT) [3]. Soon after, other methods of reprogramming somatic cells to pluripotency including fusion with ESC [4] and treatment with ESC extracts [5] were reported.

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These studies demonstrated the possibility of taking adult somatic cells from patients, returning them to a pluripotent state and then differentiating them to desired cell types for transplantation, thereby avoiding immune rejection. While this is an ideal strategy, the use of embryonic material such as oocytes and the technical, logistical, and ethical difficulties involved hindered its realization.

Takahashi and Yamanaka's [6] landmark study turned the field of reprogramming on its head by showing that simple ectopic expression of four genes, Oct4, Sox2, Klf4, and c-Myc (OSKM), in mouse fibroblasts was sufficient to reprogram them to pluripotency. These cells were coined induced pluripotent stem cells (iPSC) as they possessed many characteristics of ESC including expression of ESC genes, epigenetic hallmarks, and ability to form teratomas in immune-compromised mice and form chimeric mice with contribution to the germ line [7]. The ensuing generation of human iPSC brought the idea of autologous cell therapies a step closer [8, 9]. Despite rapid advances in iPS-based reprogramming, the efficiency of deriving iPSC remains low, with efficiencies varying from 0.01 to 0.1 % (reviewed by [10]). Interestingly, many studies have shown that the choice of somatic cell type can greatly influence its reprogramming efficiency. Aoi and colleagues [11] were the first to find that murine stomach and liver cells could form iPS colonies with greater efficiency than tail-tip fibroblasts. The most convincing evidence of a relationship between somatic cell type and reprogramming efficiency was shown by Wernig and colleagues [12]. Using their dox-inducible secondary iPS system, genetically homogenous mice were produced from two primary iPSC lines, each with different proviral integrations. Subsequently, various somatic cell types were isolated from one of these mice. Given that all the somatic cells are homogeneous and carry the "correct" combination of proviral integrations, the efficiency of iPSC generation was expected to be approximately 100 %. Upon dox induction, such secondary iPSC could be generated from many somatic cell types including neural progenitor cells, adrenal gland cells, keratinocytes, muscle cells, intestinal epithelium cells, MSCs, hematopoietic cells, mouse embryonic fibroblasts (mEFs), and tail-tip fibroblasts. Surprisingly, the reprogramming efficiencies reported were vastly different between the different cell types with the highest reprogramming efficiency observed at only 4 % using mEFs. This led researchers to believe that the formation of iPSC is a stochastic process requiring additional rare epigenetic events.

Indeed, it appears that certain somatic cell types possess an epigenetic signature that is more primed for reprogramming. Both human keratinocytes and neural stem cells have been shown to form iPS colonies at greater efficiency than their fibroblast counterparts [13, 14]. The high levels of endogenously expressed Klf4 and Sox2 in these cell types, respectively, are thought to complement the expression of exogenous transgenes and thus enhance the efficiency of reprogramming. However, neural stem cells are not a

suitable somatic cell type for generating iPSC lines, since they are difficult to obtain from patients while the use of keratinocytes is hampered by the growth and quality of hair follicles which vary dramatically depending on age, genotype, and medical condition of the patients [15]. Soon after these reports, Sun and colleagues reported the high-efficiency generation of iPSC using human adipose stem cells [16]. The high endogenous expression of Klf4, Klf5, Klf2, Esrrb, and c-Myc in human adipose stem cells was thought to contribute to the high efficiencies observed [16]. Furthermore, using adipose cells has several advantages over other cell types; firstly, the liposuction procedure for isolating the cells is a well-established, relatively safe medical procedure. Secondly, large numbers of adipose cells can be obtained from a single liposuction procedure and therefore do not need to be expanded for many weeks prior to iPS induction. Lastly, human adipose stem cells can be derived from patients of various ages without affecting their reprogramming ability [16]. These advantages coupled with high-efficiency reprogrammability suggest that adipose stem cells are an ideal somatic cell source for generating iPSC and may decrease the time needed to establish iPSC lines, which may be critical for therapeutic intervention. Our demonstration of the high-efficiency generation of mouse iPSC using mouse adipose tissue-derived cells (ADCs) further solidified a place for adipose cells as an ideal candidate for future cell therapies [17]. In this chapter, we describe a protocol for isolating ADCs from adult mouse adipose tissue and the establishment of iPSC lines using a modified protocol of Yamanaka's four-factor retroviral induction [18].

2 Materials

2.1 Equipment

1. Centrifuge.
2. Inverted fluorescence microscope.
3. Stereomicroscope.
4. Water bath.
5. Hemocytometer.
6. Cryo freezing container, such as Mr Frosty (Nalgene).
7. Flow cytometer, such as CyAN.
8. Nanodrop ND1000 Spectrophotometer (Nanodrop™, ThermoScientific).
9. PCR cycler such as BioRad.

2.2 Mouse Adipose Tissue Collection and Digestion

1. OG2 mice (Bl6 background mice containing an Oct4-GFP transgene) [18].
2. Hanks' balanced salt solution (HBSS) (Life Technologies).

3. Collection medium: HBSS containing 1× penicillin-streptomycin (Life Technologies) and 1× amphotericin B (Sigma-Aldrich).
4. Penicillin-streptomycin (10,000 U penicillin and 10,000 µg/ml of streptomycin) 100× liquid solution (Life Technologies). Aliquot and store frozen at −20 °C.
5. Amphotericin B (250 µg/ml) (100×) solution (Sigma-Aldrich). Aliquot and store frozen at −20 °C.
6. Digestion medium: 0.1 % (w/v) Collagenase type IA (Sigma-Aldrich) and 10 ng/ml DNase I (Sigma-Aldrich) in HBSS. Dissolve 10 mg of DNase I in 10 ml of HBSS to make a 1 mg/ml solution, aliquot any unused solution, and store at −20 °C. Dissolve 50 mg of collagenase in 50 ml of HBSS. To this, add 500 µl of 1 mg/ml DNase I solution. Filter sterilize solution using a 0.2 µm filter and store aliquots at −20 °C.
7. ADC medium containing DMEM/F12 (1:1) media with glutaMAX (Life Technologies), 20 % fetal bovine serum (JRH Biosciences), and 1× penicillin-streptomycin. ADC medium is sterilized using a 0.2 µm filter into a pre-sterilized bottle. ADC medium is stable if kept at 4 °C for 2 weeks.

2.3 Growth and Maintenance of Mouse Adipose Tissue-Derived Cells (ADC) and MEFs

1. Dulbecco's Phosphate-Buffered Saline with calcium and magnesium, 1× (PBS) (Life Technologies).
2. TrypLE Express (1×) with phenol red (Life Technologies). Aliquot and store at 4 °C.
3. ADC medium (*see* Subheading 2.2, **item 7**).
4. Mouse embryonic fibroblast (MEF) medium: DMEM with 10 % FBS, 1× glutaMAX, 1 mM NEAA, 1× penicillin-streptomycin. Filter sterilize using a 0.2 µm filter into a pre-sterilized bottle. MEF medium can be stored at 4 °C for up to 2 weeks.

2.4 Cryopreservation of Mouse Cells

1. Dimethyl sulfoxide (DMSO) Hybri-Max™ sterile filtered, suitable for cell freezing (Sigma-Aldrich). Store at room temperature.
2. 1.5 ml Cryovials (Nunc).
3. ADC medium (*see* Subheading 2.2, **item 7**).
4. MEF medium (*see* Subheading 2.3, **item 4**).
5. mESC medium (*see* Subheading 2.7, **item 10**).
6. Freezing medium: 20 % DMSO in fetal bovine serum. Filter sterilize 8 ml of FBS with a 0.2 µm filter; to this add 2 ml of DMSO. Store at 4 °C until used.

2.5 Inactivation of Mouse Embryonic Fibroblasts (MEFs) for Use as Feeders

1. MEF medium (*see* Subheading 2.3, **item 4**).
2. Mouse embryonic fibroblasts (MEFs) for cell culture as feeder cells (*see* Chapter 13).
3. Mitomycin C powder, suitable for cell culture (MMC) (Sigma-Aldrich) for inactivation of feeders (*see* Chapter 13).
4. Gelatin type B 2 % solution in water, tissue culture grade (Sigma-Aldrich).
5. 0.1 % Gelatin solution: Leave 2 % gelatin solution at room temperature until no clumps are visible and solution is homogenous. For 500 ml, add 25 ml of 2 % gelatin solution to 475 ml of PBS.

2.6 Maintenance of Platinum E Packaging Cell Line

1. Platinum E packaging (Plat E) cells are derived from 293T cells and contain the ecotrophic env-IRES-puro^R and gag-pol-IRES-blasticidinR cassettes driven by EF1α promoter (Cell Biolabs).
2. Dulbecco's modified eagle medium, high glucose (DMEM) (Life Technologies).
3. GlutaMAX™ liquid 100× (Life Technologies).
4. Nonessential amino acids 10 mM solution (NEAA) (Life Technologies).
5. Blasticidin S Hydrochloride (Sigma-Aldrich).
6. Puromycin dihydrochloride (Sigma-Alrich).
7. Blasticidin 10× stock: To 25 mg of powder, add 2.5 ml of tissue culture-grade water to make a 10 mg/ml stock solution. Aliquot and store at −20 °C.
8. Puromycin 10× stock: To 25 mg of powder, add 25 ml of tissue culture-grade water to make a 1 mg/ml stock solution. Aliquot and store at −20 °C.

2.7 Reprogramming of ADCs

1. Dulbecco's phosphate-buffered saline without calcium and magnesium, 1× (PBS) (Life Technologies).
2. 2-Mercaptoethanol solution (Life Technologies).
3. ESGRO mouse LIF 10^7 units (Millipore).
4. Millex-HV filter unit 33 mm 0.45 μm PVDF (Millipore).
5. FuGENE® 6 transfection reagent (Roche).
6. Hexadimethrine bromide (Polybrene) (Sigma-Aldrich).
7. Plat E packaging cells (Cell Biolabs).
8. pMXs-Oct4, pMXs-Sox2, pMXs-Klf4, pMXs-cMyc (OSKM), pMXs-GFP plasmids deposited by Shinya Yamanaka (Addgene).
9. Polybrene stock: Dilute 0.8 g of hexadimethrine bromide with 10 ml of tissue culture-grade water to give a stock solution of 80 mg/ml. Aliquot and store frozen at −20 °C. For working

stocks, thaw a 1 ml aliquot of 10× stock and dilute with 10 ml of tissue culture-grade water to make a working stock concentration of 8 mg/ml. The working stock can be stored at 4 °C for 1 year.

10. Mouse embryonic stem cell (mESC) medium: DMEM with 15 % FBS, 1 mM glutaMAX, 1 mM NEAA, 1× penicillin-streptomycin, 0.1 mM β-mercaptoethanol, and 1,000 U/ml of mouse LIF. Filter sterilize using a 0.2 μm filter into a pre-sterilized bottle. Complete mESC medium can be stored at 4 °C for up to 2 weeks.

2.8 Clonal Expansion, Growth and Maintenance of Mouse ADC-Derived iPSC

1. Insulin syringe or fine-tip plastic Pasteur pipette.
2. mESC medium (*see* Subheading 2.7, **item 10**).
3. Freezing medium (*see* Subheading 2.5, **item 4**).

2.9 Characterization of Mouse ADC-Derived iPSC

1. Alkaline phosphatase staining kit (Millipore).
2. Anti-mouse Oct3/4 primary antibody (Santa Cruz Biotechnologies).
3. Anti-mouse Nanog primary antibody (Millipore).
4. Anti-mouse SSEA-1 (Millipore).
5. Alexa 488 conjugated goat anti-mouse IgG (Life Technologies).
6. Alexa 594 conjugated goat anti-mouse IgM (Life Technologies).
7. Vectashield containing DAPI (Vector Laboratories).
8. 4-Well glass culture slides (BD Falcon).
9. 4 % Paraformaldehyde (Sigma-Aldrich).
10. Goat serum (Life Technologies).
11. Bovine serum albumin (BSA) fraction V, 7.5 % solution (Life Technologies).
12. Triton X (Sigma-Aldrich).
13. Blocking solution: 5 % Goat serum and 0.1 % BSA in PBS. For 50 ml of blocking solution, add 2.5 ml of goat serum and 0.67 ml of BSA to approximately 47 ml of PBS, store at 4 °C for up to 1 month.
14. Genomic DNA isolation kit (Qiagen).
15. RNeasy isolation kit (Qiagen).
16. Superscript III cDNA conversion kit (Life Technologies).
17. GoTaq green master mix 2× (Promega).
18. Histochoice fixative (Amresco).
19. SCID mice 5–8 weeks old.

3 Methods

All of the following procedures, unless otherwise specified, are performed in biosafety hoods using standard laboratory practices to minimize exposure to animal pathogens. All cells were cultured in tissue culture-coated flasks or plates at 37 °C in humidified incubators in 5 % CO_2/95 % air. We used a dedicated biosafety hood and incubators for all primary tissue handling to avoid cross contamination with established cell lines. All animal work was conducted in accordance with the Monash Medical Centre Animal Ethics committee.

3.1 Mouse Adipose Tissue Collection and Digestion

1. Warm all reagents and media in a 37 °C water bath.
2. Sacrifice 4–5 OG2 mice (>8 weeks old) and spray the abdomen with 70 % ethanol (v/v). Using forceps pull up the hide to separate it from the peritoneum and using scissors cut the hide towards the rear legs to reveal the peritoneum.
3. Using forceps pull up the peritoneum to separate it from the organs and using scissors cut towards the rear legs to locate the inguinal fat pads (the fat pads should be white masses of tissue). Dissect the inguinal fat pads and transfer to a 50 ml tube containing 30 ml of collection medium (on ice). The adipose tissue should be floating.
4. Take the adipose tissue to a biosafety hood and aspirate the collection medium by placing a 25 ml serological pipette to the bottom of the 50 ml tube and pipetting up. Discard the supernatant and add 30 ml of fresh collection medium. This constitutes one wash. Wash adipose tissue two more times.
5. Using tweezers transfer adipose tissue to a 10 cm Petri dish. Finely mince the adipose tissue using a scalpel. Add 5 ml of digestion medium to the minced tissue and transfer to a 50 ml tube.
6. Use another 10 ml of digestion medium to rinse the petri dish and collect any remaining minced tissue. Place the digesting solution in a 37 °C water bath for 1–1.5 h. Shake the solution intermittently.
7. Once adipose tissue appears to form a homogenous solution with no clumps, add 30 ml of ADC medium and mix gently by pipetting up and down.
8. Pass the entire digestion solution through a sterile 70 μm cell strainer and then centrifuge at 400 × *g* for 10 min. Aspirate the supernatant and resuspend the pellet in ADC medium supplemented with 1× amphotericin B solution.
9. Count the live cells using trypan blue and plate at a density of 10^4 cells/cm^2. Leave in the incubator overnight to allow cells to settle.

10. The next day, aspirate the medium and add fresh ADC medium supplemented with 1× amphotericin B solution. Change the medium every second day until 80 % confluency is reached.
11. ADCs are passaged or frozen as required. After the first passage, supplementing the medium with amphotericin B is no longer necessary.

3.2 Growth and Maintenance of ADCs and MEFs

1. Aspirate the culture medium and add PBS to wash the cells of medium. Aspirate the PBS and add sufficient trypLE express to cover the flask/plate surface. Leave in a 37 °C incubator for 5–10 min. Gently tap the flask/plate until the cells are dislodged. If many cells are still attached, leave in the incubator for another 5 min and check intermittently for detachment.
2. Neutralize trypLE by adding twice the volume of FBS-containing medium. Rinse the flask/plate of cells, collect cell suspension, and then transfer to a centrifuge tube. Centrifuge for 5 min at 400 × *g*.
3. Aspirate the supernatant and resuspend pellet in 1 ml of medium. Perform a 1:3 to 1:5 split depending on how quickly the cells divide. Refresh medium every second day.

3.3 Cryopreservation of Cells

1. Harvest cells following Subheading 3.2 (for ADC, MEF, and Plat E) and Subheading 3.8 (for mESC). Perform a live cell count using trypan blue and determine the number of aliquots to be frozen.
2. Adjust the volume of the cell suspension using the appropriate medium to obtain 0.5 ml of cell suspension for each aliquot. For example, if freezing 5 aliquots, adjust cell suspension to 2.5 ml total volume.
3. Slowly add the same volume of freezing medium to the cell suspension (1:1, so that final DMSO concentration is 10 %). Gently pipette the entire cell suspension up and down to mix. Then immediately transfer 1 ml to each cryovials. Transfer cryovials to a cryo freezing container and leave in a −80 °C freezer overnight.
4. The next day, transfer the frozen vials to liquid nitrogen tanks for long-term storage.

3.4 Inactivation of Mouse Embryonic Fibroblasts (MEFs) for Use as Feeders

1. Expand MEF cultures to passage 3 or 4 in T-175 flasks (*see* Chapter 13). Pour or aspirate medium from flasks. Add 25 ml of fresh MEF medium to each flask.
2. Dissolve 2 mg of MMC powder with 5 ml DMEM to generate a stock solution of 0.4 mg/ml. Add 0.75 ml of this MMC stock to each T-175 flask to generate a final concentration of 12 μg/ml. Incubate cells with MMC for 2.5 h.

3. Pour or aspirate MMC containing medium, and then cells wash once with PBS (*see* **Note 1**). Add 3 ml of trypLE express and leave to 5–8 min in the incubator.
4. Gently tap the side of the flask to dislodge cells, then add 6 ml of MEF medium, and rinse the flask by pipetting up and down. Transfer cells to a tube and centrifuge at 400 × *g* for 5 min. Resuspend pellet in 1 ml of MEF medium and perform a viable cell count using trypan blue.
5. If using immediately, plate inactivated MEFs at a concentration of 1.25×10^4 cells/cm^2 onto tissue culture ware coated with 0.1 % gelatin. These feeder plates are ready for use the following day and can be kept for up to 7 days.
6. Inactivated MEFs that are not used immediately should be frozen as per Subheading 3.3.

3.5 Maintenance of the Platinum E Packaging Cell Line

1. Remove a cryovial of Plat E cells from liquid nitrogen and keep on dry ice while in transit. Submerge half the cryovial into a 37 °C water bath (ensure that the lid does not touch the water) until >70 % of ice crystals are dissolved. Remove the cryovial from the water bath and spray with 70 % (v/v) ethanol.
2. Using a 1,000 μl pipette, transfer the entire cell suspension to a 15 ml tube. Slowly add 10 ml of pre-warmed MEF medium dropwise to the cell suspension. Centrifuge at 400 × *g* for 5 min.
3. Aspirate supernatant and resuspend the cell pellet in 12 ml of MEF medium. Transfer to a T-75 flask and leave overnight in the incubator.
4. The next day, refresh the MEF medium supplemented with 1 μg/ml of puromycin and 10 μg/ml blasticidin (*see* **Note 2**).
5. Continue to culture the Plat E cells until they reach 80 % confluence (typically 2–3 days), then passage at a 1:6 to 1:10 split or freeze as needed.

3.6 Reprogramming of ADCs

1. Day 1: Coat five 10 cm tissue culture dishes (one plate per reprogramming factor and GFP) with 5 ml of 0.1 % gelatin and leave for a minimum of 30 min in the incubator (gelatin-coated plates can be left in the incubator for up to 1 week). Passage growing Plat E cells following Subheading 3.5. Resuspend Plat E cells in MEF medium (without puromycin or blasticidin supplemented) and perform a viable cell count using trypan blue. Adjust cell suspension to a concentration of 8×10^5 cells/ml. Plate 8×10^6 cells/10 cm dish and incubate overnight.
2. Day 2, transfection: Keep plasmids on ice at all times. To five 1.5 ml Eppendorf tubes, add 0.3 ml of DMEM, equilibrated to room temperature. Add 27 μl of Fugene 6 to each tube and leave at room temperature for 5 min. Next, add 9 μg of each plasmid DNA (OSKM and GFP, *see* **Note 3**) to the DMEM/

Fugene6 mixture and incubate for 15 min at room temperature. Add the entire Fugene6/DNA complex dropwise to each plate of Plat E cells (one factor per 10 cm plate of cells). Swirl plate to mix and leave in the incubator overnight.

3. Day 3: The next day, aspirate medium from each transfected plate and add 10 ml of fresh MEF medium. On the same day, passage ADCs as per Subheadings 3.2.1–3.2.3 and seed 5×10^4 ADCs per well of a 6-well plate (*see* **Note 4**). We routinely seed six wells (two wells for GFP transduction, two wells for four-factor transduction, and two wells as controls, *see* **Note 5**). Incubate cells overnight.
4. Day 4, transduction: The next day, collect and pool the four-factor retroviral supernatants. Filter the pooled four-factor viruses and GFP virus alone using a 0.45 μm cellulose acetate (or low protein binding) filter. Add polybrene to the filtered virus to a final concentration of 4 μg/ml. To 3 ml of MEF medium, add polybrene to generate a final concentration of 4 μg/ml (this will be used for diluting GFP virus). To two wells of plated ADCs, add 2 ml of four-factor reprogramming viruses/polybrene supernatants. To another two wells of plated ADCs, add 0.5 ml of GFP virus/polybrene supernatant. To these same wells, add 1.5 ml of MEF medium/polybrene. Remaining two wells of ADCs will act as un-transduced controls. Leave cells overnight in the incubator.
5. Day 5: The next day, aspirate the viruses from each well and add 2 ml of MEF medium. Seed two 60 mm tissue culture dishes (coated with 0.1 % gelatin) with feeder cells. Leave in incubator overnight.
6. Day 6: For the two wells infected with GFP virus and one control well of cells, harvest as per Subheadings 3.2.1–3.2.3 and take for flow cytometric analysis. Set forward scatter/side scatter histogram to gate for live cells, and forward scatter/pulse width histogram to eliminate cell doublets and cell count/GFP filter histogram. Set GFP gates using un-transduced ADC control cells. The percentage of ADCs transduced with GFP virus is used to calculate the reprogramming efficiency. For cells infected with four-factor reprogramming viruses, harvest as per Subheadings 3.2.1–3.2.3, resuspend pellets in 5 ml of mESC medium, and transfer infected ADCs to a 60 mm dish previously seeded with feeders (one well of infected ADCs per 60 mm dish of feeders). Leave overnight.
7. Days 8–12: Change fresh mESC medium every day. At day 12, count the number of Oct4-GFP-positive colonies using an inverted microscope to determine the reprogramming efficiency (*see* **Notes 6** and **7**).

3.7 Clonal Expansion and Growth of Mouse ADC-Derived iPSC (Fig. 1)

1. The day prior to picking iPS clones, seed feeders into a 24-well plate.
2. The next day, aspirate MEF medium from feeders and add 1 ml of mESC medium to each well. Mark Oct4-GFP-positive iPS colonies using an inverted UV microscope. Then transfer to using a stereomicroscope under a biosafety cabinet. Find marked iPS colonies and use an insulin syringe or fine-tip plastic Pasteur pipette to scrape away cells surrounding the putative iPS colony. Then using a 2–20 μl pipette set at 10 μl, draw up the putative iPS colony and transfer immediately to one well of a 24-well plate coated with feeders. Pick between 10 and 24 different iPS colonies (*see* **Note 8**).
3. The next day, aspirate the mESC medium from each well and add 1 ml of PBS. Aspirate the PBS, then add 200 μl of trypLE, and leave in the incubator for 2–3 min. Gently pipette the trypLE up and down to break the iPS colony into single cells and add 2 ml of mESC medium to neutralize the trypLE. Leave overnight.
4. Change fresh mESC medium to each well every day until iPS colonies are ready for passaging (usually 5–6 days). The day prior to passaging iPSC, seed a 6-well plate with feeders.
5. On the day of passage, aspirate medium from the well and add 1 ml of PBS. Aspirate the PBS and add 0.5 ml of trypLE. Leave in the incubator for 3–5 min. Gently pipette up and down to break the iPS colonies into single cells, then add 1 ml of FBS containing medium (either MEF or ADC medium), and transfer the cell suspension to a 15 ml tube. Centrifuge for 5 min at 400×*g*. Aspirate the supernatant and resuspend the pellet in 2 ml of mESC medium. Transfer the entire cell suspension to one well of a 6-well plate seeded with feeders. Leave overnight.
6. Change fresh mESC medium every second day until the next passage (mouse iPSC are usually passaged every 3–4 days or until 70 % confluent, *see* **Note 9**).

3.8 Maintenance of Established Mouse iPSC

1. The day before mouse iPSC are ready for passaging (usually when iPSC are 60–70 % confluent), coat tissue culture dish/plate with 0.1 % gelatin and seed inactivated feeders as per Subheading 3.4. Leave in the incubator overnight. Feeders can be prepared days in advance; however, do not use feeders that have been seeded for longer than 6 days.
2. On the day of passaging, aspirate medium from iPSC and wash with PBS. Add sufficient trypLE to cover the surface of the tissue culture dish and leave in the incubator for 5–7 min. When iPS colonies begin to separate into single cells, use a 1,000 μl tip to gently pipette cells up and down to dislodge cells. Add

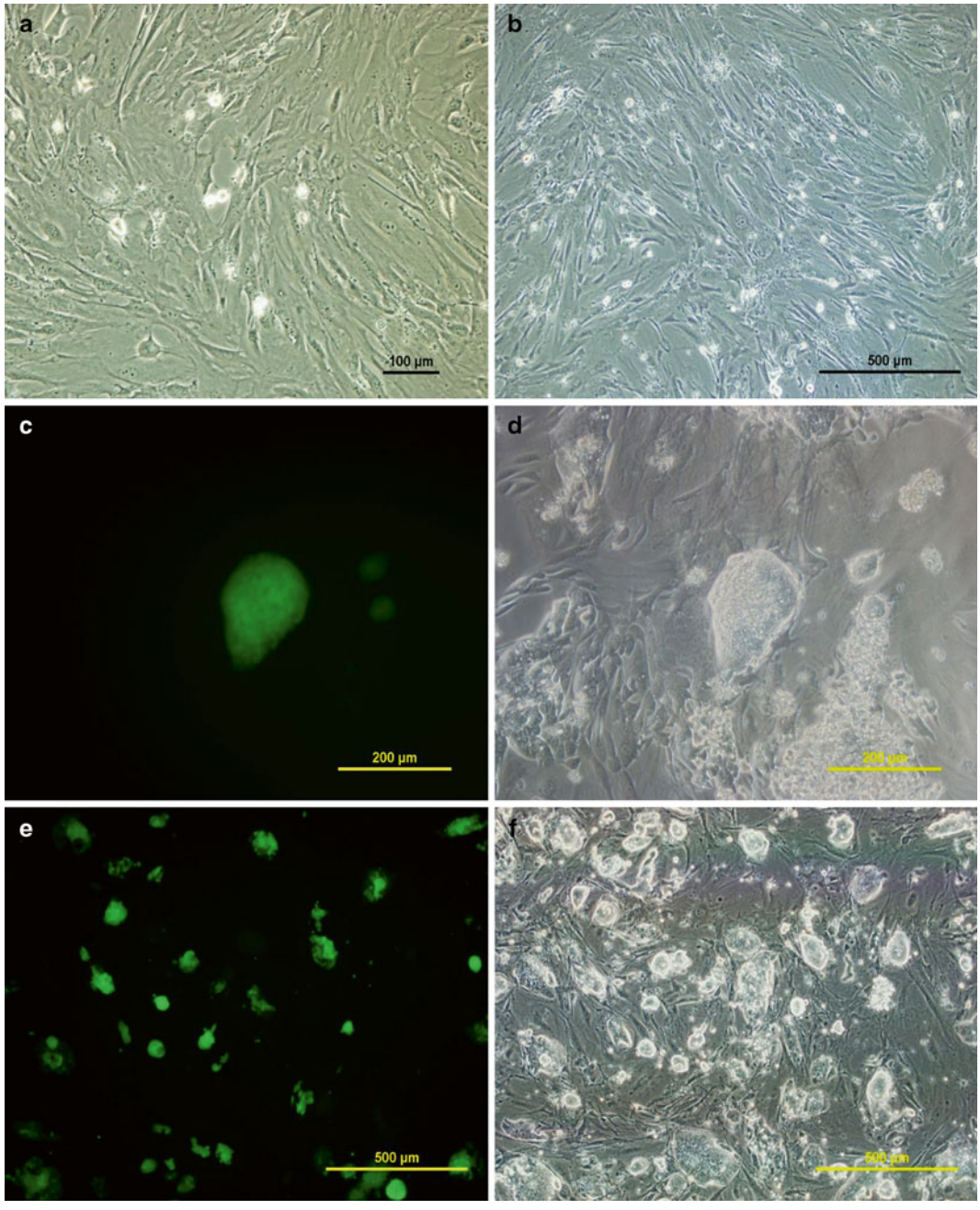

Fig. 1 Morphology of ADCs and ADC-iPSC colonies. (**a**) Primary ADCs, 6 days after initial isolation (passage 0). (**b**) Established ADCs at passage 4. (**c**) Oct4-GFP-positive ADC-iPSC colonies at day 12 post-induction. GFP fluorescence image. (**d**) Phase image of (**c**). (**d**) An established ADC-iPSC line. GFP fluorescence image. (**f**) Phase image of (**d**)

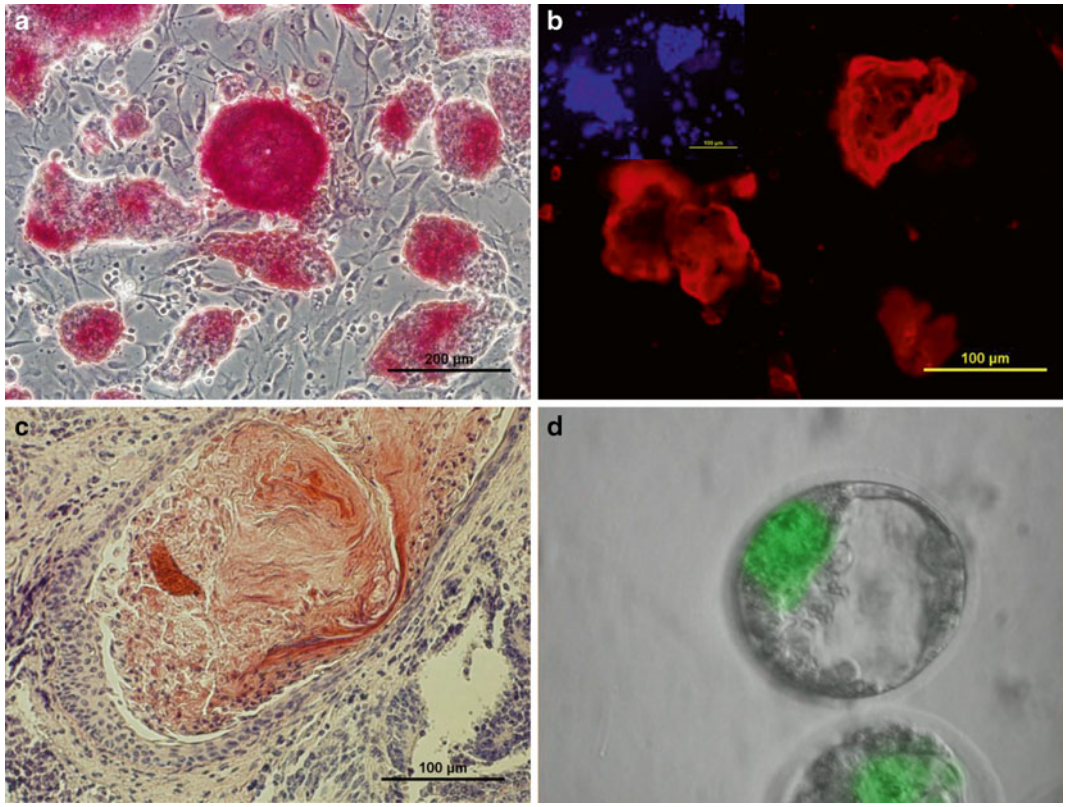

Fig. 2 Characterization of ADC-iPSC. (**a**) Alkaline phosphatase staining. (**b**) Immunostaining, SSEA-1 (Alexa 594) DAPI nuclei staining (*blue*), (**c**) H&E staining of teratoma formed after injection of ADC iPSC into SCID mice. (**d**) Upon injection into four-cell-stage embryos, ADC-iPSC contribute to the inner cell mass of blastocysts

twice the amount of mESC medium to neutralize the trypLE and transfer to a 15 ml tube. Centrifuge at 400 × *g* for 5 min.

3. Aspirate medium and resuspend pellet in 1 ml of mESC medium. Perform a live cell count using trypan blue if needed. Established mouse iPS lines are usually split at 1:10 to 1:50 depending on the growth characteristics of the line.
4. The next day, change fresh mESC medium and then change the medium every second day until ready for passaging (mouse iPSC are passaged every 3–4 days depending on the cell line).

3.9 Characterization of Mouse iPSC Lines (Fig. 2)

1. Immunostaining: Culture iPS lines on feeders in 4-well glass chamber slides for 3–4 days. Aspirate medium, wash once with PBS, and then add 4 % paraformaldehyde (PFA) for 20 min. Remove PFA and wash twice with PBS (*see* **Note 10**). Store fixed cells in PBS and cover with parafilm if not performing staining immediately. To stain cells for surface antigens, such as SSEA-1, aspirate PBS and add 0.5 ml of blocking solution for 1 h at room temperature. To stain for intracellular antigens

such as Oct3/4, add blocking solution that contains 0.1 % of Triton-X. Remove blocking solution and add primary antibody diluted 1:100 in blocking solution (minimum of 0.25 ml). Leave at 4 °C overnight in a humidified chamber. The next day, remove primary antibody, wash twice with PBS, and add the appropriate secondary antibody diluted 1:1,000 in blocking solution. Leave at room temperature for 1 h. Remove secondary antibody and wash twice with PBS. Remove PBS and plastic chamber wells. Add 2–3 drops of Vectashield (containing DAPI) and mount with a glass cover slip. When dry, seal the coverslip edges with nail polish. Take images immediately.

2. Alkaline phosphatase staining: Fix cells as per Subheading 3.6, **step 1**, with the exception that cells should only be treated with PFA for 1–2 min, as longer fixation times result in the inactivation of alkaline phosphatase. Mix kit reagents and stain cells according to the manufacturer's instructions.
3. RT-PCR expression analyses: Snap freeze cell pellets using dry ice and store at −80 °C. Thaw frozen cell pellets on ice and perform RNA extraction using RNeasy kit according to the manufacturer's instructions. Measure the amount and purity of extracted RNA by absorbance at 260/280 nM on a Nanodrop Spectrophotometer. If not used immediately, store RNA at −80 °C. For cDNA conversion, first treat 2 μg of RNA with DNase before conversion using Superscript III according to the manufacturer's instructions. Store cDNA at −20 °C and use 1–2 μl for subsequent PCR reactions. Use GoTaq green mastermix according to the manufacturer's instructions for RT-PCR reactions. For primer sequences *see* Table 1 (*see* **Note 11**). Perform PCR amplification with a total 30 cycles of denaturation at 94 °C for 45 s, annealing at 55–58 °C for 45 s, and extension at 72 °C for 45 s. Include a first denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 5 min. Run PCR products on a 1 % agarose gel at 80–100 V for 2 h.
4. Integration of retroviral transgenes: Perform DNA extraction using the DNeasy blood and tissue kit according to the manufacturer's instructions and store genomic DNA at −20 °C. Measure the amount and purity of extracted DNA by absorbance at 260/280 nM on a Nanodrop Spectrophotometer. Use GoTaq green mastermix with 100–200 ng of DNA according to the manufacturer's instructions for genomic PCR reactions. Use a total of 30 amplification cycles of denaturation at 94 °C for 30 s, annealing at 55–58 °C for 30 s, and extension at 72 °C for 1 min. Include a first denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 5 min. For primer sequences, *see* Table 1. Run PCR products on a 1 % agarose gel at 80–100 V for 2 h.

Table 1
PCR primer sequences

Gene	Annealing temperature (°C)	Primer sequence
Oct3/4 Transgene	58	F: TTG GGC TAG AGA AGG ATG TGG TTC
Oct3/4 Endogenous	58	F: TCT TTC CAC CAG GCC CCC GGC TC R: TGC GGG CGG ACA TGG GGA GAT CC
Sox2 Transgene	58	F: GGT TAC CTC TTC CTC CCA CTC CAG
Sox2 Endogenous	56	F: GGT TAC CTC TTC CTC CCA CTC CAG R: TTG CCT TAA ACA AGA CCA CGA AA
Klf4 Transgene	58	F: GCG AAC TCA CAC AGG CGA GAA ACC
Klf4 Endogenous	58	F: GCG AAC TCA CAC AGG CGA GAA ACC R: TCG CTT CCT CTT CCT CCG ACA CA
c-myc Transgene	58	F: CAG AGG AGG AAC GAG CTG AAG CGC
c-myc Endogenous	58	F: AAG TTT GAG GCA GCA GTT AAA ATT ATG GCT GAA R: TGA CCT AAC TCG AGG AGG AGC TGG AAT C
pMXs		R: GAC ATG GCC TGC CCG GTT ATT ATT
Rex-1	60	F: GGA CTA AGA GCT GGG ACA CG R: GCT GCT TTC CTT CTT GAA CAA T
Nanog	58	F: TCA AGG ACA GGT TTC AGA AGC A R: GCT GGG ATA CTC CAC TGG TG
β-Actin	55	F:GGA ATC CTG TGG CAT CCA TGA AAC R: AAA ACG CAG CTC AGT AAC AGT CCG

5. Embryoid body (EB) formation: Harvest 10^6 iPSC as per Subheading 3.8 and resuspend the cell pellet in 10 ml of mESC medium without LIF. Transfer cell suspension to a 10 cm Petri dish (non-tissue culture coated) and leave undisturbed in the incubator for 4 days. After 4 days in culture, small EBs should have formed. Gently transfer the EBs to a 50 ml tube using a serological pipette and allow EBs to settle by gravity for 10–20 min. Carefully remove the supernatant without aspirating the settled EBs. Gently resuspend the EBs in 40 ml of mESC medium without LIF and transfer to four 10 cm petri dishes (1:4 split). 7–10 days after initial plating, transfer individual EBs to a single well of a 24-well tissue culture plate coated with 0.1 % gelatin and containing 1 ml of MEF medium. Refresh MEF medium every second day and score for beating muscle 7–10 days post-plating. Plated EBs can also be fixed and immunostained for early markers of the different germ

lineages or RNA extracted for expression analyses to determine upregulation of differentiation markers and downregulation of key pluripotency genes.

6. Teratoma formation: Harvest $2–5 \times 10^6$ cells as per Subheading 3.8 and resuspend the cell pellet in 100 μl of mESC medium (*see* **Note 12**). Transport iPSC on ice. Using a 29 gauge needle, inject cell suspension into the hind leg of an SCID mouse. Monitor mice for teratoma formation between 3 and 6 weeks post-injection. Excise teratomas when they reach 10–15 mm in diameter and fix in Histochoice. Send fixed tissue to a histology facility for embedding, cutting, and hematoxylin and eosin (H&E) staining. Advice of a certified pathologist is required to ascertain the presence of tissue derivatives indicative of the three germ lineages.

4 Conclusion

GFP transduction efficiencies of ADCs should be greater than 75 % as high-efficiency transduction is critical for iPSC generation. Low-efficiency transduction suggests that ADCs are not proliferating well and is detrimental for iPSC formation. Using freshly isolated ADCs no higher than passage 3 will improve transduction efficiencies given that retroviruses can only infect actively dividing cells. ADC-iPSC lines should express many key endogenous pluripotency genes as shown by RT-PCR and immunostaining. Demonstrating the silencing of transgenes is critical for the generation of *bona fide* iPSC lines as the transgenes are driven by the monkey moloney leukemia virus (MMLV) promoter, which is naturally switched off in a pluripotent state. Furthermore, studies have shown that partially reprogrammed or "intermediate" iPSC are obtained if the transgenes continue to be expressed [19, 20]. Where transgenes are still being expressed, prolonged passaging and time in culture can result in their eventual silencing. The characterization assays described in this chapter are not exhaustive. Further studies could include but are not limited to bisulfite analysis of endogenous pluripotency gene promoter regions, microarray analysis, reactivation of the X chromosome in female cells, telomere lengthening, and/or telomerase activity. Additionally, the strictest test of pluripotency available for mouse cells is the generation of germline-competent chimeric mice but the utility of this assay may not be absolutely essential since there is no equivalent test for human pluripotent cells. Also, it has been previously suggested that the ability to generate germline chimeras is not the most relevant property as contribution to the specialized germ cell lineage is non-informative for in vitro studies [21]. Using the protocol described here, we were able to generate mouse iPSC at a reprogramming efficiency of 1.14 ± 0.77 % using ADCs which is

approximately 38-fold greater than using the mEF standard [17]. Careful attention to the protocol is required at each step to obtain similar efficiencies.

5 Notes

1. MMC is toxic; please follow local rules for hazardous waste disposal.
2. Do not add puromycin and blasticidin to the medium when passaging Plat E cells as this will prevent the cells from attaching to the tissue culture surface. Puromycin and blasticidin are only added to medium the day after passaging, once the cells have attached.
3. Dilute plasmids to the same concentration of 1 μg/ml to avoid pipetting errors.
4. The quality and condition of ADCs are vital for efficient reprogramming. ADCs must be actively proliferating and best used before passage 5. Do not use ADCs that are over-confluent, as this will cause growth arrest and subsequently lower the reprogramming efficiency.
5. Retrovirus GFP is included in parallel experiments to measure the transduction efficiency of ADCs. We routinely obtain >75 % GFP transduction efficiency of ADCs as measured by flow cytometry. No iPS colonies will be obtained if the GFP transduction efficiency is lower than 75 %.
6. Some Oct4-GFP-positive, putative mouse iPS colonies at day 12 may not be homogenously green; these will need to be subcloned later to obtain pure GFP-positive populations.
7. The reprogramming efficiency is calculated as follows:

   Reprogramming efficiency % = No. of Oct4-GFP-positive iPS colonies observed at day 12/no. of ADCs transduced with all four-factor viruses × 100

   where no. of ADCs transduced with all four-factor viruses = GFP virus transduction efficiency^4
8. Not all Oct4-GFP-positive colonies can be established into stable iPS lines, as some are only partially reprogrammed. Therefore, several colonies need to be expanded initially. By passage 5–6, only the iPS lines showing the best morphology and homogenously expressing Oct4-GFP need to be expanded.
9. Do not let iPS colonies grow so large that the edges of different colonies begin to touch each other, as this will result in differentiation.
10. PFA is toxic. Perform experiments in a fume cupboard and follow local rules for disposal.

11. RT-PCR analyses must include primers for endogenous and transgenic forms of the four factors, OSKM. For *bona fide* iPSC, transgenic expression of OSKM should not be detected. Transgene expression is usually detected in early passage iPSC; however it should not be detectable at late passages (>passage 20). If four-factor transgenes remain expressed at late passages, the iPSC are only partially reprogrammed and therefore are not *bona fide* iPSC. Collect ADCs at day 6 post-transduction for use as a positive control for transgene expression.
12. iPSC can also be resuspended in 70 % Matrigel to aid teratoma formation.

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Chapter 15

Pdx1 (GFP/w) Mice for Isolation, Characterization, and Differentiation of Pancreatic Progenitor Cells

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Abstract

It is well known that human cells are diverse with respect to their epigenome, transcriptome, and proteome. In the context of regenerative medicine, it is important for the transplanted cells or tissues to faithfully recapitulate their intended tissue type in each of these respects. Whether the cells chosen for such an application are embryonic, postnatal, or induced pluripotent stem cells, the transplanted product must behave in a predictable and reliable manner to be a safe and effective treatment option. Irrespective of the choice of cells used in such an application, the characterization and understanding of the developmental cues responsible for establishing and maintaining the desired cell phenotype are essential.

Animal models are extremely important in understanding the development of a specific tissue, which can then be subsequently extrapolated to human studies. Generation of transgenic animal models with whole-body gene knockout, conditional knockout, constitutive fluorescent gene reporters, and Cre-Lox-based conditional and lineage reporters has revolutionized the field of developmental biology. An intrinsically complex network of the actions and interactions of the multitude of different signalling cascades is required for development. A thorough understanding of such networks, gained through studies on transgenic animal models, is essential for the development of the techniques necessary to reliably differentiate a given stem or progenitor cell population into a specific cell type, such as an islet-like, insulin-producing cell aggregate.

In this chapter, we describe the use of GFP (green fluorescent protein)-based reporter mice for isolation of cells of choice, analyzing gene expression in those cells as well as their use for screening signalling molecules to understand their effect on differentiation.

Key words Pdx1, GFP reporter, Pancreatic lineage, Pancreatic progenitor cells, Cell differentiation

1 Introduction

In spite of significant advances in medical disciplines, there remains a collection of human diseases that cannot be cured with conventional therapies. Diseases such as Diabetes and Myocardial Infarction can be treated using islet or heart transplantation, respectively. However, these options carry significant risks. Surgical complications, subsequent immunosuppression, and limited availability of donor tissues

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hinder the success and restrict the widespread implementation of these techniques. This has led researchers to explore alternative therapies to treat these conditions.

Regenerative medicine represents a novel approach for the development and implementation of biological solutions to address such conditions. Regenerative medicine combines the collective knowledge of physiology, cell biology, genetics, and engineering to apply cells (cell therapy), biomaterial scaffolds, and cytokine growth factors to treat diseases, such as Type 1 Diabetes Mellitus. The successful implementation of a regenerative medicine strategy Requires a thorough understanding of the developmental biology of relevant cells and systems, such that the appropriate developmental cues can be applied for the differentiation of progenitor cell populations.

Embryogenesis is an extremely complicated process that results in the generation of a healthy individual through the coordination of the growth, differentiation, and organization of cells. Organs and tissues are organized through a complex heterogeneous patterning involving multiple cell lineages, where each cell must achieve one of multiple potential phenotypes. The complexity in developmental systems is reflected by the vast number of diverse molecular mechanisms responsible for establishing and coordinating these specific phenotypes.

Tissue specificity is determined by a combination of spatial and temporal patterns in gene expression, which are controlled by transcriptional regulatory networks [1, 2]. These networks comprise many interactions between transcription factors, chromatin modifiers, and cofactor proteins, which act at the promoter regions of specific genes to induce or repress transcription [3, 4]. The term "epigenetics" refers to the study of inherited characteristics that regulate gene expression without altering the underlying nucleotide sequence [5]. Epigenetic mechanisms including histone post-translational modifications and DNA methylation [6], along with noncoding RNAs [7–9] and nuclear dynamics [10, 11], also contribute to the phenotype exhibited by differentiated cells. The characterization of these interactions is essential for understanding tissue identity for application in regenerative medicine. Elucidating the mechanisms that control cellular identity is a comprehensive undertaking, which requires collecting extensive mRNA expression profiling, genome-wide active promoter mapping, and promoter motif analysis data. However, advances in next-generation sequencing technologies have made genome-scale approaches to understanding such interactions far more accessible and affordable. The study of human development is restricted by ethical constraints and is therefore largely limited to descriptive studies. However, the use of animal models provides us with a mechanistic understanding of embryonic morphogenesis, the results of which can be extrapolated to provide insight into human developmental processes.

This information can subsequently be applied to different facets of regenerative medicine research, such as identifying and profiling the mechanisms responsible for fate determination in progenitor cell populations.

Animal models carrying fluorescent reporters can provide significant information regarding the expression of a gene of interest, with respect to its expression pattern during development or in isolated cells under specific culture conditions. Green fluorescent protein (GFP) is the most commonly used reporter of gene transcription or protein expression and localization patterns. The molecule's intrinsic stability allows for reliable estimations of protein localization and quantification in situ. In this chapter, we explore the application of GFP for cell isolation, characterization of gene expression during embryogenesis, and monitoring of the effect of different signalling molecules on cell differentiation in vitro.

The presented method utilizes cells cultured from transgenic animals containing a GFP reporter under the promoter region of a key pancreatic gene, Pdx1 (pancreatic and duodenal homeobox 1). Presence of GFP during embryogenesis is used for cell isolation and gene expression analysis. When these isolated cells are cultured, GFP expression is turned down due to inactivity of the conjugate promoter (Pdx1). However, when stimulated to differentiate into an endocrine pancreatic lineage, promoter activity increases in response to the altered conditions, manifesting as an increase in GFP expression proportional to promoter activity. By quantifying the intensity of the GFP reporter signal at regular intervals, the activity of the associated promoter can be measured as an index of commitment to a specific phenotype. Variations to the differentiation protocol, in terms of use of different signalling molecules that may enhance or suppress the differentiation process, can be validated from changes in GFP reporter signal during the differentiation process.

2 Materials

Routine laboratory chemicals are ordered from Sigma, unless otherwise mentioned.

2.1 Cell Isolation from Embryonic Tissues

1. 1× PBS with calcium and magnesium (Gibco).
2. Antibiotic solution: Penicillin-streptomycin (Gibco) is used at a final concentration of 50 U/mL penicillin and 50 μg/mL streptomycin.
3. Collagenase type I (Sigma) is used for digestion of embryonic tissues (*see* **Note 1**).
4. Serum-containing (10 % foetal bovine serum (FBS) from Gibco) culture media. Media type dependant on the tissue of

interest (CMRL-1066 (Gibco), DMEM/F12 (Gibco), and low glucose DMEM (Gibco)) for inactivating collagenase and washing the cells.

2.2 Cell Isolation Using Fluorescent Reporters

1. FACS media: 1× PBS without calcium and magnesium (Gibco) (*see* **Note 2**) containing 2 % foetal bovine serum (Gibco).
2. Collection media dependant on the tissue of interest (CMRL-1066 (Gibco), DMEM/F12 (Gibco), and low glucose DMEM (Gibco)) with 10 % FBS (Gibco) (*see* **Note 3**).

2.3 Cell Culture

1. We use CMRL-1066 (Gibco) media for islet-derived cultures, DMEM/F12 (Gibco) media for gallbladder epithelial cultures, and low glucose DMEM (Gibco) for adipose-derived cell cultures. All these media contain 10 % FBS (Gibco) for cell proliferation in vitro. The choice of media should be made based on experience and familiarity with the cell system of interest.
2. GlutaMAX-1 (Gibco) is used at final concentration of 2 mM.
3. Antibiotic solution: Penicillin-streptomycin (Gibco) is used at a final concentration of 50 U/mL penicillin and 50 μg/mL streptomycin.
4. Trypsin-EDTA is used for dissociation of cells during subculture. A 500 mL portion of trypsin-EDTA contains 4 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 0.5 g D-glucose, 0.29 g sodium bicarbonate ($NaHCO_3$), 0.25 g bovine pancreatic trypsin (Calbiochem, EMD Chemicals), and 0.25 g EDTA. Reagents are dissolved in Milli-Q water, sterile filtered through 0.2 μm filters, and stored at −20 °C.

2.4 RNA Isolation, cDNA Synthesis, and PCR Using TaqMan Low-Density Arrays

1. TRIzol (Life Technology).
2. Chloroform (Sigma).
3. Isopropanol (Sigma).
4. Ethanol (Merck).
5. Nuclease-free water (Promega).
6. High-capacity cDNA Archive Kit that includes 10× RT buffer, 10× random primers, 25× dNTP mix, and MultiScribe reverse transcriptase enzyme (Life Technology).
7. TaqMan Universal PCR master mix (2×) (Life Technology).
8. TaqMan Low Density Arrays (TLDA) in custom or preconfigured formats for 384-Well Microfluidic Cards (Life Technology).
9. Assay-on-demand probe and primer mix for genes of interest. These can be used to design the customized TLDA cards.

2.5 Detecting GFP Expression During Differentiation In Vitro

Media conditions described are used to facilitate differentiation of progenitors into islet-like cell aggregates (ICAs) using a method previously described [12–16] with few modifications (*see* **Note 4**). Day 0 media is abbreviated as D0, day 4 as D4 and day 7 as D7.

1. Serum-free D0 (1) media: DMEM/F12 media (Gibco), 1 % (w/v) BSA (Sigma), 1× ITS (Gibco).
2. Serum-free D4 (1) media: DMEM/F12 media (Gibco), 1 % (w/v) BSA (Sigma), 1× ITS (Gibco), 0.3 mM taurine (Sigma).
3. Serum-free D7 (1) media: DMEM/F12 media (Gibco), 1 % (w/v) BSA (Sigma), 1× ITS (Gibco), 3.0 mM taurine, 100 nM GLP-1 agonist (exendin-4; Sigma), 1 mM nicotinamide (Sigma).
4. 6-Well Low Adherence Plates (Corning).

2.6 Quantitation of GFP Expression During Differentiation In Vitro

Media conditions described are used to facilitate differentiation of progenitors into islet-like cell aggregates (ICAs) using a method previously described [12–16] with few modifications (*see* **Note 4**).

1. Serum-free D0 (2) media: DMEM/F12 media without phenol red (Gibco) (*see* **Note 5**), 1 % (w/v) BSA (Sigma), 1× ITS (Gibco) + additives (*see* Table 1).
2. Serum-free D7 (2) media: DMEM/F12 media without phenol red (Gibco), 1 % (w/v) BSA (Sigma), 1× ITS (Gibco), 6.0 mM taurine (Sigma), 200 nM GLP-1 agonist (exendin-4; Sigma), 2 mM nicotinamide (Sigma).
3. Black opaque 96-well flat-bottom microplates for luminescence and fluorescence assays (Corning).

Table 1
Common growth/differentiation factors for endocrine pancreatic differentiation

Condition	Test compound/condition	Stock concentration	Final concentration
01	100 mM Sodium butyrate (Sigma)	1 M	10 mM
02	100 mM Nicotinamide (Sigma)	1 M	10 mM
03	500 ng/mL Activin A (Sigma)	5 µg/mL	100 ng/mL
04	500 ng/mL FGF2 (Sigma)	5 ng/µL	100 ng/mL
05	100 µM 5-Azacitidine (Sigma)	10 mM	10 µM
06	1 µM Exendin-4 (Sigma)	100 µM	100 nM
07	1 µM Exendin-(9-39) (Sigma)	100 µM	100 nM
08	Control (serum-free media)	–	–

3 Methods

Embryo dissection protocols described in this manuscript are adapted from those described by Shea et al. [17]. Several protocols described in detail herein are adapted, modified, and optimized from analogous protocols previously published from our laboratory [18].

3.1 Laboratory Animals

1. Pdx1(GFP/w) mice are housed within a Physical Containment Level 2 (PC2) facility, using IVC caging systems. Successful embryos are generated by mating healthy wild-type (w/w) female mice with heterozygous [Pdx1(GFP/w)] mice at an optimum breeding age of 8–12 weeks (*see* **Note 6**).
2. Elements of the physical and social environment of the animals are vital factors in optimizing a successful breeding outcome in transgenic mouse models. Animals are housed together for company where possible. Enrichment is provided in the form of tissue papers and cardboard boxes placed inside the cages to provide important stimuli to encourage natural breeding behaviour and to provide a secure feeling environment for the pregnant female mice. Animals are monitored daily, but handling and noise disturbance is kept to a minimum to reduce any unnecessary distress to the animals that may adversely affect the breeding outcomes.
3. The transgenic mouse model for Pdx1-GFP is available to monitor the expression of the pancreas and duodenal homeobox gene 1 in vivo and during differentiation of cells into an endocrine pancreatic lineage. Pdx1(GFP/w) mice are kindly provided by Prof. Edouard Stanley and Prof. Andrew Elefanty (Murdoch Children's Research Institute and Monash University, Melbourne) and are maintained by the group of A/Prof. Anand Hardikar. Progenitor populations isolated from these Pdx1 (GFP/w) mice express GFP under the control of Pdx1 promoter as they commit to a pancreatic lineage. They also continue to express GFP as cells begin to transcribe insulin, since Pdx1 is also transcribed by mature insulin-producing cells. For further details regarding the transgenic animal presented in this manuscript, please refer to **Note 7**.

3.2 Cell Isolation from Embryonic Tissues

Male breeding animals are introduced into cages containing virgin female animals just prior to the end of the daily light cycle. Conception status was determined by the presence of an ejaculatory plug in the vagina of successfully mated animals. Presence of a plug is indicative (but not necessarily confirmation) of pregnancy. Pregnant animals are separated and marked with the date of breeding and considered e0.5 of gestation. Embryo implantation normally occurs between e4 and e5 in the mouse and embryos can be

harvested for analysis as early as e6.5. However, later time points (beyond e9.5) may provide more discrete organs for isolation and analysis. Please note that animals more than e15 gestational age must also be independently euthanized, most commonly by decapitation.

1. Euthanize the pregnant animals using cervical dislocation, wipe the skin with 70 % ethanol, and place in a supine position. Make a superficial ventral incision in the skin and open the peritoneum with a second ventral incision to expose the abdominal cavity. The reproductive organs in the ventral region of the body cavity are identified and the two uterine horns, oviduct and ovaries, are withdrawn from the abdominal cavity.
2. Remove the uterine horns by severing below the oviduct and dissecting it from the local mesometrium, followed by placing these in cold sterile PBS.
3. Isolate each embryo by cutting between their respective implantation sites.
4. Bluntly dissect the muscular uterine lining from the enveloped decidua tissue using fine forceps.
5. Gently tease apart the exposed decidua using fine forceps and carefully invert to release the contained embryo. The Reichert's membrane and ectoplacental cone (trophoblast) may still be associated with the embryo, requiring additional careful dissection to isolate the embryo.
6. Image the transgenic embryos containing a fluorescent reporter using a fluorescent stereo dissecting microscope (such as a Zeiss Stereomicroscope SteREO Discovery V8 with LED-based illumination for GFP). This provides spatial information about the tissue-specific expression of the fluorescent reporter in the developing embryo. Isolated embryonic tissues can also be independently imaged following harvesting from the intact embryo (Fig. 1).
7. Sever the heads and tails of isolated embryos and dissect the remaining embryonic tissue along the neural tube and ventral midline. Carefully remove the limb buds and internal organs using blunt dissection. Locate the tissue(s) of interest (identified using a fluorescent stereo dissecting microscope), carefully remove the desired material, and place it in cold PBS for further sorting or cell culture.
8. Transfer the collected organs to sterile tissue culture plates with minimal liquid and mechanically mince the tissue into fine pieces using sterile tweezers and scissors. Dissociate cells using enzymatic digestion (1 % (w/v) collagenase 1 prepared in serum-free media at 37 °C). The duration of enzymatic digestion can vary between different tissues and should be closely

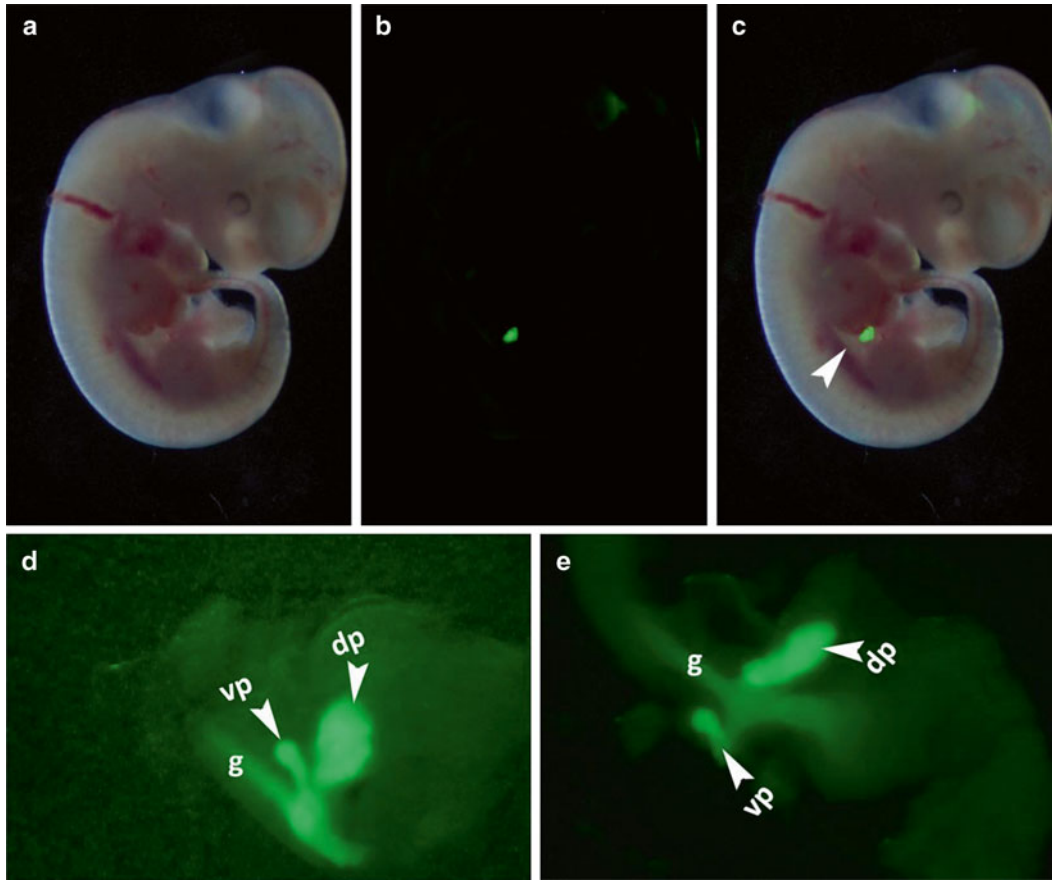

Fig. 1 Pdx1-GFP mouse embryo: Visualization of GFP fluorescence through the intact e10.5 Pdx1(GFP/w) embryo, showing phase (**a**), GFP (**b**), and merged images (**c**) of a representative embryo. The *arrowhead* (**c**) indicates expression of the GFP reporter in the developing pancreas. The developing pancreatic buds are shown in (**d**) and (**e**). Abbreviations: *dp* dorsal pancreas, *vp* ventral pancreas, *g* gut-tube/duodenum

monitored throughout the process until an optimal digestion time has been established. Samples containing fully digested tissues typically appear as turbid solutions containing fine, granular particles.

9. Neutralize the digestion enzymes by adding serum-containing media and centrifuge samples at 1,500 × *g* for 4 min at 4 °C.
10. Remove the supernatant and wash the cell pellets twice with serum-containing media.
11. Resuspend the cell pellets in serum-containing media (determined by the tissue type) and seed the cells into flasks for culture. The serum-containing media used to culture isolated cells typically contains 5.5 mM glucose, 2 mM L-glutamine, and 10 % (v/v) foetal bovine serum, but may require further supplementation depending on the cell types present. The efficiency of digestion can be determined by visualization of isolated cells in flasks.

12. Culture the cells in a humidified 37 °C incubator (with 5 % CO_2) for 3–4 hr for analysis. Cells cultured for extended periods will require media replacement with fresh serum-containing media at regular intervals (alternate days to every 2 days), based on culture confluence, cell growth, and cell proliferation characteristics. Maintain the cells until the flask appears confluent, which can be determined using an inverted phase-contrast microscope. Established adherent populations from embryonic tissues typically form a confluent monolayer in the original culture flask.
13. Subculture cells in confluent flasks using a standard technique with trypsin-EDTA as the dissociating agent. Remove the existing media and expose the confluent cells to warm (37 °C) trypsin-EDTA for 2–3 min or until cell detachment is confirmed through visual observation using an inverted phase-contrast microscope. Neutralize the trypsin-EDTA using serum-containing media and pellet the dislodged cells by centrifugation at 1,500 × *g* for 4 min at 4 °C. Resuspend the cell pellet in serum-containing media.
14. Count the cells using a hemocytometer and seed the cells at the desired cell density. Alternatively, cells should be subcultured at a predetermined subculture ratio. We typically subculture primary cells using 1:2 subculture ratios.

3.3 Cell Isolation Using Fluorescent Reporters

This protocol represents an alternative step prior to cell culture, exploiting the expression of intrinsic fluorescent reporters for isolation and quantitation of specific subpopulations of cells. Embryonic tissue from control and transgenic animals can be harvested and prepared as described in Subheading 3.2 (**steps 1–10** above). Cells are prepared and maintained in low-light conditions (avoiding direct light exposure) to preserve the fluorescent signal.

1. Resuspend the digested cell pellets in serum-containing media (determined by the tissue type). The serum-containing media used to resuspend isolated cells typically contains 5.5 mM glucose, 2 mM L-glutamine, and 10 % (v/v) foetal bovine serum.
2. Wash the cells twice with FACS media and thoroughly resuspend the isolated cells in 200 μL FACS media. This volume will vary based on cell density.
3. Pass the isolated cells through a 70 μm cell strainer to remove any tissue clumps or larger cell clusters. We recommend adding propidium iodide (PI) to the cell suspension at a final concentration of 1 μg/ml to gate on live cells.
4. Acquire and characterize the isolated cells using a Becton Dickinson FACSAria or a similar flow cytometer. Gating can be implemented after profiling control and transgenic cell samples based on GFP fluorescence in FL1 channel (Fig. 2).

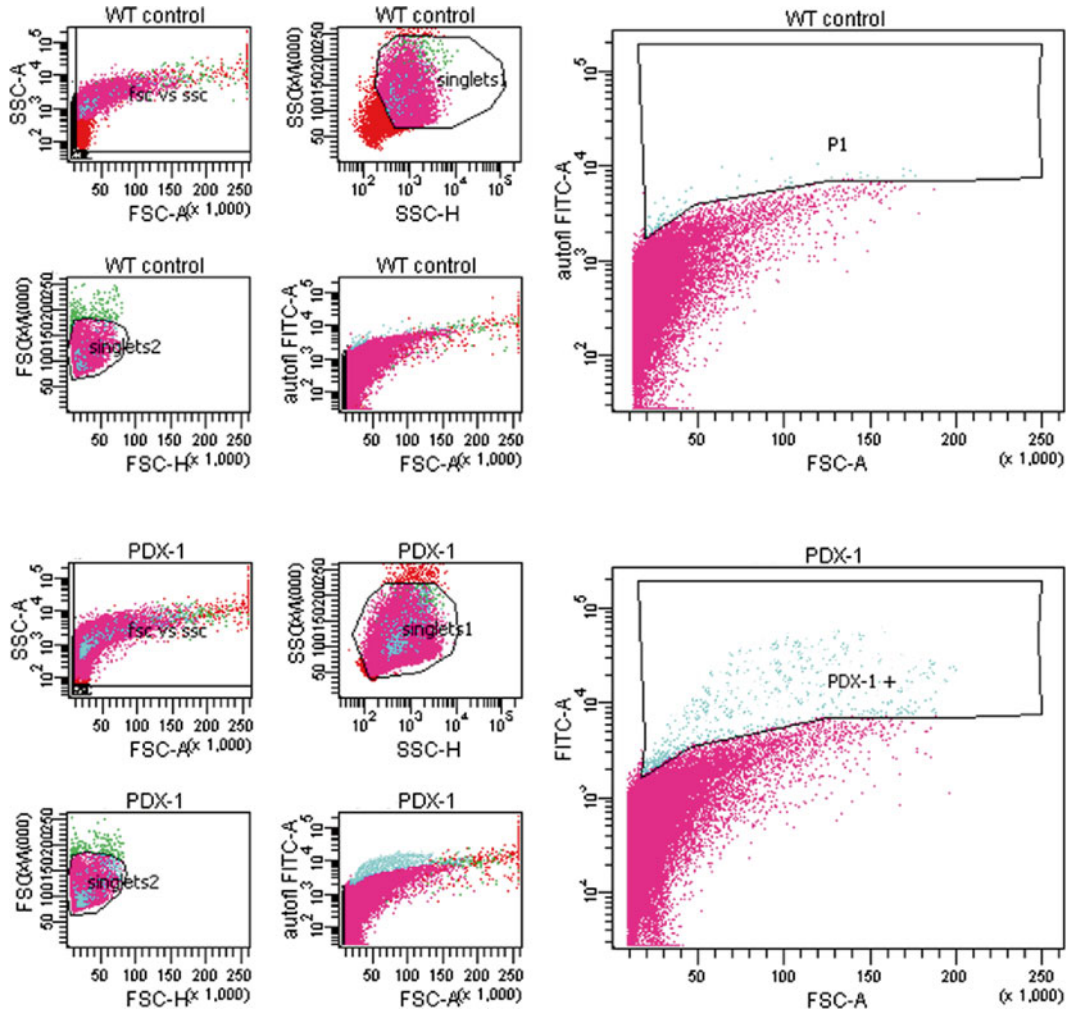

Fig. 2 Cell sorting using flow cytometry: A representative cell sorting profile for Pdx1 (GFP/w) and Pdx1 (w/w) pancreas is presented here. Live, single-cell populations are gated initially. Further gate is selected in FL1 channel to sort GFP^{high} cells. Wild-type controls do not contain any GFP-positive cells (*upper panels*), unlike those in Pdx1 (GFP/w) mice (*lower panels*) indicating active transcription of Pdx1 gene during embryonic development in pancreatic tissue

5. Collect the sorted cells in the desired serum-containing media and maintain them on ice.
6. Centrifuge the sorted cells at 1,500 × *g* for 4 min at 4 °C and decant the supernatant. Cells can then be resuspended in serum-containing media (described above) and seeded into flasks for culture or dissolved in TRIzol reagent for gene expression analysis.
7. Culture the sorted cells using **steps 12–14** of the protocol described in Subheading 3.2 (see above) or alternatively.
8. Analyze the sorted cells with respect to gene expression using the protocol described in Subheading 3.4 (see below).

3.4 RNA Isolation, cDNA Synthesis, and PCR Using TaqMan Low-Density Arrays

For transcript analysis of embryonic tissues, organs are snap-frozen in liquid nitrogen and stored at −80 °C. For transcript analysis of cultured embryonic cells and sorted GFP-expressing cells, dry pellets are stored at −80 °C. Alternatively, cell pellets can be dissolved in 1 mL TRIzol reagent and stored at −80 °C until further processing:

1. Homogenize the embryonic tissue and dissolve the resulting material in 1 mL TRIzol reagent.
2. Add 200 μL of chloroform to each tube and shake vigorously for 5 min to thoroughly mix the chloroform and TRIzol reagent.
3. Centrifuge the samples at 12,000 × *g* for 15 min at 4 °C.
4. Carefully transfer the upper aqueous layer (~400–500 μL) to a fresh tube and add 500 μL of isopropanol to this aqueous layer.
5. Mix the samples containing isopropanol by inversion (7–10 inversions) and incubate samples for 10 min at room temperature.
6. Centrifuge the samples at 12,000 × *g* for 15 min at 4 °C to pellet the precipitated RNA. To avoid loss of the RNA, the position of the RNA pellet in the tube should be predetermined by placing the tubes with the hinge end of the lids towards the outer rim of the centrifuge. The side of the tube with the hinged end is where the RNA pellets form after centrifugation. This placement facilitates maximum recovery of precipitated RNA, which is particularly relevant when handling low amounts of starting material.
7. Carefully aspirate and discard the supernatant taking care not to disturb the precipitated RNA.
8. Resuspend the precipitated RNA in 1 mL freshly prepared 75 % ethanol and vortex samples briefly for 10 s.
9. Centrifuge samples at 12,000 × *g* for 15 min at 4 °C to pellet the precipitated RNA.
10. Carefully aspirate and discard the supernatant taking care not to disturb the precipitated RNA.
11. Leave the samples to partially dry for 5 min at room temperature. However, allowing an RNA pellet to completely dry can adversely affect its solubility, resulting in loss of precipitated RNA. If most of the supernatant is removed at the previous step, then 5–7-min drying at room temperature should be adequate.
12. Resuspend the precipitated RNA pellets in appropriate volumes of nuclease-free water.
13. Quantify the solubilized RNA using a NanoDrop 1000 Spectrophotometer, which is a popular tool to quantify RNA/DNA. However, for more precise concentration measurements, alternative fluorometric techniques, such as Qubit assay kits for

use with the Qubit 2.0 Fluorometer, or RNA kits for use with the Agilent 2100 Bioanalyzer, may be used.

14. Prepare cDNA using a High Capacity cDNA Reverse Transcription Kit from Life Technology. We typically prepare cDNA from 1 μg of RNA in 10 μL of total reaction volume.
15. Analyze the samples using quantitative real-time PCR for a panel of genes using TaqMan Low Density Arrays with TaqMan Universal PCR master mix.
16. Dilute 30–1,000 ng of cDNA in 50 μL of nuclease-free water and combine this with 50 μL of TaqMan Universal PCR master mix.
17. Load each 100 μL sample into an individual port of the TLDA card. Using this setup, 8 samples can be analyzed for up to 48 different genes on 1 TLDA card.
18. Centrifuge loaded cards to facilitate loading of samples from the loading port to the PCR reaction wells and run on standard mode using the 7900 HT, ViiA7, or QuantStudio 12K system from Life Technology.
19. Collect the data and normalize the results to GAPDH/18S transcript expression, obtained as cycle threshold (Ct) values using the specific software. Calculate the fold difference over the detectable Ct value (Ct value of 39 considered to be undetectable) using the ΔΔCt method. In a preliminary analysis to estimate assay efficiencies, a linear relationship between transcript abundance and detection cycle is observed until a Ct value of 38, for 5 μL reaction volumes in 96-well plates. A Ct value over and above 39 is therefore considered to be undetectable and transcript abundance is plotted as the fold over detectable using the ΔΔCt method. However, for TLDA-based real-time PCR, with lower reaction volumes (1 μL), anything above Ct value of 33 could be considered undetectable. Users need to look at the individual amplification curves during PCR to identify and eliminate any false positives.

3.5 Detecting GFP Expression During Differentiation In Vitro

Differentiation of pancreatic progenitor cells to an endocrine pancreatic lineage is induced using a coordinated strategy with specific signalling molecules. Our standard differentiation protocol uses variations of serum-free media intended to elicit specific effects in the cultured cells to direct and promote their differentiation into an endocrine pancreatic lineage while achieving the morphology of islet-like cell aggregates (ICAs). Differentiation into an endocrine pancreatic lineage is monitored using transgenic cells isolated from Pdx1 (GFP/w) mice. As these cells adopt the desired pancreatic phenotype during differentiation, activation of the Pdx1

promoter drives expression of the GFP reporter, which can then be imaged to infer promoter activity.

1. Trypsinize subcultured cells into a single-cell suspension and centrifuge the cells at $1{,}500 \times g$ for 4 min at 4 °C. Decant the trypsin-containing supernatant (*see* **Note 8**).
2. Resuspend the cell pellet in serum-free D0 (1) media and dispense this single-cell suspension into low-adhesion tissue culture 6-well plates, with each well containing cells suspended in 2 mL of serum-free D0 (1) media. The cell density required for efficient differentiation has been found to vary between cell types, requiring some optimization for the cell type of interest. As a starting point, at least 1.2×10^6 cells should be placed in 2 mL of serum-free media in a single well of low-adhesion 6-well plates.
3. Replace the media after 24 h with fresh serum-free D0 (1) media. All manipulations of ICAs are performed using 10 mL serological pipettes to reduce the effects of turbulence on the fragile ICAs, which may be caused by using smaller bore size (1–2 mL) pipettes. During the initial 24 h of cell culture on low-adhesion plates, pancreatic progenitors migrate into ICAs and up to 10 % of the original cells may undergo cell death during this time. It is therefore essential to change the media after 24 h to remove the dead or the dying cells.
4. Replace the media in each well by transferring the contents of the 6-well plate to labelled 15 mL conical tubes. Add 500 μL of serum-free media from the sides of each well to maintain any adherent cells that are not transferred to the 15 mL conical tube. Allow the tube containing the ICAs to stand vertically for ~5–7 min so that the ICAs sink and collect at the bottom of the tube under normal gravity.
5. Carefully aspirate and discard the media above the ICAs (collected at the bottom of the 15 mL tube). This media would generally contain single cells or dead cells that are not part of any ICAs. Users can check this by placing the aspirated media in a fresh plate. Gently resuspend the collected ICAs in fresh serum-free media and transfer the ICAs back to the well containing the 500 μL of previously added replacement serum-free media.
6. Transfer the ICAs into serum-free D4 (1) media after 4 days in culture, using the media exchange method described in **steps 4** and **5**.
7. Transfer the ICAs into serum-free D7 (1) media after 7 days in culture. The serum-free D7 (1) media needs to be added when stable and compact cell aggregates are visible. If this is not observed, then the addition of D7 (1) media can be delayed by 1–3 days until the ICAs have adopted the necessary phenotype. Replace the D7 (1) media every 2 days until the conclusion of the experiment.

8. Image differentiating cells at regular intervals during their culture in serum-free media with a FLoid Cell Imaging Station (or any similar fluorescence microscope) using appropriate excitation and emission filters.
9. Collect the ICAs for quantitative PCR analysis at the conclusion of the experiment to confirm the expression of essential pancreatic transcription factors, using the techniques described in Subheading 3.4 (above).

3.6 Quantitative Analysis of GFP Reporter Activity During In Vitro Differentiation

Assays that involve quantitation of GFP reporter signals during differentiation are carried out using an amended protocol. The general principles of the differentiation protocol are maintained, but the volume of the media/additives is reduced. Using a live-cell fluorescence detection system, the expression of GFP during the differentiation process is measured in real time, where GFP intensity is used as an index to determine Pdx1 promoter activity. If small molecules/test compounds that are used for assessment using this protocol facilitate the differentiation process, it would be reflected by an increase in GFP intensity (Fig. 3). Quantitative assessment of GFP intensity over the entire duration of differentiation can provide a real-time measure of the Pdx1 promoter activity and its response to the various signalling molecules tested.

1. Trypsinize subcultured cells into a single-cell suspension and centrifuge the cells at 1,500 × *g* for 4 min at 4 °C. Decant the trypsin-containing supernatant (*see* **Note 8**).
2. Thoroughly resuspend the cells in serum-free D0 (2) media and add this to a 96-well flat-bottom microplate, such that each well contains cells suspended in 50 μL of serum-free D0 (2) media. The cell density required for efficient differentiation has been found to vary between cell types, requiring some optimization for the cell type of interest.
3. Add growth or differentiation factors (representative examples supplied in Table 1) to specific wells in quadruplet. These growth or differentiation factors should be diluted in 50 μL of serum-free media, as described in Table 1, to achieve a final volume of 100 μL in each well of the 96-well plate.
4. Analyze the microplates at 24-h intervals using a fluorescence detection system, such as the POLARstar OPTIMA multidetection microplate reader. Quantify and record the intensity of GFP exhibited by the cells. The reporter intensities obtained at predetermined time points during differentiation should then be normalized to the basal GFP intensity measured at day 0 of the differentiation protocol. It is advisable to include controls that would allow for detection of false-positive spikes in GFP intensities, which may be seen as a result of changes (feeding) of culture media.

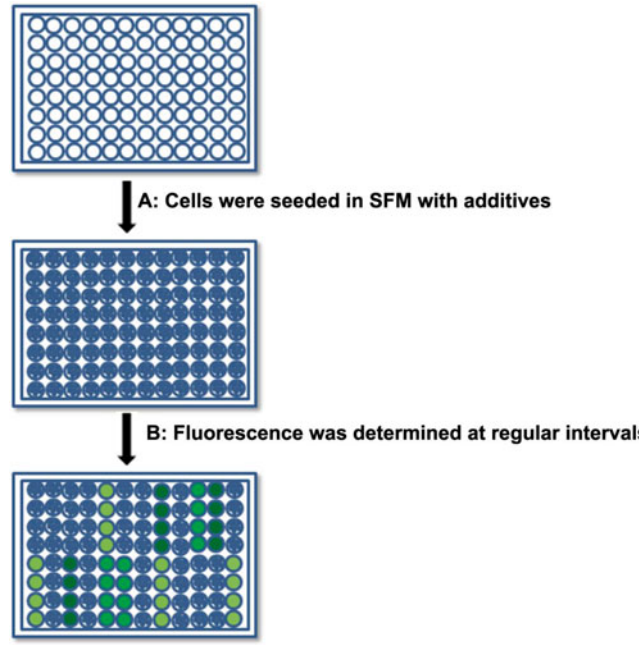

Fig. 3 Monitoring GFP expression during in vitro differentiation: Cells derived from Pdx1-GFP mice are used to assess the effect of different signalling molecules on in vitro differentiation towards pancreatic lineage. Cells are trypsinized and seeded in 96-well culture plate in serum-free medium (SFM) (A) along with additives (small molecules/test compounds). Please refer to text for details on composition of SFM and experimental plan. Since the cells lose Pdx1 expression after prolonged periods of in vitro culture, they do not produce GFP (shown here as *blue* color). When fluorescence is measured in the GFP channel at around 7 days in the presence of additives (B), cells that have started transcribing Pdx1 demonstrate GFP expression at different intensities. The quantitative readout of this fluorescence indicates the efficiency of the test compounds in differentiating progenitor cells towards endocrine pancreatic lineage

5. Analyze the microplates on day 7 of differentiation and subsequently add 100 μL of serum-free D7 (2) media to each well. This media contains additives at twice the usual concentration, such that the final 200 μL volumes in each well of the 96-well microplate would correspond to the desired concentrations of 3.0 mM taurine, 100 nM exendin-4, and 1 mM nicotinamide. A set of 100 μl "control" wells should also be measured at this time to avoid any false-positive differences because of changes in experimental volumes. For laboratories that are equipped with a micromanipulation device capable of withdrawing samples from multi-well plates, we recommend changing the media carefully with 100 μl of fresh D7 (1) media. If such facilities are unavailable, then increasing the volume with fresh D7 (2) media may be the best option to proceed.

6. Continue analyzing the microplates at 24-h intervals until the conclusion of the experiment, 14 days from induction of differentiation.

4 Notes

1. Collagenase enzyme needs calcium for its action and hence is dissolved in plain media (without serum). Optimal enzymatic digestion occurs at 37 °C.
2. Cells prepared for flow cytometry-based sorting should be prepared using calcium- and magnesium-free PBS to maintain single-cell suspension to prevent clumping.
3. Sorted cells should be collected in serum-containing media to relieve them from cellular stress and reduce cell death.
4. The differentiation protocols described in this chapter have been used extensively by our group to demonstrate the differentiation of progenitors into islet-like cell aggregates (ICAs) using a method previously described [12, 13]. However, the concept behind the GFP imaging protocol described herein could be applied to any number of differentiation systems using transgenic animals containing the GFP reporter under the control of a relevant promoter. Any tissue from such mice (for example, adipose tissue) can be isolated, cultured and tested in such a 96-well plate system for validating differentiation factor combinations that induce Pdx1 promoter activity, which is required for differentiating these cells to an endocrine pancreatic lineage.
5. Since phenol red interferes with fluorescence detection, especially in the green channel, a phenol red-free media is used and highly recommended for all fluorescence visualization and measurements.
6. Ethics: All animal and human work presented and discussed in these protocols needs to be approved by the relevant animal and/or human ethics committees. Animals should be handled in accordance with relevant national guidelines for the care and maintenance of laboratory animals, as designated by the relevant institution, such as the National Health and Medical Research Council (NHMRC) in Australia.
7. The Pdx1 promoter-GFP [Pdx1(GFP/w)] transgenic mice used in this study have been developed and studied by Holland and colleagues in 2005 [19]. These transgenic mice were generated through targeting of the Pdx1 locus by homologous recombination. The Pdx1-GFP-targeting vector comprises a 2.8 kb DNA fragment containing sequences upstream of the Pdx1 initiation codon, positioned 5′ of a cassette encoding

GFP and a hygromycin resistance gene (HygroR) flanked by flp recombinase target sites. The 3.3 kb 3′ targeting vector contained sequences from exon1 to a site immediately 5′ of exon2. The targeting vector was electroporated into W9.5 ES cells and successful recombination events were identified using PCR. Chimeric embryos were generated by injecting Pdx1-GFP transgenic ES cells into C57/BL-6 blastocysts and bred to generate a line of stable, Pdx1-GFP transgenic mice. The GFP signal is present in heterozygous Pdx1-GFP transgenic embryos. Significant expression of the GFP reporter is observed in the developing pancreas that is distinct from the surrounding tissues. The pancreas from an 8-month-old mouse demonstrated regions of bright GFP fluorescence reflecting focal areas of high Pdx1 expression within the islets of Langerhans.

8. Important: Do not use any serum at any of the steps during the differentiation process, as this would inhibit differentiation to an endocrine pancreatic lineage.

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Chapter 16

Generation of Transgenic Mouse Fluorescent Reporter Lines for Studying Hematopoietic Development

Andrei M. Vacaru, Joseph Vitale, Johnathan Nieves, and Margaret H. Baron

Abstract

During the development of the hematopoietic system, at least eight distinct lineages are generated in the mouse embryo. Transgenic mice expressing fluorescent proteins at various points in the hematopoietic hierarchy, from hematopoietic stem cell to multipotent progenitors to each of the final differentiated cell types, have provided valuable tools for tagging, tracking, and isolating these cells. In this chapter, we discuss general considerations in designing a transgene and survey available fluorescent probes and methods for confirming and analyzing transgene expression in the hematopoietic systems of the embryo, fetus, and postnatal/adult animal.

Key words Hematopoiesis, Transgenic mice, Knock-in, Green fluorescent protein, Fluorescent reporter

1 Introduction

Since the discovery and cloning of the first fluorescent protein (FP), wild-type green fluorescent protein (wtGFP) from the bioluminescent jellyfish *Aequorea victoria* [1] and the subsequent creation of spectral variants [2–4], FPs have become indispensable for imaging cellular differentiation and function at high resolution and in real time (reviewed in refs. [3, 4]). Gene-specific regulatory elements can be used to drive targeted expression of FP reporters, with spatial and/or temporal specificity, in virtually any cell type of a transgenic animal. The hematopoietic system has benefitted enormously from this approach, which made it possible to explore the emergence, expansion, migration, and differentiation of progenitors for the erythroid, myeloid, and lymphoid lineages. It is now possible to label and track the development of distinct hematopoietic cell types in vivo and to isolate these cells directly, using fluorescence-activated cell sorting (FACS). In this chapter, we discuss the steps involved in the generation and analysis of transgenic

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lines in which fluorescent reporters are expressed in hematopoietic lineages of the mouse, the most genetically tractable model for mammalian development.

1.1 Ontogeny of the Mouse Hematopoietic System

Hematopoiesis is a precisely orchestrated, stepwise process that leads to the formation of all lineages of the blood [5]. Primitive erythroid cells (EryP) are the first hematopoietic lineage to be detected in the mouse embryo (reviewed in ref. [6]). They are generated late in gastrulation, in the blood islands of the yolk sac (YS), along with macrophages and megakaryocytes [7]. The first definitive hematopoietic cells, comprising erythroid, megakaryocytic, and myeloid lineages, also arise in the YS, shortly after the appearance of EryP (reviewed in refs. [6, 7]). Hematopoietic stem cells (HSCs) form in the aorta-gonad-mesonephros (AGM) region of the embryo, in the large arteries and placenta, and, very likely, in the YS (for a review, *see* ref. [6]). They do not differentiate in these sites but instead seed the fetal liver (FL), where they expand and produce progenitors that give rise to definitive erythro-myeloid and lymphoid lineages. Late in gestation, HCSs migrate from the fetal liver to the bone marrow, which becomes the main blood production center in the postnatal animal [8]. The general hierarchy of hematopoietic development is shown as a "snapshot" in ref. [9].

1.2 General Considerations in Designing a Transgene

Transgenic mouse lines expressing fluorescent proteins (FPs) are invaluable tools for studying the development of the hematopoietic system. Careful design is necessary to achieve the desired expression of the reporter protein. The main components of a fluorescent reporter transgene are the promoter (and, usually, other upstream regulatory sequences), sequences encoding the fluorescent protein, and splice/polyadenylation signals (Fig. 1). A cartoon outlining the most commonly used approach for creation of a transgenic mouse line is presented in Fig. 2.

1.2.1 Regulatory Elements

The promoter is the region of a gene from which mRNA transcription is initiated and is essential for controlling both the spatial and temporal expression of a transgene. A number of hematopoietic specific promoters have been used successfully to drive the expression of fluorescent reporters in different hematopoietic lineages (*see* Table 1). The transgene construct should include a translational start codon (ATG), a Kozak sequence either upstream from or coupled to the start codon [10], and a translational stop codon (Fig. 1). Posttranscriptional regulatory elements may be included to enhance mRNA stability (e.g., *see* ref. [11]).

Additional regulatory elements should accompany the promoter to drive the desired transgene expression pattern. The most commonly used regulatory elements are enhancers or other upstream regulatory elements [12], an intron which provides splice donor and acceptor sites, and a polyadenylation signal (Fig. 1).

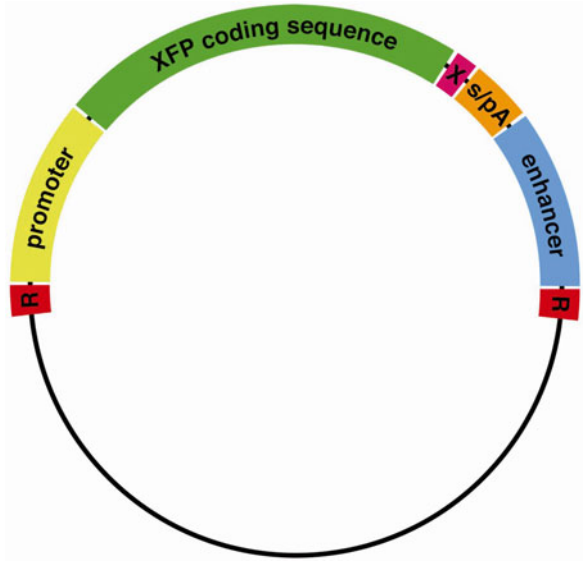

Fig. 1 Basic design of a fluorescent transgenic construct. "R" marks the restriction enzyme sites used for removal of the bacterial backbone before transgene injection. s/pA represents the splicing and polyadenylation signals. A stop codon (denoted by "X") should also be included in the construct design. The enhancer may be positioned upstream or downstream from the promoter and sequences encoding the FP and regulatory signals. For additional details, see text

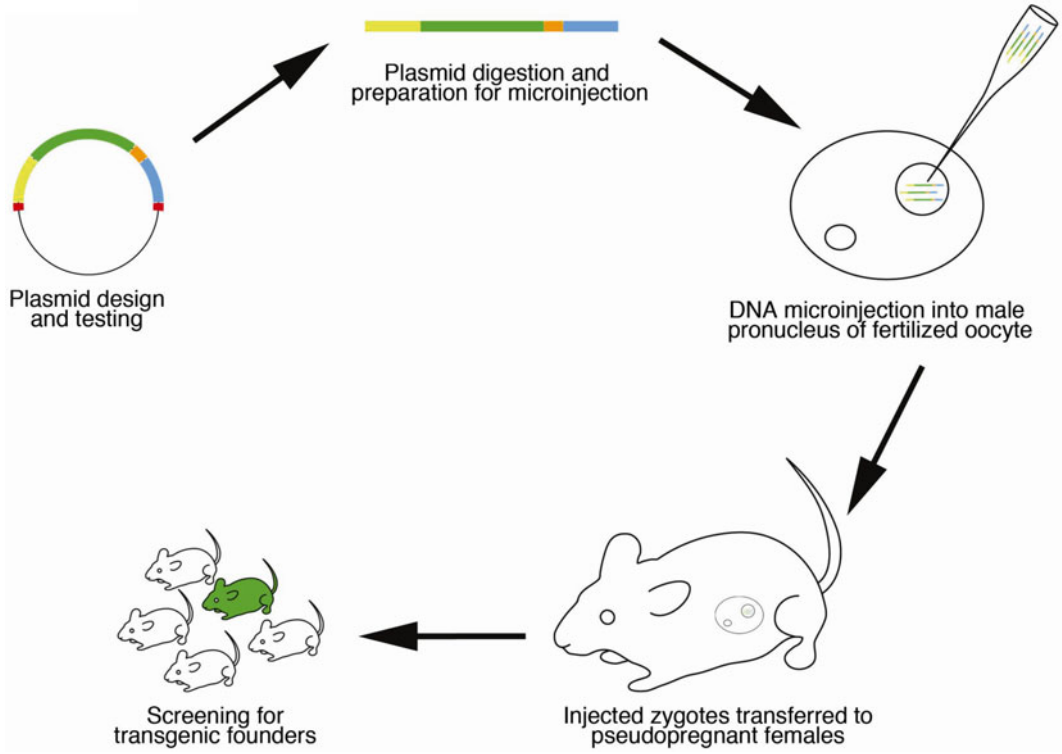

Fig. 2 Basic steps in the generation of a fluorescent transgenic reporter mouse line. Pronuclear injection of the transgene is shown in this cartoon but transgenic mouse lines can also be generated by blastocyst injection or embryo aggregation with genetically modified ES cells (see text)

Table 1
Transgenic mouse lines expressing fluorescent reporter proteins in the hematopoietic system

Gene	Reporter	Lineage labeled	Reference
Gata2	GFP (Ki)	HSC	[70]
Hoxb4	YFP (Ki)	HSC	[71]
Ly-6A	GFP (Tg)	HSC	[72]
Bmi1	GFP (Ki)	HSC[a]	[73]
Abcg2	IRES-GFP (Ki)	HSC, erythroid	[74]
c-Kit	GFP (Tg)	HSC, progenitors	[75]
c-Kit	GFP (CKO)	HSC, progenitors	[76]
Runx1	GFP (Tg)	HSC, progenitors	[77]
Pu.1	IRES-GFP (Ki)	HSC, lymphoid, and myeloid progenitors	[78]
CD41	Farnesyl-YFP (Tg)	HSC, progenitors, megakaryocytes, platelets	[79]
Gfi1B	GFP (Ki)	HSC, erythroid, and myeloid progenitors	[80]
Etv2	GFP (Tg)	Hematopoietic and endothelial progenitors	[81]
Etv2	EYFP (Tg)	Hematopoietic and endothelial progenitors	[82]
Gata1	GFP (Tg)	Hemangioblast, EryP, EryD, megakaryocytes	[83]
EpoR	GFP-Cre (Ki)	Erythroid progenitors, endothelial	[84]
Lysozyme M	EGFP (Ki)	Myelomonocytic cells, including macrophages and granulocytes	[85]
MafB	GFP (Tg)	Myelomonocytic lineages of hematopoietic cells, peritoneal macrophages	[86]
c-fms	EGFP (Tg)	Macrophages, dendritic cells, myeloid cells	[87]
β-Globin	ECFP (Tg)	MEP, EryD	[50]
miR-144/451	EGFP (Ki)	EryD	[26]
Eklf	GFP (Tg)	EryD	[88]
ε-Globin	KGFP (Tg); H2B-GFP (Tg)	EryP	[23, 24]
γ-Globin β-Globin	EGFP DsRed	EryP EryD	[89]
Langerin	IRES-EGFP (Ki)	Langerhans cells	[90]
TCRb	GFP (Tg)	Lymphoid progenitors	[91]
Runx1	IRES-GFP (Ki)	Lymphoid, myeloid, lower levels in erythroid	[29]
CD2	EGFP (Tg)	T lymphoid cells	[92]
Rag2	GFP (Ki)	T and B lymphoid cells	[93]

(continued)

Table 1
(continued)

Gene	Reporter	Lineage labeled	Reference
FoxP3	GFP (Ki)	T regulatory lymphoid cells	[94]
Ror (gT)	EGFP (Ki)	T helper 17 lymphoid cells	[95]
Pax5	EGFP (Ki)	Pre-B, B lymphoid cells	[96]
Rag1	GFP (Ki)	B lymphoid cells	[97]
Blimp1	IRES-EGFP (Ki)	B lymphoid cells, plasma cells	[98]
CX3CR1	GFP (Ki)	Macrophages, monocytes, NK cells, dendritic cells, microglia	[99]
CD45	YFP (Ki)	Widespread hematopoietic	[100]

Abbreviations: *Tg* transgenic, *KI* knock-in, *CKO* conditional knockout, *GFP* green fluorescent protein, *IRES* internal ribosomal entry site, *EGFP* enhanced GFP, *YFP* yellow fluorescent protein, *CFP* cyan fluorescent protein, *HSC* hematopoietic stem cell, *EryP* primitive erythroid, *EryD* definitive erythroid, *MEP* megakaryocyte-erythroid progenitor, *NK* natural killer cells

[a]GFP expression is highest in hematopoietic stem cells (HSCs) and is downregulated during lineage commitment and differentiation

The intron may be taken from the same gene as the promoter. Low transgene expression may be significantly increased through the use of a generic intron [13].

It is well documented that prokaryotic sequences in the vector perturb the frequency and extent of transgene expression [14–16]. Therefore, restriction enzyme sites flanking the transgene should be included to allow removal of the vector backbone before microinjection.

Following microinjection into the male pronucleus of a fertilized egg (Fig. 2), the transgene is inserted randomly, and often in multiple copies into the genome [16]. The neighboring chromatin may influence expression of the transgene, leading to undesired effects such as ectopic expression or even silencing [16, 17]. To avoid these effects, chromatin insulators can be used. These DNA elements, together with the proteins that bind to them, impair interactions with neighboring chromatin [18]. Enhancers are used to stimulate transcription and may do so in an orientation- and position-independent manner [12]. Locus control regions (LCRs) from *globin* or *CD4* genes have been used as enhancers for transgene expression in erythroid or T cells, respectively [12, 19–21]. For example, a minimal human *ε-globin* promoter combined with a truncated human *β-globin* LCR, a regulatory element that controls the erythroid-specific expression of all cis-linked *globin* genes [22], has been used to generate mouse lines expressing GFP in the primitive erythroid lineage [23, 24] (Fig. 3a).

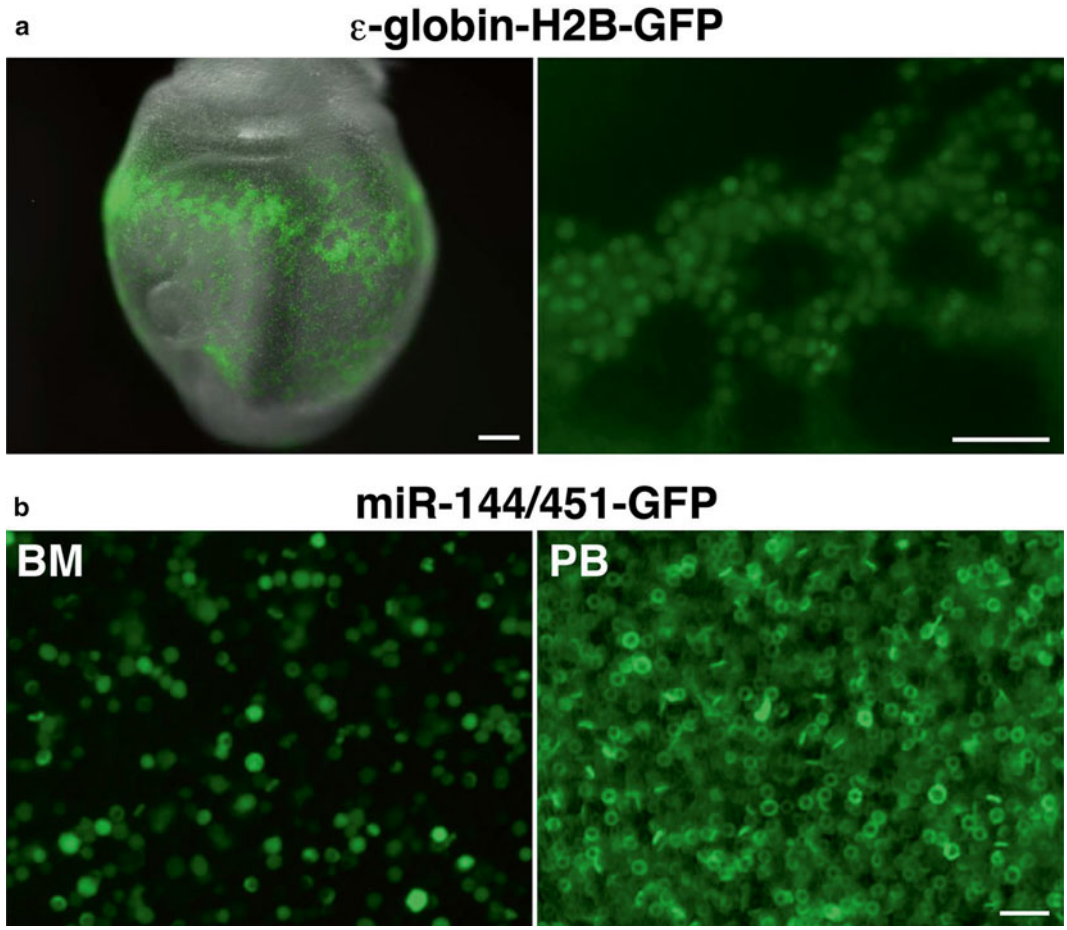

Fig. 3 Tagging of primitive and definitive erythroid lineages using GFP reporters. (**a**) GFP expression in the primitive erythroid cells of an embryonic stage (E) 8.5 *ε-globin*-H2B-GFP embryo [24] (*left panel*, whole embryo, scale bar 200 μm; *right panel*, magnified view of yolk sac, scale bar, 50 μm). Embryos were photographed on a Zeiss Lumar V12 stereomicroscope equipped with epifluorescence illumination and a NeoLumar S 1.5X FWD 30 mm objective. (**b**) Wet preparation of green fluorescent erythroid cells from the bone marrow (BM) and peripheral blood (PB) of an adult *miR-144/451*-GFP knock-in mouse [26]. The cells were photographed on a Zeiss Axio Observer Z1 inverted microscope with epifluorescence illumination and a Plan-Apochromat 20×/0.8 objective. Scale bar, 20 μm

We recommend that the expression of a newly designed transgene be tested in cultured cells before moving forward with the generation of the transgenic mouse line. This precaution helps to ensure that the transcriptional regulatory elements are functional and that the reporter sequence is translated to a functional protein.

1.2.2 Insertion of Exogenous DNA into the Genome

The most commonly used approaches for generation of fluorescent reporter mice are microinjection of DNA (plasmid or BAC; see below) into a fertilized egg (Fig. 2) and targeted insertion ("knock-in"). Insertion of a transgene into the mouse genome can also be achieved

by viral infection of the egg or injection of genetically modified embryonic stem (ES) cells into blastocysts [25].

In the "knock-in" method, DNA sequences are engineered to integrate directly into a defined locus within the genome, using site-specific recombination [16]. The knock-in approach avoids problems related to random insertion, as expression of exogenous sequences is controlled by the endogenous regulatory elements of the target gene. A recent example of a knock-in transgenic mouse is the *mirR-144/451*-GFP line, in which GFP is expressed in adult and fetal liver erythroid cells [26, 27] (Fig. 3). A concern for the knock-in approach is that it may result in haploinsufficiency, influencing not only expression of the FP reporter but also the phenotype of the resulting animal. For example, loss of one *Runx1* allele affected the distribution of HSCs in the embryo [28]. This problem was overcome by linking the sequences encoding the FP to the endogenous gene through an internal ribosomal entry site (IRES) [29] to create a dicistronic fusion mRNA [30].

Conventional transgenes are often too small to accurately reproduce the endogenous expression of the promoter/enhancer elements. Bacterial artificial chromosomes (BAC) accommodate large DNA sequences, allowing cloning of all or most of the endogenous regulatory elements required to recapitulate the normal pattern of gene expression when linked to other sequences [31] such as those encoding a FP.

1.2.3 Alternative Approaches to Drive Hematopoietic Lineage-Specific Expression of Fluorescent Protein Reporters

Inducible expression of fluorescent reporters can be achieved using the Cre-loxP or the FLP-FRT systems. For example, a mouse line in which the fluorescent reporter sequences are preceded by a floxed STOP cassette (e.g., refs. [32–34]) can be mated with a deleter line designed to express Cre recombinase under the control of a promoter active in the cells of interest. Such targeted recombination approaches have been of great utility for lineage-tracing studies (for a recent example, *see* ref. [35]).

Depending on the system chosen, recombinase activity may be constitutive or inducible. Inducible recombination activity can be triggered by tamoxifen in the case of a Cre-estrogen receptor (ER) fusion gene or by doxycycline when Cre expression is controlled by a tetracycline (Tet)-responsive element (reviewed in ref. [36]). The Mx1-Cre deleter line can be used for targeting of definitive hematopoietic lineages. In this system, Cre expression is activated by injection of interferon or synthetic double-stranded RNA polyinosinic-polycytidylic acid (poly I:C) [37].

1.3 Fluorescent Protein Reporters

A wide range of FPs covering nearly the entire visible spectrum can be used for generating transgenic reporter mice (reviewed in refs. [2–4]). Since the discovery of wtGFP (*see* Subheading 1), investigators have sought to generate other FPs with reduced phototoxicity, improved brightness, and photostability over broad ranges of pH and temperature [2–4]. Site-directed mutagenesis of wtGFP has

been employed for the generation of FPs with not only improved functionality but also diverse spectral characteristics, for example cyan FP (CFP), blue FP (BFP), and yellow FP (YFP) [2–4].

FPs with emission peaks in the red and far-red spectra have been especially useful for live-cell or whole-animal imaging, owing to their long wavelength emission and, consequently, reduced phototoxicity [2, 38]. Initially, the tetrameric DsRed was cloned from the nonbioluminescent sea anemone *Discosoma striata* but was found to be toxic to cells [39]. Subsequently, the monomeric variant mRFP was engineered and could be expressed ubiquitously in mice without deleterious effects on development [40].

Directed mutagenesis of mRFP was used to create several variants, including the orange mTomato and the red mStrawberry and mCherry FPs [38]. Far-red fluorophores such as mPlum, genetically engineered from a blue chromoprotein of the sea anemone *Actinia equina* [41], offer deep tissue penetration and reduced autofluorescence. Spectral properties of various FPs are discussed in detail in refs. [2–4].

1.3.1 Fluorescent Fusion Proteins

FPs can be engineered for localization to specific subcellular regions [42]. For example, to localize the FP to the nucleus, a nuclear localization signal is incorporated into the construct or the FP is fused to histone H2B sequences. Histone H2B fusion FPs bind to chromatin and are present through all phases of the cell cycle [43, 44]. Unlike cytoplasmic GFP, which is diluted during subsequent cell divisions, H2B-FPs are stably expressed and permit monitoring of both the cell cycle and apoptosis [44]. A histone H2B-GFP expressed under the control of a human *epsilon-globin* promoter and truncated LCR has been used for labeling the nuclei of primitive erythroid cells [24] (Fig. 3a). Cell morphology and migration can also be observed by labeling the outer or the inner leaflet of the cell membrane with lipid-tagged FP fusions (e.g., containing a glycosylphosphatidylinositol (GPI) anchor or a myristoylation sequence, ref. [45]).

1.3.2 Photomodulatable FPs

Photoactivatable fluorescent proteins (PAFP) are stimulated by lights of specific wavelengths, intensities, and durations, allowing for spatiotemporal labeling of live cells, organelles, and molecules [2–4]. There are two types of PAFP functions: photoactivation (PA) and photoswitching. Photoactivators convert from a non-fluorescent to a bright fluorescent state and can be either irreversible or reversible. PA-GFP is a GFP variant that is irreversibly converted to an anionic form, resulting in a 100-fold increase in its emission intensity [46]. Tetrameric kindling FP (KFP) can be reversibly or irreversibly photoactivated, depending on the intensity of the activating light [4, 47]. It converts to a red fluorescent state following exposure to green light and returns to a non-fluorescent state in the absence of stimulation [47]. Photoswitchers change their fluorescent state and emit at

a different wavelength (such as cyan-green for PS-CFP or green-red for EosFP, Kaede, and Kikume Green-Red, KikGR) upon exposure to transient but intense light [3].

1.3.3 General Consideration for Choosing Fluorescent Protein Reporters

When choosing an FP reporter protein, the investigator should consider whether it will be used for multicolor analysis in combination with other FPs or in immunofluorescence studies with a fluorophore of a different color. The availability of fluorescent variants allows the researcher to select combinations that minimize spectral overlap. For example, the combinations of GFP/CFP and GFP/YFP exhibit significant emission overlap, whereas CFP/YFP does not [48]. Bright reporters in the red or the far-red spectra increase the possibilities of combining different reporters to mark cells of different lineages or to mark different regions of the same cell. Due to the lower phototoxicity of the excitation light required by red or far-red fluorophores, these reporters are more suitable for live imaging studies [49]. While imaging or flow cytometric analysis of double-transgenic mice expressing FPs with overlapping spectra can be challenging [48], the judicious choice of excitation light and filters will allow optimal separation of reporter signals, as observed for the simultaneous imaging of erythroid cells expressing ECFP and myeloid cells expressing EGFP [50].

1.4 Confirmation and Analysis of Transgene Expression

1.4.1 Mouse Background

The choice of genetic background should be carefully considered in planning the generation of a transgenic mouse line. For microinjection, zygotes of mixed or outbred background are often used [16]. Microinjection of zygotes from inbred mice is more difficult and embryo viability is lower [16].

Only a relatively small number among the many available inbred strains (e.g., C57BL/6 or 129/Sv) are routinely used to create transgenic or knock-in mice. In the context of the present discussion, inbred strains would be desirable if the transgenic animals will be mated with knockout mice known to have a background-dependent phenotype or if tissue from the genetically modified animals will be used for HSC or other transplantation studies [51]. Genetic background effects (variable penetrance or expressivity) are caused by modifier genes [51]. ICR (CD1) mice are the most widely used outbred mouse strain. Unlike inbred mice, ICR mice display interindividual genetic variation. However, ICR mice are inexpensive, have excellent reproductive and maternal characteristics, and yield relatively large litter sizes [16].

1.4.2 Breeding

Once founders are identified (*see* Subheading 3.2), the colony should be expanded. Female founders should be bred so that they can give birth to at least 1 l before being sacrificed. Male founders should be placed in a cage with two nontransgenic females and plugs checked daily. Ideally, the male founder should plug 6–8 females in the first few weeks [16].

The gold standard for assessing transgene integration is germline transmission to the F1 generation. By this metric, founders should transmit the transgene to 50 % of their progeny. If transmission is not observed, the founder genotype should be reanalyzed. If the founder is positive for the transgene and germline transmission does not occur, it is likely that the founder is mosaic for the transgene and, therefore, either transmits the transgene through the germline at very low levels or not at all. In certain scenarios, transgenes will integrate at multiple loci resulting in progeny that inherit the transgene at unusually high frequencies [52].

In contrast with mouse lines created by gene targeting, each transgenic founder is distinct because of the random nature of transgene integration. Therefore the decision to eliminate a transgenic mouse line from a colony will be irreversible. To reduce costs, the investigator may choose to maintain an active colony of a few mating pairs or a small number of males that can be mated periodically to produce a younger generation. This is a relatively inexpensive approach but carries the risk that transgene expression may decrease in later generations or as the animals age; this phenomenon has often been seen for *globin* transgenes [53]. Transgene silencing may be avoided through preserving the line as frozen embryos or sperm (so that in vitro fertilization can be performed at a later date) [51]. Cryopreservation services are provided by some institutional core transgenic mouse facilities and by mouse suppliers such as Taconic Farms and Charles River Laboratory.

1.5 Analysis of Fluorescent Protein Expression Using Microscopy

The fluorescence of embryos, tissues, or cells from transgenic reporter mice can be analyzed using epifluorescence or confocal laser scanning microscopy. Confocal microscopes offer several advantages over epifluorescence microscopes. Whereas in epifluorescent microscopy the entire field is illuminated by light emitted by a mercury or xenon UV lamp, in confocal microscopy, light emitted by the laser is focused through a pinhole, creating point illumination. Out-of-focus signals are thereby eliminated and resolution is increased [54]. The confocal microscope images thin sections of the specimen that can be combined using the microscope's software into accurate 3D reconstructions of the sample. Confocal microscopes also have an increased level of sensitivity due to light detectors that can amplify the signals received from the specimen. Another advantage of confocal microscopy is that it is less invasive, resulting in reduced photobleaching [54]. The illumination provided by high-power lasers, combined with their reduced light scattering properties, allows imaging of thick, semitransparent sections, live tissues, or embryos [54]. Newer model epifluorescent microscopes use LED light sources that are more suitable for live imaging than are classical mercury lamps.

For live imaging of explanted embryos or tissues, temperature and gas composition must be carefully controlled using an environmental chamber. Inverted microscopes are typically used to image live material. For a discussion of imaging mouse embryos using confocal microscopy, *see* refs. [55–57].

Regardless of the type of microscope used, it is essential to select the appropriate light source and filters for analysis of the fluorescent specimen [54]. The identity of the cells expressing the FP may be determined using immunofluorescence, by staining for cell type-specific markers. The staining can be performed on live or fixed cells in solution or on fixed cells deposited on microscope slides. The fixation and permeabilization method should be carefully optimized for each cell type. An overview of different fixation and permeabilization options is reviewed in ref. [54].

1.6 Analysis of Fluorescent Protein Expression Using Flow Cytometry

Analytical flow cytometry is a fundamental technique for assessing fluorescence in cells from transgenic FP reporter mice. Once tissues have been dispersed into single-cell suspensions, fluorescent reporter expression from a transgene, combined with antibody staining for specific cell surface markers, can be analyzed using a flow cytometer to identify the cell surface characteristics of the component cell populations. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry that permits the physical separation of a heterogeneous sample into distinct cell populations based on their fluorescence. The ability to isolate labeled cell populations in a single step provides a valuable tool for elucidation of their developmental potentials, cell cycle, and other properties, and for culture ex vivo. For detailed protocols and reviews, *see* ref. [58].

2 Materials

2.1 DNA Purification for Microinjection

1. Agarose (Invitrogen; Cat # 16500).
2. Ethidium bromide (Sigma Aldrich; Cat # E7637).
3. Injection buffer: 10 mM Tris–HCl, pH 7.4, 0.2 mM EDTA.
4. QIAquick Gel Extraction Kit (Qiagen).
5. Restriction enzymes.
6. NanoDrop spectrophotometer (Thermo Scientific).

2.2 Isolation of Genomic DNA

1. DirectPCR Lysis Reagent (mouse tail) (Viagen; Cat # 102-T).
2. Proteinase K (Invitrogen; Cat # 25530): Stock solution prepared at 10 mg/ml in Tris–HCl 20 mM, pH 8. Aliquots are stored at −20 °C.

2.3 Polymerase Chain Reaction (PCR)

1. Plastic tubes for PCR, 0.2 ml (Denville Scientific).
2. TaKaRa ExTaq DNA polymerase, supplied with 10× ExTaq buffer and dNTP mix (Clontech).
3. Thermal cycler (available from a variety of companies).
4. For agarose gel electrophoresis: Agarose, gel-casting tray, comb with desired number of teeth, power supply, ethidium bromide, DNA size ladder.

2.4 Dissecting Tools

1. Dissecting scissors (Roboz Surgical Instrument Inc) and forceps (Sigma Aldrich).
2. Watchmaker's forceps, Dumont #5 and #55 (Roboz).
3. Sterile plastic transfer pipettes 3 ml (VWR).
4. Stereomicroscope with transmitted and reflected light sources (Zeiss, Leica, or Nikon).

2.5 Glassware and Plasticware

1. Nunclon tissue culture plates, 24 well (Nunc, Thermo Fisher Scientific).
2. Circular coverslips, 12 mm, no. 1 (Thermo Fisher Scientific).
3. 3 and 5 ml syringes (BD Biosciences).
4. Syringe needles, 20G and 25G (BD Biosciences).
5. Polypropylene tubes, 15 and 50 ml (Corning).

2.6 Embryo Dissection and Cell Preparation for Flow Cytometry

1. Phosphate buffered saline (PBS) pH 7.4 (GIBCO Invitrogen).
2. Iscove's Modified Dulbecco's Medium (GIBCO Invitrogen).
3. Fetal Bovine Serum (FBS; Hyclone, Thermo Fisher Scientific).
4. Dissection medium: IMDM +10 % FBS.
5. Heparin (Sigma Aldrich): Dissolve in PBS to 12.5 mg/ml to produce a stock solution (100×).
6. BD Falcon 40 and 70 μm cell strainers (BD Biosciences).
7. Cell Dissociation Buffer (GIBCO Invitrogen).
8. Collagenase (Sigma Aldrich): Stock solution prepared at 100 mg/ml in medium supplemented with 20 % serum. Aliquots are stored at −20 °C.

2.7 Flow Cytometry

1. FACS buffer: Heat-inactivated FBS diluted in PBS (*see* **Note 1**).
2. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Sigma Aldrich).
3. Propidium iodide (Sigma-Aldrich): Dilute powder in distilled water to prepare 1,000× stock solution.

2.8 Immunostaining and Microscopy

1. 4 % Paraformaldehyde (PFA) obtained by dilution of 16 % PFA (Electron Microscopy Sciences) in PBS.
2. Washing buffers: PBS with 0.05 % Tween-20 (v/v) (Sigma Aldrich) (PBST); PBST with 0.05 % no-fat skim milk powder (Carnation) (PBSMT).
3. Vectashield with DAPI (Vector Labs) or without (Vector Labs).
4. Primary and secondary antibodies of choice.
5. Triton X-100 (Sigma Aldrich).
6. Bovine serum albumin (BSA) (Sigma Aldrich).
7. Blocking buffer: 2 % BSA, 0.1 % Triton X-100 in PBS.

3 Methods

3.1 DNA Preparation for Microinjection

Transgenes are designed so that the gene to be microinjected can be excised and purified away from plasmid sequences. Prokaryotic sequences from the plasmid do not appear to influence the efficiency of transgene integration but they may impair the expression from eukaryotic sequences [14–16]. Therefore, the plasmid backbone sequences should be excised from the transgene construct. It is important to include a final purification step to remove particulate material that may clog the injection needle. The following protocol yields clean DNA for microinjection from plasmids smaller than 20 kb. Plasmids larger than 20 kb require a different purification procedure [59] that will not be discussed here.

1. Digest plasmid DNA to completion with the appropriate restriction enzyme(s).
2. Separate the restriction fragments by electrophoresis through an agarose gel (*see* **Note 2**).
3. Using an ultraviolet transilluminator, identify and isolate the band containing the transgene to be microinjected.
4. Extract and purify the DNA according to the manufacturer's protocols for the QIAquick Gel Extraction Kit.
5. Elute the DNA from the QIAquick column using injection buffer (*see* Subheading 2) and measure the DNA concentration using a NanoDrop spectrophotometer.
6. Pronuclear injection is typically performed in an institutional core transgenic mouse facility. For a detailed protocol, *see* ref. [60].

At our institution, the purified DNA (1–2 μg, 50–100 ng/μl) is submitted to the Mouse Genetics and Gene Targeting Shared Resource Facility. Filtration and final dilution of the DNA to be used for microinjection are performed by the facility, as is transfer of the injected zygote into pseudopregnant females [16].

3.2 Genotyping

Offspring born from injected zygotes are termed "founders" and are usually screened for the presence of the transgene. Genomic screening is most commonly performed using polymerase chain reaction (PCR) analysis of DNA from biopsied tissue (for comments on primer design, *see* **Note 3**). In most cases, the microinjected DNA will be stably integrated at the one-cell stage. However, the foreign DNA may integrate at a later (e.g., four- or eight-cell) stage, resulting in mosaic expression of the transgene and disruption of germline transmission [16]. In addition, silencing of the transgene may occur following integration into or near heterochromatin (*see* **Note 4**).

It may be possible to identify founders by microscopic analysis of biopsied tissues (*see* **Note 5**).

3.2.1 Preparation of Tissue Samples for Genotyping

1. Tail tips <0.5 cm may be biopsied from pups ≤21 days old without the use of an analgesic. Adult mice must be anesthetized according to federal and Institutional Animal Care and Use Committee (IACUC) regulations. Using scissors cleaned with 70 % ethanol, cut a 1 cm section of the tail tip of the founder mouse. Be sure to wipe the scissors with 70 % ethanol before cutting the next tail, to prevent DNA cross-contamination between samples. Place the tail tip samples into labeled 1.5 ml Eppendorf tubes. Samples can be stored frozen at −80 °C and DNA isolated at a later date.
2. For DNA isolation, add 195 μl of DirectPCR Lysis Reagent (mouse tail) to each tube, followed by 5 μl Proteinase K (10 mg/ml).
3. Incubate the tubes at 55 °C using mild agitation for 4 h or overnight.
4. Heat inactivate the samples at 85 °C for 45 min.

3.2.2 Genomic PCR

1. For PCR, pipette 1 μl DNA into a clean 0.2 ml tube containing 0.5 μl of 10 μM stocks of forward and reverse primer (*see* Table 2), 2 μl of 10× ExTaq Buffer containing $MgCl_2$ (supplied with the polymerase), 1 μl of 2.5 mM dNTP mix, 0.2 μl TaKaRa ExTaq DNA polymerase, and water to a total volume of 20 μl. For multiple reactions using the same forward and reverse primers, it is advisable to prepare a master mix containing all components except for the genomic DNA. *See* Table 2 for fluorescent reporter primer sequences used in our laboratory.
2. Conditions for PCR in a standard thermal cycler: (a) initial denaturation at 95 °C for 5 min; (b) 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and (c) final extension at 72 °C for 5 min. Samples are then maintained at 4 °C until ready for analysis using standard agarose gel electrophoresis (e.g., ref. [61]). Annealing temperature is generally close to the melting

Table 2
PCR primer pair sequences for commonly used fluorescent reporters

Reporter	Primer sequences	T_m (°C)	Product size (bp)
GFP	Forward—5′-CAT GAG CAA GGG CGA GGA ACT-3′ Reverse—5′-CAG CAG CGG TCA CAA ACT CC-3′	55	750
ExFP (for GFP, CFP, YFP)	Forward—5′-CAC CAT CTT CTT CAA GGA CGA C-3′ Reverse—5′-TTC TCG TTG GGG TCT TTG C-3′	53	350
mCherry	Forward—5′-GAT ACT CGA GCC ACC ATG GTG AGC AAG GGC GAG G-3′ Reverse—5′-CAT ACT CGA GTT ACT TGT ACA GCT CGT C-3′	53	720
tdTomato	Forward—5′-ATG GAG GGC TCC ATG AAC G-3′ Reverse—5′-CCC ATG GTC TTC TTC TGC-3′	50	370

Abbreviations: *bp* base pairs, T_m annealing temperature

temperature for the primers and may need to be optimized. The extension time may also need to be optimized according to the length of the PCR product.

3.3 Dissection

Following identification of founders using genomic PCR, expression of the reporter is examined in whole embryos, dissected tissues, peripheral blood, or bone marrow using microscopy or flow cytometry.

3.3.1 Embryo Dissection

Typically, the analysis of hematopoietic lineages in the embryo/fetus is performed from E7.5 through E16.5. Below we present a general protocol for dissection of E9.5–E14.5 embryos. For more detailed information about dissection of earlier stage embryos, *see* refs. [16, 62].

1. Euthanize pregnant mother by CO_2 asphyxiation followed by cervical dislocation.
2. Spray the abdomen of the mouse with 70 % ethanol. Pinch the fur on the abdomen and make a midline incision to open the abdominal cavity and expose the uterine horns.
3. Use a forceps to lift up the ends of the uterine horns and carefully remove the attached connective tissue and fat. Wash off the maternal blood in a 10 cm Petri dish containing PBS supplemented with 5 % FBS.
4. Carefully cut each conceptus and transfer it to a 10 cm Petri dish containing PBS. Rinse thoroughly with PBS to remove maternal blood and then transfer to a Petri dish containing dissection medium.
5. Using a pair of #5 or #55 watchmaker's forceps, peel away the uterine tissue, starting from the incision created in **step 4**.

6. Gently detach the Reichert's membrane by holding the embryo with one pair of forceps and removing the membrane with a second pair of forceps.
7. The embryo is visible inside the yolk sac, with the placenta attached. The placenta can be separated using forceps or fine scissors. Once the placental vessels are cut, the embryonic blood will be released into the medium. Placental dissociation is performed as described in Subheading 3.3.4. The YS can be removed from the embryo by carefully peeling it away using forceps.

3.3.2 Isolation of Peripheral Blood from Embryos

Peripheral blood can be obtained from ~E9.5 onward [63], when the embryonic circulation is well established [64].

1. Each embryo (YS and placenta intact) is transferred to a well of a 24-well dish containing dissection medium with 0.5 % heparin. Fluorescence can be easily evaluated using a fluorescence stereomicroscope.
2. After selection of the desired embryos, peripheral blood can be collected. Grab the region between the embryo and the placenta using a #5 watchmaker's forceps and cut umbilical and vitelline vessels with another #5 forceps. Peripheral blood cells will be released into the medium in large numbers.
3. Allow the embryos to exsanguinate (~10 min). Blood cells will collect at the bottom of the well.
4. Remove the embryos and debris from each well and resuspend the blood cells in dissection medium, using a P1000 Pipetman.
5. Filter the blood cell suspension through a 40 μm cell strainer and then collect the cells by centrifugation at 1,200 rpm ($100 \times g$) in an Eppendorf microcentrifuge.
6. Resuspend the cells at the desired concentration in a buffer appropriate for the intended application.

3.3.3 Dissection of Fetal Liver

The FL does not develop a tightly adherent epithelial structure and can be easily dispersed mechanically, allowing simple isolation of hematopoietic cells through ~E16.5.

1. Carefully dissect the FL from the embryo using a pair of #5 watchmaker's forceps.
2. Transfer the FL to a 1.5 ml Eppendorf tube containing 0.5 ml dissection medium.
3. Disperse the FL by pipetting in dissection medium using a P1000 Pipetman until obtaining a homogeneous suspension.
4. Filter the cell suspension through a 70 μm cell strainer, collect the cells by centrifugation at 1,200 rpm ($100 \times g$) in an Eppendorf microcentrifuge, and resuspend in PBS for cytocentrifugation or in FACS buffer for flow cytometry.

3.3.4 Dissection of Yolk Sac and Placenta

YS and placenta contain endothelial cells with adherent junctions and endodermal cells with tight junctions, therefore requiring more vigorous dissociation steps than for FL prior to isolation of hematopoietic cells.

1. Dissect the YS [62] and placenta [65] from each embryo at the desired stage.
2. Place each tissue into an individual 1.5 ml Eppendorf tube containing 1 ml collagenase. If two or more YSs or placentae will be collected from embryos at the same stage, they can be pooled in a 15 ml conical tube containing 4 ml collagenase. Dissociation to single cells is more efficient if narrow dissection scissors are used to macerate the YS or the placental tissue.
3. Incubate at 37 °C for at least 20 min. Shake vigorously every 5 min until a uniform cell suspension has been obtained (no large clumps remain). Filter the sample through a 70 μm cell strainer, collect by centrifugation in an Eppendorf microcentrifuge at 1,200 rpm ($100 \times g$), and resuspend in PBS or FACS buffer, as described above.

3.3.5 Isolation of the AGM Region

Dissection of the AGM region has been described in detail, and presented in a video, by others [66].

3.3.6 Isolation of Adult Bone Marrow

1. Euthanize the adult mouse by CO_2 asphyxiation followed by cervical dislocation.
2. Spray the abdomen of the mouse with 70 % ethanol. Make a midline incision to open the abdominal cavity and expose the hind legs.
3. Remove all the muscle and connective tissue from the bones using scissors and then cut the tibia and femur from the joints.
4. Cut the ends of the bones and flush the bone marrow with 1 ml buffer of choice (according to the subsequent analysis) into a collection tube stored on ice, using a 25G needle and a 5 ml syringe.
5. Homogenize the bone marrow suspension by pipetting up and down and pass the cell suspension through a 70 μm cell strainer.

3.4 General Immunofluorescence Protocol

1. Fix and permeabilize the cells using the appropriate reagents (e.g., 4 % PFA in PBS; *see* **Note 6**). Wash the cells twice for 5 min in PBS.
2. Block the cells for 15–30 min in blocking buffer.
3. Dilute primary antibody in blocking buffer and add 100 μl per slide. Incubate for 1 h at room temperature.
4. Wash three times for 5 min with PBS + 0.01 % (v/v) Triton X-100.
5. Dilute secondary antibody in blocking buffer and add 100 μl per slide. Incubate for 45 min.

6. Wash three times for 5 min with PBS + 0.01 % (v/v) Triton X-100. Rinse in PBS and then in water to avoid crystallization of the salts from PBS.
7. Mount cover slips using Vectashield mounting medium with or without DAPI (*see* **Note 7**).

3.5 Flow Cytometry

3.5.1 Labeling of Cells for Flow Cytometry

1. Count cells using a hemacytometer and dispense 1×10^6 cells into a 1.5 ml Eppendorf tube. Collect by centrifugation at 1,200 rpm ($100 \times g$) in an Eppendorf microcentrifuge and aspirate supernatant.
2. Dilute fluorescently conjugated antibody into 10 % FACS buffer (*see* **Note 1**). We generally use 2 μg of antibody per 100,000 cells; however, each antibody should be titrated for optimal results. Antibodies conjugated to different fluorochromes should be combined in the same "cocktail" to minimize cell loss due to additional incubation and washing steps.
3. As EryP do not express Fc receptors, they exhibit low background binding to antibodies. We have not found that treatment of cells with normal mouse serum or FcBlock is necessary. Cells expressing the Fc receptor should be treated with normal mouse serum or purified anti-mouse CD16/CD32 to prevent nonspecific binding.
4. Resuspend cells in a 1.5 ml Eppendorf tube containing 100 μl of the antibody cocktail and incubate on ice in the dark for 20 min, inverting the tubes every 5 min.
5. Wash with 1 ml 10 % FACS buffer and collect cells by centrifugation at 1,200 rpm ($100 \times g$) in an Eppendorf microcentrifuge.
6. If primary antibodies are unconjugated or biotin conjugated, treat cells with fluorescently conjugated secondary antibodies or streptavidin antibodies in a final volume of 100 μl in 10 % FACS buffer. Resuspend the cell pellet in 100 μl of diluted streptavidin. Incubate on ice in the dark for 20 min, inverting the tube every 5 min.
7. Wash with 1 ml 10 % FACS buffer and collect cells by centrifugation at 1,200 rpm ($100 \times g$) in an Eppendorf microcentrifuge.
8. Resuspend the cell pellet in 400 μl of 3 % FACS buffer containing DAPI (*see* **Note 8**) if a UV laser available or PI if a UV laser is not available. Transfer the cell suspension to a 5 ml round-bottom tube (*see* **Note 9**).
9. Analyze using a flow cytometer.

3.5.2 Preparation of Cells for Sorting by FACS

1. Label cells for FACS as described above in Subheading 3.5.1.
2. Prepare collection tubes for sorted cells. If the sorted cells are to be cultured, collect into a 5 ml round-bottom tube containing 1 ml sterile medium.
3. FACS instruments are typically operated by trained personnel. The investigator will advise the operator regarding the populations to be sorted.
4. Tubes containing sorted cells are kept briefly on ice until the cells can be collected by centrifugation. Cell pellets can be stored frozen at −80 °C prior to isolation of RNA.

4 Notes

1. FACS buffer is PBS-containing proteins (e.g., FBS). The proteins in FBS reduce cell loss by decreasing their interaction with plastic during the antibody staining procedure and, more importantly, the plastic tubing of the fluidics system of the flow cytometer. During antibody staining, we routinely use PBS containing 10 % heat-inactivated FBS (10 % FACS buffer). After the last washing step, the cells are resuspended in PBS containing 3 % heat-inactivated FBS (3 % FACS buffer).
2. Some investigators prefer to stain the DNA with crystal violet to avoid toxicity of ethidium bromide.
3. PCR sequences for common fluorescent reporters such as GFP are available in the literature (refer to Table 2 for primer sequences used in our laboratory). If more than one FP transgenic reporter mouse line is maintained in the colony, it is advisable to use a primer specific for sequences outside the FP-coding region. For example, one primer would hybridize with a sequence in the FP-coding region while the second would hybridize with an external sequence such as the promoter or the 3′-UTR.
4. Microinjected DNA can be transcriptionally silenced as a result of integration in or near heterochromatin, a process called variegation. Variegation is a heterocellular pattern of gene expression sometimes observed in transgenic mice. It is commonly age dependent and also results from progressive breeding of the mice [53]. This phenomenon is often seen with both the alpha- and beta-*globin* genes. Variegation may be suppressed if the transgene is linked to certain *globin* gene enhancer elements [67, 68].
5. Identification of founders may be possible using direct microscopic analysis of tissue biopsies from pups (up to 21 days old) if transgene expression is sufficiently bright. For example, we genotype *Flk1*-H2B-YFP transgenic mice [69] by examining

ear clips from 10-day-old newborn mice. The *Flk1* promoter is active in endothelial cells of the skin at this stage. This simple genotyping approach can be applied to other transgenic fluorescent reporters that are expressed in tissues that are easily collected by biopsy.

6. Exposure of the FPs to fixation reagents (e.g., formaldehyde, glutaraldehyde, methanol, acetone) denatures protein, leading to loss of fluorescence. Therefore, the fixation method (reagent and time of exposure) must be optimized. When using PFA or glutaraldehyde as fixative, quenching for 15 min with 0.1 M glycine (final concentration) following fixation will help to reduce FP denaturation. If fluorescence is lost during the fixation step, the FP can be detected by immunofluorescence after staining with an FP-specific antibody.
7. The use of Vectashield mounting medium with DAPI may result in increased background fluorescence. This may present a particular problem when the intensity of reporter fluorescence is weak. It may, therefore, be desirable to stain with DAPI, followed by washing of the slide and mounting the cover slip using Vectashield without DAPI.
8. To exclude dead cells, which may bind nonspecifically to antibodies and produce a false-positive signal, we routinely resuspend the final cell pellet in FACS buffer containing 0.2 mg/ml DAPI. DAPI is a poorly cell-permeable DNA-binding dye that is excited by the violet laser in the flow cytometer. DAPI is soluble in water but not in PBS. Therefore, we prepare a 1,000× stock solution in deionized water. The stock is then diluted into FACS buffer.
9. Sorting time and efficiency vary widely due to concentration of cell suspension and the degree of cell death. A highly concentrated sample of cells and significant amount of cell death will increase the "abortion rate"—the rate at which droplets containing single cells are not selected by the FACS machine to be sorted—resulting in lower recovery of viable sorted cells. The abortion rate can be reduced and sample purity increased by diluting the sample and/or by modifying the cell dissociation technique.

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Chapter 17

Analyzing Gene Function in Adult Long-Term Hematopoietic Stem Cells Using the Interferon Inducible Mx1-Cre Mouse System

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Abstract

Long-term hematopoietic stem cells (LT-HSCs) have the ability to self-renew and differentiate into all blood cell lineages. Understanding the genetic networks that regulate LT-HSC function in the adult bone marrow requires inducible gene targeting and bone marrow transplantations. In this chapter we describe the use of the inducible Mx1-Cre mouse model to delete genes in LT-HSCs and methodologies for examining the function of LT-HSCs following deletion.

Key words Long-term hematopoietic stem cells, Self-renewal, Conditional gene deletion, Inducible Cre, Mx1-Cre, Transplantation

1 Introduction

Due to its well-defined nature, the hematopoietic system is widely used as a model system to study the function of genes involved in stem cell self-renewal and maintenance as well as to study genes important for lineage determination [1, 2]. At the top of the hematopoietic hierarchy is the long-term hematopoietic stem cell (LT-HSC) which has the ability to self-renew and also differentiate into all hematopoietic lineages through short-term hematopoietic stem cell (ST-HSC), multipotent progenitor (MPP), and subsequent lineage-restricted progenitor cell stages (Fig. 1). Numerous genes with diverse functions have been identified as critical regulators of LT-HSCs, progenitor cells, and/or at later stages of hematopoietic development [2]. For instance, the transcription factor Evi1 and the cyclin-dependent kinase inhibitor cdkn1c (p57) have been shown to be essential for LT-HSC quiescence and self-renewal [3–5] whereas the transcription factor Gata1 is critical for erythroid development and the transcription factor Ebf is critical for B-cell development [6].

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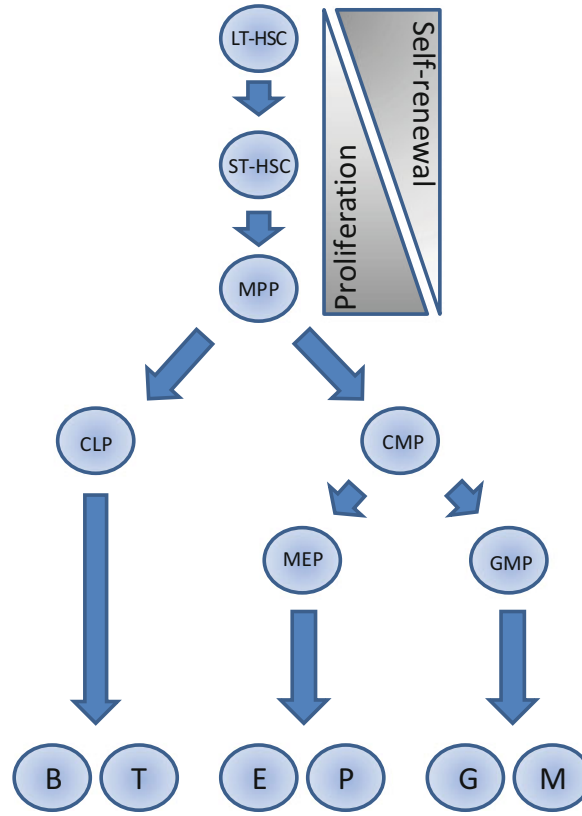

Fig. 1 The hematopoietic stem cell hierarchy. Long-term hematopoietic stem cells (LT-HSC) have the ability to self-renew and differentiate into all hematopoietic cell lineages. Upon differentiation, the LT-HSC first goes through short-term hematopoietic stem cell (ST-HSC) and multipotent progenitor (MPP) stages losing self-renewal capability but gaining increasing proliferation potential. The MPP bifurcates into common lymphoid progenitors (CLP) that differentiate into B and T-cells and common myeloid progenitors (CMP) that differentiate into erythrocytes (E) and platelets (P) through megakaryocyte erythroid progenitors (MEP) and granulocytes (G) and macrophages (M) through granulocyte macrophage progenitors (GMP)

Since many of the genes important for LT-HSCs have critical functions in other cell types, gene targeting in mice can lead to a lethal phenotype during embryonic development precluding analysis of hematopoietic cell function in adult animals [7, 8]. Therefore, it is more productive to construct conditional mouse knockout models in which genes can be deleted by Cre-lox or Flp-frt mediated recombination in a time and cell type specific manner. To date, numerous mouse Cre strains have been generated in which activity of the recombinase is either inducible or controlled by a hematopoietic specific promoter (*see* **Note 1**). The inducible strains include the Mx1-Cre (interferon inducible) and ERT-Cre (tamoxifen inducible) strains and the hematopoietic specific strains include Vav-iCre (fetal HSCs and progenitors), Lys-Cre (myeloid), CD19-Cre (B-cells),

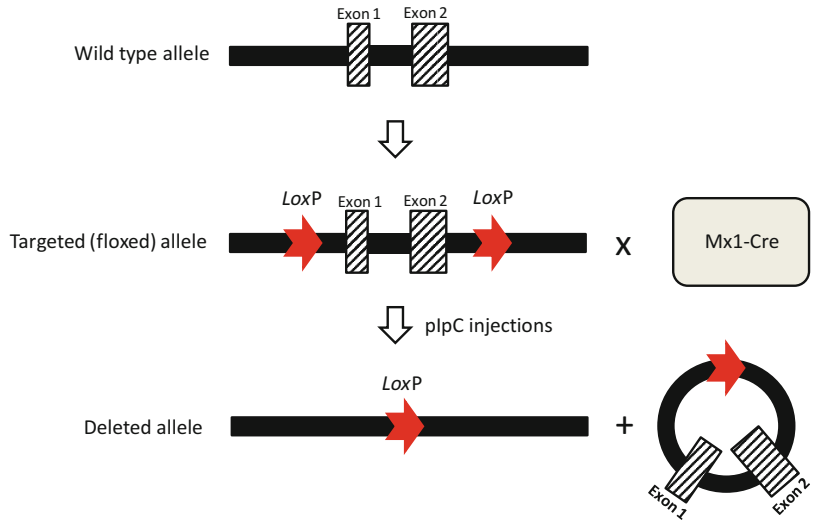

Fig. 2 The Mx1-Cre system. In this hypothetical model a targeted construct is generated by inserting 2 *LoxP* sites flanking exons 1 and 2 of a particular gene (GeneX). The targeted (floxed) mice ($GeneX^{FL/FL}$) are bred with Mx1-Cre mice to generate $GeneX^{FL/FL}$, Mx1-Cre+ mice. Upon intraperitoneal injection of $GeneX^{FL/FL}$, Mx1-Cre+ mice with pIpC, transcription of Cre recombinase is induced leading to recombination between the *LoxP* sites and deletion of exon 1 and 2

and Lck-Cre (T-cells) strains. So far, no Cre strains specific for adult LT-HSCs have been generated. Therefore, to study the function of a gene in adult LT-HSCs inducible Cre strains have to be used. The Mx1-Cre strain was one of the first inducible Cre mouse strains that were developed and has been widely used to study gene function in the hematopoietic system [9]. In these mice, expression of Cre recombinase is under the control of the tightly regulated interferon inducible *Mx1* promoter which can be activated by treatments with polyinosinic–polycytidylic acid (pIpC) [9]. To induce gene deletion in LT-HSCs, mice conditionally targeted for the gene of interest are crossed to Mx1-Cre mice and progenies carrying homozygous conditional alleles and *Mx1-Cre* subsequently treated with pIpC (Fig. 2). Since *Mx1* promoter is also active in bone marrow stromal cells, pIpC injection in the primary animals will induce gene deletion in the bone marrow microenvironment in addition to the LT-HSCs. Therefore, it is important to analyze the cell-autonomous function of your gene of interest by transplanting the targeted cells into wild type microenvironment. To this end, two approaches can be used: (1) Transplanting bone marrow cells from pIpC treated animals into lethally irradiated recipients. The limitation of this approach is that the deletion could potentially affect homing and/or lodging of the LT-HSCs into the bone marrow microenvironment and would not give any indications on stem cell function, i.e., their ability to self-renew and/or differentiate. (2) Transplanting bone marrow cells from targeted animals and treating recipients with pIpC following adequate recovery period (generally 4–8 weeks).

With this approach, stem cell function is more adequately analyzed. In this chapter, we will describe the use of the Mx1-Cre strain for studying gene function in LT-HSCs both in primary animals as well as in a transplantation setting.

2 Materials

2.1 Mouse Strains

1. Mx1-Cre and CD45.1 recipient mouse strains are available from Jackson Laboratories (www.jax.org).
2. B6.Cg-Tg(Mx1-Cre)1Cgn/J.
3. B6.SJL-Ptprca Pepcb/BoyJ (CD45.1).

2.2 Tail lysis buffer

Reagents (final concentration)	Stock	Stock (ml) needed to make 100 ml
100 mM Tris–HCl pH 8.5	1 M	10.0
5 mM EDTA	0.5 M	1.0
0.2 % SDS	10 %	2.0
200 mM NaCl	5 M	4.0
100 µg/ml proteinase K[a]	20 mg/ml	0.5
dH_2O		82.5

[a]Add proteinase K just before use.

2.3 Genomic DNA Collection

1. SSC buffer (20×) (National Diagnostics).
2. Glass capillary tube (Fisher Scientific).

2.4 Primer Pairs for Genotyping Mx1-Cre Mice

1. Generic Cre-F: GCG GTC TGG CAG TAA AAA CTA TC.
2. Generic Cre-R: GTG AAA CAG CAT TGC TGT CAC TT.

2.5 PCR

1. GoTaq Flexi DNA Polymerase Reagents (Promega).
2. PCR amplification tubes and caps (Applied Biosystems).
3. Thermal cycler (Eppendorf Mastercycler Gradient).
4. Agarose LE (Roche).
5. 10× TBE buffer (National Diagnostics).
6. Ethidium Bromide 10 mg/ml (Invitrogen).

2.6 pIpC Injections

1. Polyinosinic Acid Polycytidylic Acid, Sodium Salt (pIpC) (Calbiochem).
2. 0.22 µm sterile syringe filters (Millex-GP).
3. Syringes U-100 28G×1/2 (Becton Dickinson).
4. Alcohol pads (Triad Group Inc).

2.7 Bone Marrow Harvesting

1. Refrigerated centrifuge (Eppendorf).
2. 70 % EtOH solution.
3. Tweezers and scissors (Roboz Surgical Instruments).
4. Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco).
5. 5 ml syringes (Becton Dickinson).
6. 26G×1/2 Needles (Becton Dickinson).
7. 40 μm cell strainers (Becton Dickinson).
8. 50 ml polypropylene conical tubes (Becton Dickinson).

2.8 Preparation of B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) Recipient Mice

1. Acidified water.
2. Amoxicillin (West-ward Pharmaceutical Corp).

2.9 Bone Marrow Transplantation

1. Irradiator (Cesium source).
2. Rodent Irradiation Cage system (Braintree Scientific).
3. Mouse restrainer (Stoelting or Harvard Apparatus).
4. 1 ml syringes with 28G×1/2 needles (Becton Dickinson).
5. DPBS.
6. Warming lamp (Ledu Professional Fluorescent Clamp-On Magnifying Lamp).

2.10 Flow Cytometric Analysis of LT-HSCs

1. Refrigerated centrifuge (Eppendorf).
2. Fetal bovine serum (FBS) (Atlanta Biologicals).
3. DPBS/1 % FBS.
4. 15 ml polypropylene conical tubes (Becton Dickinson).
5. Antibodies: anti-mouse Gr1 (Clone RB6-8C5, BD Pharmingen), anti-mouse Mac1 (Clone M1/70, BD Pharmingen), anti-mouse CD4 (CloneRM4-5, BD Pharmingen), anti-mouse CD8 (Clone53-6.7, BD Pharmingen), anti-mouse B220 (Clone RA3-6B2, BD Pharmingen), anti-mouse Ter119 (Clone Ter119, BD Pharmingen), anti-mouse CD127 (Clone A7R34, eBioscience), anti-Rat PE-Cy5 (Invitrogen), anti-mouse CD45.2 FITC (Clone 104, BD Pharmingen), anti-mouse Sca1 APC (Clone D7, BioLegend), anti-mouse c-Kit APC-eFluor 780 (Clone ACK2, eBioscience), anti-mouse CD48 Biotin (Clone HM48-1, BioLegend), anti-mouse CD150 PE-Cy7 (Clone TC15-12F12.2, BioLegend), anti-mouse CD34 Alexa Fluor 700 (Clone RAM34, eBioscience), anti-mouse Flk2/Flt3 PE (Clone A2F10, eBioscience), Pacific Orange-Streptavidin (Invitrogen).
6. Sytox Blue (Invitrogen).
7. OneComp eBeads (eBioscience).

3 Methods

All experimental protocols should be reviewed and approved by your Institutional Animal Care and Use Committee.

3.1 Breeding Scheme for Generating Conditionally Targeted Mx1-Cre Mice

1. Assuming that you have generated or have acquired mice conditionally targeted for a gene of interest, the next step is to breed them with the Mx1-Cre strain to generate mice that are homozygous for the conditional allele and are Cre positive. A breeding scheme where this can be achieved in two generations is presented in Fig. 3 (*see* **Note 2**).

3.2 Genotyping Conditionally Targeted Mx1-Cre Mice

1. After you have obtained progeny from the breeding scheme, perform genotyping to identify animals carrying the targeted (floxed) allele and the Mx1-Cre transgene.
2. Just before use, prepare a master mix of tail lysis buffer by adding proteinase K to the previously prepared lysis buffer solution (*see* **Note 3**). Digest tail clippings in 0.5 ml tail lysis buffer in 1.5 ml tubes overnight at 55 °C. Pipette up and down a few times to completely dissociate the digested tissue. Centrifuge at 13,000 × *g* for 5 min. Transfer solution to a new 1.5 ml tube containing equal volume of Isopropanol (*see* **Note 4**). Invert the tube a few times to precipitate the DNA (*see* **Note 5**). Recover DNA on a glass capillary tube and

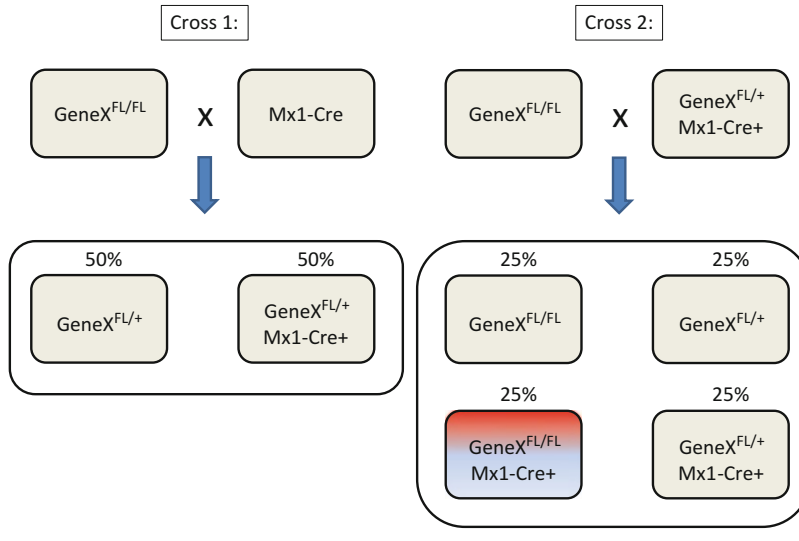

Fig. 3 A breeding scheme to generate GeneX$^{FL/FL}$, Mx1-Cre+ and control mice. In the first cross GeneX$^{FL/FL}$ mice are bred with Mx1-Cre mice producing 50 % GeneX$^{FL/+}$ mice and 50 % GeneX$^{FL/+}$; Mx1-Cre+ mice. The GeneX$^{FL/FL}$ mice are then bred with the GeneX$^{FL/+}$; Mx1-Cre+ mice generating 25 % GeneX$^{FL/FL}$; Mx1-Cre + mice. The other mice from the cross can be used as experimental controls

resuspend in 75 μl 0.1× SSC buffer in 1.5 ml tubes. Incubate sample at 37 °C for 1 h, briefly vortex to mix. Samples can be stored at room temperature.

3. Set up PCR according to your method of choice. As a reference, we use the following scheme for Cre genotyping (*see* **Note 6**):

Master mix for genotyping	
Reagents	**One reaction**
5× Colorless buffer	5 μl
$MgCl_2$	3 μl
dNTPs	0.5 μl
Generic Cre-F (15 μM)	1.0 μl
Generic Cre-R (15 μM)	1.0 μl
GoTaq	0.25 μl
dH_2O	14 μl
Total volume	24.75 μl

Add 0.25 μl DNA template to the reaction mix.

PCR Program:

95 °C	2 min	
95 °C	30 S	25 cycles
60 °C	45 S	
72 °C	45 S	
72 °C	3 min	
4 °C	Hold	

4. Run PCR products on 1.0 % TBE (0.5×) agarose gels containing ethidium bromide (add 3 μl of 10 mg/ml ethidium bromide solution to 100 ml gel solution).

3.3 Injecting Conditionally Targeted Mx1-Cre Mice with pIpC

1. After identifying animals with the correct genotypes, proceed to pIpC injections.
2. Resuspend pIpC powder in 5 ml DPBS buffer to generate 2 mg/ml stock solution. Mix until fully dissolved. Filter the solution through a 0.22 μm filter syringe. Inject 0.4 mg/mouse (*see* **Note 7**).
3. For intraperitoneal injection, hold mouse by scruff and secure tail between pinky and ring finger. Turn mouse over and sterilize

the abdominal area by wiping down with alcohol pad. Insert needle into the lower right or lower left abdominal area and inject pIpC solution.

4. Repeat injections every other day for a total of five injections (*see* **Note 8**).
5. Analyze LT-HSCs by flow cytometry, e.g., at 1, 2, and 4 months after the last pIpC injection (see protocol below). During each harvest, reserve some bone marrow cells for determining the recombination efficiency by PCR.

3.4 Preparation of B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) Recipient Mice for Bone Marrow Transplantation

To determine if a gene has an intrinsic function in LT-HSCs, bone marrow transplantation has to be performed (Fig. 4).

1. Start pre-treating the appropriate number of B6.SJL-Ptprca Pepcb/BoyJ recipient mice with acidified water and antibiotics (amoxicillin 125 mg/250 ml acidified water) 5 days before transplantation.

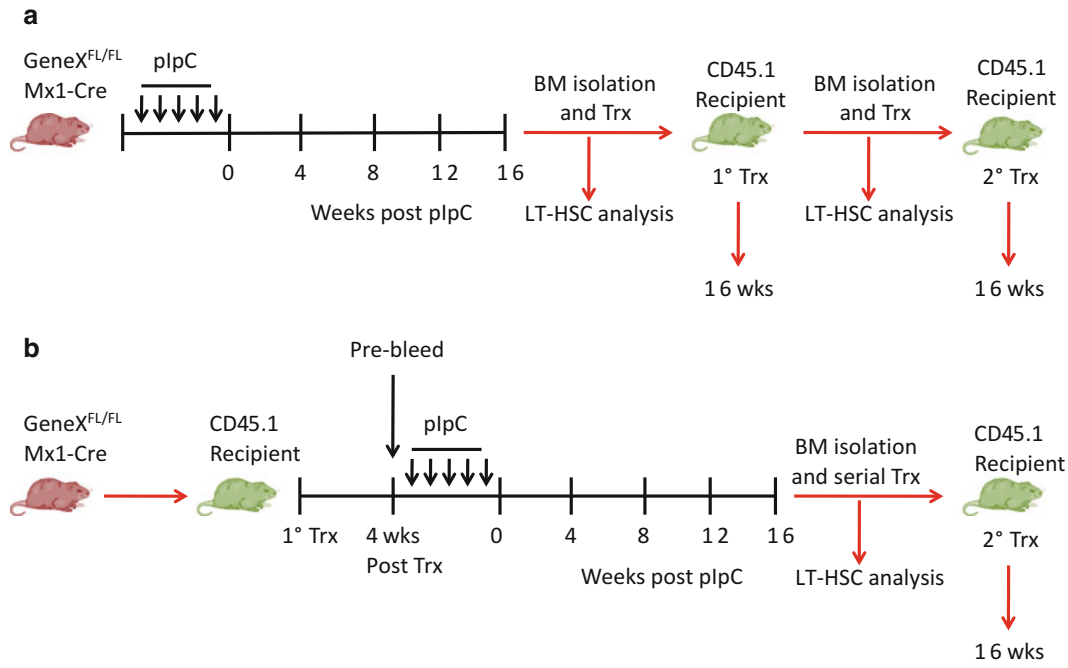

Fig. 4 Experimental schemes for analyzing LT-HSC function in GeneX$^{FL/FL}$, Mx1-Cre+ mice. (**a**) pIpC injection of GeneX$^{FL/FL}$; Mx1-Cre+ mice. Bone marrow is harvested 16 weeks after the last pIpC injection for transplantation and for phenotypic analysis of LT-HSC by flow cytometry. LT-HSCs are analyzed again after 16 weeks in the primary recipient and serial transplantation performed. (**b**) pIpC injection of CD45.1 recipient mice previously transplanted with bone marrow cells from GeneX$^{FL/FL}$; Mx1-Cre+ mice. Bone marrow is harvested 16 weeks after the last pIpC injection for transplantation and phenotypic analysis of LT-HSCs by flow cytometry. LT-HSCs are analyzed again after 16 weeks in the secondary recipient and serial transplantation performed. Both experimental schemes can be adjusted for studying LT-HSC function in a competitive setting. In that case, reconstitution is analyzed by flow cytometry 4 weeks post-transplantation by retro-orbital bleeding and staining for CD45.1+ (recipient) and CD45.2+ (donor) cells to assess baseline values before deleting

3.5 Harvesting and Preparation of Bone Marrow Cells from Donor Mice

1. Sacrifice gene targeted and competitor animals if needed (*see* **Note 9**) using a CO_2 chamber followed by cervical-spine dislocation (*see* **Note 10**).
2. Spray animal, tweezers, and scissors with 70 % EtOH solution to sterilize.
3. To harvest femur cut through subcutaneous layer of skin laterally across lower abdominal region. Pull skin apart to expose femur and remove muscle tissue using scissors. To loosen femur, cut above hip joint and below knee joint. Place femur in ice-cold DPBS in 10 cm dishes. Keep on ice.
4. To maintain cell preparation sterile, perform bone marrow flushing under a level 2 biosafety cabinet. Attach 26G×1/2 needle to a 5 ml syringe containing 3 ml ice-cold DPBS. Hold femur using tweezers, insert needle into bone marrow cavity, and flush cells into 10 cm dishes (*see* **Note 11**). Draw the bone marrow cell clumps back into the syringe a few times to dissociate into single cells.
5. Place a 45 μm cell strainer into 50 ml conical tube. Collect the bone marrow cell solution into a new syringe and filter through the cell strainer. Rinse the cell strainer once with ice-cold DPBS.
6. Collect cells by centrifugation in a chilled centrifuge (1,500 × *g*, 5 min).
7. Pour off supernatant and resuspend cells in 3 ml DPBS. Count nucleated cells using WCCF solution and a hemocytometer. Make a stock solution to allow injection of 1×10^6 cells/recipient in 0.2 ml DPBS (*see* **Note 12**).

3.6 Bone Marrow Transplantation

1. Lethally irradiate recipient mice with a total dose of 1,100 rads provided in two equal doses of 550 rads. Allow 3 h of resting between irradiations and after the last irradiation before injection.
2. Place recipient mice into a restrainer and put the tail under a warming lamp to dilate lateral tail vein. Inject 0.2 ml bone marrow cell suspension into tail vein using 1 ml syringes with 28G×1/2 needle.

3.7 Staining LT-HSCs for Flow Cytometry

1. For detecting LT-HSCs, harvest bone marrow cells 4 months post-pIpC. Use at least 5×10^6 mouse BM cells per analysis (*see* **Note 13**). Antibody master mixes are prepared in ice-cold staining solution (DPBS/1 % FBS). Use 0.5 μg antibody/1×10^6 cells.
2. Harvest bone marrow cells as described in Subheading 3.5.
3. Lyse red blood cells by incubating the cells in 3 ml ice-cold ACK lysis buffer for 2–3 min in 15 ml conical tube on ice.

4. Fill tube with ice-cold DPBS and collect cells in a chilled centrifuge at 1,500 × *g*, 5 min.
5. Resuspend in 5 ml ice-cold DPBS and collect in a chilled centrifuge at 1,500 × *g*, 5 min.
6. Resuspend cells in 3 ml DPBS and count as described in Subheading 3.5.
7. Transfer 5×10^6 bone marrow cells into a 15 ml conical tube and collect in a chilled centrifuge at 1,500 × *g*, 5 min.
8. Resuspend bone marrow cells in 0.5 ml antibody master mix containing anti-mouse Mac1, anti-mouse Gr1, anti-mouse CD4, anti-mouse CD8, anti-mouse B220, anti-mouse Ter119, anti-mouse CD127, and anti-mouse CD48 Biotin.
9. Incubate on ice in the dark for 30 min.
10. Add 5 ml DPBS/1 % FBS to each tube and collect cells in a chilled centrifuge at 1,200 × *g*, 5 min.
11. Pour off supernatant, resuspend in 5 ml DPBS/1 % FBS, and collect cells in a chilled centrifuge at 1,200 × *g*, 5 min.
12. Resuspend cells in 0.5 ml antibody master mix containing anti-rat PE-Cy5.
13. Incubate on ice in the dark for 30 min.
14. Add 5 ml DPBS/1 % FBS to each tube and collect cells in a chilled centrifuge at 1,200 × *g*, 5 min.
15. Pour off supernatant, resuspend in 5 ml DPBS/1 % FBS, and collect cells in a chilled centrifuge at 1,200 × *g*, 5 min.
16. Resuspend cells in 0.5 ml antibody master mix containing anti-mouse CD45.2 FITC (*see* **Note 14**), anti-mouse Sca-1 APC, anti-mouse c-Kit APC eFluor 780, anti-mouse CD150 PE-Cy7, anti-mouse CD34 Alexa Fluor 700, anti-mouse Flk2 PE, and Streptavidin Pacific Orange.
17. Incubate on ice in the dark for 30 min.
18. Add 5 ml DPBS/1 % BSA to each tube and collect cells in a chilled centrifuge at 1,200 × *g*, 5 min.
19. Pour off supernatant, resuspend in 5 ml DPBS/1 % FBS, and collect cells in a chilled centrifuge at 1,200 × *g*, 5 min.
20. Resuspend cells in 0.5 ml DPBS/1 % FBS and keep on ice in the dark.
21. Prepare the relevant controls to set up the flow cytometer (*see* **Note 15**).
22. Just before running samples add 0.5 μl Sytox blue viability stain to the sample (*see* **Note 16**) and incubate 1 min.
23. Run samples on a flow cytometer that has been set up with the correct filters. Analyze data using the appropriate software (e.g., FlowJo). An example of LT-HSC analysis is shown in Fig. 5.

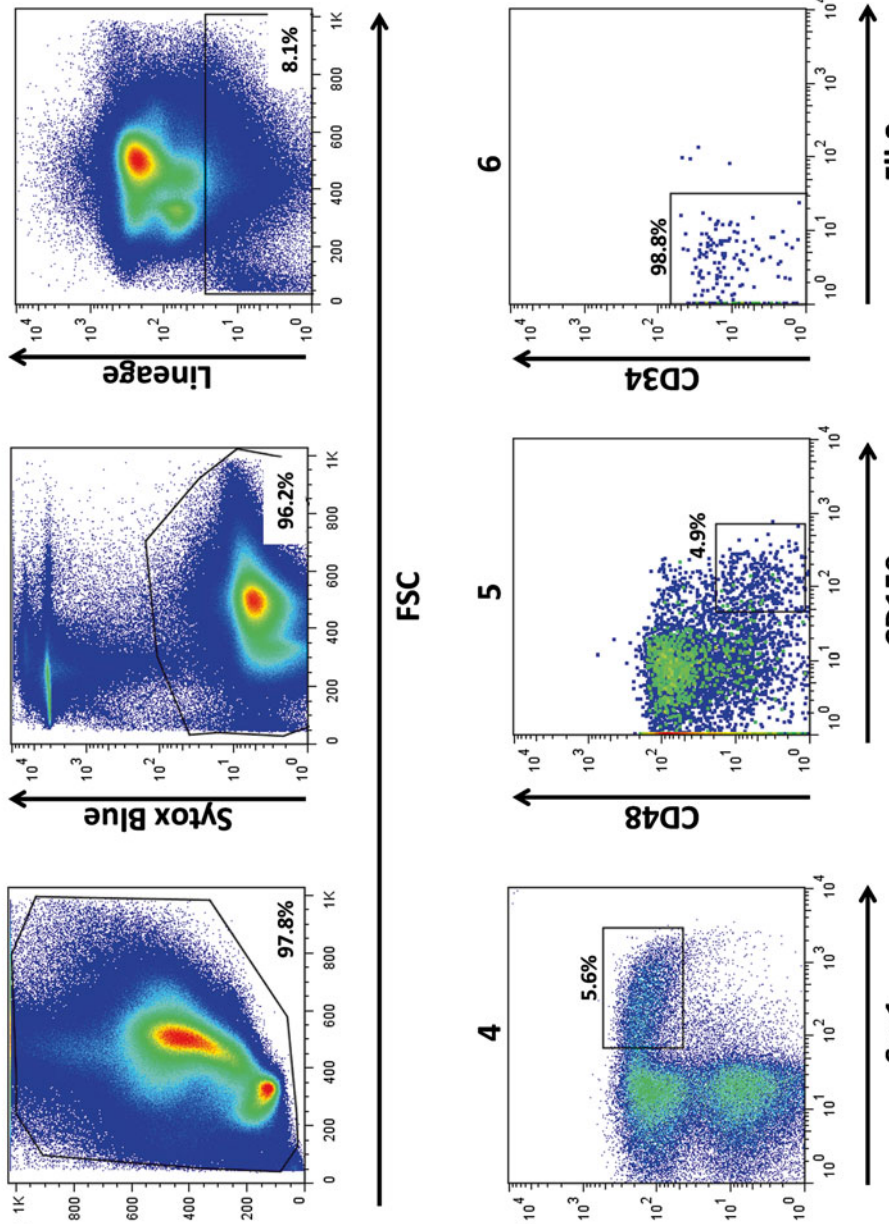

Fig. 5 LT-HSC analysis in mouse bone marrow by flow cytometry. Following cell aggregate elimination by FSC-A vs. FSC-H, single bone marrow cells are gated by FSC vs. SSC (panel *1*) followed by exclusion of dead cells by Sytox Blue (panel *2*). Next, lineage negative cells (panel *3*) are analyzed for Sca1 and c-Kit expression (panel *4*) and lineage negative, Sca1+, c-Kit (LSK) cells gated and analyzed for CD48 and CD150 expression (panel *5*). The LSK, CD48-CD150+ population, which contain the LT-HSCs, can be analyzed for CD34 and Flk2 expression (panel *6*). The majority of LSK, CD48-CD150+ cells are CD34low/- and Flk2-

4 Notes

1. For more information on Cre strains go to the Jackson Laboratory website (www.jax.org) or the Cre-X-Mice: A Database of Cre Transgenic Lines (http://nagy.mshri.on.ca/cre_new/index.php).
2. It is possible to obtain the relevant animals with other breeding combinations such as crossing GeneX$^{FL/+}$ mice with GeneX$^{FL/+}$; Mx1-Cre+ mice.
3. It is also possible to add proteinase K to the tail lysis solution, aliquot, and store at −20 °C. Tail tissue can also be stored at −20 °C until ready to digest.
4. This is easily done by pouring the solution directly into the tube containing the isopropanol without disturbing the pellet.
5. In case the DNA does not precipitate, spin the tube at full speed (14,000 × *g*) in an Eppendorf centrifuge for 15 min at room temperature. Remove supernatant and resuspend pellet in 75 μl 0.1× SSC buffer.
6. For typing the targeted allele, design primers that flank one of the *lox*p sites. A PCR product containing a *lox*p sites is 34 bp larger than a wild type product. The two can be easily distinguished on a 1.5–2 % agarose gel.
7. Do not inject more than 0.2 ml into the abdominal cavity.
8. In some protocols up to seven pIpC injections are performed [10].
9. For competition experiments, mix at least 0.5×10^6 bone marrow cells from each donor (1:1) to transplant a total of 1×10^6 cells.
10. Following the CO_2 treatment, perform the cervical-spine dislocation to make sure the animals are dead.
11. If bone marrow cavity is closed, use scissors to cut a small piece away from either end of the femur.
12. Generally, $0.5–2 \times 10^6$ bone marrow cells are injected per recipient.
13. These cell numbers are needed in order to acquire enough events by flow to do meaningful analysis of the HSC compartment.
14. Only add anti-CD45.2 antibody to the cocktail if detection of donor cells is needed.
15. To set up the flow cytometer, we run unstained bone marrow cells and OneComp eBeads for each antibody. Also, while testing the antibody cocktails, run an isotype control. For dead

cell exclusion, we prepare a 1:1 mixture of live and dead cells (prepared by incubating in 70 % EtOH for 10 min at room temperature and washing in DPBS).

16. Make a 1:3 fold dilution of Sytox in DMSO. Protect from light. Add 0.5 μl of this solution to 0.5 ml sample and incubate 1 min before running on the flow cytometer. Do not incubate more than 30 min.

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Chapter 18

Generation of Transgenic Mice by Exploiting Spermatogonial Stem Cells In Vivo

Lalit Sehgal, Abul Usmani, Sorab N. Dalal, and Subeer S. Majumdar

Abstract

The protocols in this chapter describe two techniques for the generation of transgenic mice by in vivo manipulation of spermatogonial stem cells (SSCs) with a high rate of success. SSCs in prepubescent animals can either be infected in vivo with recombinant lentiviruses expressing the transgene of interest or DNA can be injected into the testis followed by the application of an electric current resulting in integration of the linearized DNA containing a transgene downstream of the appropriate promoter into SSCs. All male pre-founder mice produced transgenic pups using both protocols with the transgene being heritable. Further, the pre-founder mice could be used in multiple mating experiments resulting in the generation of multiple progeny. These protocols could be extended to perform over-expression/knockdown screens in vivo using bar-coded lentiviruses/plasmid constructs, thus permitting the design of genetic screens in the mouse. Further, these protocols could be adapted to achieve transgenesis in other laboratory animals resulting in the generation of model systems that closely approximate human development and disease.

Key words Transgenic mice, Sperm mediated gene transfer, Spermatogonial stem cells, Lentivirus, Electroporation

1 Introduction

The generation of genetically modified transgenic mice has spurred great advances in our understanding of various aspects of growth and development. While several groups have used inducible and tissue specific knockout mice [1–9] to study various problems of interest to biologists, the increased use of RNA interference (RNAi) to study somatic cell genetics and to perform genetic screens has resulted in an increase in the interest in transgenic technologies that permit the establishment of limited genetic screens in small mammals. However, previous transgenic technologies were not suitable for performing genetic screens as they have used either injection into a one celled embryo followed by implantation into uterus of a surrogate mother [10] or stem cell aggregation techniques to generate

Lalit Sehgal and Abul Usmani have contributed equally to this Chapter.

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either knockout [11] or knockdown mice [12]. These procedures involving assisted reproduction are expensive, labor-intensive, time-consuming and require several female donors and hence are not suitable for performing limited genetic screens. In addition, these technologies require several steps to ensure that the transgene enters the germ line, further limiting the ability of these protocols to perform genetic screens in the animal.

One possible way to circumvent the hurdle of germ line transmission of the transgene is to modify germ cells in vivo and use these modified cells as sources of the transgene. Therefore, various groups have tried to modify spermatogonial stem cells (SSCs), which are responsible for the production of spermatozoa [13] and are an appropriate target for germline modification [14]. To summarize some of these efforts, Nagano et al. [15] have generated transgenic mice by infecting SSCs in vitro with recombinant retroviruses and then transplanted these cells into the testis of a male mouse [15]. However, some of the recipient mice were unreceptive to the donor SSCs [15] and the overall success rate was low. Similarly, transduction of SSCs with retroviral constructs carrying a lacZ gene in vivo resulted in a poor success rate of 2.8 % [16], probably due to the inability of retroviruses to stably infect non-dividing cells. Therefore, the low success rates post implantation precluded these techniques from replacing embryonic injection. Similar experiments using lentiviruses resulted in better success rates [17]. Other methods employed in the recent past require infection of fertilized eggs in vitro with recombinant lentiviruses, followed by implantation of the embryo into pseudopregnant females [18]. While these methods provided better success rates, the implantation experiments are technically cumbersome, require several female donors, and therefore are difficult to use to perform genetic screens in animals. A recent report has also described the generation of recombinant spermatozoa in organotypic cultures that could be used to generate transgenic mice [19]. While this method generated transgenic animals at high efficiency, generating organotypic cultures is not trivial and is not performed in most laboratories.

The two technologies described in this chapter focus on the generation of transgenic mice by modifying SSC's in vivo. The first resulted in the generation of transgenic mice after electroporation of DNA into the testes of a 30-day-old mouse. Sixteen of 17 fore founder mice generated in that study were able to sire transgenic pups [20]. The other technique used in vivo viral transduction of the EGFP-f transgene into undifferentiated spermatogonia. These fore-founder male mice were mated with wild type female mice to generate transgenic pups. The rate of transgenesis was greater than 60 % and the transgene was inherited in the germ line [21]. These protocols for the generation of transgenic mice could be used to design and perform over-expression/knockdown screens in the whole animal, thus enhancing our knowledge of disease and development.

2 Materials

All solutions should be made in Milli-Q grade water that is suitable for tissue culture. For most solutions, it is best to work with autoclaved water to maintain sterility.

2.1 Infection of SSCs with Lentiviral Particles In Vivo

2.1.1 Chemicals

Trypan blue dye (0.04 %), Tris–HCl (pH 8.0, 50 mM), 1 % sodium dodecyl sulfate (SDS), sodium chloride (100 mM NaCl), 100 mM EDTA pH 8.0, proteinase K 0.5 mg/ml, 4 % paraformaldehyde in 2 % glucose, glycerol, ampicillin (sodium salt) and kanamycin monosulfate (Sigma). dNTPs (Promega). Taq polymerase and molecular weight markers (New England Biolabs). ViraPower packaging mix and HEK293-T cells (Invitrogen). The HEK-293 cells are cultured in DMEM (Invitrogen) containing 10 % Fetal Bovine Serum (FBS) (Invitrogen), 100 U of penicillin (Nicholas Piramal), 100 μg/ml of streptomycin (Nicholas Piramal), and 2 μg/ml of amphotericin B (HiMedia). Suitable reagents from other companies can be used for cell culture. Cells are grown on tissue culture grade plasticware (BD Falcon). Cells were transfected for virus production using lipofectamine (Invitrogen). Virus particles are resuspended in Dulbecco's phosphate buffered saline (PBS) (Invitrogen) prior to injection.

2.1.2 Animals

Twenty-eight to 32-day-old mice Crl:CFW(SW) weighing about 20–25 g are used in this protocol (*see* **Note 1**). The animals are housed in a controlled environment with the temperature and relative humidity being maintained at 23 ± 2 °C and 40–70 % respectively and a day night cycle of 12 h each (7:00–19:00 light; 19:00–7:00 dark) and fed an autoclaved balanced diet prepared in-house as per the standard formula and sterile water ad libitum. Mice are housed in the Individually Ventilated Cage (IVC) system provided with autoclaved rice husk bedding material available locally.

2.1.3 Lentivirus Constructs and Lentivirus Suspension

Any commercial VSVG pseudotyped lentiviral vector can be used for making lentivirus particles for injection. For example, we have used pLKO.1 EGFP-f in our experiments [21]. Commercially available packaging constructs such as the ViraPower mix from Invitrogen can be used to package the lentiviral vector in HEK293T (ATCC) cells. A suspension of lentiviruses (10^5–10^6 TU/ml) expressing the transgene can be used for these experiments.

2.1.4 Anesthesia

A mixture of ketamine hydrochloride (50 mg/ml stock solution) and xylazine hydrochloride (20 mg/ml stock solution) is used to anesthetize the animals. The stock solutions are mixed and diluted with normal saline to achieve a final concentration of 1.6 μg/ml of xylazine hydrochloride and 9 μg/ml of ketamine hydrochloride.

2.2 In Vivo Testicular Electroporation of Spermatogonial Stem Cells of the Testis

2.2.1 Animals

FVB/J mice of 30 ± 2 days of age can be bred and used for this protocol. All animals are usually kept at 24 ± 2 °C under 14 h light and 10 h dark cycle. Please note that we used animals as per the National Guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA). Any other strain of mice with similar age can also be used for this protocol.

2.2.2 Transgene Constructs

1. Plasmids containing gene of interest cloned downstream of an appropriate promoter particularly which are functional in mammals should be used for transfection.
2. Lysis buffer (50 mM Tris–HCl, 1 % SDS, 100 mM NaCl, 100 mM EDTA).

2.2.3 Bacterial Strains

DH5α strain of *E. coli* procured from Stratagene was used for this protocol and can be procured from any suitable supplier.

2.2.4 Media for Growing Bacteria

LB broth and LB Agar may be obtained from Pronadisa. This medium should be sterilized by autoclaving at 15 lbs/in.^{2} (121 °C) for 20 min.

2.2.5 Molecular Biology Reagents and Other Chemicals

1. Plasmid isolation kits (both mini and maxi-scale) and gel extraction kit were purchased from QIAGEN or MDI for this procedure but they can also be procured from other suitable suppliers. DNA restriction enzymes can be purchased from New England Biolabs. Taq DNA polymerase (Bangalore Genie). Proteinase K (Invitrogen). Oligonucleotide primers were custom-made (Sigma Aldrich) and can be procured from any suitable supplier. The 100 bp DNA ladder and 1 kb DNA ladder (New England Biolabs).
2. Other chemicals: Sodium acetate, trypan blue, oligos for PCR amplification, ampicillin (sodium salt), kanamycin monosulfate, ethylene-di-amine-tetraacetic acid (EDTA), Tris-base ethidium bromide, bromophenol blue, xylene cyanol, calcium chloride, sodium dodecyl sulfate (SDS), (Sigma). Ethanol, isopropanol, methanol, iso-amyl alcohol, acetic acid, formaldehyde, and glycerol (Merck). Agarose from AMRESCO.

2.2.6 Anesthesia

1. Use a mixture of ketamine hydrochloride and xylazine hydrochloride; we use stock solutions of 50 and 20 mg/ml respectively.
2. Mix these solutions and dilute with normal saline to get a final concentration of 160 μg of xylazine hydrochloride and 900 μg of ketamine hydrochloride per 100 μl.

2.2.7 Equipment

1. A P-97 Horizontal pipette puller (Sutter Instruments Co) was used in all experiments. An electric pulse generator, Electroporator-ECM2001 from BTX Instrument Division,

Harvard Apparatus, Inc., may be used for electroporation. A tweezer type electrode works better for holding and in vivo electroporation of the testis. A Peltier Thermal Cycler PTC-200 with heated lid (MJ Research) or a machine of a similar type can be used in all experiments. The UV-2450, UV–VIS Spectrophotometer (Shimadzu Corp) was used for all experiments.

3 Methods

3.1 Infection of SSCs with Lentiviral Particles In Vivo

3.1.1 Preparation of High-Titer Lentiviral Particles

1. LVs were produced as described previously [21]. At the final step, the viruses are resuspended in Dulbecco's PBS. The biological titer of lentiviral vectors was determined by the infection of HEK-293 cells with different virus dilutions.
2. Usually a minimum titer of 10^5–10^6 TU/ml is required for efficient infection (*see* **Notes 2** and **3**).

3.1.2 In Vivo Transduction of the Desired Gene in the Testis

1. Inject 28-day-old Crl:CFW(SW) male mice intraperitoneally with a ketamine and xylazine solution (*see* **Note 4**) at a dose of 0.015 ml/g body weight .
2. Remove hair from inguinal area of mice and clean the area with Betadine. Anterior to the penis, make a single midline cutaneous incision of approx. 1–1.5 cm length using sterile surgical scissors under aseptic conditions. A central incision is the preferred surgical approach to get adequate access to both testes with a single cut.
3. After making the incision in the muscles, pull the dorsal fat pad associated with the testis from the lower side of the abdominal cavity with the help of curved forceps.
4. Use a 30-G needle to puncture outer covering of the testicular tissue to facilitate the insertion of syringe containing LV. Deliver solution of LV containing trypan blue dye (0.04 %) in the inter-tubular spaces of testis through 30-G syringe. Trypan blue is added to monitor the accuracy of the delivery in the testis.
5. Inject about 10–20 μl of lentiviral suspension per testis (*see* **Note 5**).
6. Replace the injected and surgically remove the contra-lateral testis by hemicastration (*see* **Note 6**). Both the internal and external wounds should be closed using sutures.
7. Apply Neosporin (Neomycin and Polymyxin B sulfates and Bacitracin zinc powder; GlaxoSmithKline) at the site of sutures and place the mice on a thermal plate for about 1 h for easy revival of the animal from anesthesia.

*3.1.3 Establishment of Transgenic Lines (See **Note 7**)*

1. After 30 days of LV injection, cohabitate the injected animals (pre-founders) for mating with 2-month-old wild type (WT) females. Pups are born usually within 22–30 days of cohabitation.
2. Prepare tail biopsies (2–3 mm) from 3-week-old pups and incubate them at 55 °C for 16 h in high salt digestion buffer (50 mM Tris–HCl, 1 % SDS, 100 mM NaCl, 100 mM EDTA, and 1,200 μg/ml Proteinase K) for lysis (*see* **Note 8**).
3. Process the lysate for DNA isolation by phenol–chloroform extraction followed by ethanol precipitation.
4. Quantify the DNA concentration of the extracted DNA by UV absorption at 260 nm and check the purity by *A*260/*A*280 nm ratio using a UV spectrophotometer.
5. Dilute the isolated genomic DNA to a final concentration of 200 ng/μl and use as stock solution for running performing polymerase chain reactions (PCR) using oligonucleotide primers that are specific for the transgene.

3.1.4 Screening of Transgenic Pups Using Polymerase Chain Reaction (PCR)

1. Prepare reaction mixture containing 1× Taq buffer, 0.2 mM each dNTPs, 0.25 μM of each reverse and forward transgene specific primer, 0.06 U Taq DNA polymerase and 20 ng of plasmid as a positive control or 200 ng of gDNA as template. Mix well and spin down briefly. The reactions can be performed in a standard thermal cycler such as a Peltier Thermal Cycler PTC-200 with heated lid (*see* **Note 9**).
2. Before performing PCR reactions on genomic DNA, we recommend that a gradient PCR reaction be performed to determine the optimal temperature for annealing of the primer. The PCR products can be resolved on agarose gels of the appropriate percentage made in either TAE or TBE.
3. PCR positive pups from F1 generation are mated with WT females to generate next generation of progeny. A homozygous line can be generated by inbreeding the transgene positive littermates.

3.1.5 Timing

1. Lentivirus production: 7 days.
2. Surgery and LV injection: 30 min.
3. Mating: 30 days post-LV injection (when age of animal is about 60 days).
4. Pups generated: Within 30 days of cohabitation.
5. Total time: 70 days for generating several transgenic pups.

3.2 Generation of Transgenic Mice Through In Vivo Testicular Electroporation Mediated Through the Spermatogonial Stem Cells of the Testis

3.2.1 Preparation of Glass Micropipette

1. Pipettes for micro injection of DNA are pulled by the P-97 micropipette puller from Sutter Instrument Co. For this, keep the borosilicate glass capillary in the heating chamber with the middle part surrounded by a platinum heating element. The instrument electrically heats up the glass capillary with a heating element and a horizontal linear force pulls the heated glass apart to produce two needles.
2. For the present protocol, make pipettes having the tip diameter of about 40–60 μm. Fire-polish the tip.

3.2.2 Bacterial Transformation and PLASMID Isolation

1. Perform transformation of DH5α competent cells with plasmid containing gene of interest and appropriate promoter. In our case, we have used IRES2-Egfp plasmid vector.
2. Inoculate a colony of transformed bacteria in LB broth and add appropriate antibiotic (for e.g. Ampicillin, Kanamycin etc.). Culture at 37 °C with shaking at 220 × *g* for 12–14 h.
3. Plasmid isolation can be performed from 6 or 250 ml of LB broth (mini or maxi scale) using the plasmid isolation kit following the instruction provided by manufacturer kit.
4. Check isolated plasmid on 1 % agarose gel. Quantify the plasmid in UV–Vis Spectrophotometer.
5. Cut the plasmid with suitable restriction endonucleases to take out the fragment with promoter and gene of interest. If plasmid is smaller than 5 kb then single cut can be performed just to linearize the plasmid. In case it is bigger, double cut is given to remove unnecessary portion of the plasmid.
6. Resolve the digested sample on 1 % agarose gel along with 1 kb molecular weight marker. Excise the portion of the gel containing the DNA fragment of interest (transgene).
7. Extract the DNA from the gel with the help of gel extraction kit following the instruction provided by the kit manufacturer.

3.2.3 In Vivo Electroporation of the Transgene in the Testis

1. Anesthetize the animal (male mice) by giving anesthesia (160 μg of xylazine hydrochloride and 900 μg of ketamine hydrochloride per 100 μl) intraperitoneally (100 μl/20 g body weight).
2. Remove the hair from the lower abdominal area surrounding the scrotal sac. Clean the shaved skin with Savlon and Betadine solution. Under aseptic environment, give a cutaneous cut, ventrally, in lower abdomen using sterile surgical instruments. A central cut in lower abdomen is the preferred surgical approach to get adequate access to both testes with a single

cut. Pull the fat pad associated with the testis from the lower side of the abdominal cavity with the help of curved forceps. Place both exteriorized testes on a sterile autoclaved paper.

3. Add about 5 μl of 0.04 % trypan blue to the DNA solution before injection. Trypan blue is added to monitor the accuracy of the injection in the testis. Deliver the DNA solution in the intertubular spaces of testis through a glass micropipette. Injection can be done from three different sites to ensure the maximum distribution of DNA solution in the testis (to facilitate the entry of the glass micropipette in the testis, prick the outer covering with the help of 30 G needle). Inject about 10–12 μg of DNA.
4. After DNA delivery, hold the testis with a sterile tweezer-type electrode attached to an electroporator and deliver mild electric pulses to the testis. For optimal result, inject 20–35 μl of linearized DNA (0.5 μg/μl) into the testis of 30 ± 2-day-old FVB male mice, followed by electroporation using 8-square 40 V electric pulses in alternating direction (changing pole of the electrode after four pulses) with a time constant of 0.05 s and an inter-pulse interval of about 1 s.
5. Hemicastrate the non-electroporated testis after tying blood vessels and place back the electroporated testis in scrotal sac. Suture both the internal and external skin. Apply Neosporin powder on the cut site. Allow the animal to recover from anaesthesia under warm environment.

3.2.4 Establishment of the Transgenic Lines

1. After 30 days of electroporation, transfected male mice are mated with wild type adult female mice. Mouse litters are obtained generally within 25 days after cohabitation (Mouse gestation period is about 20 days).
2. Take tail biopsies (2–3 mm) from 3-week-old pups and digest them in tissue lysis buffer along with 1,200 μg/ml proteinase K at 55 °C for 8–10 h.
3. Isolate the genomic DNA from the above lysate by standard Phenol–Chloroform extraction protocol.
4. Check the quality of gDNA in 0.8 % agarose gel. Quantify the gDNA at 260 nm in UV–Vis spectrophotometer.
5. Perform PCR with 100 ng of gDNA samples with transgene specific primers.
6. PCR positive pups from F1 generation are mated with WT females to generate next generation of progeny (F2). Make homozygous line by inbreeding of transgenic littermates.
7. The protocol presented above allows generation of transgenic mice within a short period of time. This deathless technique does not require highly trained manpower and expensive infrastructure, hence, can be used under standard laboratory conditions easily by junior and senior level bench workers.

4 Notes

1. Any strain of mice can be used. Thirty-day-old males are most suitable for better outcome of this protocol with the advantage that a large proportion of the testicular germ cells can be easily accessed from the interstitial side of the testis in this age group of mice. The same protocol may be tried with minor modifications for animals of different age and species.
2. It is important that the viral particles are stored in small aliquots at −80 °C. The virus suspension can be stored at −80 °C for at least 1 year without significant change in virus titer. Do not refreeze virus suspension after thawing. The virus suspension can be stored at 4 °C if an experiment is planned within a week.
3. Lentiviruses are capable of infecting human cells including nondividing cells through contact. Gloves and protective clothing are required for working with lentiviruses. Extra caution should be taken to avoid spill and splash when handling lentivirus-containing material. Lentiviruses are labile and easily decontaminated by ethanol, detergent or bleach. Working area should be decontaminated with ethanol or bleach after any spill and after any experiments involving virus handling are completed.
4. All animal studies should be performed with relevant institutional guidelines, permissions, and regulations. All steps described below should be performed in a sterile laminar flow hood. Care should be taken to prevent an overdose of the anesthetic as an overdose could lead to death of the animal.
5. After each injection, wait for 30 s before pulling out the syringe to prevent the back flow of DNA suspension or the LV solution. The total volume of fluid injected into the testis should not be more than 20 μl. Injecting large volumes of fluid may rupture the testis as might injecting the testis more than once.
6. During hemicastration, do not remove fat or any other tissue along with the testis. Tie the blood vessels with sterile nylon thread before hemicastration to prevent bleeding. Try to be careful when you handle the testis, so as to not crush it.
7. In mice, it takes about 30 days to complete a cycle of spermatogenesis during which period a spermatogonia differentiates into sperm. Hence, transgenic sperm produced after 30 days of LV/DNA injection presumably originates from the spermatogonial cells in which permanent integration of the transgene occurs at the time of LV injection.
8. Prepare 20 mg of proteinase K in 1 ml of nuclease-free water and use it as a stock. Aliquot proteinase K in volumes of 200 μl or less and store at −20 °C. Multiple freeze–thaw cycles may alter proteinase K activity.

9. In each experiment, PCR reactions using genomic DNA from wild type mice as a template and a reaction without DNA (no template) should be included as negative controls. Purified plasmid containing the respective transgene can be used as a positive control for DNA amplification.

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Part III

Mouse Genetics in Modeling Human Disease

Chapter 19

Methods for the Detection of Genome Instability Derived from Replication Stress in Primary Mouse Embryonic Fibroblasts

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Abstract

Replication stress, with its subsequent genome instability, is a hallmark of cancer from its earliest stages of development. Here, we describe assays that are sufficiently sensitive to detect intrinsic replicative stress and its consequences in primary mouse embryonic fibroblasts. First, we explain the non-denatured DNA fiber assay, a powerful tool to directly measure DNA replication kinetics via the dual-labeling of active replication forks. Then, we describe the cytokinesis-block micronucleus assay, which can be combined with detection of 53BP1 nuclear bodies to measure the levels of replication-associated genome instability carried over into G1 phase of the cell cycle.

Key words Replication stress, Genome instability, Primary MEFs, DNA fiber assay, Micronuclei, 53BP1 nuclear bodies

1 Introduction

It is becoming increasingly evident that DNA replication stress is associated with cancer development from its earliest stages [1–3]. In this chapter, we detail methods that can be used to detect genomic instability derived from replication stress. These techniques have all been optimized for use with *primary* mouse embryonic fibroblasts (MEFs), as we think it is important to use non-transformed primary cells to gain a better understanding of the mechanism by which replication stress drives cancer at early stages.

The first of these methods, the non-denatured DNA fiber assay, is used to directly monitor the progression of DNA replication forks. Decades ago, the autoradiographic detection of DNA synthesis was first conducted using H3-thymidine [4] but this method required months to obtain images of DNA fiber tracts. Modern DNA fiber techniques typically utilize the fluorescent detection of dNTP analogs like BrdU, CldU and IdU, substantially

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shortening the experimental time frame [5, 6]. However, this assay requires a DNA denaturing step for the detection of images, frequently causing newly synthesized DNA tracts to appear spotty or heterogeneous. In this chapter, we describe the use of biotinylated or digoxigenin-conjugated dUTPs for the DNA fiber assay, which enables the detection of DNA fiber tracts without the denaturing step [7]. The resulting DNA fiber tracts also look smoother with continuous staining. Moreover, the gradual exhaustion of dUTPs within the cells results in a gradual decrease in staining intensity thereby revealing the direction of fork movement. Using this technique, we describe basic procedures to detect replication fork speed, the density of active replication origins and the frequency of replication fork stalling in unchallenged S phase [8, 9].

Recently, our laboratory has provided evidence that persistent stalled replication forks can lead to the formation of micronuclei (MN) [8]. As stalled forks can leave sister chromatids physically interconnected via unreplicated regions, they induce sister chromatid non-disjunction, leading to the formation of MN in the subsequent G1 phase [10]. Using the cytokinesis-block MN assay [11], MN can be efficiently identified in recently divided cells by scoring those which are binucleated. Optional staining with the centromeric maker CENP-A [12] can then also be used to determine whether resulting MN are derived from either lagging chromosomes (include centromeric DNA) or acentric chromosome fragments. In addition to MN, it has also recently been found that unresolved replication intermediates can rupture during their passage through mitosis, with the resulting lesions being marked in the daughter nuclei in specialized compartments containing p53-binding protein 1, or 53BP1 [13, 14]. These structures, called 53BP1 nuclear bodies (53BP1-NB), often appear strikingly symmetrical in terms of their number, size and appearance within the daughter nuclei, supporting the idea that they come from unreplicated loci shared by sister chromatids. Using the same cytokinesis blocking method as in the MN assay, it is possible to efficiently measure the levels of such 53BP1-NB in G1 phase nuclei. Together, these analyses are powerful tools for determining how replication stress results in genome instability.

2 Materials

It is recommended to prepare all solutions using ultrapure water unless otherwise indicated.

2.1 General

1. Culture medium: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin Streptomycin (Gibco).
2. Trypsin: TrypLE (Gibco).

3. Phosphate Buffered Saline (PBS).
4. Parafilm.
5. Antifade mounting medium: VECTASHIELD HardSet Mounting Medium (Vector Laboratories) or ProLong® Gold Antifade Reagent (Life Technologies).
6. DAPI (4′,6-diamidino-2-phenylindole): Prepare a stock solution at 5 mg in 1 ml PBS.
7. Frosted end microscope slides: Gold Seal Rite-ON Micro Slides (Gold Seal).

2.2 The DNA Fiber Assay

1. Hypotonic solution (10× concentrated stock): 100 mM HEPES pH 7.3, 300 mM KCl. Filter-sterilize and store at room temperature (stable for 1 year). Dilute with ultrapure water to prepare a 1× solution as needed.
2. Fixative: Add 50 ml of acetic acid to 150 ml of methanol (1:3). Make fresh every time.
3. Lysis buffer: 0.5 % SDS, 50 mM EDTA, 200 mM Tris–HCl, pH 7.0.
4. Washing buffer: 4× SSC, 0.1 % Tween 20.
5. Blocking buffer (10× concentrated stock): Use Blocking Reagent (Roche) and follow the manufacturer's instructions. Just before use, dilute with washing buffer to obtain a 1× concentration.
6. Detection buffer: Dilute the concentrated stock of blocking buffer with washing buffer to obtain a 0.5× concentration.
7. Modified dUTPs: Biotin-dUTPs (Biotin-16-2′-deoxy-uridine-5′-triphosphate) 50 nmol/50 µl (Roche), Digoxigenin-dUTPs 125 nmol/125 µl (Roche).
8. Streptavidin, Alexa Fluor® 488 Conjugate: 0.5 ml (Life technologies).
9. Anti-Digoxigenin-Rhodamine, Fab fragments from sheep: (Roche).
10. Microscope coverslips (24 × 60).
11. 48-Well dish and 10 cm dishes.
12. Coplin jars.
13. Tubes: 15 ml conical tubes, 2 ml screw cap tubes.
14. Two water baths: one set at 37 °C and the other at 42 °C.

2.3 The Cytokinesis-Block MN and 53BP1-NB Assays

1. Cytochalasin B: Prepare a stock solution of 600 µg/ml with dimethyl sulfoxide (DMSO).
2. Fixative: formalin, 10 % w/v.
3. Image iT™ FX signal enhancer (Invitrogen) (*see* **Note 1**).

4. Blocking/permeabilization solution: PBS with 0.3 % Triton X-100 and 1 % bovine serum albumin (BSA). Store at 4 °C.
5. Antibodies: Rabbit monoclonal anti-CENP-A antibody (Cell Signaling). Rabbit polyclonal anti-53BP1 antibody (Abcam). Goat anti-rabbit secondary antibody conjugated with Alexa Fluor® 594 (Invitrogen).
6. High salt PBS: PBS supplemented with 0.4 M NaCl.
7. 70 % ethanol.
8. 22 × 22 coverslips.
9. 6-well plates.

3 Methods

3.1 Generation and Culture of MEFs

Generate primary MEFs from mid-gestation embryos using a standard method. Plate a homogenized embryo onto a 10 cm dish (p0), re-plate onto a 15 cm dish (p1), and freeze in three vials. For experiments, thaw one vial and culture the cells on a 10 cm dish for a few days (p2). The resulting 6–9 × 10^6 cells (p3) are then re-plated for experimental use below.

3.2 The DNA Fiber Assay

The original protocol [15, 16] was kindly provided to us by Dr. Kazuto Sugimura. Here, we provide a modified protocol that has been optimized for use with primary MEFs.

3.2.1 Labeling

1. Seed 2.5 × 10^4 cells in a well of a 48-well plate. Additionally, plate 1 × 10^6 cells in a 10 cm dish for use as non-labeled cells. Note that for each well of cells, one 10 cm dish of non-labeled cells is required. Culture cells for 2 or 3 days at 37 °C in a 5 % CO_2 incubator.
2. Prepare a total volume (30 μl) of labeling mixture of biotinylated- or digoxigenin-conjugated dUTPs as follows: 21 μl ultrapure water, 3 μl 10× concentrated stock of hypotonic solution, 6 μl of either biotinylated or digoxigenin-conjugated dUTPs (1 mM). This makes a final dUTP concentration of 200 μM.
3. Remove culture medium and wash cells with 300 μl 1× hypotonic solution.
4. Gently drop 30 μl of the labeling mixture of digoxigenin-dUTPs onto the center of the well.
5. Incubate cells for 10 min in a CO_2 incubator to allow dUTPs to be incorporated into cells (*see* **Note 2**).
6. Wash cells with culture medium once, add 300 μl of culture medium and incubate for 20 min in a CO_2 incubator (*see* **Note 3**).

7. Remove culture medium and wash cells with 300 μl of 1× hypotonic solution.
8. Gently drop 30 μl of labeling mixture of Biotin-dUTPs and incubate cells for 10 min (*see* **Note 4**).
9. Wash cells with culture medium, add 300 μl new medium and incubate for 30 min.
10. Remove culture medium, wash cells with PBS and add 300 μl of trypsin. Simultaneously, trypsinize non-labeled cells plated in the 10 cm dish. Incubate for 3–5 min.
11. Add trypsinized dUTP-labeled cells to the 10 cm dish containing trypsinized non-labeled cells (*see* **Note 5**).
12. Add 5 ml of culture medium to the dUTP-labeled/non-labeled cell suspension above and pipette up and down several times to thoroughly dissociate cells. Transfer cell suspension to a 15 ml tube.
13. Spin down cells at 1,000×*g* using a tabletop centrifuge for 3 min and remove supernatant.
14. Resuspend cells in 300 μl of culture medium.

3.2.2 Fixation

1. Add 300 μl fixative to cells while vortexing gently.
2. Add 1 ml fixative with gentle vortex a total of eight times to reach a final volume of 8.3 ml fixative.
3. Centrifuge fixed cells at 1,000×*g* for 3 min and remove supernatant.
4. Add 10 ml fixative, mix well and centrifuge cells again.
5. Remove supernatant, resuspend the cells in 1 ml fixative and transfer cell suspension to a 2 ml screw cap tube (*see* **Note 6**).

3.2.3 Extension of DNA Fibers

1. Drop 20 μl of the fixed cell suspension onto an area directly adjacent to the frosted end of a microscope slide (*see* **Note 7**). Make several slides per sample.
2. Dry cells on slides for 2 min at room temperature.
3. Put the slides into a Coplin jar filled with lysis buffer. Incubate them for 15 min at 37 °C.
4. Set up a high-humidity chamber using a water bath (Fig. 1).
5. Take the slides out from lysis buffer and place them in the chamber. The slides must be leaned with the frosted end up (as shown in Fig. 1). Let them sit for 20 min to allow extension of DNA fibers.
6. Take the slides out from the chamber, carefully wipe excess buffer from the other end of the slides and dry them.
7. Dip the slides into a Coplin jar filled with fixative for 2 min.
8. Dry the slides. These slides can be stored for a week at room temperature (*see* **Note 8**).

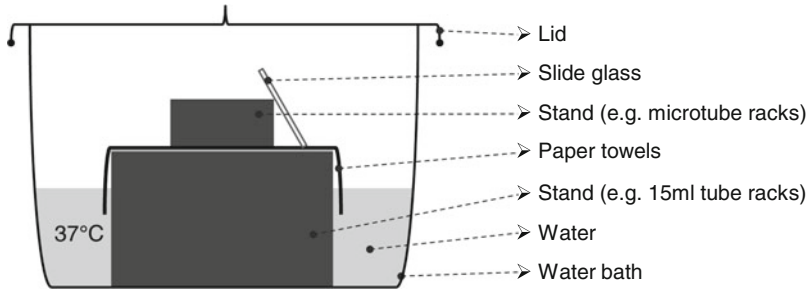

Fig. 1 Schematic presentation of the high humidity chamber for DNA fiber extension. Inside of a water bath set at 37 °C, assemble the chamber using tube holders and paper towels as shown. Immediately following the lysis step, slides are tilted on the stand at a 70° angle

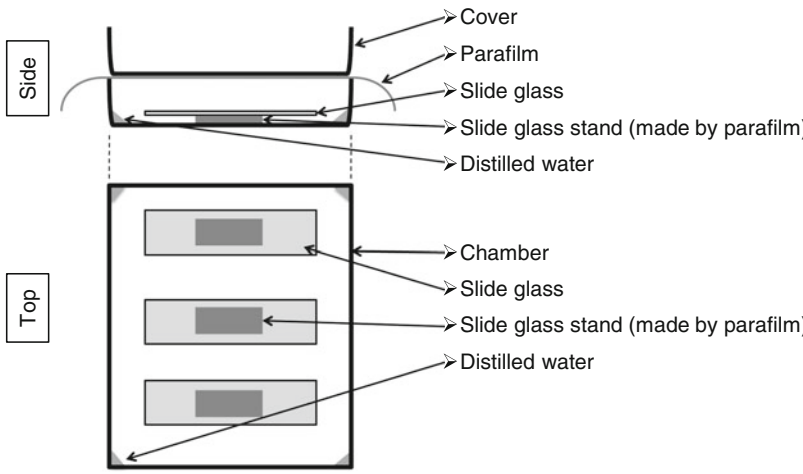

Fig. 2 Schematic presentation of a humidity chamber for DNA fiber staining. Shown is a 9 × 12 cm plastic tray with three stands assembled from piles of Parafilm within. The stands should support the slides evenly. A few drops of distilled water are placed at each corner of the chamber to maintain humidity. Place a sheet of Parafilm and another 9 × 12 cm plastic tray on top to seal the chamber tightly

3.2.4 Staining DNA Fibers

1. Wash the slide with PBS once, remove excess liquid, drop 150 µl of 1× blocking buffer onto the slide and cover with a 24 × 60 mm coverslip.
2. Incubate the slides at 37 °C in humidified conditions (Fig. 2) for 30 min (*see* **Note 9**).
3. Remove coverslips and excess buffer from the slides by waving off, and immediately drop 100 µl of detection buffer supplemented with antibody (anti-digoxigenin: 1/30, avidin: 1/40) and cover with new coverslips (*see* **Note 10**).
4. Incubate the slides for 1 h at 37 °C in humidified conditions. In the meantime, warm up washing buffer to 42 °C.
5. Remove coverslips and wash the slides with washing buffer in Coplin jars at 42 °C.

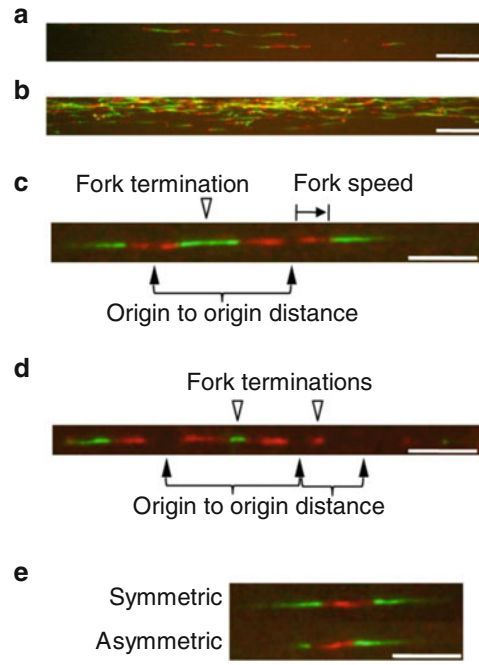

Fig. 3 Examples of stained DNA fiber tracts for analysis. (**a**) An example of properly spread DNA fiber tracts. (**b**) An area not suitable for analysis, as multiple fibers are overlapping. (**c**) An area of stained fiber tracts containing two adjacent origins with a fork termination event between them. Fork speed is obtained by measuring the distance from the start of the *red tract* to the start of the *green tract*. (**d**) An example of clustered origins with two fork termination events. (**e**) Examples of symmetric and asymmetric forks. Scale bars are 15 μm for **a** and **b** and 5 μm for **c**–**e**, respectively

6. Change wash buffer every 3 min for three total washes.
7. Take the slides out from washing buffer, remove excess liquid by waving off, drop 80 μl of antifade mounting medium supplemented with DAPI (1 μg/ml) and cover with coverslips (*see* **Note 11**).

3.2.5 Analysis of Labeled Fiber Tracts

First, check if DAPI-stained DNA fibers are extended well using a microscope at a lower magnification (e.g., 10× objective lens). Then, switch to a higher magnification (63× objective lens is recommended) to find areas where individual red-green fiber tracts are properly spaced (*see* Fig. 3a). Avoid areas with multiple, overlapping fiber tracts (Fig. 3b). Capture as many suitable images as possible for the following analyses.

1. *Fork speed*: Identify fibers with continuous red to green staining. Measure the distance from the beginning of the red tract to the point just before green labeling starts (Fig. 3c) using the ruler tool in Photoshop or other software. Afterwards, convert these

values from pixel units to kb of DNA. In our hands, 1 μm is approximately equal to 3.5 kb of DNA when determined with fluorescence in situ hybridization using a defined length of a specific probe, consistent with results published elsewhere [7]. However, it is strongly encouraged that this conversion be confirmed in each laboratory condition. Finally, divide these values by the labeling time (i.e., 20 min) to obtain fork speed values in kb/min.

2. *Origin to origin distance*: Origins are often identified as unstained areas sandwiched by a pair of red–green forks traveling in opposite directions (Fig. 3c). Alternatively, origins can be identified as the center of red tracts with two green tracts extending in opposite directions (*see* Fig. 3e). Find two or more pairs of such bidirectional forks adjacent to each other (presumed to be on the same fiber) and measure the distance between the centers of the two unstained areas or red tracts (origins). As seen in Fig. 3c, d, two green tracts from adjacent origins often merge, resulting in a fork termination event. Although rare, a short red tract can sometimes be found between two red–green tracts traveling in opposite directions (Fig. 3d). This short red tract is most likely formed by the merging of two red forks (before forming green tracts) coming from two adjacent origins, thus showing another type of termination point. Therefore, unstained areas surrounding this short red tract should also be counted as origins.
3. *Frequency of asymmetric forks*: Fork stalling events during the second labeling period can result in the asymmetric green labeling of bidirectional forks. To quantify asymmetric forks, first identify red–green bidirectional forks (Fig. 3e). Forks that have green labeling overlapping with adjacent bidirectional forks (*see* Fig. 3c for example) cannot be used. Measure the lengths of the two green tracts and compare them to each other by dividing the smaller by the larger. If a green tract length is less than two-thirds of the other, such forks can be considered asymmetric [8]. Determine the frequency of this event for the total number of forks analyzed.

3.3 Cytokinesis-Block MN and 53BP1-NB Assays

3.3.1 Plating Cells

1. Dip 22 × 22 coverslips in 70 % ethanol and set into wells of a 6-well plate.
2. Trypsinize cells and seed 0.25×10^6 cells per well.
3. Incubate for 2 days at 37 °C in a CO_2 incubator.

3.3.2 Cytochalasin B Treatment, Harvest and Fixation

1. Add a final concentration of 0.72 μg/ml cytochalasin B to each well for 4–5 h (*see* **Note 12**).
2. Wash the wells with 2 ml PBS and proceed to fixation using 2 ml 10 % formalin for 10–15 min.

3. Wash the wells twice with 2 ml PBS to remove remaining formalin.
4. Cells can now proceed to the next step or be stored at 4 °C for up to 1 week in the 6-well plate.

3.3.3 Antibody Staining

1. If performing the MN assay and not staining for CENP-A or 53BP1, proceed directly to Subheading 3.3.4.
2. Put small drops of Image iT™ FX signal enhancer onto Parafilm (one for each coverslip); Place coverslips cell side-down onto drops and incubate at room temperature for 30 min (*see* **Note 13**).
3. Return coverslips to PBS in the 6-well plate (*see* **Note 14**).
4. Prepare humidity chambers for primary antibody staining by inserting a piece of Parafilm inside of an old pipette tip box (with water in the bottom) (*see* **Note 15**).
5. Use the anti-CENP-A antibody in a 1/800 dilution, or the anti-53BP1 antibody in a 1/500 dilution with the blocking/permeabilization solution. Place in 60 μl drops onto Parafilm for each coverslip (maximum six per box); place coverslips cell side-down on drops and incubate overnight at 4 °C.
6. Return the coverslips to PBS in the 6-well plate and wash twice with fresh PBS.
7. Dilute the anti-rabbit secondary antibody to a 1/1,000 dilution in the blocking permeabilization solution; place 60 μl drops onto Parafilm for each coverslip; incubate at room temperature for at least 1 h, protected from light.
8. Return the coverslips to PBS in the 6-well plate.

3.3.4 DAPI Staining and Slide Preparation

1. Prepare a 1/5,000 dilution of DAPI in PBS (2 ml per well). Replace PBS with DAPI solution and incubate at room temperature for 10 min, protected from light.
2. Wash the coverslips 3–4 times in high salt PBS (2 min washes).
3. Place ~12 μl drops of antifade mounting medium onto new slide glasses (can fit two coverslips per slide). Mount coverslips and allow to dry.

3.3.5 Analysis of MN or 53BP1-NB

1. At higher magnification (use of 63× objective lens is recommended), use a DAPI filter to locate binucleated cells (cells with two nuclei that are touching or are very close together and that share a similar size/shape). Score all such binucleated cells for nearby MN (Fig. 4a). Up to 300 binucleated cells can easily be scored for each slide. If CENP-A staining was performed, up to 40 centromeric foci should be present in most cells. Switch between the DAPI filter and a red filter (e.g., Texas Red) to determine if a given MN contains a

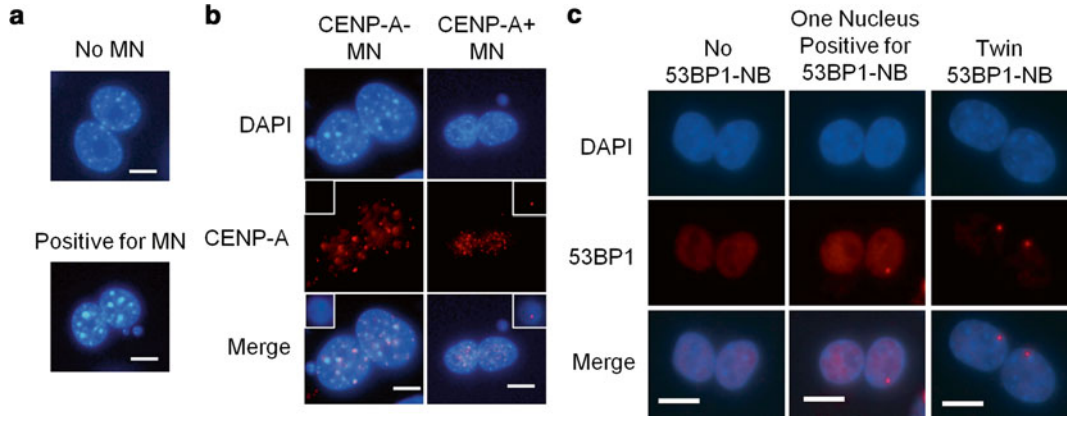

Fig. 4 Analyzing G1 phase binucleated cells for the presence of MN (CENP-A+ or CENP-A–) or 53BP1-NB. (**a**) Examples of binucleated cells negative (*top*) and positive (*bottom*) for MN are shown. (**b**) CENP-A staining (*red*) can be used to determine which MN do (*right*) and do not (*left*) contain centromeres. Embedded boxes show enlarged images of individual MN. (**c**) 53BP1 staining (*red*) reveals binucleated cells that have no nuclei positive for 53BP1-NB (*left*), one nucleus positive for a single 53BP1-NB (*middle*) and one with both nuclei positive for single 53BP1-NB (*right*). Notice the exquisitely symmetrical appearance of the 53BP1-NB in the image on the right. Nuclei are stained with DAPI (*blue*). All scale bars are 10 μm

CENP-A signal or not (Fig. 4b). If 53BP1 staining was performed, score the individual nuclei of binucleated cells for the presence of discrete 53BP1-NB (Fig. 4c) using a red filter (e.g., Texas Red). Often times both nuclei will be positive for 53BP1-NB in a symmetrical pattern.

4 Notes

1. This product comes free with purchase of the anti-rabbit secondary antibody from Subheading 2.3 as of May 2013.
2. Hypotonic treatment helps bulky molecules such as biotinylated and digoxigenin-conjugated dUTPs be incorporated into cells [17]. An alternative way for dUTP incorporation is the use of beads as described elsewhere [15].
3. As the modified dUTPs are incorporated into newly synthesized DNA, the pool of free modified dUTPs decreases. Accordingly, the intensity of the labeling gradually fades, enabling us to determine the direction of fork movement.
4. Just 30 μl of the mixture should sufficiently cover the surface of a well. If not, it can be increased to 40 or 50 μl at the expense of additional reagents.

5. Non-labeled cells act as carriers to allow for the extension of labeled DNA fibers. The ratio of non-labeled to labeled cells in this protocol is 40:1, though this can be altered depending on the desired density of labeled fibers.
6. The fixed cells can be stored for several hours. However, overnight incubation causes excess fixation, leading to insufficient cell lysis and poor DNA fiber extension. Therefore, it is strongly recommended to prepare the slides within the same day the cells are fixed.
7. Cells will stack on the edge of the frosted area during lysis and extension. This prevents DNA fibers from running off the slide and helps them to extend in a straightened manner.
8. After fixation, concentrated threads of DNA can usually be seen as faint white stains on slides. If not, it is recommended to stain one slide with DAPI to confirm the presence of stretched DNA fibers on it before proceeding.
9. We use small 12×9 cm plastic trays with three small stands assembled by piles of Parafilm within. Slides are placed on top of each stand so that each tray can hold up to three slides. Put a few drops of distilled water in the corners of each tray to keep it humidified and cover the top with a piece of Parafilm, sealing it tight by placing another tray on top. Alternative containers can also be used if kept level and in a humidified environment.
10. Red staining can be substantially weaker than green staining. If this is the case, alternative staining using a sheep anti-digoxigenin primary antibody and a secondary antibody for anti-sheep IgG conjugated with Alexa 594 can be used to intensify the red signal.
11. Stained slides can be stored at 4 °C for several weeks.
12. With longer incubations, it seems as though binucleated cells continue to progress through the cell cycle anyway, which may disrupt the ability to analyze cells that have just completed mitosis.
13. Excess liquid can be removed from the coverslips by touching the edges with a dry KimWipe before placing them for incubation. This can be done prior to antibody staining as well.
14. To avoid losing cells in this process, it is best to add ~0.5 ml of PBS to the edges of each coverslip before lifting them off the Parafilm.
15. We prepare the humidity chamber using old 1,000 μl tip boxes from TipOne. The region beneath the tip holder is filled about 1/4 full of distilled water. Then the tip holder is put back into place before a piece of Parafilm is placed on top.

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Chapter 20

Transgenic Nude Mice Ubiquitously Expressing Fluorescent Proteins for Color-Coded Imaging of the Tumor Microenvironment

Robert M. Hoffman

Abstract

We have developed a transgenic green fluorescent protein (GFP) nude mouse with ubiquitous GFP expression. The GFP nude mouse was obtained by crossing nontransgenic nude mice with the transgenic C57/B6 mouse in which the β-actin promoter drives GFP expression in essentially all tissues. In the adult mice, many organs brightly expressed GFP, including the spleen, heart, lungs, spleen, pancreas, esophagus, stomach, and duodenum as well as the circulatory system. The liver expressed GFP at a lesser level. The red fluorescent protein (RFP) transgenic nude mouse was obtained by crossing non-transgenic nude mice with the transgenic C57/B6 mouse in which the beta-actin promoter drives RFP (DsRed2) expression in essentially all tissues. In the RFP nude mouse, the organs all brightly expressed RFP, including the heart, lungs, spleen, pancreas, esophagus, stomach, liver, duodenum, the male and female reproductive systems; brain and spinal cord; and the circulatory system, including the heart, and major arteries and veins. The skinned skeleton highly expressed RFP. The bone marrow and spleen cells were also RFP positive. The cyan fluorescent protein (CFP) nude mouse was developed by crossing nontransgenic nude mice with the transgenic CK/ECFP mouse in which the β-actin promoter drives expression of CFP in almost all tissues. In the CFP nude mice, the pancreas and reproductive organs displayed the strongest fluorescence signals of all internal organs, which vary in intensity. The GFP, RFP, and CFP nude mice when transplanted with cancer cells of another color are powerful models for color-coded imaging of the tumor microenvironment (TME) at the cellular level.

Key words Nude mouse, Transgenic, GFP, RFP, CFP, Tumor microenvironment, Color-coded imaging

1 Introduction

A number of attempts have been made to visualize the tumor–host interaction. Fukumura et al. [1] and Brown et al. [2] have used transgenic mice that express the green fluorescent protein (GFP) under the control of the human vascular endothelial cell growth factor (VEGF) promoter and observed that the VEGF promoter is activated by the tumor microenvironment [1, 2]. However,

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these models did not enable simultaneous imaging of tumor and host cells.

Okabe et al. [3] produced transgenic mice with GFP under the control of a chicken β-actin promoter and cytomegalovirus enhancer. Most of the tissues from these transgenic mice, with the exception of erythrocytes and hair, fluoresce bright green.

Nagy's group developed an RFP variant, DsRed.T3, enabling them to produce a transgenic red fluorescent protein (RFP) mouse [4]. The organs of the RFP mouse are exceedingly bright.

The cyan fluorescent (CFP) mouse was also developed by Nagy's laboratory [5]. CFP is driven by the β-actin promoter similar to the GFP and RFP transgenic mice. In the CFP mouse, the pancreas is the most fluorescent compared to the other organs and to the rest of the GI tract. There is differential expression of blue fluorescence among the various organs [6].

2 Materials *(See Notes 1–3)*

2.1 Transgenic Green Fluorescent Protein Nude Mice

1. Transgenic C57/B6-GFP mice were obtained from Prof. Masaru Okabe (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan).
2. C57/B6-GFP mice express GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer [3].

2.2 Transgenic Red Fluorescent Protein Nude Mice (See Note 4)

1. Transgenic C57/B6-RFP mice expressed RFP (DsREDT3) under the control of a chicken beta-actin promoter and cytomegalovirus enhancer.
2. All of the tissues from this transgenic line, with the exception of erythrocytes, were red under blue excitation light.

2.3 Transgenic Cyan Fluorescent Protein Nude Mice

1. Transgenic CK6/CFP mice express cyan fluorescent protein under the control of the chicken β-actin promoter coupled with the cytomegalovirus (CMV) immediate early enhancer [5].
2. Expression of CFP is variable in these mice, with the pancreas having the brightest fluorescence.

2.4 Animal Care

1. Transgenic athymic nu/nu nude mice and transgenic nu/nu GFP, REP, and CFP nude mice were maintained in a barrier facility on high efficiency particulate air (HEPA)-filtered racks.
2. The animals were fed with autoclaved laboratory rodent diet (Teklad LM-485; Western Research Products).
3. Anesthesia for surgical procedures and imaging used intramuscular injection of 0.02 ml of a solution of 50 % ketamine, 38 % xylazine, and 12 % acepromazine maleate.

2.5 In Vivo Imaging with the OV100

1. For in vivo imaging based on fluorescent proteins [7–10], the Olympus OV100 Small Animal Imaging System (Olympus Corp) was used. The Olympus OV100, which contains an MT-20 light source (Olympus Biosystems) and DP70 CCD camera (Olympus), was used for cellular imaging in live mice.
2. The optics of the OV100 fluorescence imaging system has been specially developed for macro-imaging as well as micro-imaging with high light-gathering capacity.
3. The instrument incorporates a unique combination of high numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 10^5-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal.
4. The OV100 has the lenses mounted on an automated turret with a high magnification range of 1.6–16× and a field of view from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens).
5. Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell® (Olympus Biosystems) [11].

2.6 In Vivo Imaging with the IV100 Laser Scanning Microscope

1. The Olympus IV100 is a scanning laser microscope. A 488-nm argon laser was used. The novel stick objectives (as small as 1.3 mm) were designed specifically for this laser scanning microscope.
2. The very narrow objectives deliver very high-resolution images.
3. A PC computer running FluoView software (Olympus Corp.) was used to control the microscope.
4. All images were recorded and stored as proprietary multilayer 16-bit Tagged Image File Format files [12].

2.7 In Vivo Imaging with the Fluoview FV1000 Confocal Laser Scanning Microscope (See Note 5)

1. A Fluoview FV1000 confocal laser scanning microscope with a XLUMPLFL .20 (0.95 numerical aperture [NA]) water immersion objective was used for imaging. GFP was excited at 488 nm, and RFP was excited at 559 nm with an Argon laser.
2. Images were produced with FV10-ASW Fluoview software (Olympus) and ImageJ (NIH) and were not modified beyond the standard adjustment of intensity levels [13].

2.8 In Vivo Imaging with the UVP iBox Small Animal Imaging System

1. The iBox (UVP) was equipped with a BioChemi HR 500 CCD camera and a BioLite automated multispectral light source (UVP) delivered to the dark imaging chamber via fiber optics and was used for imaging the CFP mouse [6].

2.9 Fluorescence Microscopy

2. An Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-W lamp power supply was used.
3. To visualize both GFP and RFP fluorescence at the same time, excitation was produced through a D425/60 bandpass filter, 470 DCXR dichroic mirror, and emitted fluorescence was collected through a long pass filter (Chroma Technology).
4. High-resolution images of 1,024×724 pixels were captured with a Hamamatsu C5810 three-chip cooled color charge-coupled device camera (Hamamatsu Photonics Systems) and directly stored on an IBM personal computer. Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus 4.0 software (Media Cybernetics) [14].

3 Methods

3.1 Transgenic Green Fluorescent Protein Nude Mice

1. Cross the GFP female C57/B6 mice with 6–8-week-old BALB/c *nu/nu* or NCR *nu/nu* male mice.
2. Cross male F_1 mice with female F_1 C57/B6 GFP mice to obtain GFP nude mice. When female F_2 immunocompetent GFP mice are crossed with male GFP nude mice or when F_2 GFP nude male mice are back-crossed with female F_1 immunocompetent GFP mice, approximately 50 % of their offspring are GFP nude mice. GFP nude mice can be consistently produced by the methods described above [14].

3.2 Transgenic Red Fluorescent Protein Nude Mice

1. Cross 6-week-old transgenic RFP female mice with both 6–8-week-old BALB/c *nu/nu* and NCR *nu/nu* male mice, respectively.
2. Male F_1 fluorescent nude mice are crossed with female F_1 immunocompetent RFP mice. When female F_2 immunocompetent RFP mice are crossed with male RFP nude or using F_2 RFP nude male back crossed with female F_1 immunocompetent RFP mice, approximately 50 % of their offspring were RFP nude mice.
3. RFP nude mice are then consistently produced using the methods described above [15].

3.3 Transgenic Cyan Fluorescent Protein Nude Mice

1. Cross 6-week-old transgenic CFP female mice with 6–8-week-old BALB/c *nu/nu* or NCR *nu/nu* male mice.
2. Male F_1 CFP nude mice are crossed with female F_1 immunocompetent CFP mice.
3. When male F_2 *nu/nu* CFP mice are crossed with female F_1 or F_2 *nu/+* CFP mice, approximately 50 % of their offspring are transgenic CFP nude mice.
4. This schema can reliably and consistently produce nude CFP mice [6].

Fig. 1 GFP expression in the tissues and cells of the transgenic GFP nude mouse. Newborn and adult mice fluoresce brilliant, *bright green* under blue light excitation. The fluorescence could be detected with a simple blue-light-emitting diode flashlight with a central peak of 470 nm and a bypass emission filter [14]

3.4 Animal Care

1. All animal studies are conducted in accordance with principles and procedures outlined in the NIH Guide for the Care and Use of Animals under PHS Animal Welfare Assurance No. A3873-1 [6].

3.5 Cell Culture

1. Culture human and mouse cell lines, expressing fluorescent proteins such as GFP and/or RFP or non-expressing cell lines, at 37 °C in a 5 % incubator [16].

3.6 Tumor Tissue Sampling

1. Obtain tumor tissue at different time points after orthotopic inoculation of the tumor cells. Fresh tissues are cut into ~1-mm^3 pieces or very thin slices under the microscope.
2. Pressed sections are then made for observation and imaging.
3. For analysis of tumor angiogenesis, the tissues are digested with trypsin–EDTA at 37 °C for 5 min before examination. After trypsinization, tissues are put on a precleaned microscope slide and covered with another microscope slide [14].

3.7 Image Processing (See Notes 6–8)

1. Analyze all images using Image-J, and process for contrast and brightness using Photoshop Element-4 [6].

3.8 Representational Results Using Above Protocols

3.8.1 Development of the Green Fluorescent Protein Nude Mouse

1. In crosses between *nu/nu* GFP male mice and *nu/+*GFP female mice, the resultant embryos were green, apparently at the single-cell stage. Newborn mice and adult mice were very bright green (Fig. 1).
2. Green fluorescence could be detected with a simple blue-light-emitting diode flashlight with a central peak of 470 nm and a bypass emission filter [14].

*3.8.2 Discription of the Green Fluorescent Protein Nude Mouse (See **Note 9**)*

1. In the adult mice, the organs brightly expressed GFP, including the heart, lungs, spleen, pancreas, esophagus, stomach, adrenal gland, kidney, and duodenum. The entire digestive system, from tongue to anus, was dissected out and could be seen to fluoresce brilliant green upon blue light excitation. The male and female reproductive systems were dissected out, and all components fluoresced bright green on blue light excitation. The liver faintly expresses GFP.
2. The dissected brain and spinal cord also had brilliant GFP fluorescence. The dissected out circulatory system, including the heart and major arteries and veins, had a brilliant green fluorescence. The skinned skeleton highly expressed GFP. Pancreatic islets showed GFP fluorescence. The spleen cells were also GFP positive [14].

3.8.3 Dual-Color Imaging of Tumor–Host Cell Interaction in the Green Fluorescent Protein Nude Mouse

1. HCT-116-RFP human colon cancer cells, growing in the GFP mouse, invaded the GFP host tissue. HT1080-RFP human fibrosarcoma cells and GFP host fibroblasts and endothelial cells were observed interacting.
2. Stromal cells expressing GFP interacted with the human HT1080-RFP cells. Macrophages expressing GFP were visualized engulfing HT1080-RFP cancer cells (Fig. 2) [14].

3.8.4 Imaging Recruitment of Cancer-Associated Fibroblasts by Colon-Cancer Liver Metastasis

1. Human non-colored HCT-116-colon cancer cells were injected in the spleen of GFP nude mice. High-magnification fluorescence microscopy showed extensive GFP fluorescence in the tumor after 28 days. There was also significantly more GFP fluorescence in the liver metastasis compared to the non-tumor area of the liver. Thus, the host non-parenchymal liver cells expressing GFP were imaged being extensively recruited in the liver metastasis.
2. Expression of desmin was determined by immunohistochemistry with anti-desmin antibody and found to be positive in the tumor area. Thus, the liver metastasis contained cells co-expressing GFP and desmin, suggesting that cancer-associated fibroblasts (CAFs) were recruited and grew in the metastatic tumor [16].

3.8.5 Noninvasive Color-Coded Imaging of Tumors at the Subcellular Level in the Live Unperturbed Mouse

1. MMT mouse breast cancer cells were double-labeled expressing GFP in the nucleus and RFP in the cytoplasm and injected in the footpad of GFP nude mice and formed a tumor. Cancer cells become interdispersed between the stromal cells in the tumor.
2. The GFP-expressing stromal cells were a very significant portion of the tumor. Dividing cancer cells could be visualized by their two GFP nuclei. Blood vessels were prominently seen in the tumor and GFP-expressing lymphocytes were visualized flowing in them (Fig. 3) [12].

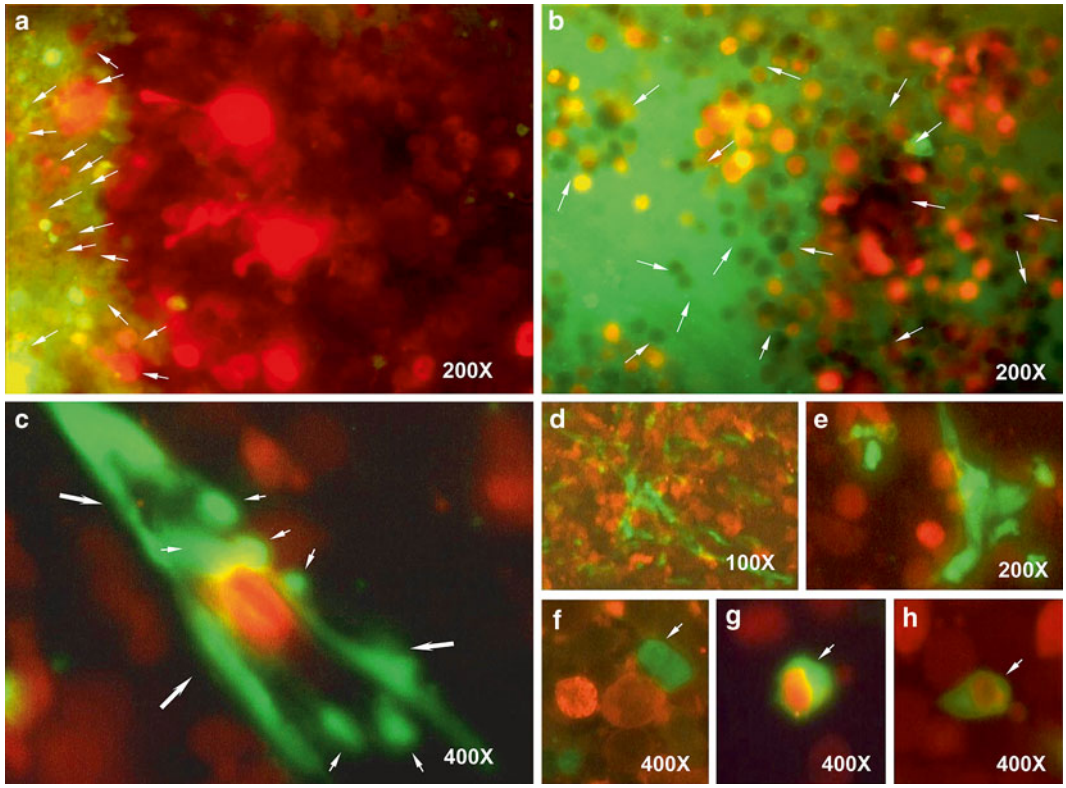

Fig. 2 Visualization of human tumor-host interaction **a** and **b**: RFP-expressing HCT-116-RFP human colon cancer cells invading the GFP nude mouse host tissue 6 weeks after implantation. (**c**) HT1080-RFP human fibrosarcoma cell surrounded by GFP nude mouse host stromal cells 6 weeks after implantation. (**d**) HT1080-RFP fibrosarcoma with host GFP stromal cells among the tumor cells. (**e**) High magnification of **d** showing intimate interaction of HT1080-RFP tumor cells with GFP-expressing host stromal cells. (**f-h**) Interaction and engulfment of HT1080-RFP tumor cells by host GFP-expressing macrophages [14]

Fig. 3 RFP nude mouse. Newborn and adult mice fluoresce brilliant *bright red* under blue light excitation. The fluorescence could be detected with a simple blue-light-emitting diode flashlight with a central peak of 470 nm and a bypass emission filter [15]

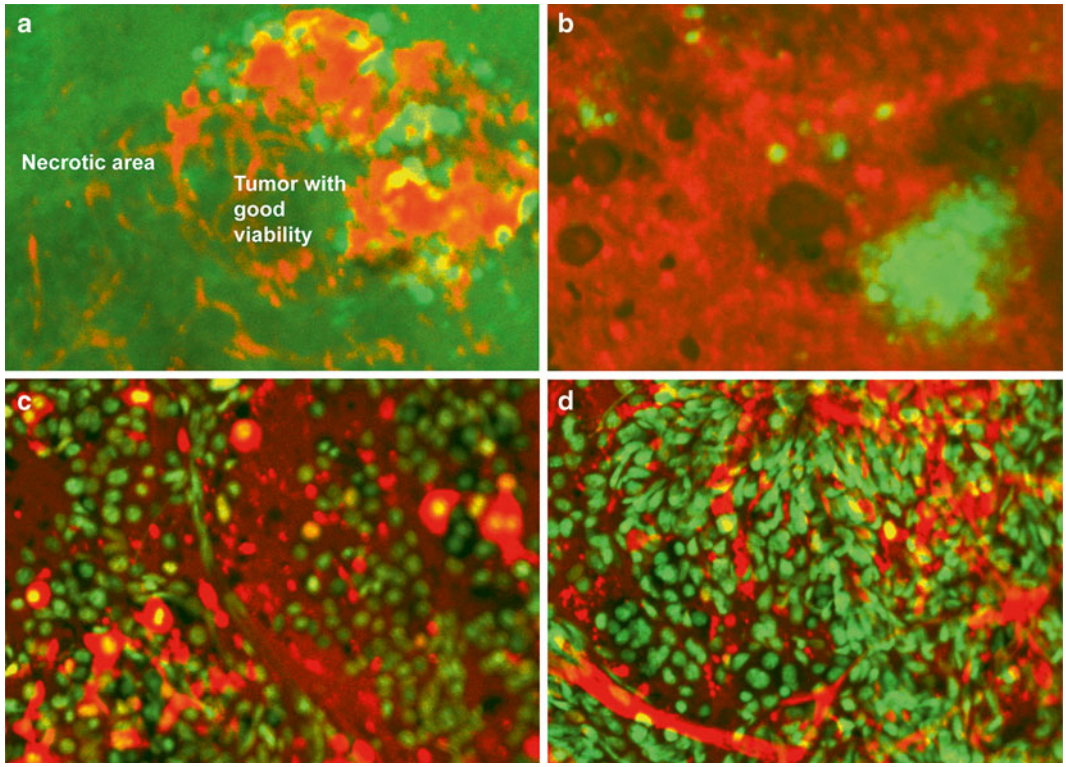

Fig. 4 Tumor-host interaction and tumor microenvironment. (**a**) Tumor vasculature in viable GFP-expressing PC-3 tumor tissue and necrotic tumor tissue in the same tumor mass. RFP-expressing tumor vasculature can be readily identified in the area where the tumor tissue maintained good viability. However, only remnants of RFP-expressing vasculature can be visualized in the necrotic area. (**b**) GFP-expressing PC-3 cancer cells can be visualized in the lung of RFP nude mouse 8 weeks after tumor implantation. (**c**) Numerous dying B16F10-dual-color melanoma cells can be visualized in the footpad in the area the tumor vasculature is lacking. (**d**) Numerous well-developed, host-derived RFP-expressing blood vessels were visualized in the footpad in the GFP-expressing-mouse melanoma 2 weeks after subcutaneous injection of B16F10-dual-color melanoma cells in the transgenic RFP mouse. Images were taken with the Olympus IV100 intravital scanning microscope using tumor tissue obtained from the footpad [15]

3.8.6 Noninvasive Color-Coded Imaging of the Effects of Chemotherapy in Real Time on Cancer and Stromal Cells

1. In the MMT footpad tumor model described above, the cancer cells are sensitive to doxorubicin. Tumor blood vessels could be visualized in the MMT tumor in the doxorubicin-treated live mouse, but they were now devoid of GFP lymphocytes (Fig. 6).
2. Twelve hours after treatment with doxorubicin, the RFP cytoplasm fragmented from the cancer cells, and the number of cancer cells was significantly reduced. The GFP host stromal cells become highly elongated [12] (Fig. 6).

3.8.7 The RFP Nude Mouse Expresses RFP Essentially in All Tissues

1. The RFP nude mouse has brilliant red fluorescence (Fig. 4).
2. All of the tissues from this transgenic line, including brain, heart and lungs, liver, the circulatory system, uterus and ovary, pancreas, kidney, and spleen, with the exception of erythrocytes, were red fluorescent [15].

3.8.8 Tumor–Host Interaction and Tumor Microenvironment (TME)

1. Dual-color fluorescence imaging enabled visualization of cancer cells expressing GFP in the nucleus and RFP in the cytoplasm, interacting with RFP-expressing host cells (Fig. 4) [15].

3.8.9 Characterization of the Transgenic Cyan Fluorescent Protein (CFP) Nude Mouse

1. The CFP nude mouse expresses blue fluorescence in nearly all its tissues but at much different intensities. The musculoskeletal system displayed strong blue fluorescence. Internal organs expressed varying levels of CFP expression.
2. The lungs, cardiovascular system, kidneys, and adrenal glands, as well as the neurological system, were very weakly fluorescent. The liver had a low level of blue fluorescence. Both female and male reproductive organs brightly fluoresced blue after a 1-s exposure. The pancreas displays the strongest CFP blue fluorescence signal [6].

3.8.10 Host–Tumor Interaction in the Tumor Microenvironment of the CFP Mouse

1. XPA-1-GFP-RFP human pancreas cancer cells expressing GFP in the nucleus and RFP in the cytoplasm were implanted in the pancreas of the CFP mouse.
2. There was a region of heavy desmoplastic activity with infiltrating XPA-1-GFP-RFP cancer cells [6].

3.8.11 Visualizing the TME of Liver and Lung Metastasis with RFP Cancer Cells in the CFP Nude Mouse

1. HCT-116-RFP cancer cells implanted in the spleen of CFP nude mice formed metastasis in the liver. HCT-116-RFP cancer cells transplanted in the tail vein of CFP nude mice, formed tumors in the lung. In both liver and lung, CFP-expressing CAFs were visualized by confocal fluorescence imaging, invading the RFP tumor.
2. The Olympus FV1000 confocal microscope enabled clear imaging of cancer-cell–stromal-cell interaction [17].

3.8.12 Four-Color-Coded TME Imaging Model in the Liver of the CFP Mouse

1. Dual-color MMT cells with GFP in the nucleus and RFP in the cytoplasm invaded the sinusoids of the CFP liver 3 days after splenic injection.
2. Non-parenchymal liver cells had bright CFP fluorescence and hepatocytes had lighter CFP fluorescence and were distinguishable from non-parenchymal liver cells.
3. Mitotic MMT-GFP-RFP cells were observed surrounded by non-parenchymal liver cells and hepatocytes (Fig. 5) [17].

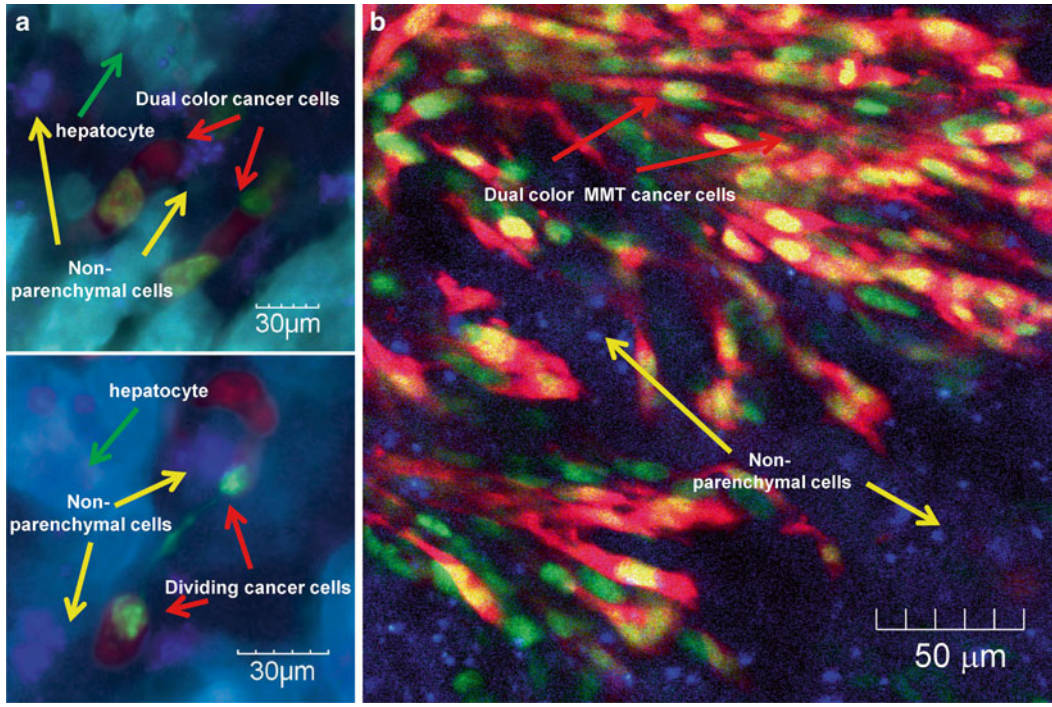

Fig. 5 Dual-color MMT cells with GFP in the cytoplasm and RFP in the nucleus, growing in the liver of a CFP nude mouse after splenic injection. (**a**) Dual-color MMT cells (*red arrows*) were observed in a sinusoid of the liver 3 days after splenic injection. Non-parenchymal liver cells had strong CFP fluorescence (*yellow arrows*). Hepatocytes had weaker CFP fluorescence (*green arrows*). The image was obtained with an FV1000 confocal microscope. (Bar = 30 μm). (**b**) Dual-color MMT cells formed tumors in the liver of a CFP mouse 28 days after splenic injection. Hepatocytes, nonparenchymal liver cells (*yellow arrows*) and dual-color MMT cancer cells (*red arrows*) were visualized simultaneously. The image was taken with an FV1000 confocal microscope. (Bar = 50 μm) [17]

4 Notes

1. The luciferase reporter technique requires that animals are anesthetized and restrained so that sufficient photons can be collected to construct a pseudo-image. Furthermore, this process must be carried out in an almost light-free environment and animals must be injected with the luciferin substrate, which has to reach every tumor cell to be useful. Detection of luciferase labeled cells in vivo is very low resolution and cellular imaging is not possible and the clearance of the luciferin results in an unstable signal. These limitations preclude studies that would be perturbed by anesthesia, restraint or substrate injection and also makes high-throughput screening infeasible [7].

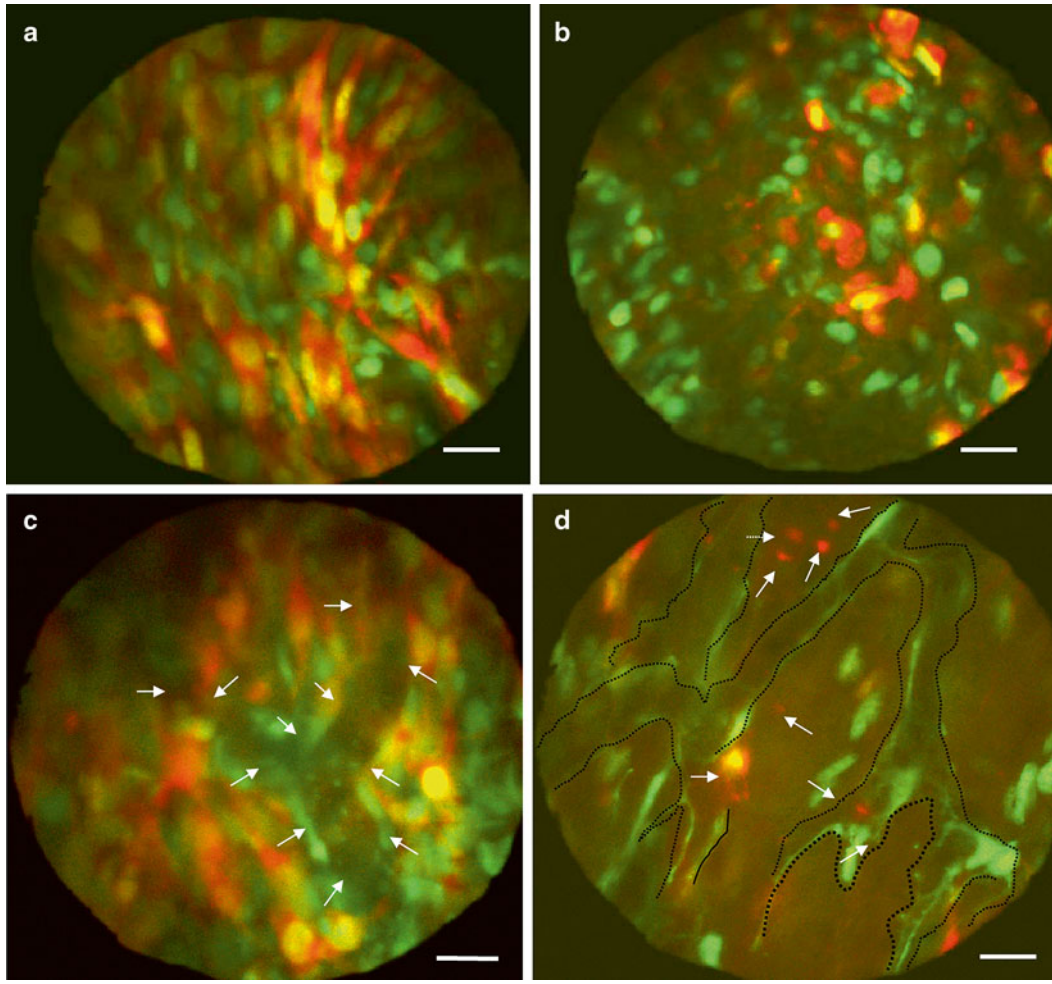

Fig. 6 Whole-body, noninvasive, subcellular imaging of drug response of dual-color mouse mammary cancer cells and GFP stromal cells in the live GFP nude mouse with and without doxorubicin. Dual-color MMT cells were injected in the footpad of GFP transgenic nude mice. (**a**) Whole-body image of untreated dual-color MMT cells in the footpad of a live GFP mouse. Note the numerous spindle-shaped dual-color MMT cells interdispersed among the GFP host cells. (**b**) Whole-body image of MMT dual-color cancer cells in a live GFP nude mouse 12 h after treatment with doxorubicin (10 mg/kg). The cancer cells lost their spindle shape, and the nuclei appear contracted. (**c**) Whole-body image of dual-color MMT tumor. Numerous dual-color spindle-shaped MMT cells interacted with GFP-expressing host cells. Well-developed tumor blood vessels and real-time blood flow were visualized by whole-body imaging (*arrows*). (**d**) In vivo drug response of dual-color MMT tumor 12 h after i.v. injection of 10 mg/kg doxorubicin. All of the visible MMT cells lost their spindle shape. Many of the cancer cells fragmented (*arrows*). Tumor blood vessels were damaged (*dashed black lines*), and the number of cancer cells was dramatically reduced 12 h after chemotherapy. Bar = 20 μm [12]

2. In comparison with the luciferase reporter, GFP and other fluorescent proteins in use have a much stronger signal and can therefore be used to image unrestrained animals—irradiation with non-damaging blue light is the only step needed. Real images can be captured using simple apparatus and

there is no need for total darkness. The fluorescence intensity of GFP and other fluorescent proteins is very strong and individual cells or even subcellular structures can be imaged in the animal [18].

3. Be careful of misconceptions in the literature. For example, the following was just published "Whole animal fluorescence imaging in vivo suffers from low signal-to-noise as a result of background autofluorescence, modeling-dependent photon quantification, photobleaching, low tissue penetration, and low resolution" [19]. Despite enormous evidence to the contrary, as outlined above, this type of misinformation continues to be published even in 2013.
4. The GFP, RFP and CFP nude mice appear to have a life span similar to that of non-transgenic nude mice, such that long-term tumor growth and metastasis studies can be carried out [14]. Thus, fluorescent proteins are not toxic to the animal.
5. The non-invasive subcellular imaging [12] is a significant improvement from the inserted-window models of Jain et al. [20] or abdominal imaging window [21] or of the skin flap models we have previously developed to image tumors [11, 22]. Cancer cells were always interdispersed among the host stromal cells in the tumor microenvironment. Such observations were facilitated by using the Olympus IV100 Intravital Laser Scanning Microscope, the GFP nude mouse as a host, and cancer cells expressing RFP in the cytoplasm and GFP in the nucleus. It is possible to image chemotherapy responses of tumors in real time at the subcellular level, which distinguishes efficacy on cancer and stromal cells as well on circulating lymphocytes [12].
6. Bleeding should be avoided at the surgical site, as hemoglobin will absorb the incident excitation light [8–10].
7. When doing a skin flap or open-biopsy procedures, it is essential to hydrate the animal by spraying normal saline on the open tissue [8–10].
8. When doing repeat procedures such as a skin flap, open biopsy or other invasive procedures, it is critical to maintain a properly sterile operation field [8–10].
9. It is important to minimize autofluorescence of tissue and body fluids by using proper filters. Excitation filters should have a narrow band as close to 490 nm as possible to specifically excite GFP whose peak is distinct from that of the skin, tissues and fluid of the animal. In addition, proper band-pass emission filters should be used with a cutoff of approximately 515 nm [8–10].

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Chapter 21

Genetically Engineered Insertional Mutagenesis in Mice to Model Cancer: Sleeping Beauty

Viive M. Howell and Emily K. Colvin

Abstract

The ability to accurately model human cancer in mice enables in vivo examination of the biological mechanisms related to cancer initiation and progression as well as preclinical testing of new anticancer treatments and potential targets. The emergence of the genetically engineered Sleeping Beauty system of insertional mutagenesis has led to the development of a new generation of genetic mouse models of cancer and identification of novel cancer-causing genes. This chapter reviews the published cancer models of Sleeping Beauty and strategies using available strains to generate several models of cancer.

Key words Sleeping beauty, Mouse models of cancer, Insertional mutagenesis, Cancer genetics, Lymphoma, Leukemia, Genetically engineered mouse model, Hepatocellular cancer, Lung cancer, Pancreatic cancer

1 Introduction

1.1 What is Sleeping Beauty?

Sleeping Beauty (SB) is a 2-transgene system of random mutagenesis able to induce cancer in mice [1]. One transgene carries an array of DNA mobile elements or transposons that can be mobilized or "cut" from the parent transgene and reinserted or "pasted" elsewhere in the genome. The second transgene expresses the SB transposase enzyme that catalyzes the cut and paste activity of the transposons (Fig. 1). When the transposase is activated in the presence of the transposon array transgene, the cut and paste activity of the transposons ensues with random reintegration of transposons elsewhere in the genome.

Reintegration of a transposon within a gene results in insertional mutation of that gene. This may lead to inappropriate gain or loss of expression of the full length or truncated transcript. The consequences of the mutation, or a series of insertional mutations, may confer an advantage to the cell such as an increased rate of proliferation, survival or loss of apoptotic responses. A selective advantage may lead to clonal expansion and tumorigenesis (Fig. 1).

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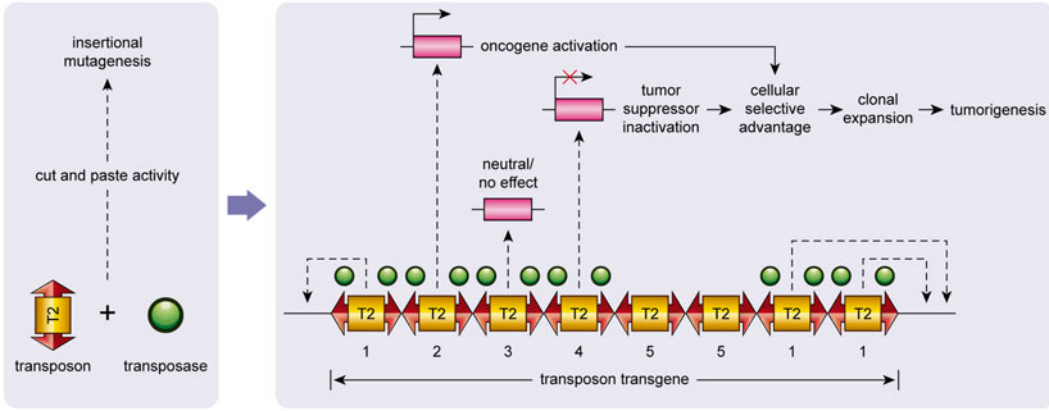

Fig. 1 Sleeping Beauty insertional mutagenesis and the induction of tumorigenesis. In the presence of transposase the transposons are mobilized and inserted elsewhere in the genome by "cut and paste" activity resulting in insertional mutagenesis. The numbers beneath the transposon transgene represent different event possibilities arising simultaneously from the parent transposon transgene: Event *1* illustrates local hopping with the transposons reintegrating into the same chromosome as the parent transgene; *2–4* show random reintegration in genes in other chromosomes leading to different mutational effects; *5* shows no mobilization due to lack of transposase activity. When the insertional mutagenesis event provides a selective advantage for the cell, as depicted in events *2* and *4*, a clonal expansion may occur and lead to the induction of tumorigenesis. This figure is reproduced from ref. 2 with permission from Elsevier

In addition to its ability as a mutagen to rapidly induce cancer in mice carrying the two SB transgenes, SB facilitates easy identification of the mutated genes as each is "tagged" with the transposon. These two advantageous features have led to the widespread use of SB and the development of a new generation of genetic SB-induced mouse models of cancer.

1.2 How Does Sleeping Beauty Work?

The SB system is a synthetic reconstruction of transposable elements identified in ancient fish genomes [3]. Each transposon is flanked by transposase binding sites to direct the transposase to the transposon to initiate the cut and paste activity (Fig. 2).

1.2.1 Reintegration Sites

TA sequences are required for reintegration of the transposon. The genomic landscape surrounding the TA site may hinder reintegration, and it is estimated that only 20 million of a possible 200 million sites are amenable to reintegration [4]. While reintegration is site specific, the number of possible sites far exceeds the number of known genes, and thus reintegration may still be considered essentially random.

1.2.2 The Transposase

The site of reintegration is also dependent on the activity level of the transposase. Different activity levels of the transposase lead to different distances travelled by the transposon. If the activity is weak (e.g., in *CAGGS-SB10*), the transposons are most likely to reintegrate close to the parent transgene; a phenomenon termed

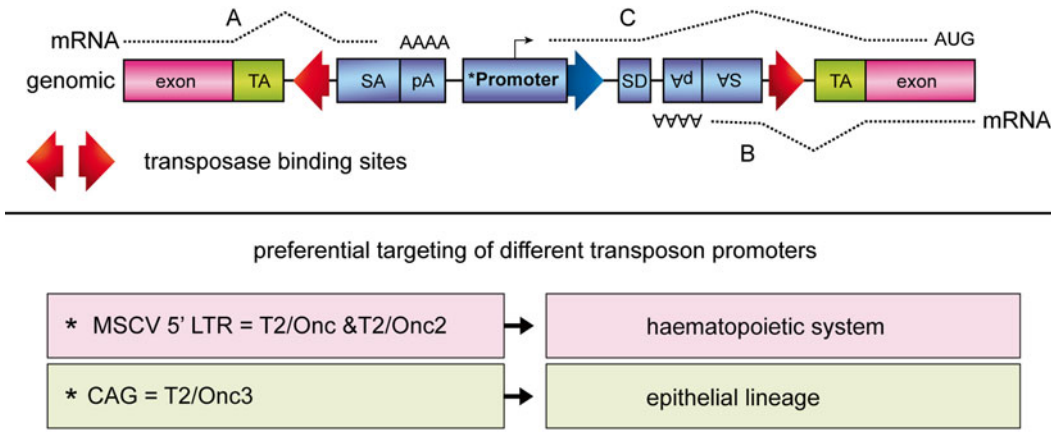

Fig. 2 Transposon actions following reintegration in the mouse genome. The transposase binding sites (*red arrows*) that flank each transposon (*blue*) are inverted repeats/direct repeats which bind to the transposase to excise and reintegrate the transposon. Reintegration requires TA sites (*green*) in the genome. These sites can be in coding or noncoding regions. The transposon is bidirectional with both loss- and gain-of-function elements so that it can mutate both tumor suppressor genes and oncogenes upon reintegration in the mouse genome. *A* and *B* show loss of function events through premature termination of the mRNA following insertion of the transposon in the forward (*A*) or reverse (*B*) orientation. In these cases the transposon may act as a genetrap if inserted within a gene. Its strong splice acceptor (SA) diverts splicing from the upstream exon (*pink*) into the transposon and the downstream polyadenylation signal (pA) in the transposon that initiates premature termination of the transposon-trapped transcript. *C* shows initiation of an mRNA by the promoter/enhancer element of the transposon. The strong splice donor (SD) of the transposon directs splicing from the transposon into downstream endogenous exons. This may result in over-expression of a partial protein if a downstream endogenous exon contains an in-frame start codon (AUG) for translation initiation. The different promoter elements (*asterisk*) used in the SB transposons (T2/Onc and T2/Onc2 and T2/Onc3) demonstrate preferential, but not complete, targeting of different cell lineages as listed. This figure is reproduced from ref. [2] with permission from Elsevier. MSCV *murine stem cell virus*; *5′ LTR* 5-prime long terminal repeat, *CAG* chicken β-actin

"local hopping" [1]. Modifying the design of the transposase (e.g., SB11, SB13), and controlling for positional effects from the endogenous promoter in a random transgenic model (*CAGGS-SB10*) by changing to a knock-in at the validated *Rosa26 locus* (*Rosa26-SB11*) increased the activity of the transposase and decreased local hopping [5, 6]. These changes to the transposase design also led to an increased penetrance of a cancer phenotype. The knock-in *Rosa26-SB transposase* was further refined by the addition of a floxed stop codon (LSL) [7]. This conditional allele enables spatial and temporal control over the SB system and facilitated the generation of tissue specific cancer models.

1.2.3 The Transposon Mutational Consequences

Once a transposon has reintegrated within a gene, the consequences of the insertional mutation can be driven by the transposon itself. Strong bidirectional elements that initiate splicing have been engineered into the transposons (Fig. 2). These can lead to splicing into the transposon from the flanking gene, and

truncation of the transcript. The transposon design includes a strong internal promoter, which can override the gene specific promoters and initiate inappropriate transcription of full length or truncated genes.

The Transposon Influences Tumor Spectrum

The transposon promoter is a major determinant of the penetrance of cancer and the resulting tumor spectrum. The early transposons (original low copy T2/Onc and optimized high copy T2/Onc2) incorporated the *murine stem cell virus 5′ long terminal repeat* (*MSCV 5′LTR*) which has strongest activity in the hematopoietic system [6]. When these promoters were used in conjunction with germ line or ubiquitous transposase expression, the tumor spectrum was biased towards hematopoietic and other soft tissue sarcomas.

T2/Onc and T2/Onc2

T2/Onc3

Recognition of the propensity of the *MSCV 5′LTR* for hematopoietic malignancies over epithelial origin carcinomas led to the development of an additional transposon. T2/Onc3 is identical to T2/Onc2 except that the promoter has been replaced by the chicken *β-actin promoter* fused to the *cytomegalovirus immediate early enhancer* (*CAG*) that has strong expression in a variety of cell types including epithelial cells [7]. In the presence of germ line transposase activity, the tumor spectrum with T2/Onc3 was the reverse of that observed for T2/Onc2; with 89 % solid tumors and only 11 % of tumors being of hematopoietic origins. Solid tumors developed in many organs, most commonly in the liver, lung, and skin.

1.3 Advantages of Using the Sleeping Beauty System

SB has gained popularity over the last few years and a large number of genetically engineered SB-induced mouse models of cancer have been published (reviewed in [2]) This is likely related to the two key advantages of SB; firstly its ability to induce cancer in mice and secondly the relative ease with which the SB-mutated genes can be identified.

In some cases, SB enables the generation of a model without knowledge of the primary oncogenic driver or drivers of the malignancy. The addition of a predisposing mutation and, or restricting activation to a specific cell type or organ may drive tumorigenesis to a particular subtype. In all models, sequencing of the transposon insertion sites has identified known and novel tumor suppressor genes or oncogenes.

1.3.1 Future Prospects

Following characterization of each line to establish its relationship to the human malignancy these models have potential as powerful platforms for preclinical drug testing. The random nature of the mutagenesis generates models in which there are different combinations of oncogenic drivers and numerous oligoclonal subpopulations. This reflects the genetic heterogeneity of human cancers. Mutational profiles that correlate with different treatment responses may highlight new targets for combination drug strategies and resistance mechanisms to the therapies under investigation.

1.4 Models of Hematopoietic Malignancies

1.4.1 Germ Line Models

The most penetrant and widely reported phenotypes following activation of SB in the germ line are hematopoietic malignancies. When activated in the germ line with no additional predisposing factors, *Rosa26-SB11* in combination with a transposon with the *MSCV 5′ LTR* (T2/Onc, T2/Onc2) induces highly penetrant lymphoma (predominantly T-cell); with differing lag times for each transposon. Table 1 lists the hematopoietic malignancies arising under these conditions. The addition of a predisposing mutation changed the genetic profile of the malignancy, enabling identification of cooperating mutations [8, 9] and in some cases altered the predominant subtype (from T-cell to undifferentiated non-CD3 non-CD4) [9], but did not significantly increase penetrance of lymphoma (Table 2).

1.4.2 Conditional Models

With the generation of a conditional lox-STOP-Lox (LSL) transposase line, SB activity could be specifically directed to particular cell types with co-expression of CRE recombinase (CRE),

Table 1

Hematopoietic malignancies following germ line activation of SB on a wild type (WT) background

Transposase	Transposon	Phenotype	Penetrance	Reference
CAGGS-SB10	T2/Onc	Nil	0 %/52w	[1]
CAGGS-SB10	T2/Onc2	Nil	0 %/52w	[1]
Rosa26^{SB11}	T2/Onc	Lymphoma	~89 %/57w ~87 %/60w	[5] [8]
Rosa26^{SB11}	**T2/Onc2**	**Lymphoma**	**~100 %/17w**	[6]
Rosa26^{SB11}	T2/Onc3	Lymphoma[a]	~11 %/70w	[7]

The most penetrant model is in *bold*, w weeks
[a]Lymphoma is the minor phenotype in this model

Table 2

Hematopoietic malignancies following germ line activation of SB on a non-wild type background

Transposase	Transposon	Background	Phenotype	Penetrance	Reference
CAGGS-SB10	T2/Onc	*Arf null*	Lymphoma[a]	~Low/50w	[1]
CAGGS-SB10	T2/Onc	*Kras $^{G12D/+}$* (pancreas specific)	Lymphoma[a]	~4 %/48w	[10]
Rosa26^{SB11}	T2/Onc	*Cadm1 null*	Lymphoma	~80 %/60w	[8]
Rosa26^{SB11}	T2/Onc	*Rassf1a null*	Lymphoma	~95 %/60w	[9]
Rosa26^{SB11}	T2/Onc	*Myc* (liver specific)	Lymphoma[a]	~29 %/16w	[11]

Kras$^{G12D/+}$ Pdx1-CRE, Kras-LSL-G12D, Myc liver-specific tet-transactivator (LAPtTA) and tet-O-MYC
[a]Lymphoma is the minor phenotype in this model

Table 3

Hematopoietic malignancies with specific *Rosa26-LSL-SB11* activation and T2Onc2

Region	Background†	Phenotype	Penetrance	Reference
Ubiquitous (β-actin-CRE)	WT	T-cell lymphoma, Erythroid tumors	100 %/12w	[7]
Germinal center B-cells (aid-CRE)	WT	B-cell lymphoma	90 %/50w	[7]
Immature thymocytes (Lck-CRE)	WT	T-cell lymphoma	100 %/48w	[12]
Late stage thymocytes (CD4-CRE)	WT	T-cell lymphoma Early T-cell ALL	90 %/52w Low/52w	[12]
HSC (vav-iCRE)	WT	T-cell lymphoma	100 %/22w	[12]
HSC (vav-CRE)	WT	Leukemia—lymphoid Erythroleukemia Leukemia—other	~70 %/52w ~35 %/52w ~15 %/52w	[13]
HSC (vav-CRE)	*JAK2*$^{V617F/+}$	Erythroleukemia Leukemia—other	>90 %/~10w 25 %/10w	[13]
B-cells (CD19-CRE)	*Tcl1*	CLL	100 %/36w	[14]

HSC hematopoietic stem cells *Lck* lymphocyte protein tyrosine kinase, *ALL* acute lymphocytic leukemia, *CLL* chronic lymphocytic leukemia, *JAK2*$^{V617F/+}$ *JAK2*^{V617F} in HSC using vav-*JAK2*^{V617F}, *Tcl1* over-expression in B cells—Eμ-TCL1

leading to the generation of models with highly penetrant B-cell malignancies as well as erythroleukemias (Table 3). The addition of a predisposing mutation increased penetrance of restricted phenotypes [13].

1.5 Models of Solid Tumors

Several solid tumor types including hepatocellular cancer were identified with germ line SB11 activation on a wild type background [5, 6, 8] (Table 4). However, these were minor phenotypes and the tumor types limited. The addition of predisposing factors and the ability to target SB changes to specific tissues increased the penetrance and expanded the spectrum of solid tumors. Targeting SB to the intestines resulted in 72 % penetrance of neoplasia with a lag time of 78 weeks. The addition of APC^{min} increased penetrance to 100 % and reduced the time to tumor development to 12 weeks (Table 5). Modification of the transposon by changing the promoter to one with higher expression in epithelial cells (T2/Onc3; Fig. 2) further improved solid tumor models. This is exemplified by *Kras*-mutant pancreatic ductal adenocarcinoma, which increased in penentrance from 50 % with T2/Onc2 to 75 % with T2/Onc3 (Table 6).

SB did not always increase the penetrance of tumor development over that with the predisposing factor alone; however, a more advanced histology or accelerated progression was often evident in

Table 4

Liver lesions induced by SB

Transposase	Transposon	Region	Background	Phenotype	Penetrance	Reference
Rosa26^{SB11}	T2/Onc	Germ line	*Myc* (liver)	HCC	65 %/16w	[11]
Rosa26^{SB11}	T2/Onc	Germ line	WT	HCC[a]	14 %/60w	[8]
Rosa26^{SB11}	T2/Onc	Germ line	*Cadm1* null	HCC[a]	9 %/60w	[8]
Rosa26$^{LSL-SB11}$	T2/Onc	Liver (*Alb-CRE*)	WT	Nodules[b]	50 %/82w	[15]
Rosa26$^{LSL-SB11}$	T2/Onc	Liver (*Alb-CRE*)	*Trp53*$^{R270H/+}$	Nodules[c]	50 %/62w	[15]
Rosa26^{SB11}	T2/Onc3	Germ line	WT	HCC[a] Adenoma[d]	37 %/69w 93 %/69w	[7]
CAGGS-SB10	T2/Onc	Pancreas (*Pdx1-CRE*)	*Kras*$^{G12D/+}$	HCC[a]	3.5 %/48w	[10]

HCC hepatocellular carcinoma; *Alb* albumin, *Pdx1* pancreatic and duodenal homeobox 1, *Myc (liver)* liver-specific tet-transactivator (LAPtTA) and tet-O-MYC, *Trp53*$^{R270H/+}$ *Trp53-LSL-R270H*, *Kras*$^{G12D/+}$ *Kras-LSL-G12D*

[a]Minor phenotype

[b]Preneoplastic lesions, adenomas, and HCC

[c]Predominantly preneoplastic lesions in male mice

[d]Most common tumor type accounting for 30 % of tumor types identified

Table 5

Intestinal lesions induced by SB

Transposase	Transposon	Region	Background	Phenotype	Penetrance	Reference
Rosa26^{SB11}	T2/Onc	Germ line	*Apc*$^{min/+}$	Oligocryptal and tubulovillous adenomas	100 %/22w	[16]
Rosa26^{Lox66SBLox71}	T2/Onc	Peritoneal (*Ah-CRE*)[a]	*Apc*$^{fl/+}$ (peritoneal)	Oligocryptal and tubulovillous adenomas	100 %/30w	[16]
Rosa26$^{LSL-SB11}$	T2/Onc	Intestine (*villin-CRE*)	*Apc*$^{min/+}$	Intestinal polyps neoplasia, adenoma	100 %/12w	[17]
Rosa26$^{LSL-SB11}$	T2/Onc	Intestine (*villin-CRE*)	WT	Intestinal neoplasia, adenoma, adenocarcinoma	72 %/78w	[18]
Rosa26^{SB11}	T2/Onc3	Germ line	WT	Colon carcinoma and adenoma[b]	<5 %/69w	[7]

[a]CRE under the *Cyp1a1* promoter inducible on administration of β-napthoflavone, delivered by interperitoneal injection

[b]Minor phenotype

Table 6

Pancreatic tumors induced using pancreatic specific SB activity directed by *Pdx1-CRE* (with the exception of germ line expression of CAGGS-SB10)

Transposase	Transposon	Background	Phenotype	Penetrance	Reference
Rosa26$^{LSL\text{-}SB11}$	T2/Onc2	WT	PDA	5 %/54w	[19]
Rosa26$^{LSL\text{-}SB11}$	T2/Onc3	WT	PDA	10 %/54w	[19]
Rosa26$^{LSL\text{-}SB11}$	T2/Onc2	*Kras*$^{G12D/+}$	PDA	50 %/54w	[19]
Rosa26$^{LSL\text{-}SB11}$	**T2/Onc3**	*Kras*$^{G12D/+}$	**Metastatic PDA**	**75 %/54w**	[19]
Rosa26$^{LSL\text{-}SB13}$	T2/Onc	*Kras*$^{G12D/+}$	PDA PanIN/papilloma Cystic lesions	64 %/48w 36 %/48w 13 %/48w	[10]
CAGGS-SB10 (germ line)	T2/Onc	*Kras*$^{G12D/+}$	PDA PanIN1 PanIN2	30 %/48w 55 %/48w 15 %/48w	[10]

The most penetrant model is in *bold*
Kras$^{G12D/+}$ *Kras-LSL-G12D*, *PDA* pancreatic ductal adenocarcinoma, *PanIN* pancreatic intraepithelial neoplasia

these cases [10, 17, 19]. A variety of SB-induced solid tumors has now been reported, although many of them at low penetrance (Tables 4–7). With the availability of SB-system transgenic strains shown to have strong expression in epithelial cells and enable tissue specific activation, it is anticipated that the range of highly penetrant models will expand.

2 Materials

2.1 Mice

1. Mice available from the National Cancer Institute Mouse Repository (Emice; http://mouse.ncifcrf.gov/available_strains.asp).

   NCI Strain No: 01XGA: double homozygous *Rosa26-LSL-SB11*; *T2/Onc2/TG6113*

   NCI Strain No: 01XT5: double homozygous *Rosa26-LSL-SB11; T2/Onc2/TG6070*

   NCI Strain No: 01XGB: double homozygous *Rosa26-LSL-SB11; T2/Onc3/TG12740*

   NCI Strain No: 01XGC: double homozygous *Rosa26-LSL-SB11; T2/Onc3/TG12775*

   NCI Strain No: 01XJ6: heterozygous *Kras-LSL-G12D*
2. CRE expressing transgenic mice from The Jackson Laboratory (JAX; http://jaxmice.jax.org/).
3. JAX Stock: 003376: *ACTB-CRE*; CRE under the promoter of human *ACTB, actin, beta* for ubiquitous expression.

Table 7

Additional tumors generated by SB (excluding hematopoietic, liver, intestinal, and pancreatic tumors that are listed in Tables 1–6)

Transposase	Transposon	Region	Background	Phenotype	Penetrance	Reference
CAGGS-SB10	T2/Onc	Germ line	*Arf null*	Sarcoma, osteosarcoma Meningioma[a]	69 %/50w Low/50w	[1]
CAGGS-SB10	T2/Onc	*Pdx1-CRE*	$Kras^{G12D/+}$ (pancreatic)	NSCLC[a] Papilloma[a] SCC[a]	24 %/48w 16 %/48w 7 %/48w	[10]
$Rosa26^{SB11}$	T2/Onc	Germ line	WT	Astrocytoma[a] Prostatic hyperplasia[a]	17 %/57w Low/57w	[5]
$Rosa26^{SB11}$	T2/Onc	Germ line	*Cadm1 null*	Miscellaneous[a] (glioma, adenoma, angiosarcoma, adenocarcinoma)	19 %/60w	[8]
$Rosa26^{LSL\text{-}SB11}$	T2/Onc	*Cnp-CRE* Schwann cell	(*EGFR* and $Trp53^{R270H/+}$) *EGFR* $Trp53^{R270H/+}$ WT	Grade 3 PNST Neurofibroma[a] Neurofibroma Grade 3 PNST[a] Grade 3 PNST[a]	~60 %/64w ~45 %/64w ~35 %/64w ~5 %/64w <1 %/64w	[20]
$Rosa26^{LSL\text{-}SB11}$	T2/Onc	C/PNS *Nes-CRE*	WT	PNET, medulloblastoma	Not stated	[21]
Math1-SB11	T2/Onc	Cerebellar Progenitor cells	$Trp53^{+/-}$ $Ptch^{+/-}$ WT	Medulloblastoma Medulloblastoma Nil	40 %/35w 97 %/10w 0 %/52w	[22]
$Rosa26^{LSL\text{-}SB13}$	T2/Onc	*Tyr-CreERt2*	$B\text{-}Raf^{V619E}$ (melanocyte)	Melanoma	Not stated	[23]
K5-SB11	T2/Onc2	Skin	*Ha-ras* ^{mutant}	Papilloma, SCC[a], BCC[a]	Not stated	[24]
$Rosa26^{SB11}$	T2/Onc2	Germ line	WT	Medulloblastoma[a] Pituitary neoplasia[a]	<5 %/16w	[6]
$Rosa26^{SB11}$	T2/Onc3	Germ line	WT	lung adenoma[a,b] SCC[a,c] Breast carcinomas[a]	34 %/69w 44 %/69w 8 %/69w	[7]

NSCLC non-small-cell lung carcinoma, *SSC* squamous cell carcinoma, *BCC* basal cell carcinoma, *PNST* peripheral nerve sheath tumors, sarcomas of Schwann cell lineage, *PNET* primitive neuroectodermal tumors, $Kras^{G12D/+}$ *Kras-LSL-G12D*, *EGFR* overexpression in Schwann cells via *Cnp-EGFR*, $B\text{-}Raf^{V619E}$, *LSL-B-Raf-V619E* corresponds to human $BRAF^{V600E}$, *Ha-ras*^{mutant} Tg.AC, *K5-SB11* SB under the control of bovine keratin K5 gene promoter to activate SB in skin cells, *Tyr-CreERt2* tamoxifen inducible CRE, *C/PNS* central and peripheral nervous systems, *Nes-CRE* Nestin-CRE

[a]Minor phenotype

[b]Eleven percentage of tumor spectrum

[c]Fourteen percentage of tumor spectrum

4. JAX Stock: 003724: *EIIa-CRE;* CRE under the adenovirus *EIIa* promoter for germ line expression.
5. JAX Stock: 008520: *CD2-CRE*; optimized CRE (iCRE) under the human *CD2* promoter and locus control region (LCR) for expression in all B cell and T cell progenitors.
6. JAX Stock: 017336: *Cd4-CRE*; CRE under the mouse *Cd4* promoter for expression in CD4-expressing T cells.
7. JAX Stock: 003802: *Lck-CRE*; CRE under the promoter of mouse *Lck*, *lymphocyte protein tyrosine kinase* for expression in T lymphocytes.

2.2 Adenoviral Delivery of CRE Recombinase

1. ADCRE: ADCREM2 (Microbix Biosystems Inc).
2. MEM: Minimal Essential Media (Sigma-Aldrich).
3. $CaCl_2{\cdot}2H_2O$: C3881 (Sigma-Aldrich).

2.3 Primers (All Given as 5′–3′)

1. CRE genotyping: CRE-1: ACCAGCCAGCTATCAACTCG; CRE-2: TTACATTGGTCCAGCCACC (CRE allele = 199 bp; from eMICE). Positive internal control primers (for IL2) to ensure DNA quality and quantity may be multiplexed or used in addition to the CRE primers. IL2-F: CTAGGCCACAGA ATTGAAAGATCT; IL2-R: GTAGGTGGAAATTCTAGCAT CATCC (Amplicon = 324 bp; from JAX).
2. *Kras-LSL-G12D* allele genotyping: Kras-1: TCCGAATTCAGT GACTACAGATG; Kras-2: ATG TCT TTCCCCAGCACAGT; Kras-LSL: CTAGCCACCATGGCTTGAGT (1+ LSL = 327 bp LSL allele, 1 + 2 = 450 bp WT allele; from Emice).
3. Excision PCR: T2x-F: TGTGCTGCAAGGCGATTA; T2x-R: ACCATGATTACGCCAAGC (excised transposon: ~225 bp; unmobilized transposon: 2.2 kb, ref. 15).

3 Methods

3.1 Generation of SB-Induced Tumors Through Breeding with a Transgenic Mouse Expressing Tissue-Specific CRE Recombinase: Highly Penetrant Lymphoma

1. To generate a model of lymphoma the double homozygous *Rosa26-LSL-SB11; T2/Onc2* mice are crossed with a transgenic mice expressing CRE according to the strategy in Fig. 3 (*see* **Notes 1** and **2**). The type of lymphoma to be generated (e.g., T-cell) will dictate the specific transgenic CRE model to be used. Table 3 lists the published models generated with either ubiquitous or restricted expression of CRE. Several transgenic CRE strains available from The Jackson Laboratory are listed in Subheading 2.1 (*see* **Note 3**). Other models can be sourced via the International Mouse Strain Resource (http://www.findmice.org/).

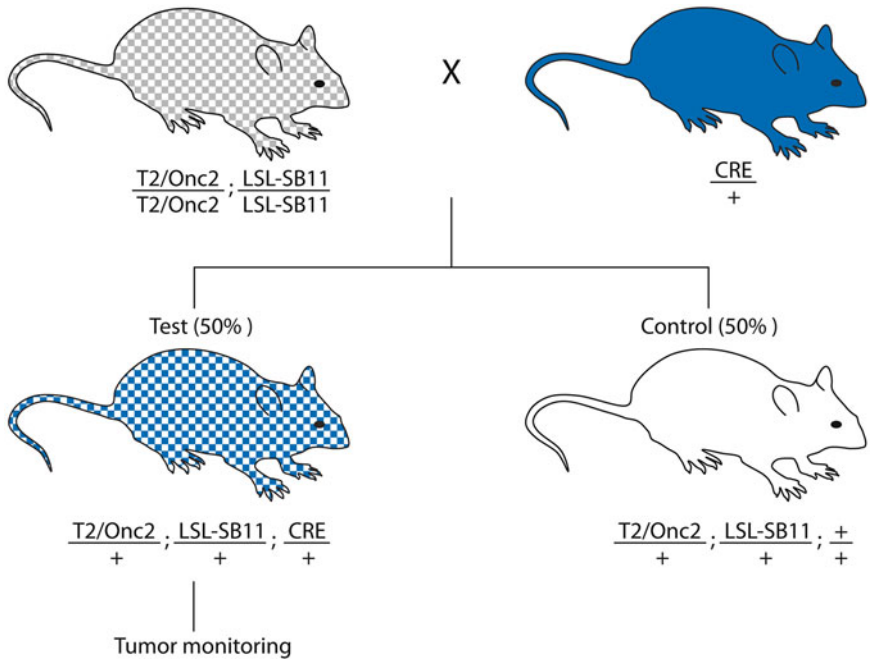

Fig. 3 Breeding strategy for the generating of mice with SB-induced tumors through breeding with a transgenic mouse expressing tissue-specific CRE recombinase

2. Resulting litters are tagged and tail snips taken for DNA extraction by standard techniques and genotyped for the CRE allele using CRE genotyping primers listed in Subheading 2.3, **step 1**. Equal numbers of CRE positive and CRE negative mice are generally expected (*see* **Note 4**).
3. CRE positive animals constitute the experimental group and need to be closely monitored for tumor development. Daily monitoring is recommended following the reported lag time as ethically defined endpoints can develop very rapidly (*see* **Note 5**). CRE negative animals are equivalent to wild type mice with regard to tumor development. They constitute a matched negative control group if required for the research question under investigation.

3.2 Generating SB-Induced Tumors Through Directed Administration of a Replication Deficient Adenovirus Expressing CRE Recombinase: Kras-Driven Lung Tumors

1. As an alternative to activating floxed alleles through breeding with a CRE transgenic mouse, CRE can be administered directly to the target site (*see* **Note 6**). SB-induced lung adenomas and *Kras*-driven non-small-cell lung carcinomas are reported as minor phenotypes in several SB-induced models (Table 7). By directing activation of both *SB* and *Kras* exclusively to the lung the penetrance of lung tumors may be increased and the development of tumors in other organs restricted. To generate mice with *Kras*-driven lung tumors, homozygous *Rosa26-LSL-SB11; T2/Onc3* mice are crossed

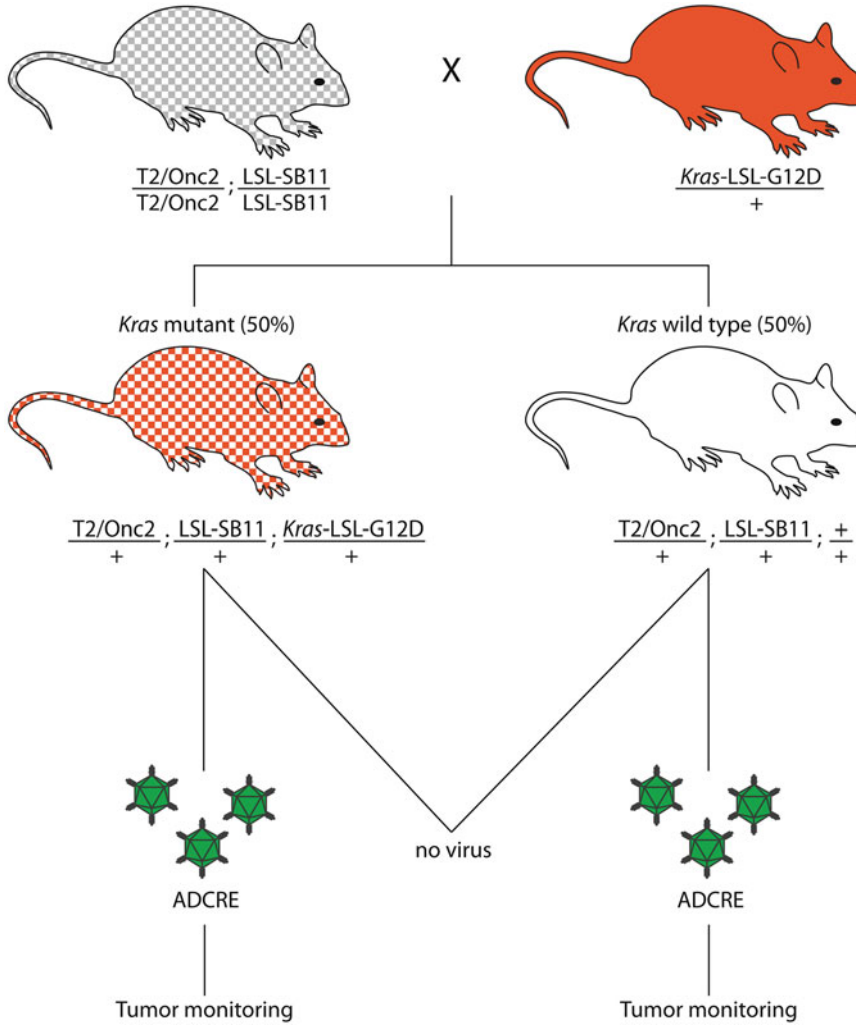

Fig. 4 Breeding strategy for generating *Kras*-driven SB-induced tumors through directed administration of a replication deficient adenovirus expressing CRE recombinase

with heterozygous *Kras-LSL-G12D* mice according to the strategy shown in Fig. 4 (*see* **Notes 1**, **2** and **7**).

2. Litters are tagged and tail snips taken for DNA extraction by standard techniques and genotyped for the *Kras-LSL-G12D* allele using primers listed in Subheading 2.3, **step 2**. Equal numbers of mice heterozygous and wild type for the *Kras-LSL-G12D* allele are expected (*see* **Note 8**). Until CRE is administered, the mice do not have an increased propensity for tumor development and may be considered as essentially "wild type" in this regard.
3. To activate both *SB* and *Kras*, CRE is administered as a solution of ADCRE. This is prepared by aliquoting the ADCRE into MEM at a concentration of 5×10^6 Plaque Forming Units

ADCRE per 125 μL dose (*see* **Note 9**). With mixing, 1 μL 2 M $CaCl_2{\cdot}2H_2O$ is added per 100 μL ADCRE in MEM and the solution incubated at room temperature for at least 20 min prior to injection (*see* **Note 10**).

4. Mice between 6 and 8 weeks of age are administered ADCRE prepared in **step 3** by intranasal instillation. Mice are anesthetized and ADCRE is administered in two drops (each 62.5 μL) 5 min apart to the same nostril [25]. If matched negative controls are required for the research question, an additional cohort of mice from these crosses should be administered MEM supplemented with 10 mM $CaCl_2$ without ADCRE (Fig. 4).
5. Following the administration of CRE, mice need to be carefully monitored. Weekly monitoring progressing to daily monitoring following the estimated lag time to overt disease is recommended as ethically defined endpoints can develop very rapidly (*see* **Note 11**).

3.3 Identification of Transposon Insertion Sites as Primary Genetic Events

1. At ethically determined endpoints, mice are euthanized and a necropsy performed to identify tumor affected tissues and metastases. Tissues of interest are collected for histological classification (into formalin) and identification of insertion sites (snap-frozen and stored at −80 °C until required for DNA extraction) (*see* **Notes 12** and **13**).
2. Prior to sequencing of tumor DNA for SB insertion sites, transposon mobilization should be assessed by PCR using the excision PCR primers listed in Subheading 2.3, **step 3** (*see* **Note 14**).
3. Ligation mediated PCR and targeted next generation sequencing of the transposon insertion sites can be performed according to methods published for two next generation sequencing platforms [15, 26] (*see* **Note 15**).

4 Notes

1. It is recommended to use more than one transposon line. This is to overcome the phenomenon of "local hopping". Transposons have a tendency to reinsert close to the parent transposon transgene. Therefore, these are considered non-random insertions and the region of each parent transgene is excluded from the downstream analysis of insertion sites. In order to identify random insertions of significance in these regions a different strain of the transposon can be used.

For T2/Onc2, there are two strains available: TG6070 in chromosome 4 and TG6113 in chromosome 1. For T2/Onc3, the two strains are TG12740 in chromosome 9 and TG12775 in chromosome 12.

2. SB mice are available as homozygous for both the conditional SB knock-in allele and the transposon and can be maintained as such. The SB allele can be genotyped using the following primers: RSB1: AAGGAGCGAGGGCTCAGTTG; RSB2: CG AGGCGGATCACAAGCAAT; RSB3: GCCCAAGGCCATAC AAGTGTTG; where RSB1 + 2 = 488 bp wild type band; RSB1 + 5 = 412 bp Rosa26-SB band (adapted from eMICE). Presence of the transposon allele can be assessed by PCR using primers T2-F: AACTCGTTTTTCAACTACTCCACA and T2-R: CTGACCCACTGGAATTGTGA (165 bp). To distinguish between one or two alleles without knowledge of the chromosomal location of the transgene, a TaqMan qPCR copy number assay can be used following the standard protocol for primer/probe concentrations and cycling conditions. For this assay the following primer and probe sets for T2/ONC2 and *Gapdh* are multiplexed: T2ONC2-F: CTTGATGGCCGCTCTAGA ACTAG; T2ONC2-R: TGTGACCATGTGGAGTCAGCTT and TaqMan MGB probe (FAM-labeled): T2ONC2-FAM: FAM-ATTGCAGCACGAAACA; GAPDH-F: CCGAGAATG GGAAGCTTGTC; GAPDH-R: TCTCGCTCCTGGAAGAT GGT and TaqMan MGB probe (VIC-labeled): GAPDH-VIC: VIC-TCAACGGGAAGCCCAT. Each sample should be run in quadruplicate with 15 ng DNA/10 μL PCR and every run requires DNA standards and controls with known zero, 1 and 2 alleles. This assay has not been assessed for the T2/Onc3 transgene; however, a SYBR Green assay has been published for quantification of T2/Onc3 copies within the transgene [7]. By using DNA standards as above, this assay may be used to differentiate the presence of one or two alleles.
3. Transgenic CRE mice are generally maintained as hemizygotes backcrossed to the background strain (e.g., C57Bl/6). This avoids possible toxicity, reported in some strains, and the difficulty differentiating between hemizygous and homozygous mice by PCR genotyping.
4. For germ line/ubiquitous expression of *Rosa26-LSL-SB11; T2/Onc2*, embryonic lethality has been reported [6], although not seen with *β-actin-CRE* [7]. Embryonic lethality will reduce the percentage of CRE positive mice in the litters.
5. Germ line or ubiquitous activation of SB results in tumor development with a very short latency [6, 7]. Daily monitoring of these mice should begin as soon as they have been identified as CRE positive by genotyping, and no later than 5 weeks of age.

6. While there are a large number of transgenic strains expressing CRE in different tissue and cell types, suitable models with specific targeting of tissues do not exist for all tissue types. For some tissues, direct administration of CRE is the preferred method of specific targeting. For lung, administration of ADCRE is by intranasal instillation [27].
7. *Kras-LSL-G12D* knock-in mice are generally maintained as heterozygotes backcrossed to the background strain (e.g., C57Bl/6). However, if required for more efficient use of litters (i.e., 100 % heterozygous for this allele) they may be bred to homozygosity, with genotypes assessed using the genotyping primers in Subheading 2.3, **step 2**. The allele is non-oncogenic until exposure to CRE.
8. Up to three different tumor cohorts of mice may be considered depending on the research question to be addressed. Lung tumors have been identified in SB mice with and without activated *Kras* (Table 7) therefore all mice from the stated cross may be of interest; i.e., *SB* with and without *LSL-Kras-G12D*. Lung tumors have also been reported in mice with activated *Kras* without SB [28]. Therefore, to determine whether SB increases the rate of tumorigenesis, penetrance or histopathological diagnosis of the resulting tumors, a separate cohort of mice with *Kras-LSL-G12D* alone can be generated by the maintenance strategy for heterozygous *Kras-LSL-G12D* mice (*see* **Note 7**).
9. The number of tumors arising over time has been shown to vary proportionally with the amount of ADCRE administered [28]. A low dose is suggested to better recapitulate the natural history of disease development; however, this can be increased if required. Prior to infecting the experimental cohort the selected dose and administration technique should be trialed for recombination with a floxed reporter (e.g., *Rosa26-LSL-LacZ*, JAX mice Stock Number: 003309) mouse. Reporter mice are sacrificed 3 days post-instillation and lungs stained for β-galactosidase activity (http://cre.jax.org/BgalProtocols.html). Positive staining confirms ADCRE-mediated recombination.
10. The addition of $CaCl_2$ has been found to enhance uptake of the virus by cells [29]. If additional experiments or a large number of animals are to be infected, the ADCRE should be expanded in HEK-293 cells (Microbix Biosystems Inc) and purified virus produced and quantified according to the manufacturer's or similar protocols [30]. Ultracentrifugation using a cesium chloride gradient is recommended over column methods to ensure a high titer of intact virus particles.
11. Hyperplastic lesions have been observed on the surface of lungs with *Kras*-activation 2 weeks following ADCRE

administration [28]. The lag time will depend on the model and dose of ADCRE used. Daily monitoring should begin no later than 20 weeks after instillation of ADCRE.

12. Tissues for collection in lymphoma models include bone marrow, spleen, thymus and lymph nodes. Hepatomegaly is a common feature in lymphoma and liver collection may also be included. In the lung model, in addition to lung tumors, metastases to the liver, kidney, peritoneal cavity and lymph nodes may be expected.
13. If there is very little tissue it is possible to identify insertion sites in DNA extracted from formalin fixed paraffin embedded tissues. However, the DNA is degraded leading to a low yield. Alternative procedures for identification of insertion sites in these samples have been reported [31].
14. SB-transposase expression may also be determined by immunohistochemical labeling of paraffin embedded tumor sections with Sleeping Beauty Transposase goat polyclonal antibody (AF2798; R&D systems) using heat retrieval (Rodent Decloaker, RD913, Biocare Medical), and an antibody concentration of 1 μg/mL.
15. If access to these platforms is not possible, traditional cloning methods may be used [32], however the yield is grossly limited compared with the newer platforms.

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Chapter 22

Generating Double Knockout Mice to Model Genetic Intervention for Diabetic Cardiomyopathy in Humans

Vishalakshi Chavali*, Shyam Sundar Nandi*, Shree Ram Singh, and Paras Kumar Mishra

Abstract

Diabetes is a rapidly increasing disease that enhances the chances of heart failure twofold to fourfold (as compared to age and sex matched nondiabetics) and becomes a leading cause of morbidity and mortality. There are two broad classifications of diabetes: type1 diabetes (T1D) and type2 diabetes (T2D). Several mice models mimic both T1D and T2D in humans. However, the genetic intervention to ameliorate diabetic cardiomyopathy in these mice often requires creating double knockout (DKO). In order to assess the therapeutic potential of a gene, that specific gene is either overexpressed (transgenic expression) or abrogated (knockout) in the diabetic mice. If the genetic mice model for diabetes is used, it is necessary to create DKO with transgenic/knockout of the target gene to investigate the specific role of that gene in pathological cardiac remodeling in diabetics. One of the important genes involved in extracellular matrix (ECM) remodeling in diabetes is matrix metalloproteinase-9 (Mmp9). Mmp9 is a collagenase that remains latent in healthy hearts but induced in diabetic hearts. Activated Mmp9 degrades extracellular matrix (ECM) and increases matrix turnover causing cardiac fibrosis that leads to heart failure. Insulin2 mutant (Ins2+/–) Akita is a genetic model for T1D that becomes diabetic spontaneously at the age of 3–4 weeks and show robust hyperglycemia at the age of 10–12 weeks. It is a chronic model of T1D. In Ins2+/– Akita, Mmp9 is induced. To investigate the specific role of Mmp9 in diabetic hearts, it is necessary to create diabetic mice where Mmp9 gene is deleted. Here, we describe the method to generate Ins2+/–/Mmp9–/– (DKO) mice to determine whether the abrogation of Mmp9 ameliorates diabetic cardiomyopathy.

Key words Ins2+/– Akita, Mmp9, Diabetes, Heart failure, Cardiomyopathy

1 Introduction

The alarming rate of diabetics [1] and its impact on cardiovascular diseases [2–8] make it a greatest challenge for medical sciences. Diabetes is a global menace [9, 10] that requires global attention. T1D are typically present in the young (therefore, also called juvenile diabetes). It is caused either due to mutation of insulin

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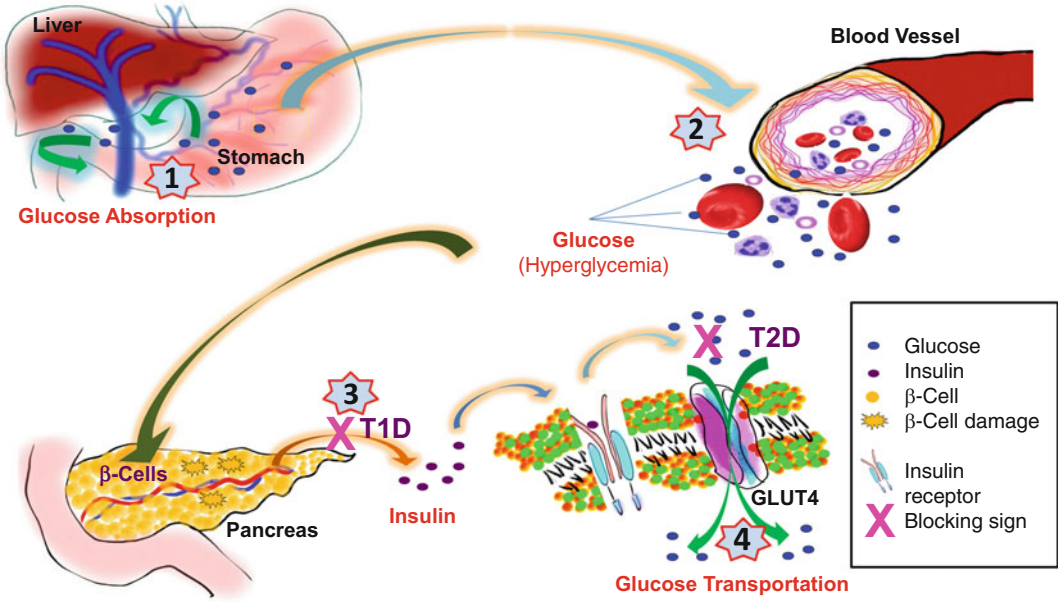

Fig. 1 Schematics showing the process of glucose metabolism and their dysfunction in T1D and T2D

gene that impairs glucose metabolism [11] or developed as an autoimmune disease, where pancreatic beta cells are targeted and destroyed by immune cells [12, 13]. The beta cells are responsible for producing insulin that regulate glucose metabolism. After food intake, blood glucose level increases that triggers signals to release insulin from pancreatic beta cells. Insulin activates Insulin receptor signaling that removes excess glucose from the blood into different cells in body using glucose transporter (GLUT4). It also helps in glucose metabolism (Fig. 1). When beta cells are less or absent due to either genetic mutation or autoimmune destruction, the insulin released in response to high blood glucose level is either very low or absolutely absent. This causes low levels of insulin and high levels of glucose in the blood, a signature of T1D (Fig. 1). T1D is less prevalent (~5 %) in the US population [14, 15]. Insulin injection before meal is the clinical treatment practiced for regulating hyperglycemia in T1D patients [12]. Insulin injection sometimes causes hypoglycemia and fluctuation of hyperglycemia to hypoglycemia has devastating impact on different organs including the heart. Heart is a sophisticated but vulnerable organ and diabetes increases the incidence of heart failure [5, 16, 17]. Therefore, investigating an intervention tool to mitigate diabetic cardiomyopathy is indispensable.

T2D results due to insulin insensitivity or resistance and is more prevalent (~90 %) [18–21]. In T2D, although beta cells release insulin in response to elevated blood glucose level, insulin is unable to trigger GLUT4 due to defective insulin receptor signaling that results into increased level of glucose in the blood (Fig. 1). High level of blood glucose triggers beta cells to release insulin constantly that results in high level of insulin in blood. Therefore, T2D is diagnosed

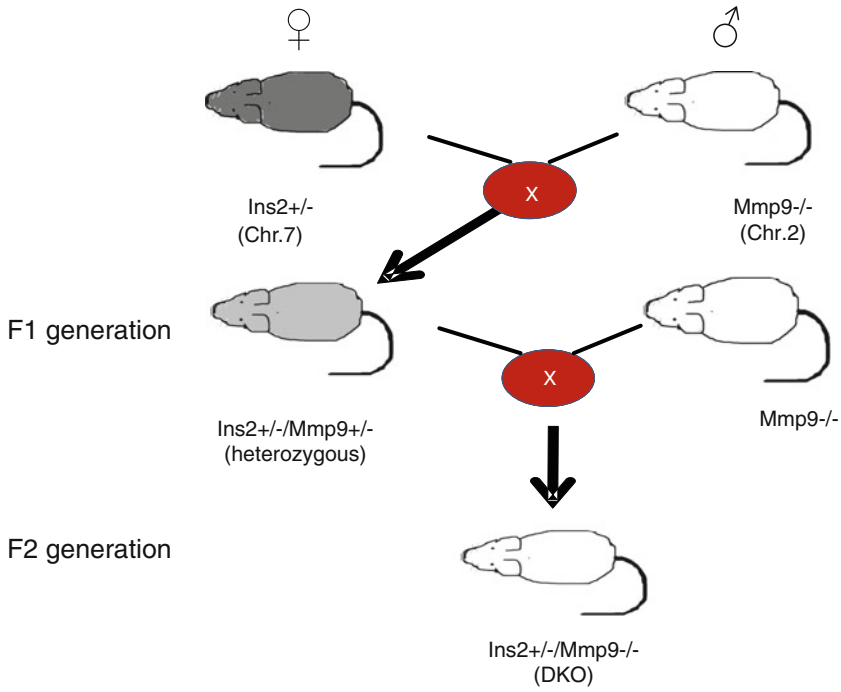

Fig. 2 Scheme for generating Ins2+/–/Mmp9–/– (DKO) from Ins2+/– and Mmp9–/– mice

by increased levels of both glucose and insulin. Chronic T2D puts extensive workload on beta cells to secrete insulin that ultimately causes their death. Therefore, long-term T2D leads to T1D.

We have reported that diabetes induces Mmp9 [22, 23] that in turn impairs contractility of cardiomyocytes [24], induces cardiac fibrosis [23, 25], and differentially expresses several miRNAs [24] that regulate cardiac functions. Recently, we have demonstrated that Mmp9 is also involved in cardiac stem cell survival and may be involved in their differentiation into cardiomyocytes [25, 26]. We also found that ablation of Mmp9 mitigates cardiac fibrosis [25]. Clinical study demonstrates that MMP9 (human matrix metalloproteinase-9) decreases survival probability and exacerbates mortality in heart failure patients with diastolic dysfunction [27]. Mmp9 is also implicated in hypertension and left ventricle remodeling [28–31]. Ablation of Mmp9 decreases the infarct size in ischemia-reperfusion injury [32].

Since Mmp9 is up regulated in diabetic hearts [23], we created DKO (Ins2+/–/Mmp9–/–) mice, where Insulin gene is heterozygous (T1D) and Mmp9 gene is abrogated to assess the specific role of Mmp9 in diabetic cardiomyopathy (Fig. 2). The process of generating DKO can be used for combination of other genes. After validating the expression of Mmp9 (Fig. 3) by genotyping, in-gel-gelatin zymography (Fig. 4), and immunohistochemistry, cardiac fibrosis was determined in the three groups: WT, Ins2+/– and Ins2+/– MMP9–/–. Interestingly, fibrosis was significantly decreased in Ins2+/–/Mmp9–/– as compared to the

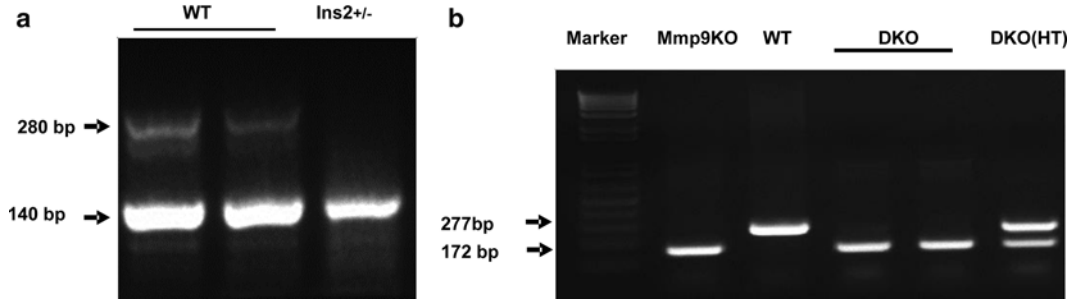

Fig. 3 The genotypes of WT, Akita, Mmp9+/–, and Mmp9–/– mice. (**a**) PCR amplification of Insulin2 gene showing two bands in WT (Ins2+/+) and one band in Ins2+/– mice. (**b**) PCR amplification of Mmp9 gene showing genotypes for Mmp9 knockout (Mmp9KO), WT (Mmp9+/+), double knockout homozygous (DKO) (Ins2+/–/Mmp9–/–), and DKO heterozygous (HT) (Ins2+/–/Mmp9+/–) mice

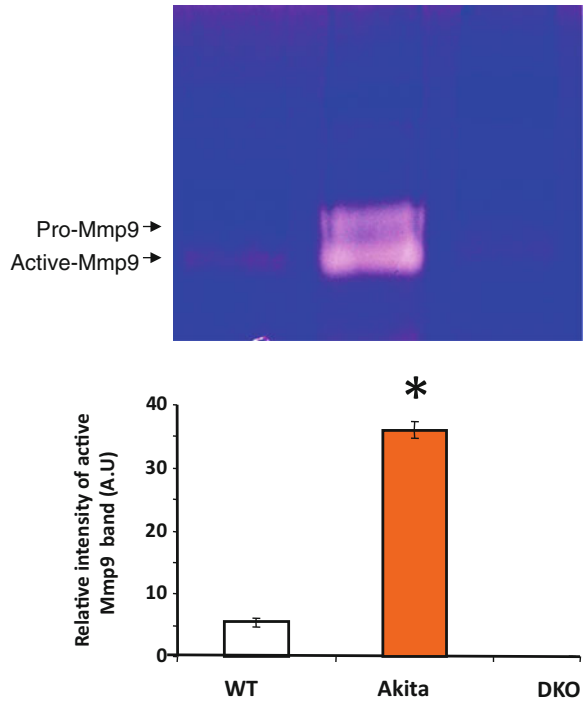

Fig. 4 In-gel-gelatin Zymography for matrix metalloproteinase-9 (Mmp9) activity showing two bands of pro- and active-Mmp9. Mmp9 activity in C57BL/6J (WT), Ins2+/– Akita, and Ins2+/–/Mmp9–/– (DKO) hearts is shown in the representative bot. The activity of Mmp9 is robust in diabetic Akita but almost absent in DKO hearts. The bar graph shows the relative band intensity of active Mmp9. Modified from Mishra PK et al. 2012, Can J Physiol Pharmacol [25]

Ins2+/– hearts [25]. We also determined the mitigation of cardiac dysfunction in DKO mice by measuring the levels of atrial natriuretic peptide (a marker of cardiac hypertrophy) (Fig. 5). The hypertrophy can also be observed by staining the cell membranes with wheat germ agglutinin (Invitrogen) (Fig. 6).

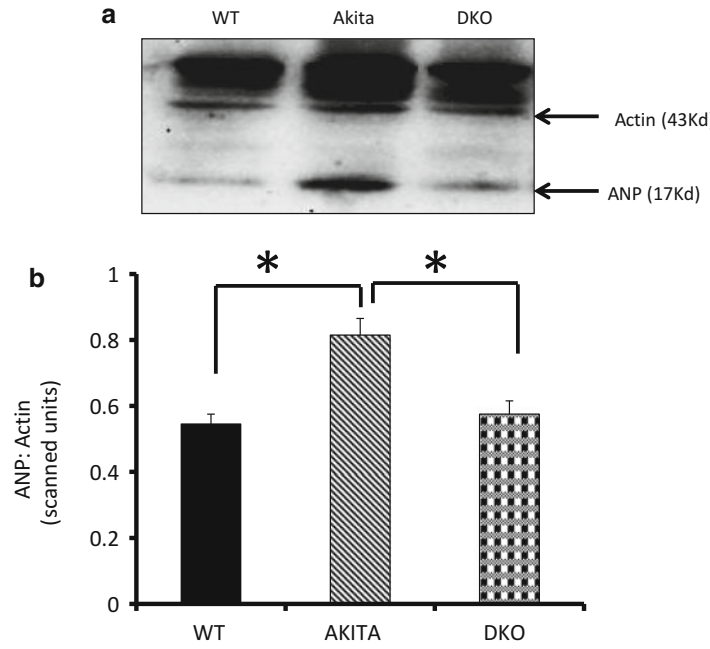

Fig. 5 The expression of Atrial Natriuretic Peptide (ANP) in the hearts of WT, Akita, and DKO mice. (**a**) Representative bands (Western blot) for the expression of ANP and actin (loading control). (**b**) Bar graph showing the relative expression of ANP in the three groups. $N = 3$, $^{*}p < 0.05$

2 Materials

Mice should be ordered from authorized vendor such as Jackson Laboratory.

1. Mice: Ins2+/– Akita, The Jackson Lab stock number is 003548 and the genetic background is C57BL/6 J and Mmp9–/– (The Jackson Lab stock number is 007084 (B6.FVB (Cg))-Mmp9^{tm1Tvu}/J).
2. Glucose measurement: Glucometer, glucose strips.
3. Genotyping: Scissors, Ice-cold ethanol (*see* **Note 1**), DNA extraction kit (Qiagen, USA).
4. Nanodrop for measuring quality and quantity of DNA, PCR instrument, PCR master mix, primers for Insulin and Mmp9 gene, nuclease free water, Agarose powder (Sigma, USA), TAE buffer (Bio-Rad, USA), Ethidium bromide (Invitrogen, USA), Gel apparatus (Bio-Rad) ChemiDoc instrument (Bio-Rad) (*see* **Note 2**).
5. RIPA (Boston BioProducts).
6. Protease inhibitor cocktail (Thermo Scientific).

7. Pierce BCA protein assay kit (Thermo Scientific).
8. 0.1 % gelatin (Sigma, USA).
9. 30 % Acrylamide/Bis solution (Bio-Rad).
10. 1.5 M Tris pH 8.8 and 0.5 Tris pH 6.8 (Bio-Rad).
11. Ammonium Persulfate (Bio-Rad).
12. TEMED (Bio-Rad).
13. 2× Laemmli sample buffer: 0.5 M Tris–HCl, pH 6.8 2.5 mL; Glycerol 2.0 mL; 10 % SDS 4 mL, 0.1 % bromophenol blue 0.5 mL and make up the final volume to 10 mL with distilled water (Bio-Rad).
14. β-Mercaptoethanol (Fisher Scientific).
15. 1× Renaturing buffer: Triton X 100–2.5 % v/v in distilled water (Acros Organics).
16. 1× Developing buffer: Tris base 1.21 g (Bio-Rad), Tris–HCl 6.3 g (Sigma), NaCl 11.7 g (Fisher Scientific), $CaCl_2$ 0.74 (Fisher Scientific), Brij 35 0.02 % (Fisher Scientific) in 1 L of distilled water.
17. Coomassie blue R-250 (0.5 % w/v in methanol) (Thermo Scientific).
18. De-staining solution: 30 % methanol (Fisher Scientific) and 10 % v/v acetic acid (Fisher Scientific).
19. Tris Buffered Saline (TBS) (Fisher Scientific).
20. Tween-20 (Fisher Scientific).
21. Tris- Glycine- SDS running buffer (Bio-Rad).
22. Tris-Glycine transfer buffer (Bio-Rad).
23. Nitrocellulose membrane (Bio-Rad).
24. ECL substrate (Bio-Rad).
25. ChemiDoc MP (Bio-Rad).

3 Methods

3.1 Cross Breeding of Ins2+/– and Mmp9–/– to Create Ins2+/–/Mmp9–/–

In mouse, there are two Insulin genes (Insulin 1 and Insulin 2) but in human, only one Insulin gene is present. Insulin2 gene of mouse is orthologous to human Insulin and mutation of this gene causes T1D. In mouse, Insulin gene is located on chromosome 7, whereas Mmp9 gene is located on chromosome 2. After genotyping of mice, Ins2+/– females are crossbred with Mmp9–/– male (female:male = 2:1). Since Ins2+/– is heterozygous, the two alleles are different (+and –). In F1 generation, the two alleles of Ins2+/– are hybridized with one allele (+) of Mmp9 to make two types of genotypes (+/+) and (+/–). For Mmp9 gene there is only "+" allele in Ins2+/– Akita and "–" allele in Mmp9–/– mice and in F1

hybrids only one genotype "+/–" are present. There are two genotypes for F1 hybrids: Ins2+/+/Mmp9+/– and Ins2+/–/Mmp9+/–. The Ins2+/–/Mmp9+/– heterozygous females are selected for backcrossing with Mmp9–/– males. There are "+" and "–"alleles for both Ins2 and Mmp9 genes in the Ins2+/–/Mmp+/– hybrids. In F2 generation, the genotypes are "Ins2+/+/Mmp9+/–", "Ins2+/–/Mmp9+/–", "Ins2+/–/Mmp9–/–(DKO)". The scheme of generating DKO mice is given in Fig. 2. The DKO are validated by genotyping as well as expression assay. For MMP9 activity, gelatin zymography are performed. Mmp9 expression is also validated at immunohistochemistry level by measuring fluorescence intensity [25].

3.2 Genotyping of Ins2+/–, Mmp9–/–, and Ins2+/–/Mmp9–/– Mice

3.2.1 DNA Extraction

1. DNA is extracted from the tail of WT, Akita, and DKO mice following the manufacturer's protocol for the kit.
2. Cut 0.2–0.4 cm length of mouse-tail and store it at –20 °C until DNA extraction.
3. Mix 180 μL buffer ATL and 20 μL of proteinase K, which are supplied with the kit, and incubate at 56 °C under rotation for overnight or until completely lysed.
4. For each tail, add 410 μL of buffer AL–ethanol (205 μL AL + 205 μL ethanol) and mix by vortexing.
5. Transfer the mixture to a DNeasy Mini spin column in a 2 mL collection tube and centrifuge at 3,300 × *g* for 10 min.
6. Discard the collection tube and the flow-through, and place the column in a new 2 mL collection tube.
7. Add a volume of 500 μL AW1 buffer to the spin column and centrifuge at 3,300 × *g* for 5 min.
8. Discard the flow through and the collection tube, and the column is placed into a new collection tube.
9. Add the same volume (500 μL) of AW2 buffer to the column and centrifuged at 3,300 × *g* for 15 min.
10. After discarding the flow through and the collection tube, place the column into another new collection tube, and add 200 μL of AE buffer for elution. Incubate it for 1 min at room temperature and then centrifuge for 2 min at 3,300 × *g*.
11. The flow through (DNA) was collected and stored at –20 °C for polymerase chain reaction (PCR).

3.2.2 Polymerase Chain Reaction (PCR)

1. For genotyping the Insulin2 and Mmp9 mutants, follow the PCR primers and programs from the Jackson Laboratory protocols.
2. For Insulin2 gene, the following primers can be used: forward, 5′-TGC TGA TGC CCT GGC CTG CT-3′; and reverse, 5′-TGG TCC CAC ATA TGC ACA TG- 3′.

3. The PCR program is: 94 °C, 3 min; 94 °C, 20 s; 64 °C, 30 s; decrease in temperature of –5 °C per cycle; 72 °C, 35 s; repeat steps for 12 cycles, 94 °C, 20 s; 58 °C, 30 s; 72 °C, 35 s; repeat steps for 25 cycles; 72 °C, 2 min; 10 °C, hold.
4. For Mmp9 gene, four set of primers: two sets for the mutant, and two sets for wild type gene. The primers for the mutant gene as follows: forward, 5′-CTG AAT GAA CTG CAG GAC GA-3′; and reverse, 5′-ATA CTT TCT CGG CAG GAG CA-3′.
5. For wild type gene of Mmp9, the primers are: forward, 5′-GTG GGA CCA TCA TAA CAT CAC A-3′; and reverse, 5′-CTC GCG GCA AGT CTT CAG AGT A-3′. The cycling conditions are as follows: 94 °C, 3 min; 94 °C, 30 s; 66 °C, 1 min; 72 °C, 1 min; repeat steps for 35 cycles; 72 °C, 2 min; 10 °C, hold.

3.2.3 Agarose Gel Electrophoresis

1. Separate the PCR product by electrophoresis using 1.5 % agarose gel, and ethidium bromide (0.008 %) and visualize the bands under UV light.
2. For 100 mL of 1.5 % agarose gel, weigh 1.5 g of agarose and dissolve in 1× TAE buffer and boil it for 2 min.
3. Allow it to cool down to ~60 °C–70 °C and then add 8 μL of ethidium bromide.
4. Pour the solution into the Agarose gel cast and allow it to solidify.
5. Perform gel electrophoresis in 1× TAE buffer at 70 V.

3.2.4 Analyses of Bands

1. In Akita mice, Insulin2 gene is heterozygous. The Insulin2 gene amplification product will show two bands of 140 and 280 base pairs.
2. The WT mice will have only 140 bp band.
3. In DKO (Ins2+/–/Mmp9–/–) mice, the PCR product for Mmp9 gene primer will show single band at 172 bp.
4. In WT mice, there will be single Mmp9 band of 277 bp, whereas in heterozygous mice there will be 2 bands of 172 and 277 bp [25] (Fig. 3b).

3.3 In-Gel-Gelatin Zymography

1. Prepare SDS–substrate PAGE with gels with 0.1 % gelatin in both the stacking and resolving gels.
2. To prepare the 0.1 % gelatin solution, weigh 0.1 g of gelatin and dissolve in 100 mL of distilled water. Gelatin will not be dissolve at room temperature. Heat the solution to 50–60 °C until the gelatin is completely dissolved (*see* **Note 3**).
3. Cool down the solution to room temperature and prepare the SDS PAGE gel with stacking (4–5 %) and resolving (7.5–10 %) gels, in place of water use this 0.1 % gelatin solution.

4. For sample preparation, take the desired concentration of protein and add 2× sample buffer.
5. Mix the protein and 2× sample buffer and keep it at room temperature for 10 min (*see* **Note 4**).
6. Load 10–30 μL protein samples and perform gel electrophoresis in 1× Tris-Glycine-SDS running buffer at 70 V.
7. Once the tracking dye passes through the bottom of gel, remove the resolving gel and wash it in distilled water.
8. Incubate the resolving gel in 1× renaturing buffer with gentle shaking for 30 min. Repeat this step two times, and each time use fresh renaturing buffer.
9. After this, keep the gels in 1× developing buffer at 37 °C at slow shaking for 48 h.
10. Wash the gels with distilled water and stain the gels with Coomassie blue R-250 (0.5 % w/v in methanol) for 30 min.
11. To see the clear bands wash the gels with distilled water.
12. De-staining solution can also be used to remove the excess stain of the gel.
13. Gelatinase activity could be seen in any Gel Doc instrument with UV light as cleared (unstained) regions on the blue background (Fig. 4).

3.4 Western Blotting

3.4.1 Western Immunoblotting

1. Protein is extracted in Radio immunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail from the whole heart of WT, Akita, and DKO mice.
2. The concentration of proteins extracted from the hearts is quantified by Bradford assay following the protocol of Pierce BCA protein assay kit.
3. Protein samples for gel electrophoresis are prepared by adding Laemmle sample buffer (2×) (*see* **Note 5**).
4. The protein and sample buffer mixture is heated for 4–5 min at 95 °C on a heating block and allowed to cool down. The samples are spin to pool it down before loading.
5. 10 % SDS-PAGE gel is prepared (*see* **Note 6**).
6. There are two parts of gel, resolving part is at the bottom and stacking part is on the top.
7. The composition of resolving gel varies with different percentage of gel (*see* **Note 7**). On the top of resolving gel, a thin layer of isopropanol is added to remove the bubbles. It is kept for polymerization for nearly 12–30 min. After polymerization isopropanol is removed from the top layer and stacking gel is loaded. On the top layer of stacking gel, different-well comb is inserted. The staking gel is also left for 12–30 min (*see* **Note 8**).
8. Remove the comb after gel polymerization and wash the wells properly with distilled water.

9. Remove the gel from the gel-preparation apparatus.
10. Transfer the gel with glass plate into a tank with running buffer (*see* **Note 9**) and test whether the electric current is passing properly.
11. After confirming the proper electric current, protein samples should be loaded into the wells of the gel. Protein marker (ladder) should also be loaded. The electric current should be 60–70 V (*see* **Note 10**).
12. Once the blue dye is passed from the gel (it takes approximately 1.5–2 h), the gel should be transferred on to a positively charged nitrocellulose or PVDF (Polyvinyl difluoride) membranes (*see* **Note 11**).
13. To make the transfer, a sandwich is prepared where gel is placed on a Whatman blotting paper supported by a cushion. Just above the gel, nitrocellulose membrane should be placed and above that, Whatman paper and cushion should be placed. All the above things should be in wet condition in transfer buffer (*see* **Note 12**).
14. The sandwich should be packed in a cassette and loaded in a transfer apparatus in a gel tank (*see* **Note 13**).
15. The transfer current should be 0.1–0.2 Ampere at 4 °C, overnight (*see* **Note 14**).
16. After transfer, the sandwich is opened and nitrocellulose membrane is removed nicely and transferred into a gel box with 1× TBS (Tris-buffered Saline) (*see* **Note 15**).
17. Allow it to incubate for 5–10 min.
18. Transfer the membrane into another gel box with blocking solution (5 % nonfat dry milk in 1× TBS) and incubate it for 30 min to 1 h at room temperature.
19. Transfer the membrane into another gel box with anti-rabbit ANP and anti-mouse-Actin primary antibody (1:1,000 dilution in 3 % milk in TBST (TBS, 0.1 % tween-20) (*see* **Note 16**).
20. Incubate in primary antibody for overnight at 4 °C or 2–3 h at room temperature on slight rotation (for homogeneous binding to the antigen).
21. Remove the membrane into fresh gel box containing 1× TBST. Incubate for 5 min at room temperature with rocking to wash the membrane.
22. Washing should be repeated three to five times (*see* **Note 17**).
23. Incubate the membrane with the recommended dilution of labeled secondary antibody (IgG-HRP) in 3 % blocking buffer in TBST for 1 h at room temperature.
24. Wash the membrane in the same manner as done for the primary antibody.

25. After washing add ECL substrate on membrane and keep it for 1–2 min (*see* **Note 18**).
26. Develop the developed using ChemiDoC instrument following their protocol.
27. Analyze the relative intensity of target bands (here, ANP) with the endogenous control (here, actin) to determine the increase or decrease in target protein (Fig. 5).

3.5 Measuring Hypertrophy by Staining the Cell Membranes with Wheat Germ Agglutinin (WGA)

1. Dehydrate the frozen transverse sections of the hearts (*see* **Note 19**) by treating with phosphate buffered saline (PBS) for 5–10 min.
2. Fix the hydrated sections with freshly prepared 4 % formaldehyde (15 min at 37 °C).
3. Wash the sections 3× with PBS for 5 min.
4. Prepare a 1.0 mg/mL wheat germ agglutinin (WGA) conjugate stock solution by dissolving 5.0 mg of lyophilized WGA conjugate in 5.0 mL of PBS. Protect it from light (*see* **Note 20**).
5. Apply 100–200 μL WGA solution (*see* **Note 21**) on to the sections and incubate them for 1 h (*see* **Note 22**) at room temperature.
6. If required, these sections can also be stained with DAPI (10 min) (*see* **Note 23**).
7. Mount the sections by applying mounting medium (*see* **Note 24**) and coverslip
8. Keep the mounted sections for sometime to dry (*see* **Note 25**) and observe it under fluorescence microscope.
9. The cell boundaries of cardiomyocytes in the sections are stained with WGA (Fig. 6).

4 Notes

1. When genotyping is done in 10–21 days old mice, ice-cold ethanol is used as local anesthesia before clipping 0.2–0.4 cm tail.
2. Any Gel Doc instrument with UV light will serve the purpose.
3. Excessive heat or boiling of sample may degrade gelatin. Since gelatin is the substrate for Mmp9, it is necessary to dissolve the gelatin without boiling it.
4. The samples should not be boiled as it alters the quaternary structure of protein and impairs the enzymatic activity of Mmp9.
5. The concentration of dye (2×, 4× or 6×) depends on protein concentration. If the protein is less concentrated, it will

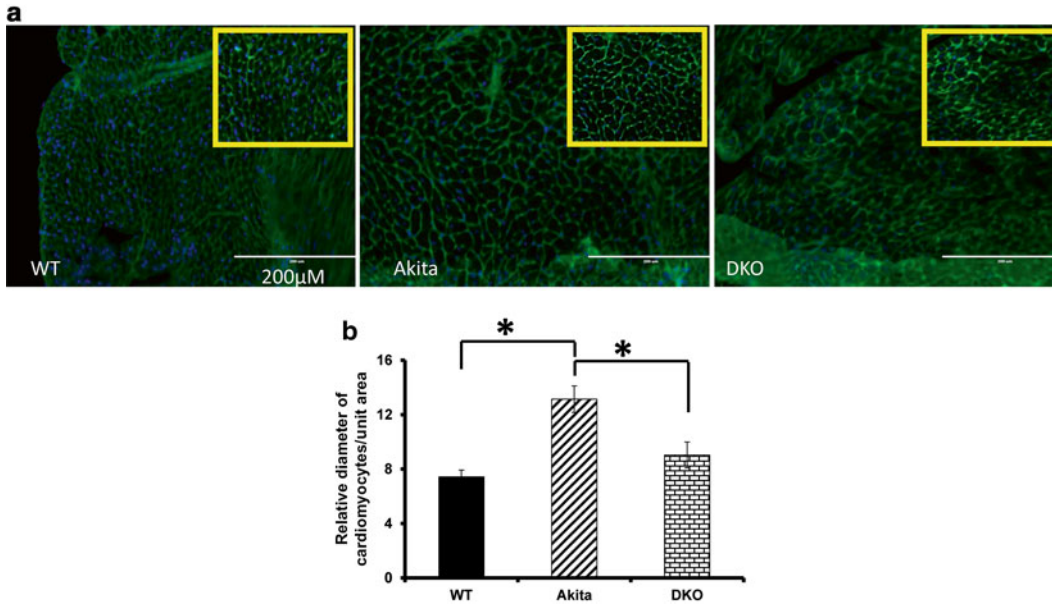

Fig. 6 Measurement of cardiac hypertrophy by WGA staining. (**a**) Fluorescent wheat germ agglutinin (WGA, *green color*) stains cell membrane and is used to demarcate cell boundaries. DAPI (*blue*) is used to stain cell nuclei. WGA staining in the transverse sections of WT, Akita, and DKO hearts, where the magnified view of size of cardiomyocytes are shown in inset. The size of cardiomyocytes is bigger in Akita as compared to WT. However, the size is relatively decreased in DKO hearts. (**b**) Bar graph showing relative increase in the diameter of the cardiomyocytes, which is used to measure cardiomyocyte hypertrophy. $N = 3$, $^*p < 0.05$

require more volume for a desired concentration. Since the total volume of a well in a SDS-PAGE gel is fixed, higher concentration (6×) dye should be used. Conversely, if the concentration of protein is high, less concentrated dye (2×) should be used. β-Mercaptoethanol is added to the sample buffer in the definite proportion as recommended in the buffer stock. It is recognized easily by its pungent smell and is essential for breaking disulfide bonds of protein that helps in linearizing the proteins.

6. The percentage of SDS-PAGE gel depends on molecular weight of target proteins. If the molecular weight of protein is higher than 150 kDa, it is recommended to use 6 % gel. On the other hand, if the molecular weight of the target protein is lower than 50 kDa, it is recommended to use 12–15 % gel. If the molecular weight is between 50 and 150 kDa, it is recommended to use 8–10 % gel. The molecular weight of Mmp9 is 92 kDa, therefore, 10 % gel is used.
7. The premade gels can be used. However, if required to prepare, glass-plate and gel-preparation apparatus is required. The composition of 5 mL of 10 % resolving gel is distilled water = 1.9, 30 % acrylamide = 1.7, 1.5 M Tris (pH 8.8) = 1.3, 10 % SDS = 0.05, 10 % APS = 0.05, TEMED = 0.002 (unit in mL). The composition

of distilled water and 30 % acrylamide changes in different percentage of gel. For resolving gel, the composition of the gel remains same and does not change with percentage of gel. The composition for 1 mL of gel is distilled water = 0.68, 30 % acrylamide = 0.17, 0.5 M Tris (pH 6.8) = 0.13, 10 % SDS = 0.01, 10 % APS = 0.01, and TEMED = 0.001 (unit in mL).

8. Different sizes of glass plates are used for making gels. Make sure that the thickness of gel glass plate (say 1.5 mm) and the size of comb spacer should match. There are different well number combs. Depending on number of samples to be loaded, different well number combs are used. The prepared gel can be stored at 4 °C for at least overnight if covered properly.
9. Readymade 10× running buffer is available that can be diluted 10 times and 1× running buffer can be used. If preparing running buffer, use 25 mM Tris base, 190 mM glycine and 0.1 % SDS. The pH of the solution should be nearly 8.3.
10. The electric current should be kept in low voltage until the protein solutions are not migrated to resolving gels. Once it is in resolving phase, if required voltage can be increased to expedite electrophoresis. However, more than 120 V are not recommended.
11. Both nitrocellulose and PVDF membranes are positively charged. However, if PVDF membranes are used, they need to be activated in methanol but nitrocellulose membranes do not need to be activated.
12. Transfer buffer can be purchased readymade. The 10× transfer buffer has same composition as running buffer except there is no SDS. To make 1× transfer buffer from 10×, the ratio should be 100 mL of 10× transfer, 700 mL of distilled water and 200 mL of methanol. They are freshly prepared and mixed well before use.
13. It is necessary to double check the orientation of sandwich, gel apparatus and electric current. General method to check is place black part of sandwich in the black part of transfer apparatus and the black wire should be placed in the black part of transfer apparatus.
14. The increase in current generates heat. Therefore, proper cooling must be done if high current is applied. The transfer time also depends on molecular weight of the target protein. For lower molecular weight, less transfer time is enough but for higher molecular weight proteins, overnight transfer at 4 °C is recommended.
15. Transferring nitrocellulose membrane into 1× TBS facilitate the membrane to mix from methanol (organic) medium to TBST (aqueous) medium.
16. The dilution of primary antibody differs for different antibodies. If the protein is abundant such as housekeeping (tubulin or

actin), the dilution should be higher. Conversely, if the protein is less abundant, less dilution of antibody is recommended.

17. This is a critical step, where more washing can reduce the antibody binding and decrease the antibody signal. On the other hand, less washing can increase the nonspecific binding of antibody and increase the noise to signal ratio.
18. ECL is light sensitive; therefore keep the membrane with ECL away from direct light.
19. The paraffin sections need to be deparaffinized and hydrated. The cryosections should be hydrated with phosphate buffered saline (PBS) or distilled water. PAP-pens are used to make a boundary surrounding the tissue. It allows the hold the liquid in the boundary and helps to keep the section wet. Generally cells are permeabilized before adding the fluorescence dye. It helps in making pore in the cell membrane that allows entrance of antibody into the cells. However, for WGA staining, it is necessary to not permeabilize the sections to keep the cell membrane intact.
20. The stock solution should be stored at –20 °C. It is good for at least a month at –20 °C. For short-term storage, add sodium azide to a final concentration of 2 mM, and store at 2–6 °C.
21. The volume of WGA depends on the area surrounding the section. It is necessary to keep the section wet for incubation period.
22. The time of incubation depends on the desired intensity of WGA color. We found that 1.0 mg/mL concentration of WGA stains cell membrane prominently within 1 h.
23. To observe the location of nucleus, these sections can be stained with DAPI.
24. Different mounting medium can be used depending on the conditions. Antifade mounting medium such as ProLong® Gold antifade reagent or Fluoromount are good for long lasting color of WGA.
25. It is recommended to keep the mounted slide for drying and observe it once it is dry. It helps to keep the coverslip on position during observation. If the mounting medium is not dry, there may be movement of coverslip that may disturb the orientation of tissue sections.

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Chapter 23

Experimental Osteoarthritis Models in Mice

Julia Lorenz and Susanne Grässel

Abstract

Osteoarthritis (OA) is a slowly progressing, degenerative disorder of synovial joints culminating in the irreversible destruction of articular cartilage and subchondral bone. It affects almost everyone over the age of 65 and influences life quality of affected individuals with enormous costs to the health care system. Current therapeutic strategies seek to ameliorate pain and increase mobility; however, to date none of them halts disease progression or regenerates damaged cartilage or bone. Thus, there is an ultimate need for the development of new, noninvasive treatments that could substitute joint replacement for late- or end-stage patients. Therefore, osteoarthritis animal models for mimicking of all OA features are important. Mice develop an OA pathology that is comparable to humans, rapidly develop OA due to the short lifetime and show reproducible OA symptoms. They provide a versatile and widely used animal model for analyzing molecular mechanisms of OA pathology. One major advantage over large animal models is the availability of knockout or transgenic mice strains to examine genetic predispositions/contributions to OA.

In this chapter, we describe three widely used instability-inducing murine osteoarthritis models. The most common two methods for surgical induction are: (1) destabilization of the medial meniscus (DMM) and (2) anterior cruciate ligament transection (ACLT). In the DMM model, the medial meniscotibial ligament is transected while in the ACLT model the anterior cruciate ligament is destroyed. In the third, chemical induced instability method, intraarticular collagenase is injected into the knee joint. Intraarticular collagenase weakens articular ligaments which cause instability of the joint, and full-blown OA develops within 6 weeks. For morphological evaluation, we correspond mainly to the recommendations of OARSI for histological assessment of osteoarthritis in mouse. For statistical evaluation summed or mean scores of all four knee areas (medial tibial plateau (MTP), medial tibial condyle (MFC), lateral tibial plateau (LTP) or lateral femoral condyle (LFC)), medial and/or lateral regions are used.

In future, not only large animal models like guinea pigs, sheep, goats, or horses will be important for a better understanding of osteoarthritis, but especially the mouse model with its rapid development of osteoarthritis and its numerous advantages by providing knockout or transgenic strains will become more and more relevant for drug development and determination of genetic predispositions of osteoarthritis pathology.

Key words Osteoarthritis, Articular cartilage, Mouse model, Destabilization of medial meniscus (DMM), Anterior cruciate ligament transection (ACLT), Intraarticular collagenase injection

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1 Introduction

Osteoarthritis (OA) is an age-related and/or trauma-induced multifactorial, slowly progressing, and primarily non-inflammatory degenerative disorder of the synovial joints culminating in the irreversible destruction of the articular cartilage. Clinical symptoms of OA including some degree of movement limitation appear in more than 10 % of the world population affecting almost everyone over the age of 65. Because of the increasing longevity and obesity in the European Community, the burden caused by OA rapidly grows substantially influencing life quality of the affected individuals with enormous costs to the health care system. Current therapeutic strategies seek to ameliorate pain and increase mobility; however, to date none of them halts disease progression or regenerates damaged cartilage. Thus, there is an ultimate need for the development of new, noninvasive treatments that could substitute joint replacement for late- or end-stage patients. Although OA is the most common musculoskeletal condition that causes significant health and social problems worldwide, its exact etiology is still unclear. Genetic disorders, limb mal-alignment and overuse as well as metabolic problems (obesity, immune responses, inflammation) play an important role in the onset of OA. Besides metabolic imbalance, activation of the whole endochondral ossification program starting with cell proliferation through articular chondrocyte hypertrophy and apoptosis has been identified as an important determinant of OA progression. Many processes associated with chondrocyte hypertrophy—such as inhibition of Sox9, collagen II, and aggrecan, induction of MMP-13 expression, chondrocyte apoptosis, ECM mineralization, and recruitment of blood vessels and osteoclasts—are reactivated and contribute to OA pathophysiology. Although the view of a generalized hypertrophy of OA chondrocytes is controversial, signalling molecules relevant for endochondral ossification may be involved in OA pathogenesis. Recent studies suggest the presence of cells, which express mesenchymal stem cell (MSC) markers and possess multilineage differentiation capacity in normal articular cartilage. The frequency of these resident chondroprogenitor cells is increased in OA implicating that these cells are involved in the pathogenesis of the disease. Reactivation of embryonic differentiation pathways and the proposed presence of multipotential mesenchymal cells in adult articular cartilage underscore the clinical relevance of chondrocyte differentiation associated processes for OA pathogenesis [1–5]. Whatever the cause, the significant burden of OA is not in question. Therefore, intensive further research is required to fully understand how OA affects an individual physically and psychological and to determine their healthcare need.

1.1 Advantages of Murine OA Models

Often OA develops from a focal lesion, which can be generated traumatically in animals and humans. Post-traumatic OA may develop more rapidly and can be reproduced by mechanical insult and surgically by creating joint instability. For better understanding, preclinical and clinical studies should be conducted in parallel. For that, animal models of OA have a wide range of severity and rate of progression of pathological changes. Naturally occurring models develop over a much longer period of the animal's life. For a variety of reasons, it is advantageous for industry to use more rapid models of OA, i.e., for drug development. Regardless of the rate of development of OA pathology, it is important to study selected sites of the joint (areas of abnormal loading/extreme overload) rather than the whole joint to ensure valid comparisons can be made to test hypotheses [6].

Mouse models may be of special use in the prevention of OA. There is a need to use knockout or transgenic mice to examine genetic predispositions/contributions to OA. Conditional genetic manipulations should be preferred, particularly those that can be induced in adult animals. The relationship between disease and activity impairment and OA onset requires more study. It should be remembered that genetically altered mice might better be considered as research tools and are not necessarily better OA models. Natural onset OA is clearly more pronounced in the STR/ort and STR/1N mouse strains and these are viewed as effective screening models. However, it is important to recall that the exacerbating effect of joint loading in human OA may not be accurately captured possibly due to scaling effects [6].

The age of mice used for OA studies should be at least 10 weeks as OA is a disease of adults and growing animals have a better capacity to manage joint damage. At that age mice are skeletal mature even if the growth plate is not closed. In rat and mice however, longitudinal long bone growth has ceased even if the growth plate never closes completely. In addition, there are differences in osteoarthritis progression and severity between male and female mice. This is partly in contrast to humans. The incidence of knee, hip and hand OA is higher in women than men and in women it increases dramatically around the time of menopause [7]. The latter finding has led investigators to hypothesize that hormonal factors may play a role in the development of OA, but the results of clinical and epidemiologic studies have not universally corroborated this. Some have shown a protective effect for estrogen or hormone replacement therapy on radiographic knee and hip OA or progression to joint replacement [8–11]. However, a recent systematic review of 16 studies found that there was no clear association between sex hormones and radiographic hand, knee or hip OA in women, although single analysis of the studies was not possible due to study heterogeneity [10, 11].

However, when using mice, gender specific effects should be considered and data need to be evaluated separately for male and female. Sex hormones play a critical role in the progression of OA in the murine medial meniscus destabilization (DMM) surgical model with males having more severe OA than females. Ovariectomized female mice had more severe OA than intact mice indicating a protective effect of female sex hormones, whereas testosterone exacerbates OA in male mice [12].

1.2 Methods of OA-Induction in Mice

There is a perceived advantage in using naturally occurring models of OA, as they are more like human OA with slower onset and progression. In general, development of spontaneous OA is linked to a particular genetic background.

1.2.1 Spontaneous/Natural Osteoarthritis

STR/ort mice spontaneously develop degenerative changes of knee joints with aging resembling human osteoarthritis, with the males being more severely affected than the females. Both cartilage degeneration and articular cytokine expression differ between sexes. The protection from cartilage degeneration in female mice correlates with an increased expression of TGFbeta1 and IL4 at 2 months of age. Thus, the increased expression of TGFbeta1 and IL4 in young STR/ort female mice suggests that the sexual dimorphism is mediated through the expression of cytokines involved in articular cartilage metabolism [13]. This is supported by a study from de Hooge et al. [14] who demonstrated that upon aging, IL-6$^{-/-}$ male mice developed more severe spontaneous OA. Reduced proteoglycan synthesis and bone mineral density (BMD) values might be indicative for an impaired repair response in IL-6$^{-/-}$ mice. This suggests a protective role for IL-6 in age-related OA in male mice [14]. Strong candidates for development of spontaneous OA are altered or missing structural extracellular matrix (ECM) molecules. Mice deficient for collagen IX, a fibril-associated collagen with interrupted triple helices (FACIT)—collagen highly specific for cartilage, develop spontaneous OA at the age of 3 months. Histologic analysis showed age-dependent OA-like changes in the knee and temporomandibular joints (TMJs) of *Col9a1−/−* mice starting at the age of 3 months. At the age of 6 months, enhanced proteoglycan degradation was observed in articular cartilage of knee and TMJs of mutant mice. The expression of matrix metalloproteinase (MMP)-13 and discoidin receptor (DDR)-2 proteins and the amount of degraded type II collagen were higher in knee joints of *Col9a1−/−* mice than in their wild-type littermates. Changes in cartilage mechanics were observed in femoral and tibial plateaus of *Col9a1−/−* mice at 6 months, including a decrease in compressive modulus and uniaxial modulus. At 3 and 6 months of age, tibial cartilage in *Col9a1−/−* mice was found to be more permeable to fluid flow, with an associated compromise in the fluid pressurization mechanism of load support. All of these changes occurred only at medial sites indicating that disturbance of

the structural integrity of the extracellular matrix is a prerequisite for developing degenerative changes in the joint [15, 16].

In addition, transcription factors may be critical for OA pathogenesis. Peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor, is suggested as an attractive therapeutic target to counteract degradative mechanisms associated with OA. This is supported by the fact that adult PPARγ knockout mice exhibited a spontaneous OA phenotype starting at the age of 6 months. This phenotype is associated with enhanced cartilage degradation, hypocellularity, synovial and cartilage fibrosis, synovial inflammation, mononuclear cell influx in the synovium, and increased expression of catabolic factors, including matrix metalloproteinase-13, accompanied by an increase in staining for matrix metalloproteinase-generated aggrecan (VDIPEN) and type II collagen (C1-2C) neoepitopes. PPARγ-deficient articular cartilage exhibits elevated expression of additional catabolic factors as hypoxia-inducible factor-2α (HIF), syndecan-4, and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) and of the inflammatory factors cyclooxygenase-2 and inducible nitric oxide synthase. This leaves PPARγ as a critical regulator of cartilage health, the lack of which leads to an accelerated spontaneous OA phenotype [17].

1.2.2 Chemically Induced Osteoarthritis

Intraarticular injection of monosodium iodoacetate (MIA) or collagenase creates an acute model for the study of acute cartilage degradation and joint pain [18]. However, it has some limitations as a model for OA. For example, since MIA is a metabolic poison, chondrocyte cell death in this model is extensive, unlike in human OA. Therefore, this method is only rarely used for induction of OA. Intraarticular collagenase weakens the articular ligaments, which causes instability of the joint, and full-blown OA develops within 6 weeks [19]. Van Osch et al. [20, 21] induced degenerative joint disease in knee joints of mice by intraarticular injection of two different stimuli: monosodium iodoacetate and highly purified collagenase. Intra-articular injection of MIA in vivo inhibited proteoglycan synthesis in cartilage from the central part of the patella. In the peripheral part of the patella, inhibition on day 1 was followed by stimulation of synthesis on days 3–30. Proteoglycan synthesis was inhibited in central parts of medial and lateral tibial plateaus. In vitro incubation with collagenase did not have a direct effect on proteoglycan metabolism of intact cartilage; this led to the assumption that osteoarthritis after injection of collagenase is caused by ligamentous injury, which leads to an unstable joint indicating that mechanical instability is the primary cause for collagenase induced development of OA. In addition, they found that the amount of cartilage loss and the degree of osteophyte formation at the medial side of the joint depended mainly on the severity of cruciate ligament damage. This is in contrast to changes at the

lateral side of the joint, which appeared not to be associated with the severity of ligament damage [20, 21]. The MIA induced OA model is mainly a good model for pain related OA research. Ogbonna et al. [22] reported that intra-articular MIA is associated with referred mechanical hypersensitivity and increased release of calcitonin-gene-related-peptide (CGRP) from primary afferent fibers in the dorsal horn where second-order neuron activation is associated with a microglial response. Antagonism of CGRP receptor activation provides a therapeutic avenue for treatment of pain in OA [22].

1.3 Mechanical Instability-Induced Osteoarthritis

Considering that mouse is now the most ideal animal for the molecular study due to recent progress in mouse genetics and the availability of transgenic and knockout mice, mechanical instability-induced OA models in mice have been established that are reproducible and resemble human OA, using a microsurgical technique to produce instability in the knee joints. In principal four slightly different methods were developed which lead to different severity grades of OA.

The first surgical model of OA described in the mouse, was the partial medial meniscectomy (PMM) and medial collateral ligament transection (MCLT) model, developed by Visco et al. [23]. This model was used to evaluate interleukin-1 beta (IL-1ß), MMP-3 (Stomelysin-1), interleukin- 1 converting enzyme (ICE), and inducible nitric oxide synthase (iNOS) knockout (KO) mice. Surprisingly, disease was not abrogated in any of these KO mice and more severe OA was observed in contralateral limbs of the IL-1ß KO. This result could indicate that other catabolic pathways were upregulated, increased weight-bearing was occurring on the non-operated limb, or that alternate models or scoring systems may be more appropriate [24]. This note supports the hypothesis of Visco et al. that mechanical instability created in the PMM and MCLT model may be too severe to overcome, and may also be impacted by differences in histological sectioning and scoring between different research groups.

The two preferred models for induction of OA in mice are the anterior cruciate ligament transection (ACLT) and destabilization of the medial meniscus (DMM). The ACLT method was first applied in mice by the group of Kamekura [25] who established four types of murine models exhibiting various speeds of OA progression which were classified as severe, moderate, mild, and medial, depending on the severity and direction of joint instability imposed by combinations of ligament transection and menisectomy. The early stage changes in the mouse articular cartilage after surgery were found to be a defect of the superficial zone and a decrease of Safranin-Orange (Safranin-O) staining, followed by progressing cartilage destruction and aggravation of joint congruency that created a vicious circle to advance the condition. These

changes were identical to human OA pathology reported as arthroscopic and histological findings [26, 27]. Along with catabolic changes, anabolic reactions such as chondrocyte proliferation, subchondral sclerosis, and osteophyte formation were also seen in human OA cartilage. Although instability was present in the whole knee joint, OA changes were much more severe in the tibial cartilage than in the femoral condyle cartilage. Since the cartilage layer is much thinner and osteophyte formation is rarely seen in the femoral condyle, it is difficult to quantify OA development with the same accuracy as in the tibial cartilage. Hence, current ACL models focused on OA mediated changes in tibial cartilage. In the three models with ACL transection, severe, moderate and mild, OA development in posterior tibial cartilage was dependent on the severity of joint instability, indicating that combinations of these models will enable to identify molecular backgrounds at various stages. The severe and moderate models appeared to be useful to evaluate reactive osseous changes like osteophyte formation which is characteristic in the late stage of human OA. Because the moderate model showed somewhat slower progress in the early stage of cartilage destruction than the severe model, this model also seemed suitable to follow the entire process including early stage changes. OA development in the mild model was limited to a partial cartilage destruction, and chondrocyte morphology was relatively preserved during the observation period, indicating that this model is useful for detailed investigations of the early stage [25].

The lab of Glasson evaluated the ACLT model because of its wide use in other species, and the DMM model, which was selected, based on earlier unpublished studies in guinea pigs where this model induced OA with great ease and reproducibility. The severity of OA was compared for both models, with the aim of identifying a mild to moderate model of OA, so that effects of disease modification with enzyme-deleted mice would not be overwhelmed by greater biomechanical damage associated with more severe models. In addition, more severe models of OA were considered to have a greater association with iatrogenic damage, variability, biomechanical unloading, or regenerative changes (such as dramatic osteophyte formation or ankylosis) that could hamper the assessment of cartilage degradation. The DMM model provided extremely good reproducibility and a slower progression of disease with mild to moderate OA progression quite comparable to the human situation, and was subsequently selected for evaluation of enzyme-deleted mice, where it was instrumental in showing that ADAMTS-5, and not ADAMTS-4, was critical for the progression of surgically induced mouse OA [28–30].

The DMM model was used subsequently in a variety of transgenic and knockout mice in order to evaluate the influence of specific genes of interest in OA development, progression and severity.

As examples, these were mice with genes deficient for structural matrix proteins (MIA/CD-RAP), proteinases (ADAMTS-4, -5), ECM receptors (Syndecan-4), growth factors (fibroblast growth factor (FGF) 2 [31]), cytokines (IL-1ß [24]), and hormone receptors (melanocortin (MC) 1-receptor (own unpublished data)).

For mice deficient in melanoma inhibitory activity/cartilage-derived retinoic acid-sensitive protein (MIA/CD-RAP), a structural component of fibrillar cartilage ECM [32], application of the DMM model, clearly showed enhanced chondrocytic regenerative capacity under MIA/CD-RAP deficiency. For the first time, proliferating chondrocytes could be determined because loss of MIA/CD-RAP clearly illustrated the highly regenerative potential in mature cartilage. Consequently, proliferating chondrocytes together with their increased matrix production not only counteracted development of lesions in MIA/CD-RAP-deficient mice as seen at day 21 of OA compared with WT but also promoted enhanced regeneration at day 42, although OA proceeded in WT. The score of MIA/CD-RAP-deficient mice 42 days after OA induction decreased to the level of the control group, which leaves no doubt to the enhanced tissue regenerative capacity by loss of MIA/CD-RAP [33].

Induction of surgical osteoarthritis in syndecan-4 knockout (*Sdc4–/–*) mice, showed typical signs of proteoglycan loss in operated knees of wild-type controls, whereas a strong Safranin-O staining in knee cartilage of *Sdc4–/–* mice was found. A higher Mankin score (12.5 points) [34] was found in operated knees of wild-type mice compared to in control mice (6.0 points) with a significant reduction of cartilage thickness and a substantial loss of proteoglycans. Syndecan-4 deficiency completely protected mice from the development of osteoarthritis-like changes. Morphometric quantification of the Safranin-O-stained cartilage area in operated knee joints showed that *Sdc4–/–* mice have less than 30 % of the proteoglycan loss of wild-type mice. During osteoarthritis, ADAMTS-4 and ADAMTS-5 activity is increased in articular chondrocytes, which changes the physiological balance between matrix synthesis and degradation and results in an enhanced aggrecan proteolysis. As the loss of syndecan-4 protected mice from early cartilage degradation, the appearance of ADAMTS-mediated aggrecan cleavage products was analyzed. Whereas wild-type mice showed a prominent aggrecan neoepitope staining throughout the arthritic cartilage, less staining for aggrecan neoepitopes in *Sdc4–/–*osteoarthritic cartilage was found. Morphometric analysis of cartilage sections showed that 80 % of wild-type but only 20 % of *Sdc4–/–* chondrocytes were surrounded by an aggrecan neoepitope–positive matrix. These data clearly show that loss of syndecan-4 protects mice from the development of osteoarthritis-like changes, a phenotype almost identical to the phenotype of *Adamts5–/–* mice [35].

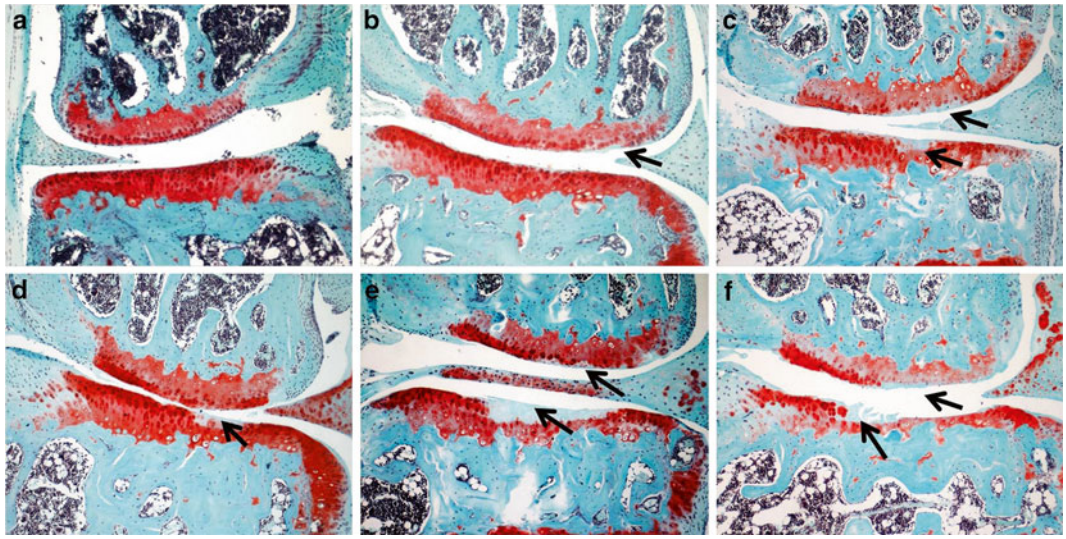

Fig. 1 Osteoarthritis progression after DMM in male C57bl/6 wild-type mice. Osteoarthritis progression is demonstrated on medial areas of Safranin-O stained sections of right hind knee joints at 2 (**b**), 4 (**c**), 6 (**d**), 8 (**e**), and 12 (**f**) weeks after DMM surgery. Left knee joints served as Sham controls (**a**). *Arrows* indicate articular cartilage damage of femoral condyle and tibial plateau. $^{**}P < 0.01$. (**a–f**) ×100 magnification

We have applied the surgical DMM method using wild-type C57bl/6 which provided reproducible results (*see* Figs. 1 and 2).

2 Materials

2.1 Basics

1. Gloves (Roth).
2. Eppendorf cups 2.0 ml (Eppendorf).
3. Pipette tips 5, 10, 50 ml (Sarstedt AG & Co).
4. Stereo microscope model 33213 (Eschenbach Optik GmbH).
5. 1× PBS sterile (Roth).
6. 100 % Ethanol (Merck).
7. 2-Propanol (Merck).
8. Paraformaldehyde (Sigma).
9. Paraplast (Surgipath).
10. Roti-Histol (Roth).
11. Roti-Histokit (Roth).

2.2 Instability-Induced Osteoarthritis

1. Mouse Dissecting Kit (World Precision Instruments).
2. Single syringe for insulin U100, 0.3 mm (Becton Dickinson).

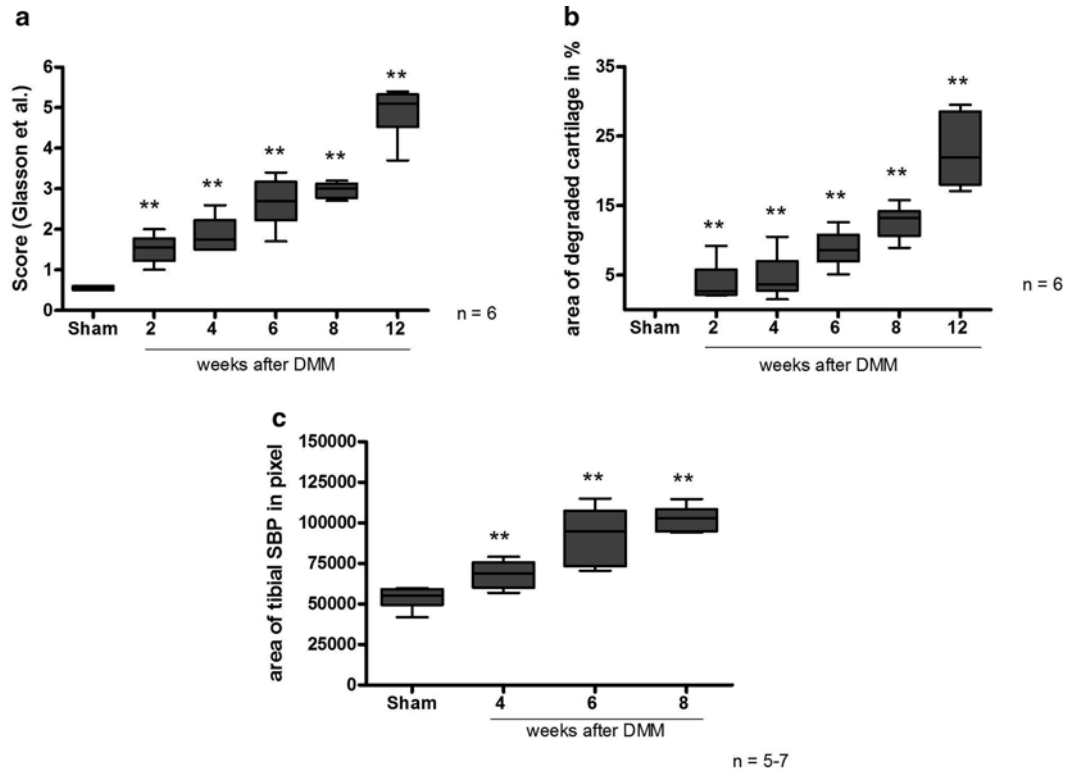

Fig. 2 Histological and morphometrical evaluation of osteoarthritis progression after DMM in male C57bl/6 wild-type mice. Osteoarthritis progression is demonstrated after histological scoring according to Glasson et al. (2010) (**a**) and after morphometric scoring (**b**) on medial areas of articular cartilage and after morphometric scoring of medial tibial areas of subchondral bone plate (**c**). 5–6 Safranin-O stained sections in 80 μm intervals through the weight-bearing area of hind knee joints were scored. Osteoarthritis was induced with DMM and sections were prepared 2, 4, 6, 8 and 12 weeks after surgery. Left hind knee joints served as Sham controls. SBP = subchondral bone plate. $^{**}P < 0.01$

3. Anesthetics: Xylazine 2 % injection solution (Serumwerk Bernburg) and Ketamine 10 % injection solution (Bela-pharma).
4. Analgesia: Buprenovet 0.3 mg/ml injection solution (Bayer vital).
5. Michel suture clips (1.75 × 1.75 mm) and applicator (11 mm) (Fine science tools).
6. Eye and ear ointment Bepanthen (Bayer vital).
7. Absorbable fissure material vicryl 8-0 (Ethicon).
8. Injection cannula 0.55 × 25; 0.45 × 13 (Becton Dickinson GmbH).
9. 0.9 % Sodium chlorid solution sterile (Braun).
10. 70 % Ethanol solution: 700 ml Ethanol; 300 ml distilled water.
11. Collagenase from *Clostridium histolyticum* (Sigma).

2.3 Specimen Preparation

1. 60 °C incubator (Binder GmbH).
2. Embedding forms metal (Roth).
3. Embedding boxes Rotilabo® 40 × 28 × 6.8 mm (Roth).
4. Embedding station EG 1150H (Leica Biosystems).
5. 4 % Paraformaldehyde solution: 40 g Paraformaldehyde; 1,000 ml PBS; plus sodium hydroxide concentrated (32 %) to pH 7.4.
6. 20 % Ethylenediaminetetraacetic acid (EDTA) solution: 200 g EDTA; 200 ml distilled water; plus sodium hydroxide concentrated (32 %) to pH 7.4; ad 1,000 ml distilled water.
7. 50 % Ethanol solution: 500 ml Ethanol; 500 ml distilled water.
8. 70 % Ethanol solution: 700 ml Ethanol; 300 ml distilled water.
9. 96 % Ethanol solution: 960 ml Ethanol; 40 ml distilled water.
10. 1:1 2-propanol–Paraplast mixture: 250 ml 2-propanol; 250 ml Paraffin.

2.4 Sectioning

1. Rotational microtome RM 2155 (Leica Biosystems).
2. Cooling block EG 1150C (Leica Bioscience).
3. Water bath TFB 45 (MEDITE GmbH).
4. Superfrost plus object slides (Menzelgläser, *see* **Note 1**).
5. 37 °C incubator (Memmert GmbH + Co. KG).

2.5 Safranin-Orange Staining

1. Weigert's iron hematoxylin solution: Stock solution A: 1 g hematoxylin; 100 ml 95 % alcohol. Stock solution B: 4 ml 29 % ferric chloride in water; 95 ml distilled water; 1 ml hydrochloric acid, concentrated. Working solution: Mix equal parts of stock solution A and B. This working solution is stable for about 4 weeks.
2. 0.5 % acid alcohol solution: 10 ml HCl, concentrated (32 %); 900 ml 70 % ethanol.
3. 0.001 % Fast green (FCF) solution: 0.01 g Fast green, FCF, C.I. 42053; 1,000 ml distilled water.
4. 1 % Acetic acid solution: 1 ml acetic acid, glacial; 99 ml distilled water.
5. 0.1 % Safranin-O solution: 0.1 g Safranin-O, C.I. 50240; 100 ml distilled water.

3 Methods

3.1 Instability-Induced Osteoarthritis

There are three widely used instability-induced osteoarthritis models for mouse described in literature (*see* Subheading 1.3).

The most common two methods for surgical induction are: (1) destabilization of the medial meniscus (DMM) and (2) anterior

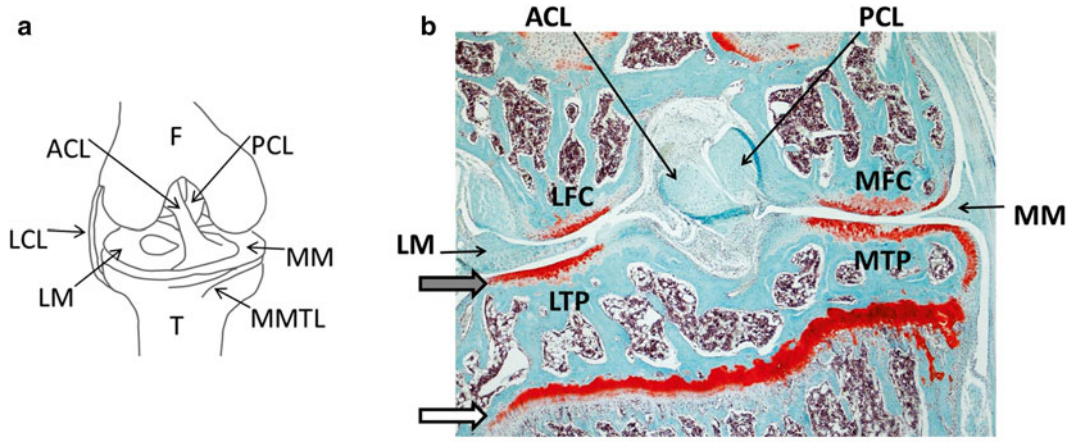

Fig. 3 Overview of a right knee joint of a mouse. (**a**) Outline of a right knee joint. The MMTL is transected to generate destabilization of the medial meniscus (DMM). The ACL is transected in the ACLT model [29]. (**b**) Overview of Safranin-O stained frontal section of a mouse knee joint 4 weeks after DMM induction. *Grey arrow* indicates articular cartilage, *white arrow* indicates growth plate. ×40 magnification. ACL: anterior cruciate ligament; F = femur; LCL = lateral collateral ligament; LFC: lateral femoral condyle; LM = lateral meniscus; LTP: lateral tibial plateau; MFC: medial femoral condyle; MM: medial meniscus; MMTL = medial meniscotibial ligament; MTP: medial tibial plateau; PCL: posterior cruciate ligament; T = tibia

cruciate ligament transection (ACLT). Both methods induce instability in mouse knee joint through transection of important ligaments. In the DMM model, the medial meniscotibial ligament is transected while in the ACLT model the anterior cruciate ligament is destroyed (*see* Fig. 3).

In the chemical induced instability method, intraarticular collagenase was injected into the knee joint. Intraarticular collagenase weakens articular ligaments, which causes instability of the joint, and full-blown OA develops within 6 weeks [19].

For all three methods, right hind knee joints are used to surgically induce osteoarthritis. Sham operated right knee joints of a seperate group served as controls (*see* **Note 2**). Sham surgery in DMM and ACLT means the direct visualization of ligaments without transection and in the chemical method an injection of physiological sodium chloride solution.

Osteoarthritis induction can start with 10 week old mice, because long bone growth is finished at this age even though the growth plates are not closed [6].

3.1.1 Destabilization of Medial Meniscus (DMM) [29]

1. Anesthetize mice intraperitoneal with Xylazine (6–8 mg/kg body weight) and Ketamine (90–120 mg/kg body weight).
2. Administer locally eye ointment and subcutaneous NaCl-infusion (10 ml/kg body weight).
3. Desinfect operation area and hind legs of the mouse with 70 % ethanol.

4. Incise 3 mm longitudinal over the distal patella to proximal tibial plateau.
5. Open the joint capsule directly medial to the patella tendon with micro-iris scissors.
6. Dissect the fat pad of the cranial horn and of the medial meniscus with forceps.
7. Visualize the intercondylar region, the meniscotibial ligament and the medial meniscus.
8. Transect the ligament with micro-iris scissors and confirm transection through lifting of the meniscus from the tibia plateau.
9. Suture the joint capsule with a continuous fissure.
10. Close the cutaneous layer with 1–2 Michel suture clips.
11. Administer subcutaneous NaCl-infusion (10 ml/kg body weight) and Buprenorphine (0.09 mg/kg body weight) 12 and 24 h after operation.

3.1.2 Anterior Cruciate Ligament Transection (ACLT) [25]

1–5. *See* DMM model
6. Dislocate the patella medially.
7. Keep the cartilage moist with saline solution.
8. Transect the ACL with a micro-surgical knife and confirm complete transection by the presence of anterior drawer.

9–11. *See* DMM.

3.1.3 Intra-articular Collagenase Model [18, 19]

1–2. *See* DMM model
3. Inject 1 U collagenase intra-articular through the patellar ligament on day 0 and 2.

3.2 Histological Evaluation

We correspond mainly to recommendations of OARSI for histological assessment of osteoarthritis in mice [36].

3.2.1 Specimen Preparation

1. Euthanize mice with CO_2.
2. Dissect knee joints free of skin and muscle.
3. Fixate specimen 18–24 h with 2 ml 4 % paraformaldehyde solution.
4. Decalcify joints with 8 ml 20 % EDTA solution for 2 weeks (*see* **Note 3**).
5. Change EDTA solution every 2–3 days.
6. Wash joints three times 30 min with PBS.
7. Dehydrate specimen with ascending alcohol row (2–3 h EtOH 50 %, 2–3 h EtOH 70 %, over night EtOH 96 %, 4 h EtOH 96 %).
8. Remove alcohol and solvent with 2-propanol (twice 2–6 h).

9. Transfer knee joints in boxes for embedding.
10. Infiltrate specimen with paraffin at 60 °C (24 h with 1:1 2-propanol/paraffin mixture, 12 h and afterwards 24 h with paraffin).
11. Embed knee joints frontal in paraffin blocks (*see* **Note 4**).
12. Harden paraffin blocks at least for 24 h at 4 °C.

3.2.2 Sectioning

1. Cool paraffin blocks at least 30 min on a 0 °C cooling block.
2. Cut paraffin embedded knee joints frontal in 5 μm sections with a rotational microtome.
3. Select sections through the entire weight-bearing area of the joint (*see* **Note 5**).
4. Bring sections from 50 °C warm water to object slides.
5. Dry sections at least 2 days at 37 °C.

3.2.3 Safranin-Orange Staining

This method is used for the detection of cartilage, mucin, and mast cell granules on formalin- fixed, paraffin-embedded tissue sections, and may be used for frozen sections as well, to determine the grade of osteoarthritis. Cartilage and mucin will be stained orange to red, and nuclei will be stained black. Cytoplasm is stained green.

1. Deparaffinize slides (two times 5 min incubation in Roti-Histol).
2. Hydrate slides with a descending alcohol row (two times 1 min incubation in 2-propanol, two times 1 min in 96 % ethanol, 1 min in 80 % ethanol, 1 min in distilled water).
3. Stain with Weigert's iron hematoxylin working solution for 6 min.
4. Dip sections in distilled water.
5. Dip sections in 0.5 % acid alcohol.
6. Wash in running tap water for 5 min.
7. Stain with fast green solution for 5 min.
8. Dip three times in 1 % acetic acid solution.
9. Stain in 0.1 % Safranin-O solution for 6 min.
10. Dehydrate with an ascending alcohol row (dip 10 times in 96 % alcohol, 20 times in 2-Propanol, incubate 1 min in 2-Propanol and 2 times 2 min in Roti-Histol).
11. Mount using Roti-Histokit.
12. Dry over night at room temperature.

3.2.4 Histological Scoring of Cartilage

There are several scoring systems for murine osteoarthritis described in literature. We correspond here to widest accepted scoring system, recommendations of the OARSI [36] (Table 1). For evaluation of osteoarthritis in transgenic/knockout mice it might be suitable to

Table 1

Semi-quantitative scoring system recommended from the OARSI [36]

Grade	Osteoarthritic damage
0	Normal
0.5	Loss of Safranin-O without structural changes
1	Small fibrillations without loss of cartilage
2	Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina
3	Vertical clefts/erosion to the calcified cartilage extending to <25 % of the articular surface
4	Vertical clefts/erosion to the calcified cartilage extending to 25–50 % of the articular surface
5	Vertical clefts/erosion to the calcified cartilage extending to 50–75 % of the articular surface
6	Vertical clefts/erosion to the calcified cartilage extending >75 % of the articular surface

The recommended scoring system is a modification from Chambers et al. [38] and is recommended to apply to all four quadrants of the joint: medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), lateral tibial plateau (LTP)

determine additional the area of destroyed cartilage and changes of the subchondral bone area (*see* Fig. 4). We used therefore the graphic tablet Bamboo from Wacom which allows to precisely draw in digital pictures and ImageJ software to determine pixel.

Scoring should be applied to all four quadrants of the joint (medial tibial plateau (MTP), medial tibial condyle (MFC), lateral tibial plateau (LTP) or lateral femoral condyle (LFC)). OA severity is expressed as summed, maximal and/or mean scores, which can be combined for the entire joint, or split out for lateral and medial areas (*see* **Note 6**).

Histological Scoring System According Glasson et al. [36]

1. Use per specimen 5–6 sections in 80 μm intervals through the weight-bearing area of the joint.
2. Score each section according to the scoring system of Table 1.

Morphometric Scoring With Bamboo Graphic Tablet (Wacom)

1. Use per specimen 5–6 sections in 60–80 μm intervals through the weight-bearing area of the joint.
2. Circuit area of a cellular cartilage without Safranin-O staining and determine pixel.
3. Circuit area of complete cartilage (healthy and destroyed) and determine pixel.
4. Calculate destroyed cartilage to complete cartilage in percent.

Histological Scoring of Bone and Synovia

If there are any abnormalities regarding bone or synovia, like osteophyte formation, synovitis, or subchondral bone changes, OARSI recommended a 0–3 scoring paradigm: 0 = normal,

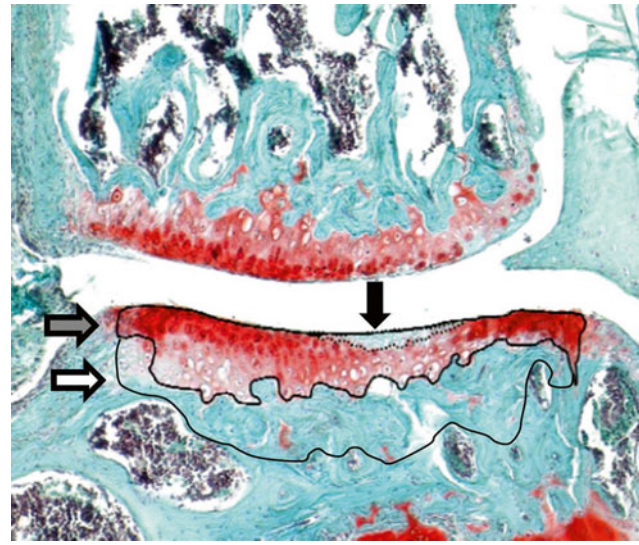

Fig. 4 A representative image for morphometrical evaluation of osteoarthritis. Morphometric evaluation is demonstrated on the medial area of a Safranin-O stained section of a right hind knee joint 4 weeks after induction of Osteoarthritis with DMM. *Black arrow* denotes area of a cellular/unstained cartilage, *grey arrow* the whole cartilage area and *white arrow* subchondral bone plate (SBP). ×100 magnification

1 = mild, 2 = moderate and 3 = severe changes (Glasson et al 2010). Especially for subchondral bone changes, above described morphometric scoring is suitable to determine the area of subchondral bone plate (SBP) and compare it between the groups (*see* Fig. 4). To analyze subchondral bone more in detail, MicroCT measurements are the appropriate choice [37]. Synovitis is not an appreciable feature of the milder surgical models of OA such as the DMM model. Notes should be made on any abnormalities such as blood presence in the joint, deposits, abnormal appearance of ligaments (chondrogenesis, degradation, etc.), meniscii, and subchondral bone cysts, etc. if they are present [36].

3.2.5 Statistical Analysis

For statistical evaluation, GraphPad Prism with the nonparametric Mann–Whitney-U test can be used if comparing two groups. If comparing multiple groups, the Kruskal–Wallis test ANOVA should be applied. Mean, maximal or summed scores of all four areas (medial tibial plateau (MTP), medial tibial condyle (MFC), lateral tibial plateau (LTP) or lateral femoral condyle (LFC)), medial and/or lateral regions can be included in statistic analysis.

3.2.6 Perspectives

In future, not only large animal models like guinea pigs, sheep, goats, or horses will be important for a better understanding of osteoarthritis, but especially the mouse model with its rapid development of osteoarthritis and its various possibilities through availability of knock out or transgenic mice get more and more relevant

for drug development and determination of genetic predispositions of Osteoarthritis.

4 Notes

1. It is highly important to use these Superfrost plus object slides, because sections get lost during staining procedure when using other slides.
2. We see OA-like symptoms in subchondral bone of sham-operated knee joints, when left knee joints served as sham controls and right knee joints received DMM. We therefore strongly recommend to use a separate control group for sham surgeries.
3. Some groups decalcify mouse knee joints at 37 °C, but we see a Safranin-O staining loss at this temperature after 2 weeks incubation time.
4. Fill the embedding form less than a half with paraffin. Put the knee joint with the white patella (is easy to observe) as the deepest point into the paraffin and adjust it vertically to the bottom of the embedding form. Cool paraffin only so much that the knee joint is stabilized at the bottom, but not completely hardened. Then fill up the embedding form completely with paraffin and cool it down slowly.
5. Sectioning requires a trained histologist/pathologist to recognize in unstained sections weight-bearing regions of the joint.
6. Morphological scoring should be executed from at least two independent persons.

Acknowledgements

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Chapter 24

Mouse Models and Methods for Studying Human Disease, Acute Kidney Injury (AKI)

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Abstract

Acute kidney injury (AKI) is serious complication in hospitalized patients with high level of mortality. There is not much progress made for the past 50 years in reducing the mortality rate despite advances in understanding disease pathology. Using variety of animal models of acute kidney injury, scientist studies the pathogenic mechanism of AKI and to test therapeutic drugs, which may reduce renal injury. Among them, renal pedicle clamping and cisplatin induced nephrotoxicity in mice are most prominently used, mainly due to the availability of gene knockouts to study specific gene functions, inexpensive and availability of the inbred strain with less genetic variability. However, ischemic mouse model is highly variable and require excellent surgical skills to reduce variation in the observation. In this chapter, we describe a detailed protocol of the mouse model of bilateral renal ischemia–reperfusion and cisplatin induced nephrotoxicity. We also discuss the protocol for the isolation and analysis of infiltrated inflammatory cell into the kidney by flow cytometry. Information provided in this chapter will help scientist who wants to start research on AKI and want to establish the mouse model for ischemic and toxic kidney injury.

Key words Ischemia–reperfusion injury, Cisplatin, Acute kidney injury, Flow cytometry, Immune cells, Biomarkers

1 Introduction

Acute kidney injury (AKI) has now replaced the term acute renal failure and is a frequent and serious complication among hospitalized patients. AKI has a poor prognosis with the mortality ranging from 10 to 80 %, depending upon the patient population studied. Patients who present with uncomplicated AKI have a mortality rate of up to 10 % [1, 2]. In contrast, patients presenting with AKI and multiorgan failure have been reported to have mortality rates of over 50 %. If renal replacement therapy is required, the mortality rate rises further to as high as 80 % [3]. AKI is no longer considered an innocent bystander merely reflecting coexistent pathologies. It has been demonstrated to be an independent risk factor for mortality [4, 5]. However, the cause for this is unclear but is possibly associated with an increased risk of "non-renal" complications

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such as bleeding and sepsis. An alternative explanation may lie in experimental work that has demonstrated the "distant effects" of ischemic AKI on other organs. In these experimental models, isolated ischemic AKI up-regulates inflammatory mediators in other organs including the brain, lungs, and heart [6, 7]. Once a patient has developed AKI, the therapeutic options are limited with the mainstay of treatment being renal replacement therapy (RRT).

The pathogenesis of AKI in humans is compound. The major causes of AKI in the intensive care unit (ICU) include renal hypoperfusion, sepsis/systemic inflammatory response syndrome (SIRS), and direct nephrotoxicity, although in most cases the etiology is multifactorial [8–11]. Furthermore, epidemiological studies involving patients undergoing cardiothoracic surgery [4, 12] or contrast administration [13, 14] have determined additional risk factors including age, preexisting hypertension, diabetes mellitus, heart failure, and prolonged and complex surgery. It follows that minimizing renal injury should confer a benefit to patients. The mechanism of AKI pathogenesis is only partially understood, and data regarding human AKI morphology is sparse. Thus, our understanding of this disorder depends, largely, on animal studies. Use of appropriate animal models is required to interpret the mechanism of the human disease and translating the finding into animals to cure the human disease. Use of inappropriate models may lead to misconceptions, adopting, and projecting irrelevant experimental findings to the clinical scenario.

The pathophysiology of various mouse models and the mode of action of tested drugs are the major determinants to be considered while designing AKI experiments. This will determine the model type, functional determination, and time course of tissue harvesting for histopathological studies. However, mouse models lack many of the procedures done in humans during the course of injury and treatments, such as repeated blood sampling, surgical manipulation and continuous physiological monitoring. In addition, physiology and renal structure are very different among different species and strains, and caution is warranted when extrapolating experimental data from an animal model to humans. Therefore, substituting large animals for disease models such as renal transplantation is recommended. There are several types of AKI disease models have been studied, such as toxic, Ischemic and Infectious/septic.

Nephrotoxic models, such as those induced by gentamycin, cisplatin, amphotericin, or mercury compounds, are the most predictable and consistent, and the kidney damage occurs due to transport-mediated accumulation of these toxins in the proximal tubular epithelial cells. Moreover, the damage is dose-dependent and can be enhanced by sodium depletion or dehydration. However, the intracellular mechanisms of tubular injury are different among the nephrotoxins. They may mediate the damage

through direct effects or reactive oxygen species (ROS) or through interruption of cellular metabolism and energy stores [15–20]. Renal hypoxia is also seen with radiocontrast and nonsteroidal anti-inflammatory drugs as well as glycerol-induced rhabdomyolysis.

Among ischemic AKI models, warm ischemia and reperfusion in rodents is the most widely used model. This model is simple and reproducible when renal blood flow is completely interrupted for a period of 25–45 min (mouse and rats). With renal venous stasis, inflammation seems to play an important role in hypoxic injury. The Outer stripe S3 segments of the proximal tubules are mostly affected in this model, suggesting their dependence on oxidative metabolism [21, 22]. Other models of selective medullary hypoxic injury are based on the intensification of physiologic medullary hypoxia [23] by the enhancement of tubular transport activity and oxygen consumption and/or by altering medullary blood flow [24, 25]. These are multi-insult hypoxic AKI models, comprising defective prostaglandin- or nitric oxide synthesis, combined with radiocontrast media, renal hypertrophy, chronic renal disease, diabetes, hyperlipidemia, altered effective blood volume, vasoconstrictive stimuli, endotoxemia, and so forth. Many of these models are replicas of clinical scenarios of AKI on top of predisposing risk factors. Medullary thick ascending limbs (mTALs) in the inner stripe of the outer medulla are mostly affected in these experimental models. S3 injury and papillary damage are noted as well [25]. It seems that in these models mTAL damage is caused by hypoxia as long as tubular sodium transport is maintained. Conceivably, mTALs are protected in the WIR model due to total cessation of urine generation and tubular transport. A comparable pattern of predominantly distal tubular injury is noted in cold ischemia and reflow (CIR) models [26]. Prolonged extracorporal cold preservation with subsequent re-implantation makes CIR models highly relevant to the clinical setup of cadaveric renal transplant. However, CIR is highly variable and is a difficult method to induce consistent injury.

Small and large animal strains markedly differ in their physiologic responses to experimental sepsis. Cecal ligation and perforation (CLP) in aged rodents have gained popularity. The experimental setting is complex and closely resembles the clinical scenario: fluid resuscitation and antibiotics are needed for a survival time long enough for the development of full-blown AKI. By contrast, endotoxin infusion, which may elicit systemic hemodynamic derangement, does not lead to progressive or established AKI. A recent excellent review article and other manuscripts [27–29] provide a more detailed description of methods and models on sepsis.

Despite some drawbacks, a warm ischemia–reperfusion injury model in mice is the most widely used, accepted model and results are consistent and reproducible. A conclusion can be drawn with a

small number of animals as compared to other ischemic models. Similarly, cisplatin-induced AKI is a widely used, accepted model for toxic AKI. It is reproducible, and conclusions can be drawn with a small number of animals. In this manuscript, we will discuss in detail on the methodology associated with these model systems in mice. Few other models are available for AKI and recent advances in early diagnosis of AKI using biomarkers.

2 Materials

2.1 Mouse Model of Warm Ischemia–Reperfusion Injury (Fig. 1)

1. Animals: We use 8–12 week old male C57BL/6 mice purchased from Jackson Laboratories and maintained in a normal room with a 12 h dark/12 h light cycle. Other strains can also be used but susceptibility and ischemic time may vary. Conditions need to be standardized for each particular strain (*see* **Note 1**).
2. Pentobarbital sodium (Sigma-Aldrich or Pharmacy grade bought through Diamond Drugs).
3. Vascular clamps (Fine Science Tools).
4. Vascular clamp applicator (Fine Science Tools).
5. Forceps.
6. Irish scissors (Fine Science Tools).
7. Sutures (Ethicon 4.0) (Fine Science Tools).
8. Needles (Fine Science Tools).
9. Thermal regulator with heating pad and rectal probe (CWE, Inc. Ardmore).
10. Normal saline (0.9 % NaCl).
11. Slide warmer.
12. Incubator capable of maintaining temperature at 33 °C.
13. Wound closure clips and applicator (Fine Science Tools).
14. Hemostats (Fine Science Tools).
15. 1 ml insulin syringe with 27 gauge needle.
16. Razor blade.

Fig. 1 (continued) applicator, and suture needle. (**c**) Rectal probe. (**d**). Shaved skin on the dorsal side of the mouse. (**e**) Renal pedicle clamped. (**f**) Animal covered with wet cotton gauze and rested on slide warmer to maintain body temperature during clamping period. (**g**) Removal of clamps and closure of wounds. (**h**) Animals left in cage outside of incubator after regaining consciousness

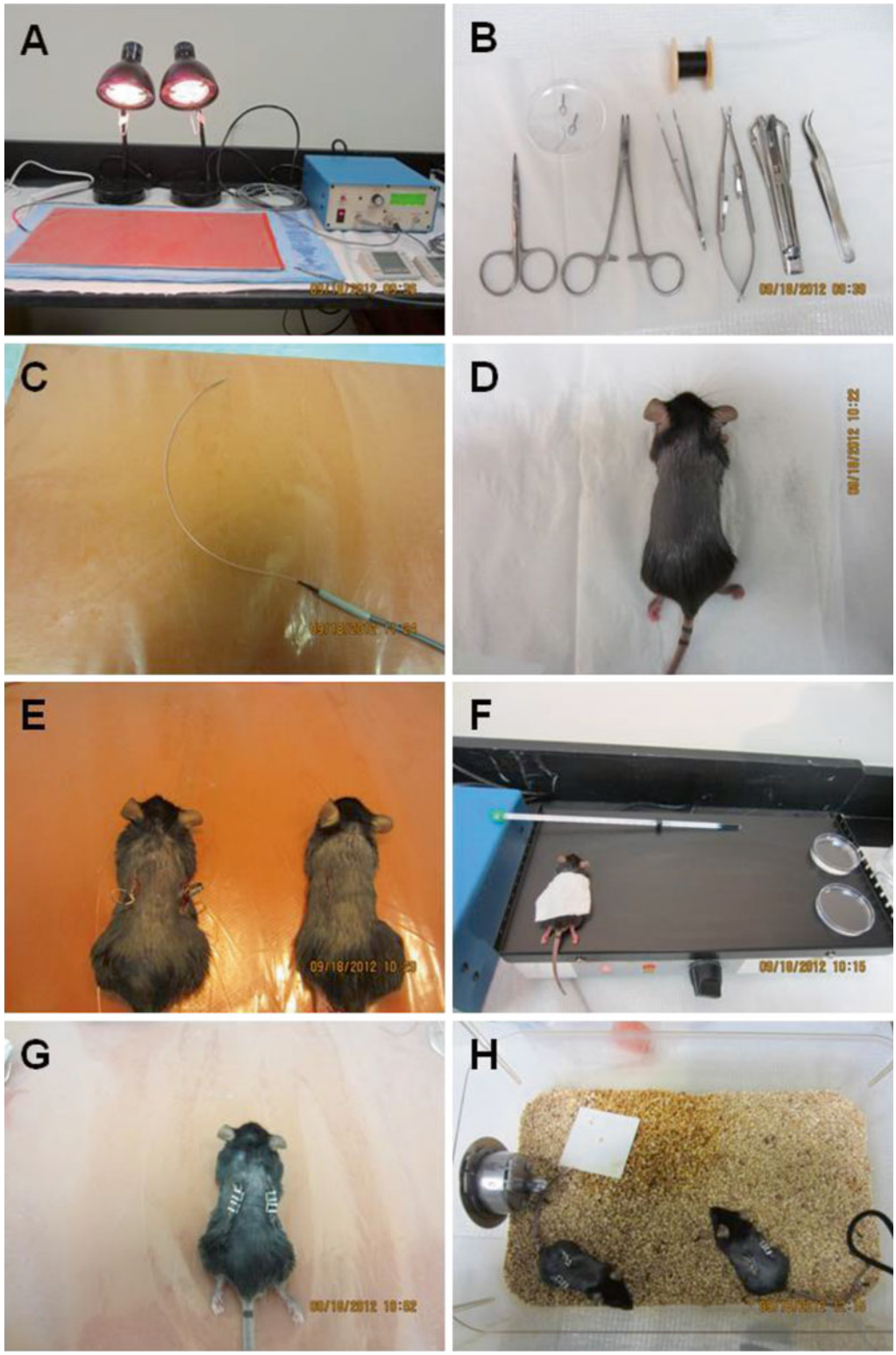

Fig. 1 Instruments and handling of animals during surgery. (**a**) Thermal controller connected to heating pad and rectal probe. (**b**) Surgical instruments such as scissors, forceps, vascular clamps, nylon threads, clamp

17. Hair cutter/trimmer.
18. Disinfectant wipes.
19. 70 % Ethanol.
20. Autoclave.

2.2 Cisplatin-Induced AKI

1. Animals: We use 8–12 week old male C57BL/6 mice purchased from Jackson Laboratories and maintained in a normal room with a 12 h dark/12 h light cycle. Other strains can also be used but susceptibility and dose may vary and condition should be standardized for each particular strain. To get consistent renal injury, mice should weigh around 25–30 g.
2. Cisplatin (MP Biomedicals).
3. Normal saline (0.9 % NaCl).
4. 1 ml insulin syringe with 27G needle.
5. Vortex machine.
6. Glass or serological pipettes.
7. 0.2 μM syringe filter.
8. 70 % Ethanol.
9. Razor blade.
10. Heating lamp.

2.3 Studying Inflammation in Acute Kidney Injury

Inflammation is major mediator of tissue injury in AKI. Inflammation in the kidney is a collection of many phenomena including increased cytokine and chemokine production from immune cells and renal tubular epithelial and endothelial cells, up-regulation of adhesion molecules and immune cell infiltration into the kidney interstitium. Quantification of cytokines and chemokines can be done by immunoassay whereas adhesion molecule expression can be determined by immunohistochemistry and Western blot analysis. Quantification of immune cell infiltration is routinely done by flow cytometry in addition to immunohistochemical analysis. The first step in the analysis of immune cells infiltration into the kidney is to prepare a single cell suspension by digesting the kidney with a combination of enzymes, labeling with antibody and finally analysis by flow cytometry. We will describe these techniques in detail.

2.3.1 Single-Cell Suspension from Kidney and Analysis of Immune Cells by Flow Cytometry

1. Collagenase Type I.
2. DNase Type III.
3. Red cell lysis buffer (ebiosciences).
4. Mouse FC receptor blocker (ebiosciences).
5. Fixation/permeabilization buffer kit (e-biosciences).
6. 100 μM and 40 μM cell strainer (Fisher Scientific).
7. Razor blade.

8. Syringe (5 ml).
9. EDTA.
10. Paraformaldehyde Sodium Azide.
11. V-bottom plate or 2 ml centrifuge tube.
12. Digestion buffer: DMEM/F12 containing 1 mg/ml of collagenase type I (Calbiochem) and 0.1 mg/ml of DNase type III (both can be bought from SIGMA or Calbiochem). With elastase (0.1 mg/ml) (Worthington) added. If the tissue is very fibrous, collagenase IV (1 mg/ml) can also be added.
13. FACS Buffer: 1× PBS; 0.1 % Sodium Azide; 10 mM EDTA; 1 % BSA.
14. Fixation buffer: 1× PBS; 0.1 % Sodium Azide; 10 mM EDTA; 0.5 % paraformaldehyde.

3 Methods

3.1 Mouse Model of Warm Ischemia–Reperfusion Injury

1. Sterilization: Before starting each experiment, sterilize all surgical tools by autoclaving or using a hot bead sterilizer. Wipe tables and other equipments with betadine or other disinfectant.
2. Heating pad and thermal regulators: Turn on the heating pad and thermal regulator at least 2 h before surgery to stabilize the temperature. Set the thermal regulator to 35 °C for the heating pad.
3. Administration of anesthetics: Prepare pentobarbital sodium solution by solubilizing or diluting it in saline at a concentration of 15 mg/ml. Wipe the animal belly with 70 % ethanol and the administer the anesthetic intraperitoneally at a dose of 60 mg/kg body weight.
4. Preparation of animals: Animals are determined to be unconscious by pressing on the animal leg. If the animal is does not responds, the back is shaved as shown Fig. 1 and then sterilized by wiping with 70 % ethanol or an alcohol pad and chlorhexidine.
5. Surgical procedure: Make an incision (1 cm) parallel to the vertebral column on both sides 1 cm below the rib cage on the dorsal side. Cut the skin first followed by the abdominal muscle. Use hemostats to hold the muscle on both sides of the incision (*see* **Note 2**).
6. Locating and clamping renal vessels: Kidneys are located on both sides and dissect the mice to remove the fat between the kidney and inferior veno cava to see the renal pedicle (care must be taken to avoid damaging the renal vessel).

Using a vascular clamp applicator, apply the clamp on the renal vessels on both sides (*see* **Notes 3** and **4**). As soon as the renal vessels are clamped, you should see change of kidney color from red to pink then to blackish.

7. Maintaining body temperature: Sterile cotton gauze is wet in saline that is equilibrated at 37 °C and is used to cover the wound. Animals are kept on the heating pad or if operated on many animals at a time, they can be transferred to a slide warmer with the temperature set at 34 °C. Monitor the body temperature using a rectal probe every 10 min; the probe tip should be wet with sterilized glycerol before being inserted into the rectum (*see* **Note 5**).
8. Releasing clamps and visual confirmation of good clamping: After the required time of clamping (generally 25–30 min should be sufficient), release the clamp using a clamp holder and remove. The kidney should change in color from dark pink to red.
9. Closing of wounds: Stitch the abdominal muscles with nylon thread (sterilized by wiping with 70 % ethanol). Three stitches should be enough on each side. Close the skin using wound clips.
10. Avoiding dehydration after surgery: Administer 1 ml of saline intraperitoneally after closing the wound to avoid dehydration.
11. Monitoring animals after surgery: Transfer the animal to a cage and kept in an incubator that is set at 33 °C until animal regain consciousness.
12. Sham surgery for controls: Since the surgical procedure alone can cause changes in kidney physiology and pathology, it is critical to use appropriate surgical controls to interpret the results properly. For sham surgery perform all procedures used for ischemia–reperfusion except that artery clamps should not be applied.
13. Collection of blood from tail vein: Warm the tail with a heating lamp, and collect a drop (50 μl) of blood into a lithium-heparin coated Microvette® CB 300 capillary tube by nicking tip of the tail. Stop the bleeding by applying pressure on the tail. Centrifuge the tube and transfer the plasma to a clean tube. Immediately measure plasma creatinine or the plasma frozen at −80 °C for future measurement.
14. Measuring kidney functions: Measure creatinine from serum/plasma using an enzyme based creatinine assay kit. Baseline serum creatinine is 0.1–0.2 mg/dl. By 6 h after reperfusion (release of the clamp), serum creatinine may reach 0.4–0.6 mg/dl and by 24 h serum should increase to 1.5–2 mg/dl, which is an indication of kidney injury.

15. Sacrificing the animals and collecting urine, blood and kidney: To assess the histopathology and changes in gene expression, sacrifice animals between 6 and 24 h after release of the clamp. Administer pentobarbital sodium (100 mg/kg body weight) to the animals. Once these animals are unconscious, make a large incision in the ventral (belly) side of the animal and remove the gut using a forceps and keep outside the body. Draw blood drawn from the inferior vena cava using a 1 ml syringe with a detachable needle. Drop the blood collected into a heparin-coated tube for plasma or a plain tube for serum. Sacrifice the animal by cervical dislocation and harvest the kidney.
16. Assessing tissue injury: Morphologic evaluation is of supreme importance for understanding the nature of AKI and assessing the effectiveness of intervention. Other methods such as reduced glomerular filtration rate (GFR) may not reflect true tubular injury because an alteration of GFR may be due to change in the renal hemodynamics or secondary to sublethal forms of tubular injury. High-quality morphologic assessment may require experience and skills in renal tubular morphology and pathology. Kidney tissue can be fixed with 10 % buffered formalin for 24–48 h and tissue sections stained for Periodic-acid Schiff (PAS) to assess histological damage in the kidney. Warm ischemia–reperfusion induced damage generally appears in the outer stripe of the outer medulla at the S3 segment of proximal tubules. Necrosis, cast formation, damage to the brush border, flattened epithelial cells, tubular dilation, and apoptosis of epithelial cells can be seen. Apoptosis can be assessed using TUNEL staining. Recent manuscripts describe in detail how to assess the tubular injury which can be referred [30–33].
17. Perfusion of organs to remove blood: Removal of circulating white cells is critical to determine the extent of immune cell infiltration into the kidney interstitium by flow cytometry and to assess the functional response of infiltrated immune cells. Organs can be perfused with saline or PBS using a perfusion pumps. Anesthetize animals by injecting pentobarbital sodium (60 mg/kg body weight) and fix on the thermocole flat surface using 22 G needles. The chest cavity is cut open and perfusion pump set up. The pump is turned on (5–10 ml/min) and the catheter needle inserted into the left ventricle. The vena cavae that supplies blood to right atrium are immediately cut. The organs begin to turn white. Stop the perfusion when it reaches 20–25 ml of saline and harvest the kidney for further studies.
18. Advantages of warm ischemia–reperfusion injury: The warm ischemia–reperfusion model is widely used: simple to carryout, stop the renal flow for a precise amount of time, causing a

highly reproducible injury in the proximal tubular epithelial cells. Therefore, conclusions can be drawn from a smaller number of animals. It is a clinically relevant model although it does not mimic organ transplantation where the organ experiences cold ischemia followed by warm reperfusion. However, the method is relatively similar to other hypoxic injuries observed during surgical intervention of different organs including cardiopulmonary bypass surgery (*see* **Notes 6–10**).

3.2 Cisplatin-Induced AKI

1. Solubilization of cisplatin. Dissolve the Cisplatin in saline (0.9 % NaCl) by vortexing for 2–5 min. If large particles settle in the bottom of the tube, disperse it with a serological pipette and vortex again until the solution appears clear.
2. Sterilization of cisplatin: Sterilize the cisplatin solution by passing through a 0.2 μM syringe filter.
3. Administration of cisplatin: Inject Cisplatin intraperitoneally using a 1 ml syringe with 26G needle at a dose of 25 mg/kg body weight. Make sure the injected solution does not leak out from the belly (*see* **Note 11**).
4. Blood collection: Warm the animals using a heat lamp for a few minutes. Sterilize the animal tail using 70 % ethanol, and male a nick at the end of the tail using a sterile razor blade. Collect 50 μl of blood into a capillary blood collection tube coated with lithium-heparin. To stop the bleeding, press the tail and hold for couple of minutes. Centrifuge the blood and transfer plasma to a labeled microcentrifuge tube.
5. Monitoring kidney function: Monitor kidney injury and function by measuring serum creatinine, using a creatinine assay kit. The baseline value of creatinine is 0.15–0.2 mg/dl. Seventy-two hours after cisplatin administration, serum creatinine should rise to 1.5–2 mg/dl.
6. Sacrificing animals and tissue harvesting: To assess the histopathology and changes in gene expression, sacrifice animals 72 h after administration of cisplatin. Inject pentobarbital sodium (60 mg/kg body weight) into the animal and make a large incision in its ventral (belly) side. Carefully remove the gut using a forceps and keep outside the body. Draw blood from the inferior vena cava using a 1 ml syringe with detachable needle. Remove the needle and collect the blood into a heparin-coated tube for plasma or a plain tube for serum. Sacrifice the animal by cervical dislocation and the kidney harvested.
7. Histopathological studies: Fix kidney tissue with 10 % buffered formalin for 24–48 h and stain tissue sections with PAS to assess histological damage in the kidney. Cisplatin induced damage generally appears in the proximal tubules of the outer and inner cortex. However, damage is also seen in the distal

tubules. Necrosis, cast formation, damage to the brush border and apoptosis of epithelial cells can be seen. Apoptosis can be assessed using TUNEL staining. Recent manuscripts can be consulted for how to assess the tubular injury [30–36].

8. Advantages of cisplatin induced AKI: Cisplatin induced AKI in animal is a highly clinically relevant model. It is simple and inexpensive to perform and produce reproducible injury pattern in the cortex. The mechanisms of injury can be studied easily and the mild injury and apoptosis detected easily.
9. Other available models for AKI: Other animal models for AKI are available and have been used by investigators, including glycerol induced AKI, gentamycin induced AKI, radiocontrast induced AKI, nonsteroidal anti-inflammatory drug induced AKI (acetaminophen and diclofenac), osmotic nephrosis (sucrose), mercuric chloride induced AKI, folic acid induced AKI, uranyl nitrate induced AKI, ifosfamide induced AKI, ferric nitro triacetate induced AKI, endotoxin induced AKI, and polymicrobial sepsis induced AKI. More recently, diphtheria toxin mediated injury in the proximal tubule also induced AKI [31]. More details on these models can be found in a recent review [15, 24, 27, 37].

3.3 Digestion of Kidney Tissue and Isolation of Single Cell Suspension

1. Before starting experiments, aerate the medium by bubbling in O_2.
2. Anesthetize mice with pentobarbital sodium (60 mg/kg body weight).
3. Perfuse 20 ml of PBS via the left ventricle and bleed the animal by cutting the vena cava that connects the right atrium.
4. Harvest the kidneys and remove the kidney capsule by squeezing between fingers. Wash the kidneys with DMEM/F12 medium.
5. Weigh and transfer the kidneys onto petri plate lid.
6. Chop the kidneys with a pair of razor blades into tiny pieces or a paste.
7. Press the kidney tissue with a syringe plunger to break it up into the smallest fragments possible.
8. Transfer the kidney pieces into 10 ml of digestion buffer (50 ml of digestion buffer for 1 g of tissue should be good enough to get complete digestion).
9. Incubate the suspension at 37 °C water bath shaker for 30 min.
10. Pass the digested tissue suspension through a 100 μM cell strainer.
11. Add 5 ml of FACS buffer and pass the suspension through a 40 μM cell strainer.

12. Centrifuge the cell suspension at 1,500 × *g* for 15 min at 4 °C.
13. Remove the supernatant, resuspend the pellet in 2 ml of red cell lysis buffer, and incubate for 2 min followed by gentle vortex (*see* **Notes 12** and **13**).
14. Dilute the red cell lysis buffer tenfold with FACS buffer and recentrifuge.
15. Resuspend the cell pellet in 1 ml of FACS buffer. Count the cells and add 10 μl of FC receptor blocker.
16. Incubate the cells at 4 °C for 15 min.
17. Centrifuge the cells are at 1,500 × *g* for 5 min. Remove the supernatant and resuspend the pellet in 0.5 ml of FACS buffer.
18. Add 100 μl cell suspension (1–3 million or at least 300–500,000) to each well in a 96 well plate or 1.5 ml tube for each leukocyte and isotype control (depending on the number leukocyte marker stained) or secondary antibody control. 100 μl of cell suspension for unlabelled control and single fluorophore for compensation.
19. Centrifuge the suspension and discard the supernatant.
20. Dilute the antibody (like CD45-FITC, CD4-PE) in FACS buffer (1:100).
21. Suspend the cell pellet in 100 μl of diluted antibody for each well.
22. Incubate the suspension at 4 °C for 30 min.
23. Add 150 μl of FACS buffer and the centrifuge mixture at 1,250 × *g* for 5 min.
24. Discard the supernatant and vortex the pellet gently.
25. Repeat **steps 24** and **25**.
26. Add 300 μl of fixation buffer and vortex the pellet gently. Transfer the labeled cells to FACS tubes.
27. Analyze the samples immediately by flow cytometry or store in the dark at 4 °C for later analysis (24–48 h).
28. Other measurements of kidney function: In addition to renal morphology, assessing perfusion of the kidney (glomerular filtration rate, GFR) is an important measure used to determine functional decline of the kidney. Any treatment that prevents a decline in GFR or that enhances the GFR is considered a success in treating kidney injury. However, reduction of GFR may not necessarily reflect an obvious tubular injury, so caution must be applied when interpreting the data. GFR in mice can be measured using FITC-inulin. Further details on that protocol can be found on the Web site for Animal Model of Diabetic Complication Consortium (AMDCC) (http://www.amdcc.org).

Recent advances in technology have to lead to identification of several candidate biomarkers that are capable of predicting the development of kidney injury much earlier than the currently used gold standard of serum creatinine levels and GFR. These include neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule (KIM-1), *N*-acetyl-glucosaminidase (NAG), liver fatty acid binding protein (L-FABP), netrin-1, and interleukin-18 (IL-18) [38–43]. These biomarkers are segment specific and reflect overall kidney dysfunction/injury in many types of kidney injury. Many preclinical and clinical studies have shown that an early rise in urine biomarkers predict the development of renal functional dearrangement later such as GFR and rise in serum creatinine level. However, not many studies have looked at histological damage and rise in biomarker concentrations in urine. If a biomarker is established as true tubular injury marker, it can be used to diagnose kidney injury early and assess the effectiveness of intervention in both a preclinical model of AKI and clinical practice.

Animal models for AKI are highly valuable tools for research to determine the pathological mechanism of disease process, clarify pathways and molecules that mediate tissue injury, determine changes in gene expression spatially and temporally during the disease processes, and conduct studies to assess the effectiveness of drug intervention. However, this requires a profound understanding of the model, its pathophysiology, and its morphologic characteristics. The need for a simple and reproducible AKI model should be weighed against the clinical relevance of these models regarding the severity of the insult, inclusion of predisposing factors, and administration of supportive treatment. Despite these concerns, the warm ischemia–reperfusion injury of the kidney and cisplatin induced AKI animal models are highly clinically relevant, simple, and reproducible. However, continuous biomarker based monitoring, inclusion of some predisposing factors in the experiments, and developing techniques to determine sublethal injuries will help to advance our understanding of the mechanisms of renal injury and lead to development of effective therapies for treating AKI.

4 Notes

1. Older and fatty animals are difficult to dissect and thus make it difficult to visualize renal pedicles.
2. Ventral (belly) approach is easier to locate the kidney and renal artery; however, it leads to increase in mortality, and chances of damaging intestine are very high which may lead to sepsis.
3. Before every surgery, clamp tension should be checked. This can be done holding an 8×11 printer paper with vascular clamp and

see whether it can hold without dropping. This is an indication of good clamp.

4. Autoclaving often causes deterioration of clamps and surgical. This can be avoided by using bead-based sterilizer.
5. The body temperature is critical during clamping and should not vary among the animals in order to obtain consistent injury.
6. One must keep in mind that the warm ischemia–reperfusion injury model has several limitations that include: except for a few examples such as organ transplantation and partial nephrectomy, most human AKI due to ischemia, will not have complete stoppage of the blood supply to the kidney.
7. The definition of AKI in humans is completely different from mouse AKI. In humans, the rise of creatinine 50 % over baseline is considered an AKI and quite a significant injury, whereas in mice, 1,000 % (10 times) the increase in serum creatinine levels is considered a mild reperfusion injury.
8. The time course of ischemic AKI after an insult is also much different. Human AKI is diagnosed based on creatinine 3–5 days after the initial insult whereas in mice, AKI can be diagnosed (based on serum creatinine levels) within 12–24 h after the initial insult.
9. Human ischemic AKI leads to injury in both proximal and distal tubules but in mice injury is seen mostly in the proximal tubules.
10. Mouse warm ischemia–reperfusion model lacks the typical comorbid conditions and manipulation done in humans.
11. Since cisplatin dose is normalized to weight of the animals, use of similar age and weight is critical for consistent response among animals and between experiments.
12. Cell suspension should be kept on the ice during labeling to reduce cell death and generation of debris, which can absorb antibodies nonspecifically.
13. It is not necessary to lyse the RBC before staining with antibodies. RBC lysis occurs when permeabilization buffer addition during intracellular staining.

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Chapter 25

Mouse Models of Acute and Chronic Colitis

Santhakumar Manicassamy and Indumathi Manoharan

Abstract

Crohn's disease (CD) and ulcerative colitis are two main clinically defined forms of chronic inflammatory bowel disease (IBD). Our understanding of IBD depends largely on rodent models. DSS-induced intestinal inflammation in mice and T cell transfer colitis in SCID mice are most widely used and accepted models that can recapitulate the human diseases. Here, we provide detailed protocols of these two mouse models of experimentally induced intestinal inflammation. We also discuss the protocols for the isolation and analysis of inflammatory T cell from the colon.

Key words IBD, DSS, Acute and chronic colitis, Flow cytometry, Immune cells, T cell transfer colitis

1 Introduction

Inflammatory bowel disease (IBD) is an important public health problem in western society, affecting more than two million Americans. Crohn's disease (CD) and ulcerative colitis are two main clinically defined forms of chronic inflammatory bowel disease (IBD) with multifactorial etiologies [1, 2]. Patients with IBD suffer from abdominal pain, diarrhea, weight loss and rectal bleeding [1]. Biopsies from IBD patients with active disease show infiltration of mononuclear and polymorphonuclear leukocyte; epithelial cell erosion, loss of goblet cells, crypt abscesses, and epithelial cell hyperplasia [3]. Although the pathogenesis of IBD is largely unknown, accumulating evidence suggest three critical interacting elements involved in the pathogenesis of IBD—the gut mucosal immune response, intestinal microflora, and host genetic susceptibility factor and environment [2]. Intestinal immune cells have emerged as principal effectors in the pathogenesis of IBD [4–6]. Thus, our understanding of IBD depends largely on animal models that can recapitulate the human diseases.

In general, animal model of IBD can be grouped as—(a) Chemical-induced colitis [7, 8], (b) T cell transfer model of colitis [5], and (c) spontaneous model of chronic colitis [9]. Chemical-induced

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colitis is based on repeated administration chemical triggers of acute or chronic intestinal inflammation that resembles human colitis. There are several widely accepted chemical-induced colitis such as dextran sodium sulfate- (DSS); the hapten 2,4,6, trinitrobenzene sulfonic acid (TNBS)-, and oxazolone-induced colitis [9]. Among these, DSS-administration in water is the most widely accepted model of IBD. These models are helpful in evaluating the efficacy of prolonged prophylactic or therapeutic treatment of colitis. Further, these models are also useful in evaluating the intestinal health effects of nutritional components. T cell transfer model of chronic colitis is based on adoptive transfer of CD4+ CD45RB^{high} T cells (naïve T cells) into T and B cell deficient mice [5, 10]. Adoptive transfer model helps investigators in studying immunological basis of both induction and progression of gut inflammation. Spontaneous model of chronic colitis are based on genetic defects in immune regulation or targeted gene deletion that are involved in immune regulation (IL-10 deficient mice). Several recent excellent review articles and other manuscripts [1, 6, 11–13] provide a more detailed description of different animal models of intestinal inflammation.

Despite some drawbacks, DSS-induced intestinal inflammation in mice and T cell transfer colitis in SCID mice are the most widely used models, and the results are useful in understanding the mechanistic basis of disease. This unit provides detailed protocols of these two most commonly used mouse models of experimentally induced intestinal inflammation.

2 Materials

2.1 DSS-Induced Inflammation

1. Animals: We use 8–12-week-old male C57BL/6 mice purchased from Jackson Laboratories and maintained in a barrier SPF room with a 12 h dark–12 h light cycle. Other strains can also be used but susceptibility and severity may vary (*see* **Note 1**).
2. 3.5 % Dextran Sulfate Sodium salt (DSS; MW: 36,000–50,000 Da; MP Biomedicals): Dissolve 17.5 g DSS powder in 500 ml of autoclaved drinking water. Store until use at 4 °C (*see* **Note 2**).
3. Sterile PBS and balance, forceps and scissors; 10 % Buffered Formalin Phosphate (Fisher scientific).
4. 70 % Ethyl alcohol (Pharmco-AAPER): add 70 ml of absolute alcohol to 30 ml distilled water.
5. Tissue path Macrosette cassettes (Fisher scientific).
6. Dissecting scissors sharp/blunt tip (VWR international).
7. Digital caliper (Fisher Scientific).

8. *o*-Dianisidine dihydrochloride (Sigma-Aldrich). 1 mg/ml of dianisidine in PBS (*see* **Note 3**).
9. Hexadecyltrimethylammonium bromide (Sigma-Aldrich). Dissolve 0.5 g hexadecyltrimethylammonium bromide in 1 ml of 50 mM PBS (pH 6.0).
10. Biotek EL808 Absorbance plate reader (Biotek).
11. Microtest plate 96-well flat bottom (Fisher).
12. 30 % H_2O_2 (Sigma-Aldrich). Add 4 μl of 30 % H_2O_2 to 96 μl of H_2O.

2.2 T Cell Transfer Model of Chronic Colitis

1. Animals: We use 8–12-week-old male Rag-1-deficient mice on C57BL/6 background purchased from Jackson Laboratories and maintained in a barrier SPF room with a 12 h dark–12 h light cycle. Other strains can also be used but susceptibility and severity may vary (*see* **Note 1**).
2. 1× PBS, Ca^{2+} and Mg^{2+} free, Hank's balanced salt solution (HBSS), RPMI, Antibiotics (penicillin and streptomycin) (Fisher Scientific).
3. 1 M HEPES, ACK lysis buffer (Lonza).
4. Fetal bovine serum (Atlanta Biologicals).
5. 0.5 M EDTA (pH 8.0) (Cellgro).
6. Trypan blue (Invitrogen).
7. DNase I (Roche): Prepare stock solution by dissolving 1 mg of DNase I in 50 ml of HBSS. Store in –20 °C freezer.
8. Collagenase VIII (Sigma-Aldrich): Prepare stock solution of 100 U/ml in complete RPMI medium. Store in –20 °C freezer.
9. Collagenase IV (Worthington): Prepare stock solution of 4,000 U/ml in complete RPMI medium. Store in –20 °C freezer.
10. Percoll (GE Healthcare): Prepare 100 % Percoll by mixing 90 ml of Percoll with 10 ml of 10× PBS. Prepare 40 % Percoll by mixing 40 ml of 100 % percoll with 60 ml of 1× PBS.
11. CD4 microbeads, LS MACS Column (Miltenyi Biotec).
12. Flow cytometry antibodies: CD45RB-PE; CD4-PE, IL17-PE, IFNγ-APC, CD45-Percp, Rat anti-mouse CD16/CD32, Cytofix/Cytoperm (BD Pharmingen).
13. 15 and 50 ml conical tubes (BD Biosciences).
14. 100, 70, and 40 μm cell strainer (BD Biosciences).
15. Brefeldin A and monensin (BD Pharmigen).
16. PMA and Ionomycin (Sigma Aldrich).
17. MACS Buffer: 1× PBS containing 2 mM EDTA and 4 % FBS.

18. FACS Buffer: 1× PBS Containing 0.5 % FBS.
19. CMF/HEPES: HBSS (Ca^{2+} and Mg^{2+} free) containing 2 mM HEPES and 2 % FBS.

3 Methods

3.1 Acute DSS-Dependent Colitis

1. Acclimatize the C57BL/6 mice for 1 week before the experiment.
2. On day 1, mark and weigh each mouse two times.
3. Replace the drinking water with 3.5 % DSS-water in appropriate mouse cages. Estimate 5 ml DSS solution per mouse per day. Control mice receive the same drinking water without DSS.
4. Refresh DSS solution on day 3 and day 5.
5. Replace the remaining DSS solution by autoclaved water on day 8.
6. Monitor body weight and disease activity index daily or every other day. These are important parameters indicating the colitis severity between experimental groups (*see* **Note 4**).

3.1.1 Sacrificing Animals and Tissue Harvesting

1. To assess the histopathology and changes in gene expression, animals can be sacrificed at the end of the experiments by institution's approved method.
2. Using standard dissecting scissors make a large incision in its ventral (belly) side. Locate and carefully remove the entire colon. Determine colon length (digital caliper) and weight.
3. Remove the fecal contents by rinsing the colon with sterile PBS.
4. Colon tissue can be fixed with 10 % buffered formalin for 24–48 h and tissue sections stained for H&E stain to assess histological damage in the colon.

3.1.2 Disease Activity Index (DAI)

1. DAI is used to evaluate the grade and extent of the intestinal inflammation using previously established scoring system [15–17]. Feeding mice with DSS in drinking water leads to the development of inflammation in colon with signs of weight loss, watery diarrhea, fecal bleeding, and infiltration of inflammatory granulocytes [17, 18].
2. To assess the extent of colitis, body weight, stool consistence, and blood in the stool are monitored daily (Table 1). The score of DAI ranges from 0 to 12 (total score).

3.1.3 Histological Grading of Colitis

1. Histological grading on the severity of inflammation is performed with the H&E staining and histological scoring of colonic tissue using previously published results [18, 19].

Table 1

Disease activity index (DAI)

Score	Weight loss	Stool consistency	Blood in the stool
0	None	Normal	Normal
1	1–5 %		
2	5–10 %	Very soft	Slight bleeding
3	10–20 %		
4	>20 %	Watery diarrhea	Gross bleeding

Table 2

Histology grading

Score	Epithelium	Infiltration	Ulcer
0	Normal morphology	No infiltrate	None
1	Loss of goblet cells	Crypt basis	
2	Loss of crypt, epithelium present	Mucosa	Positive
3	Loss of crypt and epithelium	Submucosa	
4	Loss of crypt and epithelium (large area)	Submucosa (extensive)	Positive (extensive)

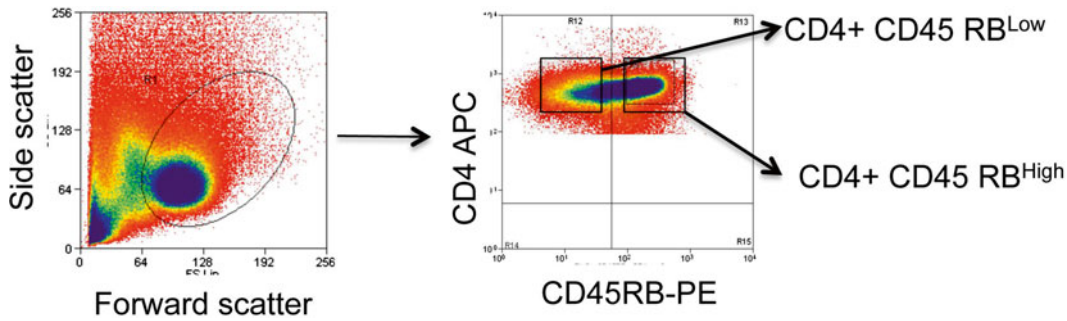

Fig. 1 FACS analysis for sorting $CD4^+$ $CD45RB^{High}$ T cells. (**a**) FACS plot analysis showing the Forward and Side scatter of $CD4^+$ T cells enriched with magnetic beads. (**b**) FACS plot analysis showing $CD4^+$ $CD45RB^{High}$ and $CD4^+$ $CD45RB^{Low}$ T cells enriched with magnetic beads

2. Administration of DSS in mice results in severe macroscopic and histological damage in colon.
3. Total severity is calculated by summing the scores for inflammation, glandular epithelial loss and erosion from proximal, middle and distal segments of colons (Table 2). The grading score ranges from 0 to 12 (Total score) (Fig. 1).

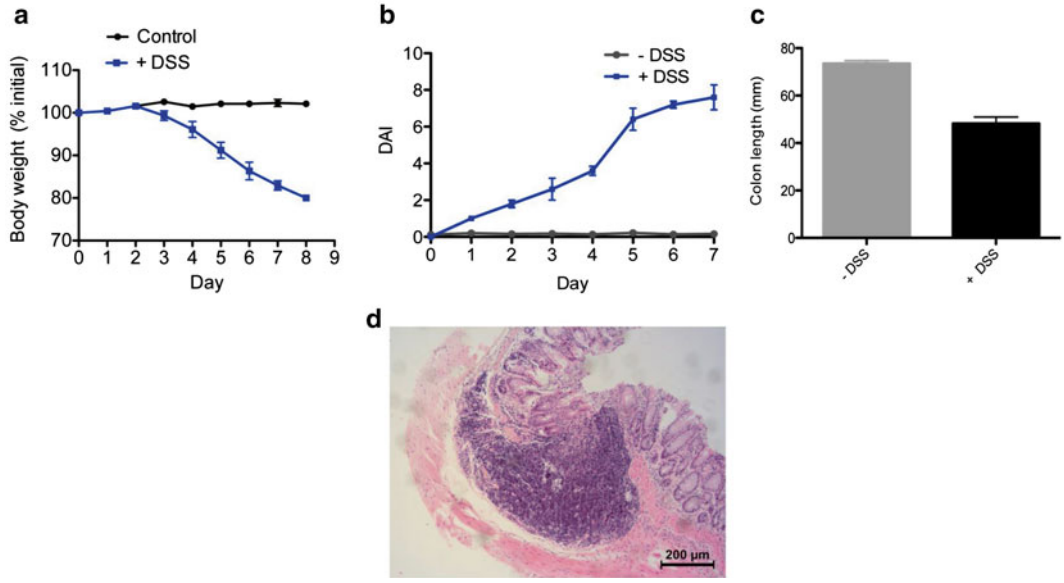

Fig. 2 Assessment of DSS-induce colitis severity using disease activity index (DAI) and histological grading of colitis. C57BL/6 mice were given 5 % DSS in drinking water for 7 days and control mice received drinking water. (**a**) Body weight change of DSS treated and control mice for 7 days (**b**) Severity of colitis in DSS-treated and control mice, as measured by stool consistency, presence of fecal blood, and weight loss. (**c**) Colon length of DSS treated and control mice. (**d**) Colon histology of DSS-treated mice showing tissue damage and infiltration of inflammatory cells

3.1.4 Colon Culture

1. Cut and open the colon longitudinally and wash four times in HBSS supplemented with penicillin and streptomycin.
2. Cut across colon into 1-cm segments and culture each piece in 24-well flat-bottom culture plates (Costar) in serum-free RPMI 1640 medium (Cellgro) supplemented with penicillin and streptomycin, L-glutamine, and nonessential amino acids.
3. After 24 h, collect the supernatant and centrifuge at 13,000 × *g* for 10 min at 4 °C and store at −20 °C for assaying cytokines by ELISA (Fig. 2).

3.1.5 Tissue Myeloperoxidase (MPO) Assay

1. MPO activity can be used as marker of inflammation. MPO is a marker for neutrophil and helps in assessing the neutrophil influx into inflamed tissue [14].
2. Take 50 mg of colon tissue in 2 ml micro centrifuge tube containing 1 ml of PBS for 30 s. Spin the homogenate at 13,500 × *g* for 5 min.
3. Decant the supernatant without disturbing the pellet. Resuspend the pellet in 1 ml of 50 mM PBS (pH 6.0) containing 0.5 % hexadecyltrimethylammonium bromide. Freeze-thaw the samples three times, sonicate (90 s), and centrifuge at 13,500 × *g* for 5 min.

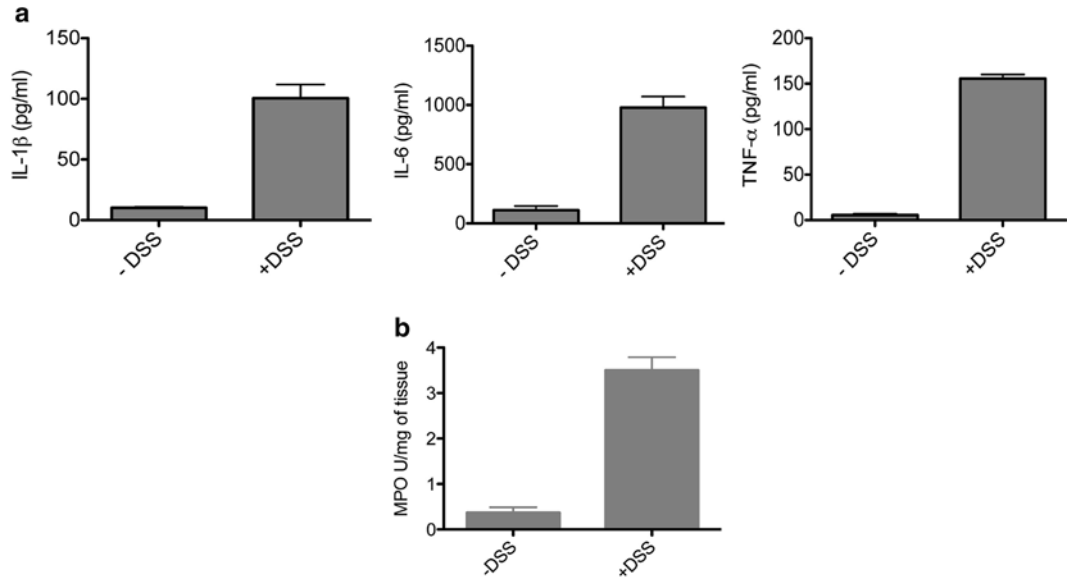

Fig. 3 Colonic inflammatory cytokine and MPO levels in response to DSS-induced colitis. C57BL/6 mice were given 3.5 % DSS in drinking water for 7 days and control mice received drinking water. Severity of DSS-induced colitis is associated with higher levels of inflammatory cytokines (**a**) and MPO activity (**b**) compared the control mice

4. Incubate the sample in water for 120 min at 60 °C. Centrifuge the sample at 13,500 × *g* for 5 min. MPO is assayed in the clear supernatant in 96-well plate in triplicates. Add 10 µl of supernatant (in triplicates) to 200 µl of 1 mg/ml of dianisidine dihydrochloride and 5×10^{-4} % H_2O_2. The change in optical density is measured at 450 nm three times at 30 s intervals using spectrophotometer.
5. Use Human neutrophil MPO (Sigma) as standard. One unit of MPO activity is defined as the amount that degraded 1.0 µmol of peroxide per minute at 25 °C (*see* **Note 5**).

3.1.6 Representative Results and Discussion

1. Disease activity index and Histological grading of colitis are most important parameter for determining the severity of colitis. DAI may evaluate colitis severity and histology-scoring using previously published scoring system (*see* Fig. 2).
2. Mice treated with 3.5 % of DSS may have significantly worsened disease compared to the control mice with water. Animal treated with DSS will show significant increase in DAI and histological score compared to the controls treated with water only. Consistent with this, colons of animals treated with DSS will show increased MPO activity compared the representative controls.
3. Further, this is associated with increase in the production of inflammatory cytokines (IL-1b, IL-6, and TNF-alpha) by DSS-treated colons (Fig. 3). MPO activity and tissue cytokines can be used as surrogate marker for colitis severity.

3.2 T Cell Transfer Model of Chronic Colitis

3.2.1 Enrichment and Fluorescent Labeling CD4+ T Cells from Spleen

1. Euthanize C57BL/6 mice or donor mice and harvest spleen by an institutional approved method.
2. Prepare single cell suspension of spleen cells in 10 ml FACS buffer (*see* **Note 6**).
3. Pellet cells by centrifugation at 400 × *g* for 10 min at 4 °C and discard the supernatant.
4. Lyse the red blood cells by adding 2 ml per spleen ACK lysing buffer, gently mix the cells, and leave for 5 min at room temperature (*see* **Note 7**).
5. Wash the cells with FACS buffer. Determine the cell viability by Trypan blue dye exclusion.
6. A single cell suspension of spleen cells is suspended at 100 μl MACS buffer per 10^7 cells. Add 5 μl rat anti-mouse CD16/CD32 and incubate for 15 min at 4 °C.
7. Add MACS anti-CD4 beads (Miltenyi Biotech) (10 μl beads per 10^7 total cells) and incubate for additional 15 min at 4 °C. Wash the cells with MACS buffer once.
8. Perform Magnetic separation with the MACS Separator (Miltenyi Biotec) as per the manufacturer's instructions (Miltenyi Biotec) (*see* **Note 8**). Pellet cells by centrifugation at 400 × *g* at 4 °C for 10 min.

3.2.2 Fluorescently Labeling of Enriched T Cells for Cell Sorting

1. Resuspend enriched CD4 pellet in 300 μl (10^7 cells) of antibody staining buffer and incubate with 10 μl CD4-APC and 10 μl of CD45RB-PE for 15 min in 4 °C.
2. Wash the cells twice in ice-cold FACS buffer.
3. Proceed to FACS-sorting to sort $CD4^+$ $CD45RB^{high}$ and CD4+ $CD45Rb^{low}$ cells as shown in Fig. 3 (*see* **Notes 9** and **10**).

3.2.3 Adoptive Transfer of Sorted CD4+ $CD45RB^{high}$ into Rag-1-Deficient Mice

1. Pellet the sorted CD4+ CD45Rbhi T cells by centrifugation at 400 × *g* at 4 °C for 10 min. Discard the supernatant and resuspend the cell pellet in 10^6 cells in 500 μl of sterile PBS.
2. Mark and weigh each mouse two times. Inject 0.5×10^6 cells (250 μl) intraperitoneally (ip) into each mouse using 1-ml syringe with the 26G3/8 needle attached.
3. Monitor the disease progression by weighing all mice weekly. Starting at week 4–5, mice will start losing weight (*see* **Notes 3** and **11**).

3.2.4 Sacrificing Animals and Tissue Harvesting

1. Sacrifice the colitic mice and perfuse with PBS. 20 ml of PBS is perfused via the left ventricle and the animal in bled by cutting the vena cava that connects the right atrium.
2. Using surgical scissors, collect MLN and colon in 5 ml of RPMI and keep on ice.

3.2.5 Isolation of T Cell from Mesenteric Lymph Node (MLN)

1. Make single cell suspension by placing MLN on a sterile 70 μm nylon mesh strainer and grind the tissue using the base of a plunger from a 1 cc syringes.
2. Add 5 ml of RPMI media and transfer 15 ml conical tube.
3. Centrifuge the cells at 500 × *g* for 5 min.
4. Decant the supernatant and cells are suspended in 5 ml of RMPI. Cells can be used for enrichment of CD4+ T cell (as described in Subheading 3.2.1), gene expression analysis, and ICS.

3.2.6 Isolation of T Cells from Colon

1. Cut open the colon longitudinally and clean fecal contents. Wash in HBSS three times. Cut across colon into small (1 cm) pieces and place into 50 ml conical tube containing 10–15 ml of CMF/HEPES and agitate gently. Filter the content using a single mesh wire strainer. Repeat this step for six times to remove the mucus and rest of fecal content.
2. Transfer the washed pieces into 50 ml conical tube. 20 ml complete RPMI/collagenase and incubate for 30 min at 37 °C. After 15 min, pipette the tissue up and down (*see* **Note 12**).
3. Pour the content of each tube through a fresh 100 μm cell strainer into 50 ml conical tube and save it. Transfer pieces back into a tube with fresh RPMI/collagenase and incubate again as in **step 5**.
4. Pool the filtrate in the 50 ml conical tubes and spin 10 min at 1,500 × *g*. Discard the supernatant. Wash the pellet once with RPMI and spin 10 min at 1,500 × *g* (*see* **Note 13**).
5. Resuspend cells in 7 ml 40 % Percoll in sterile PBS, underlay with 5 ml 80 % Percoll and spin 20 min at 1,800 × *g* at room temperature with no brake.
6. Collect cells from the interface in 14 ml tube and fill will RPMI-10. Vortex and spin 10 min at 1,500 × *g*. Discard the supernatant and wash one more time with RPMI (*see* **Note 13**). Cells can be used for enrichment of CD4+ T cell (as described in Subheading 3.2), gene expression analysis, and ICS.

3.2.7 T Cell Stimulation and Intracellular Staining for Cytokines

1. Stimulate isolated lymphocytes with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 h in the presence of Brefeldin (10 μg/ml).
2. Centrifuge the cells at 1,500 × *g* for 5 min. Remove the supernatant and resuspend the pellet in 0.5 ml of FACS buffer.
3. Add 100 μl cell suspension (1–3 million or at least 300–500,000) to each well in a 96 well plates or 1.5 ml tubes for each leukocyte and isotype control (depending on the number leukocyte marker stained). Centrifuge the suspension and discard the supernatant.

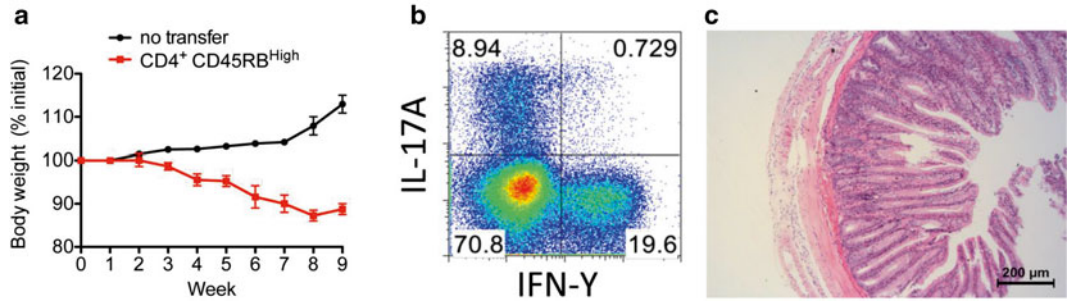

Fig. 4 Naïve T cell transfer model of chronic colitis. (**a**) Body weight change, (**b**) frequency of Th1/Th17 cells in the colon, and (**c**) Colon histology of Rag-1$^{-/-}$ mice adoptively transferred with CD4+ CD45RB^{High} T cells

4. Dilute the antibody cocktail (CD45-Percp, CD4-FITC) in FACS buffer (1:100). Suspend the cell pellet in 100 µl of diluted antibody for each well. Incubate the suspension at 4 °C for 30 min. Add 150 µl of FACS buffer and centrifuge the mixture at 1,250 × *g* for 5 min. Discard the supernatant and vortex the pellet gently.
5. Permeabilize cells using Cytofix/Cytoperm (BD Pharmingen) and stain by using IL17-PE and IFNγ-APC according to the manufacturer's protocol.
6. Analyze the samples immediately by flow cytometry or store in the dark at 4 °C for later analysis (24–48 h).

3.2.8 Colon Culture, Histological Scoring, and DAI Can Be Performed as Described in Subheading 3.1

1. Adoptive transfer of naïve T cells (CD4+ CD45RB^{High}) into mice deficient in T and B cells results in pancolitis and intestinal inflammation [5, 10].
2. Mice start showing signs of colitis after 4–5 weeks of T cells transfer with signs of weight loss, watery diarrhea and fecal bleeding (Fig. 4).

3.2.9 Representative Results and Discussion

3. Histopathological analysis of distal colon from colitis mice shows infiltration of mononuclear and polymorphonuclear leukocyte; epithelial cell erosion, loss of goblet cells, crypt abscesses, and epithelial cell hyperplasia.
4. Major advantage is that this model helps in understanding the both induction and progression of gut inflammation. Inflammation induced in this model is mediated by INF-γ/IL-17 producing T cells (Fig. 4b).

4 Notes

1. Conditions need to be standardized for each particular strain, animal facility, and environmental variability such as microflora, diet, and other environmental conditions. If a particular strain is sensitive to 3.5 % DSS, then reduce the concentration to 2–2.5 % DSS. Thus dosage and duration of DSS treatment need to be optimized for each strain.
2. Prepare DSS with animal facility water. Mice do not drink distilled water. It is important to use DSS of specified molecular weight.
3. *o*-Dianisidine dihydrochloride solution should be prepared fresh when performing assay.
4. If animals lose more than 25 % of their initial body weight, euthanize them and save the intestines for histology.
5. MPO activity assay should be performed within the first week of tissue collection. Activity decreases over time.
6. FACS buffer should be prepared fresh every time when performing the assay.
7. Do not keep cells in ACK lysis buffer for longer time, as it will affect the cell viability.
8. Although the present protocol uses Miltenyi $CD4^+$ T cell enrichment kit, other comparable $CD4^+$ T cell isolation methods can be used.
9. Cell must be kept in ice and protected from light prior to sorting.
10. Low yield of $CD45RB^{high}$ cell might be due to not sufficient number of spleens were used.
11. The reconstituted mice develop little or no disease. This might be due to several factors such as low viability of injected CD4+ $CD45RB^{high}$ T cells or low number of cell injected into mice or poor ip injection technique. Viability and purity should be greater than 90 % upon sorting.
12. RPMI/collagenase should be prepared fresh from the stock when performing the isolation. Each batch of collagenase should be tested for toxicity and activity.
13. If the cell suspension clumps together, increase the concentration of DNase I.

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Chapter 26

Manipulation and Assessment of Gut Microbiome for Metabolic Studies

Sarang N. Satoor, Deepak P. Patil, Holly D. Kristensen, Mugdha V. Joglekar, Yogesh Shouche, and Anandwardhan A. Hardikar

Abstract

The mammalian gut is inhabited by a complex and highly diverse population of bacteria. About 100 trillion microbes are present in the human gut, a number ten times more than the total number of cells in an adult human body. These microorganisms play an important role in several fundamental and crucial processes such as immunity, digestion, synthesis of vitamins, and metabolizing bile acids, sterols, and xenobiotics in the host, thereby influencing human health. Identification and manipulation of these metabolic interfaces is therefore critical. Here, we present a set of methods for manipulation and targeting the 16S rRNA based identification of rodent gut microbiota using Sanger's and next-generation sequencing platforms. Novel methods for manipulation of gut microbiota are also presented. In principle, these methods can be easily adapted to most rodent models for successful screening and manipulation of gut microbiome, to generate a better understanding of their role in metabolic disease.

Key words Gut microbiota, 16S rRNA, Pyrosequencing, Fecal transplantation, Metabolism

1 Introduction

Obesity and diabetes are two recent and prevalent metabolic disorders in humans. It is well accepted that obesity in humans is often the result of interplay between genetic factors, diet, and lifestyle of the host. In recent years, the involvement of gut microbes [1–3] in metabolic disease is also a well-recognized mechanism.

The gut of a fetus is sterile until just after birth, when it is rapidly colonized by a host of microorganisms [4]. A number of studies have shown a role for gut microbiota in host metabolism, including the development of obesity and diabetes [5–7]. Obesity is associated with a number of metabolic disorders, such as insulin resistance/type 2 diabetes, gut permeability, and an overall state of low-grade inflammation. The gut microbiota of lean and obese

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individuals differs significantly [2, 8, 9] with lean individuals possessing a higher proportion of Bacteroidetes and obese individuals hosting a larger proportion of Firmicutes. Transfer of gut microbial communities from lean to obese individuals, or modulating microbial community (through use of antibiotics) confers advantageous metabolic effects, such as reduced gut permeability [10] and inflammation [11], along with increased insulin sensitivity [12, 13]. Although certain types of bacteria have been associated with specific metabolic changes in the host [7, 14], the mechanism(s) that lead to such changes remains poorly defined. With the advent of increasingly sophisticated metagenome sequencing and characterization workflows [13, 15–17], researchers have been able to reveal the immense capabilities locked within the gut microbiome as influential on host metabolism [5].

We present here an easily adaptable protocol for manipulating the gut microbiome in rodent models, to allow for examining the effects of gut microbial transplantation on metabolic health of the host. We also present methodologies for screening gut microbiota using conventional and contemporary methods.

1.1 Manipulation of Gut Microbes

Gut bacteria are now well known to modulate several critical functions including digestion, assimilation of food, vitamin synthesis and development of immune response [18]. As a result of easy adaptation to internal milieu and shorter duplication time, the large numbers of these bacteria present in our gut can impact on several metabolic pathways [8, 19]. Once an association between the relative abundance of bacteria (say Bacteroidetes and Firmicutes) is made with a metabolic outcome, it is important to assess if changes in bacterial proportions are causal to the observed outcome(s). We present a novel systematic workflow to assess such changes. These methods are useful in modulating gut microbiome in most rodents and have been rigorously tested in our laboratory. Although they involve basic manipulations to complex surgical procedures, these methodologies provide researchers with an assessment tool to test associations between changes in bacterial metabolites and metabolic outcome in disease.

1.2 Characterization and Identification of Gut Microbes

Identification of bacteria in complex communities such as the mammalian gut is the primary step in establishing a relationship between the resident microbiome and disease. Bacterial identification using the classical culture-based method is laborious and highly limited as majority of species fail to grow in standard laboratory conditions in defined media [20–22]. Although specialized incubators are available for propagation of bacteria under anaerobic conditions, it is difficult to identify optimal settings for several thousands of bacterial species found in the mammalian gut. Modern techniques of cloning and DNA sequencing have revolutionized identification of such "difficult to culture" bacteria from any community. Most of these methods use sequence comparison of universal genes such as 16S

rRNA for identification purposes [23, 24]. Being an integral component of the 30S small subunit of bacterial ribosomes, 16S rRNA sequence is evolutionarily conserved in bacteria and plays an important role in protein synthesis. It contains several conserved and species-specific hypervariable regions that allow for identification of bacteria through sequence comparison. Such comparisons can be carried out on public databases such as NCBI (National Centre for Biological Information), KEGG (Kyoto Encyclopedia of Genes and Genomes), eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups), and GOLD (Genomes Online Database; www.genomesonline.org). Databases such as MetaHIT (Metagenomics of the Human Intestinal Tract) can be used for characterization and identification of human gut specific microbes. The 16S rRNA gene is 1.5 kb in length and comprises nine variable regions named V1 to V9. Variable regions V3 and V6 spanning 433–497 and 986–1,043 ntd are the most suitable regions that allow for identification to the genus level [25]. For many years, Sanger sequencing of 16S rRNA gene amplified plasmid clones from a bacterial community was a standard method of identification. Longer read lengths from Sanger sequencing allows for full-length sequencing of the 16S rRNA gene of any bacteria and therefore, it is a method of choice for typing, comparing, and discriminating closely related cultured-bacteria, and establishing a novel species of bacteria. However, sequencing minimal shorter regions such as V3 or V6, using next-generation sequencing platforms such as Ion-Torrent PGM are the recent methods of choice. These platforms provide several folds higher coverage of sequences with comparatively low investment of time and resources. We present three workflows for identification of gut bacteria using Sanger sequencing and Next-Generation sequencing platforms (HiSeq2000 and Ion-torrent). All methods discussed herein are based on amplification and generation of 16S rRNA library from the extracted pool of bacterial community DNA from rodent samples (feces or gut washings). The captured diversity is thus termed as uncultured bacterial diversity. Isolated DNA from material such as feces or gut contents is usually rich in PCR inhibitors [26] and needs an efficient purification step along with an inevitable step of PCR optimization. Diluted DNA samples show better PCR amplification and represent the true bacterial diversity in a DNA pool [27]. In addition, the numbers of PCR cycles is also critical in capturing the diversity. Use of the smallest and best amplifiable amount of community DNA with low cycle number tends to avoid the over-representation of dominant microbiota in the sample. It also reduces the number of chimeric sequences that are formed by hybridization of two different parent/template molecules during the annealing phase of PCR [27]. Such sequences show a low homology to the existing sequences in the database and falsely represent novel bacterial groups/species. We have incorporated several modifications based on our experience and as discussed

by other investigators [27] to avoid PCR drift, over-amplification of Taq errors, and formation of chimeras in this protocol.

Analysis of bacterial communities especially using the NGS platforms generates massive data. Computation analysis of the sequences using open source tools such as Mothur, MEGAN, Qiime, or Partek Genomics Suite can be used for efficient analysis and visualization of the data. The processing includes steps for filtering adapter sequences (vector sequences in the Sanger sequencing approach), identification and elimination of chimeric DNA sequences followed by comparison of sequences with the already known 16S rRNA sequences in the database such as RDP (Ribosomal Database Project) [28], SILVA [29], Greengenes [30], and EzTaxon [31]. Most of these databases have an automated pipeline of programs, which eventually present the data in several interesting formats such as percentage distribution and phylogenetic trees.

2 Materials

Prepare all solutions in nuclease-free water (*see* **Note 1**). Carry out all the steps in a nuclease-free workspace. Follow waste disposal as per the regulations. Use sterile deionized water (resistance of 18 MΩ cm at 25 °C) for all the steps.

2.1 Manipulation of Gut Microbiota in Mice

1. Alzet slow release pumps with accessories.
2. Gavage needle.
3. 1 ml syringe.
4. PBS (1×).
5. Custom designed cages (Fig. 1).

2.2 Isolation and Quantification of Total Fecal DNA

1. Stool DNA extraction kit, e.g., QIAamp DNA Stool Mini Kit (Qiagen, *see* **Note 2**). Other methods such as Qiagen midi kit following bead beating provide similar or better efficiency.
2. Spectrophotometer, e.g., NanoDrop2000 (Thermo Scientific, *see* **Note 3**).
3. Qubit® 2.0 fluorometer (Invitrogen).

2.3 Assessment of PCR Inhibition

1. Nuclease-free water (*see* **Note 1**).
2. Taq DNA polymerase (5 U/μl, NEB) (*see* **Note 4**).
3. 10× ThermoPol Reaction Buffer (supplied with Taq DNA polymerase, NEB): 200 mM Tris–HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1 % Triton X-100, pH 8.8 at 25 °C.
4. Oligonucleotide primers 8F-I (5′-GGA TCC AGA CTT TGA TYM TGG CTC AI-3′), 907R-I (5′-CCG TCA ATT CMT

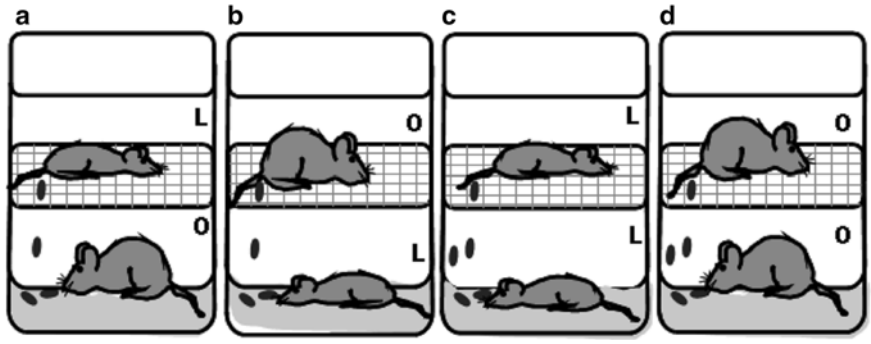

Fig. 1 A passive method for fecal transplantation: Design for passive transfer of gut microbiome from donor rats (*top cage*) to recipient rats (*bottom cage*). Specifically designed cages/boxes (see text) allow for fecal contents of the donor rats to be transferred in real time to the recipient's box. As a result of the coprophagic behavior seen in rodents, recipients exhibit allocoprophagy, thus allowing for passive transfer of donor's microbiome to recipient. The four different cage setups above (**a**–**d**) are necessary to test the effects of gut microbiome transfer from lean (L) animals to the Obese (O) animals or vice versa and include the essential controls

TTG AGT TI-3′): 10 μM stock each. (Standard desalted primers work good).

5. 10× dNTP stock (2 mM each): Dilute 1:4 times from the commercially available 10 mM stocks of dNTP mix in water. Store at −20 °C in aliquots.

2.4 Electrophoresis

1. Agarose: Molecular biology-grade agarose.
2. 5× TBE: Dissolve 54 g of Tris base, 27.5 g of boric acid, and 20 ml of 0.5 M EDTA (pH 8.0) in 800 ml of water and makeup the volume to 1 L. Autoclave 15 min at 15 psi pressure on a liquid cycle and store at room temperature (*see* **Note 5**).
3. 0.5× TBE: Dilute 1:10 times from the 5× TBE stock in water.
4. DNA loading dye: EZ-VISION® THREE 6× DNA loading dye (Amresco) (*see* **Note 6**).

2.5 Construction of Sanger Sequencing Library

1. All the reagents from Subheading 2.4.
2. PCR cleanup column (e.g., DNA Clean & Concentrator™-5-Capped Columns, Zymo Research).
3. Gel extraction column (e.g., Zymoclean™ Gel DNA Recovery Kit, Zymo Research, *see* **Note 7**).
4. TA cloning vector (pGEM-T Easy, Promega corp. OR pCR4TOPO from TOPO TA Cloning Kit for Sequencing, Life Technologies).
5. Chemically/electrocompetent *E. coli* (DH5α) cells.
6. Luria–Bertani Agar (Commercially available).
7. Antibiotic (100 mg/ml Ampicillin, *see* **Note 8**).

8. Sterile wooden toothpicks (*see* **Note 9**).
9. Vector-specific Oligonucleotide primers M13F (5′-GTA AAA CGA CGG CCA G-3′) and M13R (5′-CAG GAA ACA GCT ATG AC-3′): 10 μM stock each. (Standard desalted primers work well).
10. PEG–NaCl PCR purification mix (*see* **Note 10**): Dissolve 20 g of PEG 8000 and 14.6 g of NaCl in 100 ml of sterile water (Final strength is 20 % PEG; 2.5 M NaCl). Autoclave and store at room temperature.
11. ABI 3730 DNA Analyzer (Applied Biosystems, available commonly at Sequencing Core facilities and commercially).

2.6 Construction of PGM Sequencing Library

1. Oligonucleotide primers for 200 bp sequencing V3_Seq—314F (5′-cct acg gga ggc agc ag-3′) and V3_Seq—518R (5′-att acc gcg gct gct gg-3′). For 400 bp sequencing V6_Seq_536F (5′-gtg cca gcm gcc gcg gtr ata-3′) and V6_Seq_907R (5′-ccg tca att cmt ttg agt tt-3′): Primers 25 nM stock each diluted to 10 pM/μl as working dilution.
2. AmpliTaq® Gold™ PCR Mastermix (LifeTech, contains AmpliTaq Gold® DNA Polymerase, dNTPs, Gold Buffer, and $MgCl_2$).
3. Eppendorf Mastercycler.
4. Nanodrop Spectrophotometer (Thermo Scientific).
5. Ion Xpress™ Plus Fragment Library Kit (LifeTech) with optional Ion Xpress™ Plus Barcode adaptors.
6. Ion PGM™ Template OT2 200 Kit (200 bp sequencing); Ion PGM™ Template OT2 400 Kit (400 bp sequencing) for use with the Ion OneTouch™ 2 Systems. Although a 400 bp sequencing option in now available (and preferred) on OT2, a 200 bp sequencing strategy also provides with workable sequence information for bacterial classification.
7. Ion PGM™ Sequencing 200 Kit v2 (200 bp sequencing); Ion PGM™ Sequencing 400 Kit.
8. Agencourt® AMPure® XP Kit for DNA purification.
9. Agilent 2100 Bioanalyzer® instrument to analyze fragment length distribution during library preparation and quality assessment.
10. Agilent® High Sensitivity DNA Kit.

2.7 Construction of HiSeq2000 Sequencing Library

1. Oligonucleotide primers V3_F_modified (5′-aat gat acg gcg acc acc gag atc tac act ctt tcc cta cac gac gct ctt ccg atc tNN NNC CTA CGG GAG GCA GCA G-3′) and V3_XR (5′-caa gca gaa gac ggc ata cga gat XXX XXX gtg act gga gtt cag acg tgt gct ctt ccg atc tAT TAC CGC GGC TGC TGG-3′): 10 μM stock each (*see* **Note 11**).

2. Phusion® High-Fidelity DNA Polymerase (NEB, supplied with 5× HF Buffer).
3. 10× dNTP stock (2 mM each).
4. Gel extraction column (e.g., Zymoclean™ Gel DNA Recovery Kit, Zymo Research, *see* **Note 7**).
5. Spectrophotometer (Nanodrop from Thermo Scienctific).
6. HiSeq2000 (Illumina Inc., available commonly at Sequencing Core facilities and commercially).

3 Methods

Handle all reagents with gloves and carry out all the steps in a nuclease-free environment in pre-PCR and post-PCR working cabinets. Thaw the biological samples, DNA, and reagents on ice. During additions, maintain all reactions on ice. Mix the reaction by gentle pipetting before each incubation followed by a quick centrifugation step (12,000 × *g* for 5 s) to pool the contents.

3.1 Manipulation of Gut Microbiota in Mice

3.1.1 Transfer of Gut Microbiota

One of the major questions in this field is to assess if a specific disorder is associated with or a result of the changes in gut microbiome. The simplest answer would be to feed a specific strain of cultured bacteria. However, since most bacteria are non-cultivable and multiple species of bacteria may actually contribute to the final disease phenotype, it is important that initial studies involve a complete exchange of gut microbial contents from donor to recipient animals. We designed a strategy for efficient and passive transfer of gut microbiota from donor to recipient rodents, by exploiting the coprophagic behavior in mice. Coprophagy refers to the behavioral adaptation of eating feces. Most animal species show coprophagic behavior as a routine lifestyle; rodents consume 5–50 % of their daily diet in the form of feces. Other species are known to exhibit coprophagy under specific conditions. We studied coprophagy in rodents and validated through labeling studies that most common species (Wistar rats, Swiss and BALB/c mice) do not show selective preference for autocoprophagy (own feces), but do demonstrate allocoprophagy (same species feces).

Specially designed cages containing two compartments separated by a metallic sieve were used for this experiment, as shown in Fig. 1. Such cages can be specially ordered or prepared manually for rodent microbiome manipulations. Briefly, a bottomless cage is placed on top of a regular cage such that the top cage sits firmly on a metallic sieve (lid) of the bottom cage and clamped together from outer side. The donor animals are placed in the top cage while recipients are placed in the bottom cage. Sterile cotton gauze bedding is provided along with autoclaved hideaways for both cages.

Four such sets of cages need to be prepared for any experimentation. The first cage should contain lean mice in the top cage and obese in the lower cage. The second cage should contain obese mice in the top cage and lean mice in the lower cage. The third and fourth cages are necessary controls, in which lean/obese animals are placed in the top cages, above lean/obese animals in the lower cage (i.e. lean over lean, obese over obese). This method provides a passive transfer method in real time for transfer of donor fecal matter (top cage) to the recipient (bottom cage). Although rodents do not show preferential autocoprophagy, this can be tested by color-coding their diets with non-metabolizable food colors/additives, which render a characteristic color to the feces. Gut microbiome transplantation carried out in this way allows for passive transfer and colonization of gut microbiota. Animals can be routinely followed for metabolic changes (e.g.: biochemical and DEXA measurements), which are generally noticeable within 3–5 weeks. Alternatively, animals could be placed in metabolic cages to collect for their urine and feces, while recording the input calories and related parameters, with regular (forced) feeding using gavage needle.

3.1.2 Gavage

As an alternate protocol, fecal contents of the donor rodents can be collected and suspended in 1× PBS (1.0 ml per 10^8 bacterial cells from fecal matter) and 100 μl of the resuspended content administered to each recipient mouse by oral gavage each week in the initial month followed by once a month until conclusion of the study. Transplanted mice are maintained in sterile cages during the study (*see* **Note 12**) [32].

3.1.3 Localized Delivery of Gut Metabolites

Although experimentation related to microbial transfer can be initiated with transfer of total gut contents/fecal transplantation, such a procedure would have limited translation to practice. Subsequent studies may necessitate transfer of specific metabolites to recipient rodents. These could be metabolites that allow for or promote the growth of a specific type of bacteria and/or need to be delivered to a specific region of the gut. We have developed a unique way for delivering such metabolites to specific regions of the rodent gut. Although these procedures involve the use of specialized surgical techniques, they can be easily carried out in most species by small animal surgeons. We present a well-tested method that we have optimized for transplanting key metabolites to recipient mice using slow release osmotic pumps (Alzet pumps cat # 1002 or 2004).

1. Prepare ~250 μl of metabolites that are intended for transfer in a microfuge tube. Always keep in excess of the volumes required as the volumes used would depend on the size of the catheter as well as the capacity of the pump used.
2. Alzet slow release pumps are available in multiple sizes and offer a wide range of delivery options. For most studies related

to gut microbiome analysis, pumps with a flow rate of 0.25–1 μl/h over 2–4 weeks are suitable. Please refer to www.alzet.com for more details on specific pumps. Please note that filling and implantation of Alzet pumps should be carried out under aseptic conditions and surgical gloves should be worn at all times. Deposition of skin oils on the surface of these pumps may interfere with the performance of these pumps. If this happens, wipe clean the pump's surface with an aqueous solution of 70 % isopropanol immediately before use. Do not soak the pump in isopropanol.

3. Loading a pump: Weigh the empty pump together with its flow moderator and check the instructions (supplied by manufacturer) for the mean fill volume for the lot of pumps that you would be using. Attach a filling tube (supplied) to a 1 ml syringe and draw up the metabolite solution from its microfuge tube (brought to room temperature before loading). Draw in extra volume for spillage. With the flow moderator removed, insert the filling tube through the pump's opening until it cannot go any further. Gently push the plunger, maintaining the pump in an upright position. Avoid rapid filling as it can introduce air bubbles into the reservoir. Insert the flow moderator until the cap is flush with the top of the pump. Wipe off any excess solution from the opening of the pump and place it in a conical tube (containing saline) to prime the pump. Weigh the pump and check that the fill volume (in mg) is more than 90 % of the reservoir volume specified in the manufacturer's sheet.
4. Priming the pump: We recommend priming the pumps prior to implantation, to overcome the startup gradient phase—a time when pumps soak up fluid and achieve physiological temperature. To prime pumps loaded with the desired metabolite or saline (control), place these in a 50 ml conical tube containing saline at 37 °C for 6–12 h. Please check manufacturer's instructions as some models of pumps require extended priming.
5. Since some metabolites may directly interact with the proprietary material forming the walls of the pump reservoir, it is desirable to have the metabolite solution in a separate chamber. 2–3 mm of polyethylene tubing can be attached to the pump (before the catheter) and coiled around the pump during implantation. Coiled tubing can be prepared in water baths exposing the tubing to hot (60 °C) and cold (ice water) temperatures. The length of this tubing can be computed to accommodate the desired volume. Once the pump is loaded and primed as shown in Fig. 2, prepare for surgical procedure and implant the catheter in the desired portion of the gut. We generally implant catheters in the ileum or colon with no effect/stress observed in sham infused animals.

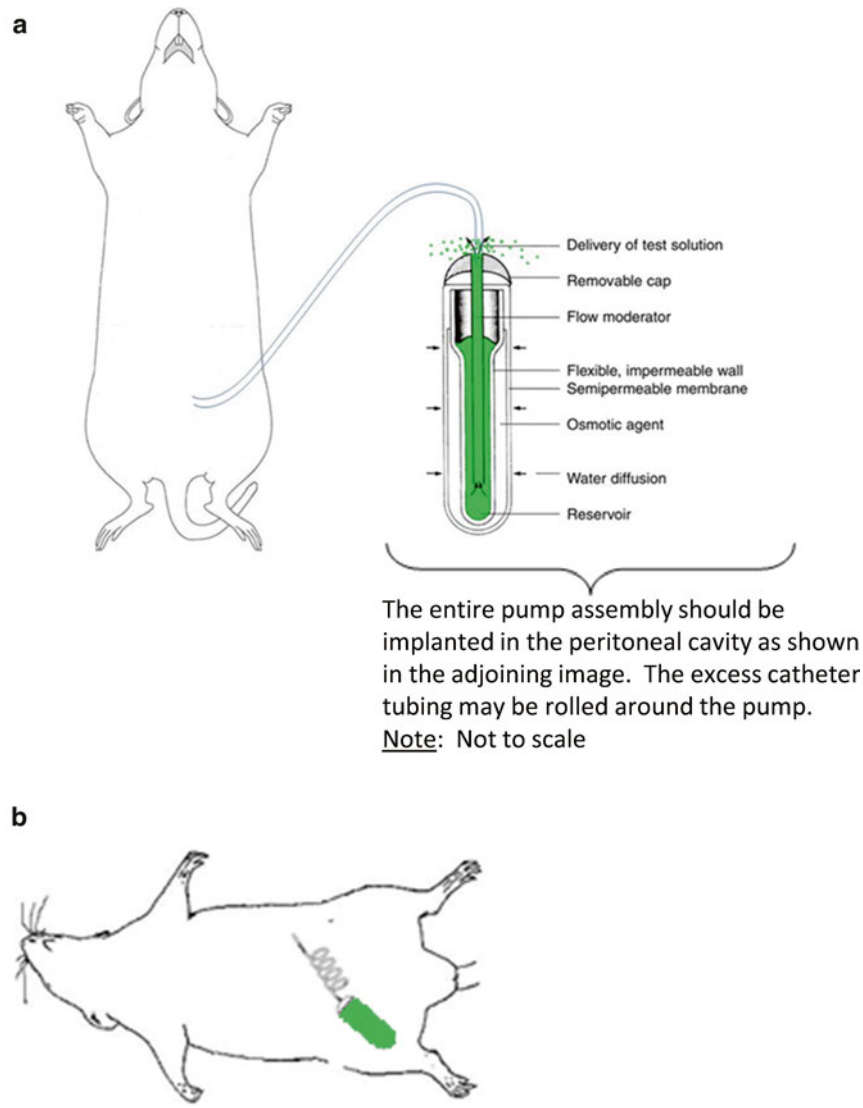

Fig. 2 Regulated delivery of microbial metabolites to recipient's gut: In order to test the effect of specific metabolites on microbial populations in the gut, approaches involving targeted delivery of such metabolites over extended periods are desired. The Alzet pumps (see text) provide the option of delivering metabolites of interest to recipient gut in order to analyze their effects on metabolic health. A mixture of metabolites that support growth of specific bacteria or a short chain fatty acid solution can be incorporated in the loading device (**a**) and delivered following transplantation to recipient animals (**b**). Animals can then be followed for changes in their metabolic profile by serial blood sampling at predefined time points and assessed for various biochemical and metagenomic screens as discussed in the text

6. Delivery of key metabolites to specific segments of the gut, as discussed in this procedure, allows for systematic assessment of their role in influencing gut microbiome and metabolic health. The effects of delivery of key metabolites or bacterial products to desired regions can then be assessed by several biochemical, epigenetic, and molecular analyses, so as to validate the role of

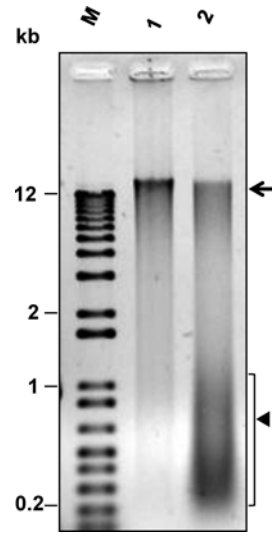

Fig. 3 A representative image for total bacterial DNA from feces. Lane "M" is 1 kb plus DNA ladder, *Lanes 1* and *2* are total bacterial DNA from two different samples. Note the small molecular weight smear (*arrowhead*) at the bottom of lane "2", which appears to be partially degraded. *Arrow* indicates position of intact high-molecular weight bacterial genomic DNA (lane "1")

specific microbial metabolites on health outcomes in this rodent model. Once DNA is isolated, using appropriate blank, e.g., Buffer AE from QIAamp DNA Stool Mini kit, estimate the concentration of isolated total genomic DNA using a spectrophotometer.

7. Analyze 100 ng of the isolated total genomic DNA on a 1 % agarose gel by electrophoresis in 0.5× TBE. A good preparation is seen as a tight smear on the gel as shown in Fig. 3.

3.1.4 Isolation and Quantification of Total Fecal DNA Using Mini Kit

1. Isolate DNA from <200 mg of mice feces/gut contents using a standard Stool DNA extraction kit for e.g., QIAamp DNA Stool Mini Kit (*see* **Note 2**).
2. Elute the DNA in 50 μl of Buffer AE instead of 200 μl as recommended by the kit.

3.1.5 Gut Microbes DNA Extraction Protocol Using Qiagen Midi Kit

1. Isolate DNA from 1 cc of gut content/feces suspension. Gut content samples extracted and stored at −80 °C are thawed to room temperature prior to use.
2. Treat the sample for 1 h with 0.2 ml of lysis buffer containing 50 mM Tris–HCl pH 8.0, 5 mg/ml lysozyme and 10 mM $MgSO_4$ in 37 °C shaker incubator.
3. Take a fresh 15 ml screw cap tube and add 100 μl of proteinase K. Add entire sample contents to this tube, mix thoroughly by vortexing for 10 s.
4. Add 1.2 ml of buffer AL, mix thoroughly by vortexing for 5 s.

5. Incubate at 70 to 95 °C for 10 min to ensure optimum DNA yield. Lysis temperature of 95 °C is recommended optimum temperature for hard-to-lyse pathogens.
6. To the pooled solution from **step 4,** add one half of Inhibit-Ex tablet, vortex, and mix well.
7. Centrifuge 15 ml screw cap tubes from **step 5** @ 3,500 × *g* for 3 min to pellet inhibitors bound to Inhibit-EX matrix.
8. Transfer all the supernatant into a new 15 ml centrifuge tube and discard the pellet.
9. Add 2 ml ethanol (96–100 %) to the sample, and mix by inverting the tube ten times.
10. Label the lid and the tube of a new QIAamp Midi spin column placed in a 15 ml collection tube (White Capped and Supplied by manufacturer). Carefully transfer the complete lysate (from **step 8**) to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at 3,000 × *g* for 2 min.

    Note: Close each spin column to avoid aerosol formation during centrifugation. If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.
11. Open carefully the QIAamp spin column and add 2 ml Buffer AW1. Close the cap and centrifuge at 3,500 × *g* for 5 min. Now discard the filtrate and place the QIAamp spin column in the same 15 ml collection tube.
12. Open carefully the QIAamp spin column and add 2 ml Buffer AW2. Close the cap and centrifuge at 3,500 × *g* for 25 min. Discard the collection tube containing the filtrate.

    Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Columns with tubes can be placed at 70 °C for 10 min to aid in evaporating all remaining ethanol. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.
13. Transfer the QIAamp spin column into a new, labeled 15 ml centrifuge tube. Spin columns need to be placed into a fresh 15 mL tube. This should be done prior to baking at 70 °C for 10 min to ensure no residual ethanol is stuck between the column and tube, which could later be spun into the eluate containing DNA. Carefully open the QIAamp spin column and pipette 300 µl Buffer AE directly onto the QIAamp membrane. Close the cap and incubate for 5 min at room temperature, then centrifuge at 3,500 × *g* for 5 min to elute DNA.

    Note: Incubating time can be increased to 10 min for maximum yield.

Table 1
PCR cycling conditions for Sanger Sequencing libraries

Step no.	Time/temperature	Remark(s)
1	3 min at 95 °C	Hold
2 3 4	10 s at 95 °C 45 s at 53 °C[b] 1 min at 72 °C[c]	20 cycles[a]
5	20 min at 72 °C	Hold[d]
6	4 °C	Hold

[a]Low cycles ensure linearity of the reaction
[b]When using a different primer combination, annealing temperature may require optimization
[c]Set polymerization time to 1 min/kb
[d]Essential for Taq-mediated addition of 3′ A onto the resulting amplicon required for TA cloning

14. Transfer the 300 μl eluate containing DNA back again in the same column and repeat the **step 11**. This step will ensure maximum yield of DNA in 300 μl eluate. Transferring back onto the column will ensure maximum concentration; pipetting an extra 50 μL of buffer AE will increase yield.
15. Store the DNA in −80 °C until analyzed.

3.2 Assessment of PCR Inhibition

1. Make a 1:2 dilution series such as 1, 1:2, 1:4, 1:8, etc. up to 1:256 of the total genomic DNA for each sample in nuclease-free water.
2. In a set of 0.2 ml PCR tubes, set PCR for amplification of 900 bp of 16S rRNA gene using the 8F-I and 907R-I primers as follows (Also set a no-template reaction, where add nuclease-free water instead of the total genomic DNA):

Components	Volumes in μl
10× Taq polymerase buffer	2.5
10× dNTPs	2.5
10 μM 8F-I primer	0.5
10 μM 907R-I primer	0.5
Diluted total genomic DNA	2.0
Taq DNA polymerase (5 U/μl)	0.25
PCR grade water	to 25

3. Mix and place the tubes on a thermocycler. Amplify the 16S rRNA gene amplicon using the cycling conditions given in Table 1 (*see* **Note 4**).

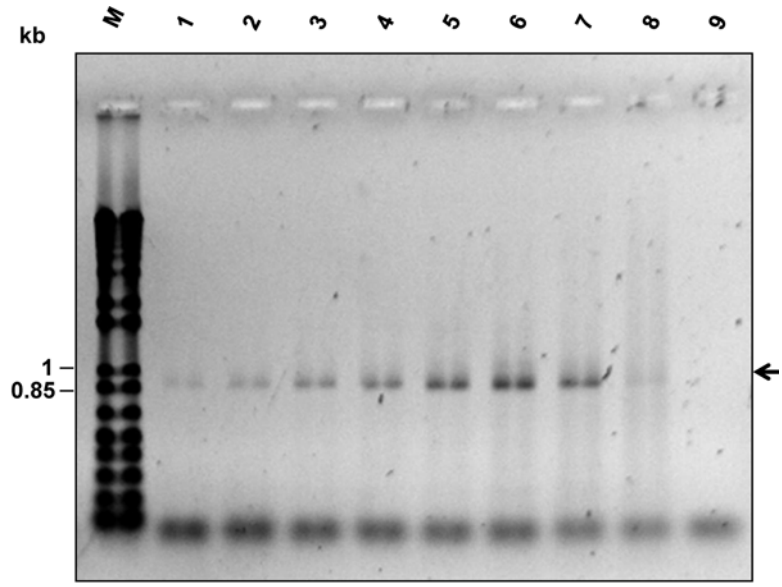

Fig. 4 Effect of fecal DNA dilution on 16S rRNA amplification. Lane "M" is the 1 kb plus DNA ladder, while *lanes 1* through *8* are twofold serial dilutions, in increasing order of total fecal bacterial DNA template for PCR. Lane "9" is a "no-template control". *Arrow* indicates the 900 bp 16S rRNA PCR product. Lane "8" is for original template DNA and lane "6" is for four times diluted template. Diluted template DNA eliminates PCR inhibition

4. Analyze 5–10 μl of the PCR mix on 1 % agarose gel by electrophoresis in 0.5× TBE and determine the dilution of DNA showing best amplification from the amplicon density. A representative image is shown in Fig. 4.

3.3 Construction and Sequencing of Sanger Sequencing Library

1. Set four replicates of 25 μl PCR reactions using the estimated best amplifiable dilution of total genomic DNA for each sample in Subheading 3.2.
2. Mix and place the tubes on a thermocycler. Amplify the 16S rRNA gene amplicon using the cycling conditions used in Subheading 3.2 but for 20 cycles.
3. Pool the reactions for each sample separately. Purify using a PCR purification column and elute the DNA in 20 μl of elution buffer.
4. Load the whole 20 μl PCR product 1 % agarose gel in a well using EZVision loading dye.
5. Visualize on a UV transilluminator and extract the piece of agarose gel containing the 900 bp band using a new (sterile) scalpel blade.
6. Purify the amplicon from the excised gel using a standard gel extraction column.
7. Elute and quantify the eluted fragment using a spectrophotometer.

Table 2
PCR cycling conditions for colony PCR for Sanger Sequencing libraries

Step no.	Time/temperature	Remark(s)
1	3 min at 95 °C	Hold
2 3 4	10 s at 95 °C 45 s at 55 °C 1 min at 72 °C	30 cycles
5	1 min at 72 °C	Hold
6	4 °C	Hold

Standard thermal cycling conditions are presented for colony PCR

8. Set ligation with a TA cloning vector with an insert to vector ratio of 3:1 as per the manufacturer's instructions.
9. Transform the ligation mix into chemical or electro-competent *E. coli* DH5α cells and spread the culture on LB agar containing 100 μg/ml of ampicillin at an appropriate dilution to obtain well-separated recombinant colonies.
10. Perform colony PCR using vector specific primers such as M13F and R for pGEM-T easy and pCR4TOPO vectors. Prepare a 25 μl PCR mix as follows and inoculate colonies using a sterile toothpick:

Components	Volumes in μl
10× Taq polymerase buffer	2.5
10× dNTPs	2.5
10 μM M13F primer	0.5
10 μM M13R primer	0.5
Taq DNA polymerase (5 U/μl)	0.25
PCR grade water	to 25

11. Mix by gentle pipetting and amplify the insert using the cycling conditions given in Table 2.
12. Add 0.6 volumes of PEG–NaCl solution to each colony PCR reaction, mix by gentle pipetting, and incubate at 37 °C for 1 h (*see* **Note 13**).
13. Centrifuge at >12,000 × *g* at 25 °C for 20 min.
14. Discard the supernatant. Pellet is generally invisible.
15. Add 4 volumes of freshly prepared 70 % ethanol, invert-mix, and centrifuge at >12,000 × *g* at 25 °C for 20 min.
16. Discard the supernatant and repeat the 70 % ethanol wash once again.

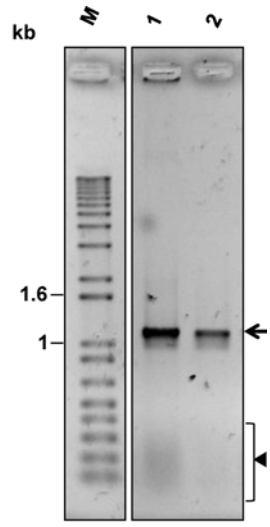

Fig. 5 Colony PCR and purification of PCR product prior to sequencing. Lane "M" is 1 kb plus DNA ladder, Lane "1" is the amplified PCR product containing 16S rRNA insert, using M13F and R primer. Lane "2" is PEG–NaCl based purification of PCR product from Lane "1". Note the loss of primer dimers or smaller PCR products in PEG–NaCl-purified sample, indicated by the *arrowhead. Arrow* indicates PCR product containing 16S rRNA insert

17. Discard the supernatant and vacuum or air-dry the pellet (*see* **Note 13**).
18. Dissolve pellet in 25 μl of nuclease-free water (*see* **Note 14**).
19. Use 5 μl of the purified PCR product (from **step 18**) alongside of 5 μl of unpurified PCR product (from **step 11**) to carry out electrophoresis on a 1 % agarose gel to verify removal of free primers or primer-dimers. A representative image is shown in Fig. 5.
20. Sequence the PCR product using appropriate primers separately using Sanger Sequencing on ABI 3730 Genetic Bioanalyzer.

3.4 Construction and Sequencing of PGM Sequencing Library

1. Set 25 μl amplification PCR (shown in table below) for each DNA sample in quadruplicate. Take 10 ng of Qiagen Midi Kit isolated DNA (as described in Subheading 3.2) in a 0.2 ml PCR tube using appropriate primer (200 or 400 bp sequencing) sets. Include a control reaction with no DNA or add nuclease-free water.

Components	Volumes in μl
Sample DNA (10 ng/μl)	1.0
10 pM F primer	0.25
10 pM R primer	0.25
AmpliTaq® Gold™ PCR Mastermix (2×)	12.5
PCR grade water	to 25

2. PCR conditions: pre-PCR incubation step of 95 °C for 10 min followed by 25 cycles of 94 °C for 1 min (denaturation), 55–60 °C for 1 min (annealing temperature, set as per primer melting temperature (T_m), higher temperature is proportional to enhanced specificity), 72 °C for 1 min (extension) and a final incubation of 72 °C for 7 min.
3. Pool the PCR products and purify using a PCR purification column. The quality and quantity can be analyzed using Agilent Bioanalyzer 2100®. Store the purified PCR products in −20 °C until processed for library preparation.
4. Prepare the DNA library of the purified PCR product for PGM sequencing following the appropriate protocol for Ion Xpress plus fragment library kit using manufacturers' instructions.

3.4.1 Construction and Sequencing of HiSeq2000 Sequencing Library

1. In a set of 0.2 ml PCR tubes, set PCR for amplification of 170–200 bp of 16S rRNA gene using the V3_F_modified and V3_XR primers for each DNA sample in triplicate using different reverse primers in each as follows (A no-template reaction is highly recommended, where instead of the total genomic DNA, add nuclease-free water. For the reverse primer, *see* **Note 11** and **Note 15**):

Components	Volumes in µl
5× HF buffer	10.0
10× dNTPs	5.0
10 µM V3_F_modified primer	1.0
10 µM V3_XR primer	0.5
Total genomic DNA (5 ng/µl)	2.0
Phusion DNA polymerase (5 U/µl)	0.5
PCR grade water	to 50

2. Mix and place the tubes on a thermocycler. Amplify the 16S rRNA gene amplicon using the cycling conditions given in Table 3.
3. For each separate reaction, load 20 µl of the amplified PCR mix on 2 % agarose gel in a well using EZVision loading dye and electrophorese.
4. Visualize on a UV transilluminator and extract the piece of agarose gel containing the 330 bp band using a fresh razor blade.
5. Purify the amplicon from the excised gel using a standard gel extraction column.
6. Elute and quantify the eluted fragment using a spectrophotometer (*see* **Note 16**).

Table 3
PCR cycling conditions HiSeq2000 libraries

Step no.	Time/temperature	Remark(s)
1	5 min at 95 °C	Hold
2 3 4	1 min at 95 °C 1 min at 50 °C 1 min at 72 °C	20 cycles[a]
5	7 min at 72 °C	Hold
6	4 °C	Hold

[a]Low cycles ensure linearity of the reaction

7. Mix equal amounts of PCR library from each replicate and sequence them on HiSeq2000 by paired-end sequencing (at any core/commercial facility).

4 Notes

1. Nuclease-free water is prepared by autoclaving glass-distilled water with a resistance of 18 MΩ cm at 25 °C. Commercially available molecular biology-grade water may also be used.
2. To improve DNA yield, an additional step of bead beading can be adopted concurrently to the addition of lysis buffer. We recommend use of sterile glass/zirconium beads of various sizes. A comparison of methods for isolation of fecal DNA is available in reference [33].
3. A regular spectrophotometer capable of reading at 260 and 280 nm is also compatible. However, fluorescence based detection has been proven to be more accurate e.g., as in Qubit from Life Technologies.
4. Another source of thermophilic DNA polymerase, which can add 3′ A on double stranded PCR products can also be used. A change in the source may require some optimization. Use of proofreading thermophilic DNA polymerase may require an additional step of Taq-mediated 3′ A tailing after gel purification of the PCR product. Addition of A's on 3′ end is necessary for TA cloning. However, blunt cloning vectors such as pCR®-Blunt II-TOPO® from Life Technologies can be directly used following the initial amplification using a proofreading DNA polymerase.
5. 5× TBE stock is more stable and does not show precipitation during storage as compared to a 10× stock.
6. DNA loading dye used here pre-stains the PCR products. Users may also use other available staining methods involving

ethidium bromide and nontoxic stains, such as SYBR GOLD. The fluorescently labeled TSP product may not be visible on gel due to a low cycle PCR.

7. We highly recommend the cleanup column from Zymo Research Corporation because of their efficient design that drastically reduces carryover of residual ethanol in the eluates. Contaminating ethanol, being lighter, causes loss of PCR product during loading of wells in the gel electrophoresis step.
8. Use of antibiotic depends on the selected vector/plasmid for cloning the PCR products.
9. Plastic (disposable) toothpicks are a better alternative to conventional wooden toothpicks.
10. PEG is insoluble at room temperature. A short incubation in lukewarm water increases its solubility. The magnetic separation based Agencourt AMPure XP PCR purification system from Beckman Coulter is commercially available alternative to the PEG–NaCl mix.
11. "N" in V3_F_modified primer is a degenerate base and represents any base at the particular location. As per the reference [34], the four bases are first read during sequencing and these generate the required diversity for identification of clusters during sequencing on HiSeq2000. In V3_XR reverse primer, "X" represent barcode/index number. "XXX XXX" in the primer sequence represents the barcode sequence, which are useful for pooling the samples for sequencing. For V3_1R the sequence is CGT GAT, 2R: ACA TCG, 3R: GCC TAA, 4R: TGG TCA, 5R: CAC TGT, 6R: ATT GGC, and so on [34].
12. Selection of donor animal and the source of bacteria for transplantation is of considerable importance. Contents from any other part of the organism may be chosen as required. Recipient is usually a dietary control or a gnotobiotic mouse.
13. During centrifugation, keep the hinge side of the microfuge tube on the outer side of the rotor. This helps to identify/locate the pellet position as the pellet may be invisible. Pipetting should be done from top to bottom of the supernatant. Decant the maximum volume of liquid from the tube using a sterile pipette tip. Give the tube a flash spin ($10{,}000 \times g$ for a min or so). You will see some more volume of liquid gathered with the pellet. Collect this liquid by means of a fine pipette tip. This protocol reduces the time of pellet drying.
14. Usually for sequencing on ABI 3730, 3–5 ng of PCR product is enough for a sequencing PCR reaction. Further dilution or concentration may be required before sequencing.

15. Choice of the barcoded-reverse primer is critical, especially, when pooling four or less samples per lane during sequencing. Please consult Illumina Inc. or your sequencing facility.
16. Quantification of PCR product is very critical to obtain equal number of sequencing reads per library. Estimation of concentration by fluorometric assays as by Qubit (Life Technologies) and by Agilent Bionalyzer is also recommended.

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