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Chimeric Mouse Generation by ES Cell Blastocyst Microinjection and Uterine Transfer

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Abstract

The ability to generate chimeric mice through microinjecting embryonic stem (ES) cells into blastocysts is a critical step for the conventional ES cell-mediated knockout technology. In recent years, designer nuclease-based methods, especially the CRISPR technology, have substantially decreased the needs for blastocyst microinjection. However, this method has still remained as a valuable technique for generating sophisticated genetic models as well as for stem cell research. In this chapter, we describe the detailed procedures used in our laboratory on how to use ES cells to produce chimeric mice, including derivation and inactivation of MEF feeder cells, culturing and handling of mouse ES cells, collection and microinjection of blastocysts, and finally implantation of injected blastocysts into the uteri of pseudopregnant surrogate mothers.

Keywords

Blastocyst microinjection; Embryo transfer; Mouse ES cells; Knockout; Chimeric mice; MEF feeders

1 Introduction

Two decades of research converged on a method that matured in the late 1980s, and won a Nobel Prize yet another two decades later in 2007 for developing the revolutionary mouse knockout technology. DNA homologous recombination technology started in the 1960s by Oliver Smithies [1], combined with mouse embryonic stem (ES) cell derived from embryos by Martin Evans [2] made it possible to modify genes in the ES cells [3, 4]. With embryo manipulation work developed earlier [5], a general strategy for knocking out mouse gene was developed by Mario Capecchi and colleagues [6]. Several years later, conditional knockout mouse using cre-lox method was developed [7, 8].

Using these methods, a great majority of mouse genes have been conditionally modified in mouse ES cells through internationally coordinated consortia [9], and these ES cell lines are available from publicly funded repositories for reasonable fees (<https://www.komp.org>, <http://www.mousephenotype.org>, <https://www.mmrrc.org>). Converting these valuable ES cell lines into live mice requires the generation of chimeric mice by mixing ES cells with early embryos. Although a few alternative methods for generating chimeric mice have been

reported, such as ES cell-embryo aggregation [10] and morula injection methods [11], the most widely used method is blastocyst microinjection as outlined in Fig. 1. In this method, ES cells are injected into blastocyst stage embryos using a pulled micro glass needle, and then the injected blastocysts are surgically implanted into the uteri of pseudopregnant surrogate mothers. If the injected ES cells are pluripotent, they will contribute to various lineages of the embryos, including the germline (usually sperm but can sometimes be eggs), which can pass the modified gene to the next generation.

In the past decade, designer nuclease-based technologies, including ZFN (zinc finger nucleases) ([12] and Chapter 17 of this book), TALEN (transcription activator-like effector nucleases) [13], and CRISPR (clustered regularly interspaced short palindromic repeats) [14, 15], have been developed and increasingly used for precisely manipulating the genome of mice and a variety of other species. The CRISPR technology has emerged as the predominant method for engineering mouse lines due to its simplicity and extraordinary efficiency. It has also dramatically shortened the timespan for gene knockout and small knockins because the CRISPR reagents can be directly injected into zygotes without using ES cells and chimera generation. However, the conventional blastocyst microinjection method is still a valuable tool because: (1) at this time the efficiency for using CRISPR to generate conditional knockouts and large knockins are still low and unpredictable, so a significant number of facilities still use the conventional methods for complicated projects; (2) floxed ES clones are already available for a majority of mouse genes, which can be obtained and used for generating conditional knockout mice through blastocyst injection; (3) besides generating gene-targeted mice, blastocyst microinjection is a valuable method for other areas of biomedical research, such as testing pluripotency of stem cells, tetraploid complementation studies [16], interspecific chimera study ([17] and Chapter 18 of this book); (4) at the present time, there are still concerns about CRISPR's nonspecific activities at off-target sites, although most off-target mutations, if any, can be eliminated through breeding. Therefore, it is unlikely that the blastocyst microinjection method will become obsolete anytime soon.

2 Materials

2.1 Targeted Mouse ES Cells and Cell Culture Reagents

1. Germline-competent mouse ES cells: mouse ES clones with desired genetic modifications can be obtained from various repositories, such as <https://www.komp.org>, <http://www.mousephenotype.org>, and <https://www.mmrrc.org>, or engineered in individual laboratories.
2. Inactivated mouse embryonic fibroblast (MEF): MEF cells can be obtained from commercial sources or prepared from E13.5 embryos as described in Subheading 3.1.
3. Tissue culture hood.
4. Water bath.
5. Centrifuge with rotor for spinning down cells.

6. Inverted microscope for cell culture.
7. Tissue culture dishes (35 mm, 60 mm, 100 mm, and 150 mm).
8. Gelatin solution (0.1% in distilled water) for coating dishes.
9. PBS without Ca^{2+} and Mg^{2+} .
10. DMEM medium for culturing MEF.
11. Knockout DMEM medium for culturing ES cells.
12. ES cell qualified fetal bovine serum (FBS).
13. 1 M HEPES solution (pH 7.4).
14. Tissue culture grade β -mercaptoethanol (1000 $\times$).
15. 100 $\times$ pen-strep solution.
16. 100 $\times$ nonessential amino acids (AA).
17. 200 mM L-glutamine solution (100 $\times$).
18. 0.25% Trypsin-EDTA.
19. MEF culture medium: DMEM with 10% FBS, 1 $\times$ pen-strep, 1 $\times$ L-glutamine.
20. ES cell culture medium: Knockout DMEM with 15% FBS, 1 $\times$ nonessential AA, 1 $\times$ pen-strep, 1 $\times$ L-glutamine, 10 mM HEPES (pH 7.4), 0.1 mM β -mercaptoethanol, 1000 IU/mL leukemia inhibitory factor (LIF), and optional G418 (200 $\mu\text{g/mL}$).
21. Serological pipets.

2.2 Mice

1. Twenty C57BL/6 stud males: 2–12 months old; individually caged (*see* Note 1 for choosing recipient mouse strains).
2. Fifty to 100 C57BL/6 females: 4–12-week-old; up to five mice per cage.
3. Twenty vasectomized CD-1 male mice: 2–12 months old; individually caged. Vasectomy procedure can be performed in house or by the animal supplier for a reasonable fee.

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¹-Most earlier ES cell lines were derived from 129 mouse strain, which has coat colors ranging from agouti, chinchilla, and albino depending on the substrains. When these ES cells are injected into C57BL/6 (B6) blastocysts, chimeras are easily recognizable. After breeding with B6 mice, germline transmission can also be recognized through coat color because the 129 color is dominant. Due to increased robustness, ES cells derived from F1 hybrid strain (often 129 and B6 mix) are also popular for gene-targeting experiments. Various ES cell lines have also been derived from B6 mice. For easy visualization of chimerism and germline transmission, some of these ES cell lines were intentionally derived from B6 mice with the c-2J albino mutation [19], or with restored agouti locus [20]. For blastocyst injection, it is recommended to use B6 variants with a different coat color as blastocyst donors, such as albino B6 ES cells can be injected into black B6 blastocysts or vice versa, and agouti-restored B6 ES cells can be injected into black B6 blastocysts. While chimerism can always be recognized if the ES cells and recipient blastocysts are in different color, some germline transmitted pups may not be visualized if the ES cell line was derived from mice that are heterozygous on the color gene, such as the F1 hybrid ES cells and agouti-restored B6 ES cells. In these cases, half of the germline pups will show the dominant color (usually agouti) while the other half of germline pups may have the recessive color, which is usually the same color as the blastocyst donor mice. Therefore, if only a few germline offspring with the desired color are obtained, genotyping all offspring regardless of coat color can increase the number of germline offspring.

4. Fifty to 100 CD-1 females: 6–16-week-old; up to five mice per cage.

2.3 Equipment

1. CO₂ incubator: 37 °C, 6% CO₂, and humidified by a pan containing distilled water.
2. Benchtop sterilizer for autoclaving surgical instruments.
3. Hot bead sterilizer for sterilizing surgical instruments between animals when more than one animal is used during a session.
4. Heating water blanket for keeping the cage warm for aiding post-surgical recovery.
5. Stereo microscope with both top and bottom light sources for embryo collection and embryo transfer procedures.
6. Inverted microscope with 5×, 10×, 20×, and 40× objectives for blastocyst microinjection.
7. Two Eppendorf TransferMan 4r micromanipulators.
8. Two Eppendorf CellTram vario.
9. Air table for reducing vibration of the microinjection setup.
10. Optional digital video camera and computer system for training and recording.

2.4 Tools and Supplies

1. Microinjection chamber: custom made by using rubber cement to glue a standard histological glass slide to a 2 mm thick aluminum frame. Lids of tissue culture dishes (60–100 mm) can also be used, but it requires using much more mineral oil in order to fully immerse the microinjection drop to prevent evaporation.
2. Holding pipette and microinjection needles: both micropipettes are commercially available (Eppendorf VacuTip FCH, holding capillary, 25° tip angle, 60 µm inner diameter, 0.5 mm flange, sterile (5175240006); and TransferTip (ES), for ES cell transfer, 20° tip angle, 15 µm inner diameter, 1 mm flange, sterile (5175107004)). Alternatively, microinjection needles and holding pipette can be made by using a pipette puller and microforge following procedures described by Colin Stewart [18].
3. Embryo transfer pipette (Fig. 3a): the embryo transfer pipette is made by heating a long-tipped Pasteur pipette using a flame, and then pull the softened glass to a thin tube (150–200 µm in diameter) outside of the flame. The pipette is connected to a plastic mouth piece and rubber aspirator tubing (Sigma, Cat: A5177–5EA) with a disposable syringe filter to prevent accidentally sucking medium into the mouth or dripping saliva into the medium.
4. Small hair clipper for removing hair from the surgical sites of mice.
5. Betadine (Povidone-Iodine Swabsticks).

6. Alcohol swab.
7. 29G $\times$ 1/2" 1-CC syringe and 3 mL disposable plastic syringe.
8. 18G and 25G hypodermic needles.
9. Sterile surgical gloves.
10. 5–0 Vicryl absorbable suture.
11. Surgical instrument sterilization tray.
12. Micro dissecting scissors.
13. Small iris forceps.
14. Micro dissecting tweezers (#5).
15. Dieffenbach micro clamp.
16. Wound clip applier.
17. Suturing needle holder.
18. Pipette tips.

2.5 Embryo Culturing Media and Chemicals

1. M2 embryo culture medium.
2. ES cell microinjection medium: Knockout-DMEM with 10% FBS, 1% nonessential AA, 1 $\times$ L-glutamine, 25 mM HEPES (pH 7.4).
3. Embryo culture-tested mineral oil.
4. Ketamine/Xylazine anesthetics: prepared fresh by mixing 1.7 mL 0.9% NaCl with 0.2 mL Ketamine (100 mg/mL) and 0.1 mL Xylazine (20 mg/mL) (*see* Note 2 for proper storage and required record keeping).
5. Bupivacaine (0.25% injectable solution).
6. Meloxicam (5 mg/mL injectable solution).

3 Methods

3.1 Preparation of MEF Cells

MEF cells are usually derived from the carcass of E13.5 embryos. Depending on needs, MEFs can be derived from various mouse strains, such as CD-1 outbred mice for economy, or strains with single (neomycin, hygromycin, or puromycin) or multi (DR4) antibiotic resistance for culturing ES cells that need selection. Ready-to-use MEFs are also available from commercial sources.

1. Set up mating of desired mouse strain for timed pregnancy.

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²-Ketamine is a controlled substance regulated by the US Drug Enforcement Administration (DEA). A license is required for purchasing and storing this anesthetic. A lock box is needed to store these chemicals. A log book is needed to record the usage until it is depleted or expired.

2. Sacrifice females that are visually pregnant 13 days after checked positive for mating.
3. Dissect E13.5 embryos out of uteri and their yolk sacs, and wash in PBS three times.
4. Under dissecting microscope, using sharp tweezers pinch off and discard the heads. Tear open abdominal cavity and remove liver (red color) and other internal organs.
5. Use a pair of tweezers to place the embryo carcasses into the barrel of a 3 mL disposable plastic syringe with an attached 18G needle. Add 2 mL MEF medium into the syringe, and then push the plunger to force the carcasses through the 18G needle to break them into small pieces. Further triturate the pieces by drawing in and pushing out the tissues 4–5 times through the 18G needle. Up to 10 embryos can be processed in one 100 mm culture dish. Add 15 mL MEF medium to each 100 mm dish and shake to distribute the tissue pieces evenly throughout the dish.
6. Place the dishes in the CO₂ incubator undisturbed for 48 h to allow the tissue clumps to attach and fibroblasts to migrate out.
7. Aspirate off the culture medium and wash the dish with PBS twice. Add 3 mL of Trypsin-EDTA and incubate for 5 min at 37 °C to lift off attached cells and tissue clumps. Add 15 mL MEF medium and split the content into two 150 mm culture dishes. Add 20 mL MEF medium to each dish and culture for two more days in the CO₂ incubator.
8. Trypsinize the cells using Trypsin-EDTA, and add MEF medium to inactivate trypsin. Centrifuge at $270 \times g$ for 5 min to pellet the cells and then resuspend in MEF medium at $\sim 2 \times 10^6$ cells/mL.
9. Add equal volume of 2× cryopreservation medium (50% FBS, 30% MEF medium, and 20% DMSO). Aliquot 1 mL cell suspension ($\sim 1 \times 10^6$ cells) into each cryopreservation vial and label the vial as P1 MEFs. Freeze the cells at –80 °C overnight before transferring into liquid nitrogen for long term storage.
10. Prior to using, P1 MEFs can be thawed and further expanded for 3–4 additional passages (1 to 4 expansion for each passage). For inactivation, the MEFs are harvested and resuspended in MEF medium in a 50 mL sterile tube. Place the tube inside the chamber of a γ -irradiator and irradiate for 3000 RADS (exposure time is dependent on the source of the irradiation) (*see Note 3 for MEF inactivation using Mitomycin C*).

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³If a γ -irradiator is not available, MEFs can be inactivated by treating with Mitomycin C. Briefly, grow P3 or P4 MEFs to 70–90% confluency in MEF medium, and then change to MEF medium containing 10 μ g/mL Mitomycin C and culture for 2–3 h. After washing the MEFs three times with PBS, they are trypsinized, centrifuged down, and resuspended in MEF medium at the density of 4×10^6 /mL. Next, equal volume of 2× cryopreservation medium (50% FBS, 30% MEF medium, and 20% DMSO) is added, and then aliquoted into cryopreservation vials at 1 mL per vial (2×10^6 cells). The vials are placed in a freezing container and store overnight at –80 °C before transferring into a liquid nitrogen freezer for long term storage.

11. After inactivation, MEF cells can be used directly or cryopreserved at 2×10^6 per vial for later use.

3.2 Preparing ES Cells for Microinjection

ES cells are normally stored in liquid nitrogen but can be temporarily stored at -80°C . We usually thaw ES cells 2 days before injecting them, but culturing dishes need to be coated with gelatin and plated with MEF feeders prior to thawing.

1. Coat each 60 mm dish with 2 mL 0.1% gelatin at room temperature overnight or at 4°C for at least 2 h.
2. Aspirate off gelatin solution. Plate one vial of MEFs ($\sim 2 \times 10^6$ cells) in 5 mL of MEF medium and culture until the cells attach (*see* Note 4 for attaching time) at 37°C in a CO_2 incubator.
3. Take ES cell frozen vials out of the liquid nitrogen freezer and quickly immerse them in a 37°C water bath till majority of ice in the vial melt. Wipe the tube clean and disinfect outside with 70% alcohol. Transfer the content into a sterile 15 mL tube, and then gradually dilute out the cryoprotectant (DMSO) by slowly adding ES cell medium while shaking. If the cryopreservation medium is diluted by 10-folds or more ($<1\%$ DMSO), ES cells can be directly plated without centrifugation. Otherwise, *see* **step 4** below.
4. (Optional) Centrifuge the cells for 5 min at $270 \times g$. Aspirate off the supernatant and suspend the ES cells in 7 mL ES cell medium.
5. Aspirate off medium from MEF dish prepared in **step 2** above, and gently add resuspended ES cells into the dish. Culture in a CO_2 incubator at 37°C .
6. Change medium and check ES cells under the inverted microscope every day to make sure cells do not become over confluent ($<80\%$ confluency) and ES colonies are morphologically normal (spindle shaped colonies with smooth and shining surface, and borders for individual cells are hard to distinguish).
7. If necessary, split the cells by first washing with PBS, and then digest with 1.5 mL 0.25% trypsin-EDTA for 5 min at 37°C . Add 5 mL ES medium and pipet up and down ~ 10 times to break up colonies into small clumps. We usually make a 1:4 to 1:10 split depending on when the cells will be subsequently used.
8. On the day prior to the scheduled blastocyst microinjection, we trypsinize ES cells for ensuring that majority of cells are in log growing phase.

3.3 Blastocyst Collection

1. Four days prior to the scheduled microinjection, select estrus C57BL/6 female mice and mate with stud males in the afternoon (*see* Note 5). The next morning,

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⁴. Most MEF feeders will attach to gelatin-coated dishes within 2 h after plating, but we have noticed that MEFs from certain mouse strains, such as CF-1, take much longer (~ 6 h for CF-1 MEF) to attach. Under emergency situations, ES cells and MEFs can be plated simultaneously as long as LIF-containing ES medium is used.

check the females for vaginal plugs. Plugged females are group caged until needed for blastocyst collection.

2. On the day of microinjection, the females are euthanized by CO₂ exposure. The abdominal cavity of each mouse is cut open using a pair of scissors, and the entire uterus together with oviducts and ovaries are removed and placed in a 60 mm culture dish containing 3 mL M2 medium.
3. Clean the reproductive organs by dissecting out associated fat, and place the cleaned organs into a new 60 mm dish. Separate the two uterine horns by cutting with a pair of small scissors.
4. Fill a 3 mL disposable plastic syringe (with 25G needle) with M2 medium. Bend the needle to ~45° using a pair of forceps. Under a stereo microscope, insert the needle into the lumen of each uterine horn near the uterine-oviduct junction. Flush each uterine horn with 1 mL medium. Up to 10 uterine horns can be flushed in the same 60 mm dish.
5. After all uterine horns are flushed, gently swirl the dish for 30–60 s to gather blastocysts to the center area of the dish. Pick up blastocysts and morulae (some morulae will develop into usable blastocysts a few hours later) using an embryo transfer pipette and wash them through two drops of M2 medium. Culture the embryos in a M2 drop in the CO₂ incubator until needed for microinjection in the afternoon.

3.4 Blastocyst Injection

1. After finishing collecting blastocysts, trypsinize ES cells and replat them onto a gelatin-coated dish (without MEF). Culture in a CO₂ incubator for 1.5–2 h, which is enough for most feeders to reattach but most ES cells only loosely adhere to the MEFs.
2. Aspirate off the medium with unattached cells and debris. Use a 5 mL serological pipet containing 3 mL injection medium (*see* Note 6), gently rinse off the ES cells adhering to the MEF feeders. Place the collected ES cells into a 15 mL sterile tube and store on ice for the entire duration of microinjection.
3. Add a drop of injection medium into the center of the injection chamber, and immediately cover the drop with mineral oil. Transfer a few microliters of ES cell suspension into the drop (*see* Note 7). Place the injection chamber onto the microscope stage.

⁵A swollen and reddish genital area is a good indication that the female is in estrus. Estrus females can be selected from a pool of breeding age animals. On average, only ~20% of the females are in estrus, but sometimes the females in the same cage are in-sync which makes the selection process more unpredictable. An obvious alternative method is to inject the females with hormones to induce superovulation, which may or may not be better than natural mating depending on the substrains, age, and housing environments.

⁶Normal ES cell medium contains 10 mM HEPES (pH 7.4), but we normally increase its concentration to 25 mM in injection medium, because the injection drop will be out of the CO₂ incubator for an extended period of time. We also lower the FBS concentration from 15% to 10% and omit LIF from the injection medium due to possible deleterious effects of LIF and other serum growth factors on blastocysts. It is imperative to omit G418, which is often present in ES culture medium for maintaining selection, from the injection medium because the recipient blastocysts are usually not neomycin-resistant.

⁷It is important to load the injection chamber with an appropriate density of ES cells. Too few cells make it difficult to find healthy cells for loading the injection needle. Too many cells make it difficult to separate the good ES cells from the bad ones and selectively

4. Attach the holding pipette and blastocyst injection needle into the capillary holders, which are connected to the CellTram vario through Teflon tubes filled with mineral oil (*see* Note 8).
5. Lower the tip of both the holding pipette and injection needle into the drop containing ES cells. Focus the microscope to make sure the ES cells are in focus, and adjust micromanipulators to make sure the holding pipette and injection needle are in the center of the view field and nearly touch the bottom of the injection chamber.
6. Draw healthy looking ES cells into the injection needle by turning the CellTram vario (*see* Note 9 for selecting healthy ES cells).
7. Transfer about 30 blastocysts into the medium drop containing ES cells. Move the tip of the holding pipette to the vicinity of a blastocyst, and turn the CellTram vario to firmly grasp the blastocyst. Use the Eppendorf 4r micromanipulator to align the microinjection needle so that it is in the same straight line and focus plane as the holding pipette and blastocyst (Fig. 2a). Quickly insert to injection needle into the blastocyst, ideally near the boundary of inner cell mass (ICM) and trophoblasts (Fig. 2b). Turn the CellTram vario to slowly expel 5–15 ES cells into the cavity, and then withdraw the needle (Fig. 2c). Move the injected blastocyst to a different area of the medium drop.
8. After all blastocysts are injected, take them out of the injection chamber using a mouth pipette and culture at 37 °C in a CO₂ incubator until uterine transfer (*see* below).

3.5 Uterine Transfer

1. Three days prior to the scheduled blastocyst injection (1 day after mating the blastocyst donor mice), select estrus CD-1 females and set up mating with vasectomized male mice to prepare foster mothers. The next morning, check the mice for vaginal plugs, and house plugged females in groups until needed for uterine transfer (2 days after plug). As a general rule, we prefer to obtain one plugged foster mother for every 3 plugged blastocyst donor mice (*see* Note 10 for timing of embryo transfer and alternative methods).

load the good cells. Also, too many dead cells often lead to sticky needle and make it hard to aspirate and expel cells. If a high proportion of cells are lysed, adding 300 IU of DNase I into the injection drop can help in reducing stickiness. Some people prefer to use a cooling stage to keep the injection chamber at 4 °C for reducing cell death.

⁸For precisely controlling the microinjection needle, it is critical that the entire line, from CellTram vario to injection needle, is completely filled with oil. Any air bubbles inside the line will reduce the precision of moving cells in and out of the injection needle. For holding pipettes, precision is less important and therefore the line can be filled with either oil or air.

⁹Healthy ES cells are small and round, while MEFs are much larger under the microinjection microscope. Healthy undifferentiated ES cells tend to stick to each other forming aggregates. Preferentially loading small aggregates (usually 2–3 cells) into the injection needle provides another level of selection for good ES cells, which should enhance germline transmission later. We usually preload a large number of ES cells into the same needle so many blastocysts can be injected, but some scientists prefer to load just enough cells (usually 10–20) for injecting each blastocyst.

¹⁰Injected blastocysts should be implanted into the uteri of E2.5 pseudopregnant foster mothers on the same day of microinjection. However, if insufficient number of E2.5 surrogate mothers are available, E0.5 and E1.5 mother can also be used but the blastocysts should be transferred into the oviducts instead of uteri. Surgical implantation of injected embryos requires expertise and extensive experience. Transfer without surgery using ParaTechs' Non-Surgical Embryo Transfer (NSET™) Device (<http://www.paratechs.com/nset/>) or transfer tips [21] have also led to acceptable results.

2. After finish blastocyst injection (Subheading 3.4, **step 8**), anesthetize recipient foster mother by injecting (i.p.) Ketamine/Xylazine mixture at 10 $\mu\text{L/g}$ of body weight.
3. Remove hair from the dorsal lumbar area of the back using a small hair clipper. Disinfect the clipped area using alcohol swab followed by Betadine.
4. Use a pair of dissecting scissors making a small dorsal midline incision in the disinfected skin area, and then tear by spreading the back of the scissors to ~1 cm long. Separate skin and body wall using the back of the scissors.
5. Put the animal on its left side, and use iris forceps to move the skin opening to the right ovary/uterus area (the ovary associated fat pad can be seen through the muscle body wall as a slightly paler area). Cut a small incision in the body wall and expand the opening to 0.5–1 cm long with the back of scissors. Avoid cutting major blood vessels.
6. Use a pair of small iris forceps grab the fat pad and gently pull the fat and associated reproductive organs out of abdominal cavity. Hold the fat pad in place with a Dieffenbach clip (Fig. 3c).
7. Load the embryo transfer pipette with some M2 medium and a small air bubble near the tip. Then, suck the mouth piece to draw in 7 injected blastocysts followed by another small air bubble (*see* Fig. 3b). The two air bubbles mark the boundaries of the suspended embryos, which are useful for ensuring successful expelling of all embryos with minimum volume of M2 medium.
8. Under the dissecting microscope, use a 25G needle punching a hole on the uterine wall near the oviduct junction. While using a pair of fine tweezers loosely holding the uterus in place with one hand, use the other hand carefully insert the tip of the transfer pipette into the hole punched by the needle. Gently blow into the mouthpiece until the two air bubbles enter the uterus.
9. Remove the Dieffenbach clip and carefully push the uterus, ovary, and fat pad back into the abdominal cavity. Using absorbable 5–0 Vicryl suture to sew the body wall incision, and drop one drop of 0.25% Bupivacaine solution, which serve as a long-lasting local anesthetic.
10. Turn the mouse on its right side, and move the midline skin opening to the left ovary/uterus area and cut open the muscle body wall as described in **step 5**, and repeat **step 6–9** for transplanting 7 blastocysts into the left uterine horn.
11. Close the skin incision with stainless steel surgical wound clips. Inject (i.p.) the mouse with diluted Meloxicam solution (2–5 mg/kg of body weight) for reducing pain. Place the mouse in a warm cage, which is placed on top of a circulating warm water blanket.
12. After mice awake (usually 30–60 min post-surgery), the cage is returned to the animal room. Wound clips are removed 10–14 days after surgery.

13. Pups are born 16–17 days after the embryo transfer procedure. At postnatal day 5, skin patches with various darkness indicate chimerism. At 7–8 day, the coat color of the pups becomes clear, which indicates the degree of chimerism. The offspring are weaned when they are 19–21 days old.
14. Usually male chimeras with high percentage of coat color derived from ES cells are preferable for mating with wild-type C57BL/6 females for achieving germline transmission. As shown in Fig. 4, pups bear the color of the ES cells are germline pups, which may carry the gene altered in the ES cells.

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Fig. 1.

Outlining of the conventional ES cell-mediated gene knockout procedures. A targeting construct containing a selection marker gene (*neo*) and several Kb of homologous arms is electroporated into a germline-competent ES cell line. After antibiotic selection, ES colonies are picked and grown up in multi-well plates. A duplicate set of plates are used to extract genomic DNA for genotyping, while the original set of plates are cryopreserved. After genotyping, positive ES clones are thawed from the cryopreserved plates and expanded. These ES clones are microinjected into blastocyst stage embryos to generate chimeric mice,

which are used to breed with wild-type mice for passing the ES cell-derived gametes to the next generation

Fig. 2.

Blastocyst microinjection procedures. **(a)** A blastocyst is firmly grabbed by the holding pipette, and the microinjection needle loaded with ES cells is brought to the vicinity of the blastocyst. **(b)** With a sudden and forceful move of the micromanipulator, the needle is inserted into the blastocyst cavity, slowly turn CellTram vario to expel 5–15 ES cells out of the injection needle. **(c)** Slowly withdraw the needle while leaving all or most of injected ES cells inside the blastocoel

Fig. 3. Implantation of blastocysts into the uterus of a surrogate mother. **(a)** A photograph of a typical embryo transfer pipette, which consists of a pulled Pasteur pipet connected with a plastic mouth piece through tubing and a disk filter. **(b)** A magnified view of the tip of the transfer pipette showing embryos sandwiched between two air bubbles. **(c)** An anesthetized surrogate mother with her reproductive organs (including ovary, oviduct, and a segment of the uterine horn) exteriorized through a small incision on the body wall and skin. A Dieffenbach clip is clamped to the fat pad associated with the ovary for holding the organs in

place. Then, the tip of the transfer pipette is inserted into the lumen of the uterine horn through a hole punched by a sterile needle

Fig. 4.

A photograph showing germline pups born to a mating pair (C57BL/6 mother x chimeric father). The male chimera (agouti and black mix color) was produced by injecting ES cells derived from 129 mouse strain into a recipient blastocyst collected from C57BL/6 strain. After breeding the chimera with a C57BL/6 female mouse (black), those pups with agouti color are derived from microinjected ES cells and may carry the targeted gene, while the black pups are derived from the recipient blastocysts