Published in final edited form as:

Curr Protoc Cell Biol. 2009 September ; CHAPTER: Unit-19.1436. doi:10.1002/0471143030.cb1914s44.

Generation of Gene Knockout Mice by ES Cell Microinjection

Glenn Longenecker¹ and Ashok B Kulkarni^{1,2}

¹ Gene Targeting Facility, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA

² Functional Genomics Section, Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA

Abstract

This unit lists and describes protocols used in the production of chimeric mice leading to the generation of gene knockout mice. These protocols include the collection of blastocyst embryos, ES cell injection, and uterine transfer of injected blastocysts. Support protocols in the superovulation of blastocyst donor mice, generation of pseudopregnant recipients, fabrication of glass pipettes, and generation of germline mice are also included. Practical tips and solutions are mentioned to help troubleshoot problems that may occur.

Key Terms

Embryonic stem cells; Blastocyst; Blastocyst injection; Uterine transfer; Chimeric mice; Knockout mice

INTRODUCTION

The generation of knockout mice through the process of creating chimeric mice via embryonic stem (ES) cell injection is a powerful tool for understanding in vivo functions of a particular gene of interest. Knockout mouse models may be used to elucidate the molecular mechanisms underlying human diseases and could aid in the creation of new therapies to treat these diseases.

ES cell injection is the most common method used to generate these chimeras, but morula aggregation is also used. There is a very narrow time window in embryonic development for the ES cell injection procedure to be successful in the generation of chimeric mice. For this reason, all components of the procedure for generating chimeric mice—harvesting, injection, and surgical transfer of the mouse blastocysts—are usually done the same day and require good planning and coordination.

Learning all of the procedures involved requires time and practice. It also requires skills not everyone might possess. How fast and easily these techniques are learned will depend on previous experience, natural abilities, and the desire to learn them.

Note: All animal work performed must be approved by the Animal Care and Use Committee regulations of the institution where the work will be taking place. The animal facility where the mice are held should ideally be pathogen free.

COLLECTION OF BLASTOCYSTS

Embryonic day (E) 3.5 blastocysts can be found free-floating in the uterine horn. To collect these embryos, the uterine horns are generally flushed out with media. Although this is considered a straightforward technique, it does require a certain amount of manual dexterity to perform this procedure. Another important component is the ability to use a mouth pipette in the collection and washing of embryos. Without this ability it would be hard, if not impossible, to collect the mouse embryos. Even though in theory only blastocysts should be obtained from this procedure, it is not uncommon to obtain earlier stage embryos as well. Of these earlier stage embryos, morulas should be kept in culture along with blastocysts. Depending on the stage of development, the morula may become blastocysts in culture and can be used for ES cell injections if needed. When flushing out the blastocysts, a zoom setting of 1.2 is suggested, since it will allow a viewing of the uterine horn all the way to the opening of the cervix. For washing blastocysts, a zoom setting of 3.2 is recommended in that it offers enough magnification to discern the blastocyst well, but still allows for a relatively large viewing area to see the embryos on the dish.

Materials

- 10 C57BL/6NCr 3.5-day postcoitum (p.c.) female mice (see Support Protocol 1)
- 70% alcohol
- Alcohol pads
- 30 ml of injection media (see Reagents and Solutions)
- Filtered light white mineral oil (Sigma M-3516 embryo-tested or equivalent)
- Needle, 30-ga, 1/2-in. (Becton Dickson #305106 or equivalent)
- Tuberculin syringe (3 cc)
- 3-cm sterile tissue culture dish (Falcon 3001 or equivalent)
- Transfer pipette (see Support Protocol 3)
- Surgical instruments (cleaned and sterilized with alcohol pads)
 - Dissection scissors (Roboz RS-5880 or equivalent)
 - #55 forceps (Roboz RS-5063 or equivalent)
- Dissecting microscope (10×/23 eye pieces, 1.0 x objective, 0.6 to 6.6× zoom, with base illumination)
- Microdrop culture (see Reagents and Solutions)
- Bench paper (absorbent material with plastic backing)
- Mouth pipette assembly
 - Mouthpiece (Fisher Scientific #13-647-10 or equivalent)
 - Saliva trap (cotton plug from a 1-mm pipette)
 - Syringe filter, 0.22- μ m (Gelman # 4602 or equivalent)
 - Pipette insert and reservoir (Drummond microcaps #1-000-0300 or equivalent). A modified 1000- μ l pipette tip can be used as a reservoir.
 - Approximately 23 in. of natural latex tubing (I.D., 1/8 in.; wall, 1/32 in.)

Male luer, 5/32-in. (Ark-Plas Products #10-12ML016N or equivalent) (connects female end of syringe filter to rubber tubing)

Female luer, 5/32-in. (Ark-Plas Products #10-15FL016N or equivalent) (connects male end of syringe filter to rubber tubing)

Harvesting uterine horns

1. Take out injection medium from the refrigerator and allow it to warm to room temperature.
2. Place bench paper on the area where the mice will be euthanized.
3. Pour injection media into a 3-cm tissue culture dish and also load injection media into a 3-cc syringe and insert a 30-ga needle onto it. Tap the syringe to allow bubbles to travel to the plunger end.
4. Swab a pair of #55 forceps and dissection scissors with 70% alcohol pads and allow to dry.
5. Euthanize each mouse by cervical dislocation.

Note: It is suggested that all 10 blastocyst donor females be used even if a plug is not seen in some of them. These females could potentially be plugged even though a copulation plug was not seen. Failure to use all of the mice could potentially decrease the embryo yield.

6. Place each mouse on its back, and orient it such that the head and tail are in the 3 o'clock and 9 o'clock positions, respectively. With a squeeze bottle filled with 70% ethanol, spray the abdomen of each mouse.
7. Gently, with the fur placed between the index and thumb of each hand, pull the skin apart toward the head and tail of the mouse.
8. With a pair of #55 forceps and scissors, grasp the peritoneal abdominal muscle wall and make a vertical incision. Move the intestines to the anterior side away from the ovaries.
9. Next, using the #55 forceps, grasp the white fat pad of the ovary and snip the ligament latum attached to the uteri and the kidney. Carefully cut away the fat and blood vessel structure (mesometrium) running along the uterine horn and cut at the cervix. Avoid cutting the side of the uterine horn.
10. Repeat step 9 on the other horn and cut at the oviduct and place the uterus into the dish containing the injection medium.
11. Repeat steps 5 through 9 for all mice.

Flushing uterine horns

12. After all the uteri have been harvested, take the uteri out of the dish one at a time and place in another 3-cm dish that has no injection media in it.
13. On the uterine horn without the ovary attached, straddle the uterine horn with forceps, holding the outer edges.
14. Insert the 30-ga needle with 3-cc syringe into the tip of the uterine horn inside the lumen. Inject medium through the cervix while holding the uterine horn down onto the dish until it is flushed well with media.

Note: If the needle does not move freely, and the uterine wall expands, the needle is not inside the lumen. A good sign that the uterine horn is being flushed out well is that the medium can

be seen coming out of the cervix and the uterine horn expands, showing the rib-like structures of the uterine wall.

15. Repeat step 14 on the other uterine horn with the ovary still attached.

16. Repeat steps 13 and 14 for all mice.

Washing and collecting blastocysts

17. When all flushing is complete, with a polished transfer pipette attached to a mouth pipette, collect embryos on the dish while avoiding the collection of any debris in the process.

18. Transfer embryos into a second 3-cm dish containing injection media until all the embryos are mostly free of any debris or other tissue.

19. Repeat step 18 again on a third 3 cm dish containing injection media.

20. Transfer the washed embryos with the transfer pipette into a drop of injection media (30 μ l) under light mineral oil on a 3-cm plate, and place this in an incubator until ready to inject. Prepare ahead of time additional media/oil overlay plates for injected blastocysts.

Note: When flushing out through the cervix, orient the cervix opening so that it is to the side and slightly up. This allows one to see the media being flushed out and help prevent media from squirting out of the dish. Transfer pipettes used for washing the embryos should be a little larger to allow for capillary action but not so big that there is no control in the mouth pipetting of embryos.

ES CELL INJECTION OF BLASTOCYSTS

When injecting blastocysts, the optimal type of blastocyst to inject would be between the middle and late stages, with a good-sized blastocoel cavity. Larger, more developed blastocysts tend to reexpand faster after injection but are more susceptible to collapse prior to injection. Trying to inject any hatched blastocysts can be difficult because they lack rigidity from the loss of the zona and have a very sticky outer surface, so they should be avoided. See Figure 3. The morphology of ES cells to inject should be round and smooth and appear opaque in nature. It is not uncommon to have small, medium, and large cell sizes. Medium-sized cells would be the preferred choice in that they will likely give a higher chance for germline transmission, and they are less likely to be differentiated. Avoid the largest cells because they are mouse embryonic fibroblasts (MEFs). See Figure 2. Morula injections could be used instead of blastocyst injections to increase the success of generating chimeras and have higher germline efficiency. The authors have found this not to be necessary. A circulating pump may be used during the cooling stage in place of the dish to cool the ES cells and blastocysts. A suggested microinjection setup is listed in the Materials section. See Figure 9.

Materials

Blastocysts (see Basic Protocol 1)

30 ml of injection media (see Reagents and Solutions)

Filtered light white mineral oil (Sigma M-3516 embryo-tested or equivalent)

DPBS

ES cell suspension (1×10^6 /ml)

Holding pipette (see Support Protocol 3)

Injection pipette (see Support Protocol 3)

Dissecting microscope (10×/23 eye pieces, 1.0 x objective, 0.6 to 6.6× zoom, with base illumination)

Microinjection setup (see Reagents and Solutions)

Injection apparatus

Inverted microscope (Zeiss 135M or equivalent)

Course micromanipulators (microscope mounted Narishige MMN-1 or equivalent)

Fine micromanipulators (Joy stick micromanipulator Narishige #MO-202U or equivalent)

Mounting bracket for mounting Narishige course manipulators

Video camera (Dage-MTI CCD-72 or equivalent)

Video monitor (Sony PVM-137 or equivalent)

Injection line

Instrument holder- to hold injection pipette (Leica #520145 or equivalent)

Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × approximately 4-mm long Ruber tubing insert for instrument holder to connect injection pipette to PE 60 tubing

Polyethylene tubing for injection line (Becton Dickson PE 60 #427415 I.D. .030 in. × O.D. .048 in. × approximate length 2 ft. or equivalent)

Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × approximately 8 mm long or equivalent to connect between Tygon tubing AAC00002 and PE 60 tubing

Tygon tubing AAC00002: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × approximately 1 cm long or equivalent to connect Tygon AAC00001 tubing to PE 200 tubing

Polyethylene tubing for injection line (Becton Dickson PE 200 Clay Adams #427440: I.D. 0.055 in. × O.D. 0.075 in. or equivalent) approximately 3.5 cm. in length

Female luer flare type adapter (Popper # 6193 or equivalent) to connect PE 200 tubing to 3-way stopcock

3-way stopcock (Baxter #K177A or equivalent)

Tygon tubing (Cole Parmer # 6408-63: I.D. 3/32 in. × O.D. 5/32 in. × wall 1/32 in. or equivalent) for injection line between the stopcock and to either the syringe reservoir or the Hamilton syringe

Female luer with barb (Bio Rad # 7318223 or equivalent) to connect Hamilton syringes to Tygon tubing

Male luer with barb (Bio Rad # 7318226 or equivalent) to connect to luer lock connection of stopcock to Tygon tubing

Micrometer syringe (Minnetonka instruments or equivalent) with Mitutoyo syringe 0–50 mm with gas-tight syringe 50 µl (Hamilton #80920 or equivalent for injection line)

Female luer connection with barb (Cole Parmer #31507-65 or equivalent) to connect Cole Parmer 6408-63 to tuberculin syringe reservoir

Tuberculin syringe (12 cc) containing Dow Corning silicone oil 200 for reservoir

Holding line

Instrument holder- to hold holding pipette (Leica #520145 or equivalent)

Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × approximately 4 mm long or equivalent rubber tubing insert for instrument holder to connect holding pipette to PE 60 tubing

Polyethylene tubing for holding line (Becton Dickson PE 100 Clay Adams #427425: I.D. 0.034 in. × O.D. 0.06 in. × approximate length 2 ft. or equivalent)

Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × approximately 1.8 cm long or equivalent to connect between PE 100 tubing and blunted 19-ga needle

Blunted 19-ga needle to connect male luer end of stopcock to Tygon AAC00001 tubing

3-way stopcock (Baxter #K177A or equivalent)

Tygon tubing (Cole Parmer # 6408-63: I.D. 3/32 in. × O.D. 5/32 in. × wall 1/32 in. or equivalent) for injection line from the stopcock to either the syringe reservoir or the Hamilton syringe

Female luer with barb (Bio Rad # 7318223 or equivalent) to connect Hamilton syringe to Tygon tubing

Male luer with barb (Bio Rad # 7318226 or equivalent) to connect luer lock connection of stopcock to Tygon tubing

Micrometer syringe (Minnetonka instruments or equivalent) with Mitutoyo syringe 0–50 mm with gas-tight syringe 250 µl (Hamilton #81120 for holding line)

Female luer connection with barb (Cole Parmer #31507-65 or equivalent) to connect Cole Parmer 6408-63 to tuberculin syringe reservoir

Tuberculin syringe (12 cc) containing silicone oil 200 for reservoir

Cooling system

Water circulating pumps (#8329 Baxter or equivalent)

Siliconized tubing I.D. 3/16 in. tubing with plastic connections

Male luers with 1/16 in. barb (Cole Parmer # 31507-62 or equivalent) to connect plastic connections of silicone tubing to inflow and outflow

Tygon tubing

Injection dish (see Figure 4)

Tygon tubing for inflow and outflow cooling lines I.D. 3/32 in. × O.D. 5/32 in. × wall 1/32 in.

Tuberculin syringe (60 cc) with Tygon tubing to load water jacket of injection dish

Female luer with 3/16 in. to 1/4 in. barb (Cole Parmer # 31507-62 or equivalent) connected to inflow tubing as a weight in ice water

Ice bucket containing ice and water

Air suspension platform setup

Air suspension table (Kinetic Systems 9101-02-46 or equivalent)

Gas regulator (Curtis Mathis 1H-580 or equivalent)

Nitrogen gas cylinder (Roberts Oxygen # R-31 or equivalent)

Checking microinjection apparatus

The microinjection system should be functioning properly before proceeding with injection of ES cells into blastocysts. Address all problems before starting the actual microinjection procedure. Both injection and holding systems should be in working order before attempting ES cell injections. See Critical Parameters and Troubleshooting for details on problems encountered when injecting blastocysts.

1. In a 6-cm tissue culture dish, add 1× DPBS, and check the condition of both the injection and holding pipettes. They should be in good condition and not clogged or broken, with no debris sticking to the inside. The uptake of DPBS into both pipettes should be a smooth and fluid motion.
2. If using a silicone- or mineral oil-based system, check to see that there are no bubbles or leaks present in either the injection or holding line systems. If they are present, severe control problems can occur.

Preparation of injection dish

For a cooling system, we use a glass injection chamber with an internal hollow opening around the injection chamber to allow for chilled (4 °C) water to circulate. See Figure 5. There are 2 reasons to use some sort of cooling apparatus. The first is to delay the reformation of surface proteins after trypsinizing the cells so they do not become sticky. Secondly, cooling aids in the rigidity of the blastocysts. The injection dish should be free of alcohol as this can cause differentiation of the ES cells.

3. Swab the injection chamber area thoroughly with a 70% alcohol pad, and wash liberally with 1× DPBS.
4. Add a very light film of liquid soap under the bottom of the injection dish to prevent water droplets from forming during the cooling process.
5. Attach inflow tubing from the H₂O circulating pump to the input opening of the injection dish.
6. Next, insert outflow tubing into the output opening of the injection dish, which has been placed in a chilled H₂O reservoir, and then start the circulating pump.
7. Pipette injection media onto the well of the injection dish and add 30 µl of the ES cell suspension.
8. Overlay with light mineral oil.

Note: The authors recommend a cell suspension of 1×10^6 . This is more than enough cells for injection purposes. If too many cells are added to the injection dish, the cells tend to become sticky. A high ratio of ES cells to feeder cells is best for injections. Having some feeder cells can be a benefit in that they can be used to clear debris from inside the injection pipette.

Transferring embryos to microinjection dish

Blastocysts are more tolerant to pH and temperature changes than that of other earlier stage embryos such as zygotes. Cooling the temperature will delay the onset of blastocyst hatching. Keeping blastocysts in the cold injection chamber too long might be detrimental to their vitality.

9. Take the dish containing the blastocysts with mineral oil and injection media out of the incubator.
10. With a clean and polished transfer pipette, mouth pipette light mineral oil to the hub of the transfer pipette.
11. Load the blastocysts inside the transfer pipette from the dish containing the blastocysts.
12. Take the transfer pipette and gently expel the embryos onto the chamber of the injection dish, while clearing an area for injections in the cell suspension. Only transfer the number of blastocysts to the injection dish that can be injected within a half hour.

Injection of ES cells

When first learning ES cell injections, 200 $\times$ magnification should be used. Focus on the outer ring of trophoblast cells and then the tip of the injection pipette before attempting to inject inside the blastocoel cavity. This ensures that the injection pipette is in the correct plane of the z-axis, being approximately in the center of the blastocoel cavity. After becoming proficient in ES cell injections, a tapping technique, which looks at indentations on the blastocyst, can be employed to make sure the injection pipette is in the right plane. 100 $\times$ will work well with this procedure and has the advantage of a broad field of view to collect the ES cells, and changing the objective is not required. The general range for the number of ES cells that can be injected inside a blastocyst is considered to be between 10 and 20. The authors' preference is to inject 15 ES cells, which balances the chance of germline transmission versus normal homeostasis in embryonic development. It is advisable not to inject more than 20 ES cells in that it is thought to be detrimental to the developing mouse embryo.

13. Transfer blastocysts to the bottom of the inner chamber on the injection dish, and then lower the holding and injection pipettes.
14. With the injection micrometer syringe, uptake a small amount of injection media inside the injection pipette. This will allow a buffer area between the cells and the oil and help prevent injection of oil inside the blastocoel cavity.
15. Load 15 ES cells inside the injection pipette with as little injection media as possible.

Note: The cells do not have to be touching each other when inside the injection pipette. If the cells are touching, there is a possibility that the cells may stick to each other, and many of the cells may come out of the blastocyst when withdrawing the injection pipette.

16. Orient the blastocyst so that the inner cell mass (ICM) is in the 6 o'clock position. The bevel of the injection needle should be pointed down.

Note: The blastocyst can be oriented with the injection pipette by moving the joystick in either a clockwise or counterclockwise motion. Using the holding pipette to orient the blastocyst will disperse the cells away from the injection area and also other blastocysts.

17. With the holding micrometer syringe, uptake injection media inside until the blastocyst is held securely to the holding pipette. Lower the holding pipette until the blastocyst is just touching the bottom of the injection dish.
18. Focus on the zona and outer trophoblast layers of the blastocyst, and then move the injection pipette so it is also in focus. This puts the injection pipette in the correct plane of the z-axis.

Note: It is better to inject at the junction between the trophoblast cells, reducing damage to the embryo. Sometimes this junction may be hard to see.

19. With a quick, short popping and jabbing motion, insert the injection pipette inside the blastocyst cavity, being careful not to touch the ICM. Pull back the injection pipette slightly after inserting into the blastocoel cavity.

Note: Touching the ICM is considered harmful to the blastocyst. If the injection pipette is inserted too slowly, the blastocyst will likely collapse, making injection difficult. If the pipette is only partially inside the blastocoel cavity and the blastocyst collapses, media can be injected to reexpand the blastocyst, and another attempt to inject the blastocyst can be tried. Otherwise, move the blastocyst to the side and perform injections on another.

20. Gently expel the ES cells inside the blastocoel cavity onto the ICM, being careful not to inject oil into the blastocoel cavity.

21. Withdraw the injection pipette slowly out of the blastocoel cavity, preventing the expulsion of the ES cells outside of the blastocyst. Allow the injection site to close before fully withdrawing the injection pipette. Letting some media escape will enhance the settling of the injected ES cells onto the ICM.

Note: When injecting, consider the pressure inside the blastocoel cavity. If too much media is injected inside the blastocyst or too much holding force is applied by the holding pipette, the injected ES cells may be forced out of the blastocoel cavity.

22. After injection of the blastocyst, move it to an area away from the uninjected ones on the injection dish.

23. When all blastocysts on the injection dish have been injected, with a transfer pipette place the injected blastocysts on a microdrop dish with only media and oil and put back into the incubator.

24. Allow the blastocysts to reexpand in culture. Reexpansion of the blastocyst is a possible sign of its vitality.

Note: All blastocysts should be injected within a half hour of putting them on the injection dish, and the ES cell clones should be injected within 2 hours after putting them on the injection dish. If both pipettes are flushed out with oil, they have the potential to be reused for another injection day. Some people use a DNase solution on top of the oil overlay to minimize stickiness due to cells and other debris.

UTERINE TRANSFER OF INJECTED BLASTOCYSTS

Surgical transfers have to be performed the same day as that of ES cell injections, and E2.5-day-old pseudopregnant recipient females are generally used to allow for the blastocyst to catch up developmentally prior to implantation at E4.5–5.0 days. If E2.5 recipients are unavailable, the authors have had success with E1.5 and E2.0 recipients with this procedure. Pups are generally born 18 to 21 days p.c. of the pseudopregnant recipient. It should be noted that blue beads could be used as an alternative to bubbles when loading the transfer pipettes with embryos. The authors recommend a zoom setting of 3.2 for the surgical microscope, which will allow a large enough field of view of the uterine horn and still be able to see the hole in the uterine horn wall.

Materials

- 2.5-day p.c. pseudopregnant B6D2F1 female mice or equivalent (see Support Protocol 2)
- Injected blastocysts (see Basic Protocol 2)

30 ml of injection media (see Reagents and Solutions)

Filtered light white mineral oil (Sigma M-3516 embryo tested or equivalent)

Transfer pipettes (see Support Protocol 3)

Plasticene (Fisher Scientific # P148-1LB or equivalent)

10-cm tissue culture dish (Falcon 3010 or equivalent)

Alcohol swabs

Betadine swabs

2.5% Avertin working solution (see Reagents and Solutions)

Sterile cotton swabs

25-ga, 5/8 in. needle

1-cc tuberculin syringe

0.5% Marcaine or 0.5% Naropin analgesic

Sterile gauze, 2 × 2

Sterile gauze, 4 × 4

Surgical instruments (cleaned and sterilized by placing in glass bead sterilizer for 10 seconds prior to use in surgery)

- Dissection scissors (Roboz RS-5880 or equivalent)
- #7 curved forceps (Roboz RS-5064 or equivalent)
- Foerster forceps, straight tips (Roboz RS-5065 or equivalent)
- Dieffenbach clamp (Roboz RS 7422 or equivalent)
- Wound clip removing forceps (Roboz RS-9268 or equivalent)
- Wound clip applicator Reflex 9 (Roboz RS-9260 or equivalent)
- 9-mm wound clips (Roboz 9262 or equivalent)

Dissecting microscope (10×/23 eye pieces, 1.0x objective, 0.6–6.6× zoom, with base illumination)

Surgical microscope (16×/16 eye pieces, 0.63x objective, 0.8–6.6× zoom) with Diagnostics SMS6B sliding zoom stand or equivalent)

Fiber optic light source (Zeiss KL-1500 or equivalent)

Surgical stand (6 1/8 in. × 7 1/2 in. Nalgene container top with vinyl bumpers attached on bottom or equivalent)

Glass bead sterilizer (Fine Science Tools FTS 250 or equivalent)

Slide warmer (Temperature setting 37 °C)

Mouse fur clippers (Oster finisher trimmer #76059-030 or equivalent)

Mouth pipette assembly (see Basic Protocol 1)

Loading transfer pipettes with injected blastocysts

1. Take an already made transfer pipette and the plate containing the embryo, oil, and media oil overlay and load mineral oil to the hub by mouth pipette.

2. On the inside of the top section of a 3-cm tissue culture dish add a small amount of injection media to the dish.
3. Under low-power magnification, (a zoom control knob setting of 1.2 with 10× eye pieces and 1× objective), place the transfer pipette into the media and then onto the area that does not contain media, and then gently take up the media forming an air gap.
4. Immediately place the transfer pipette into the drop of media while keeping the air gap to form a bubble.
5. Repeat steps 3 and 4 to form a second air bubble.
6. Under high power (a zoom control knob setting of 6.6 with 10× eye pieces and 1× objective), take the plate containing media with injected blastocysts under oil and place the transfer pipette into the media. Take up the media until the last bubble is just out of the field of view.

Note: If an oil droplet forms on the outside of the transfer pipette upon placing it into the drop of media, gently shake the transfer pipette back and forth until the drop of oil comes off.

7. Next, uptake the injected blastocysts slowly one at a time. Allow as little amount of media as possible until all embryos are loaded into the transfer pipette.
8. Make sure the blastocysts are stabilized inside the transfer pipette so that the embryos do not move. If they do move up the transfer pipette, repeat steps 1 through 7.
9. Store transfer pipettes with loaded embryos in a 10-cm tissue culture dish containing Plasticene just prior to surgery.

Surgical transfer of embryos

10. Swab the abdomen with an alcohol pad and anesthetize the mouse by intraperitoneal injection with 2.5% Avertin, 0.015–0.017 ml/g body weight.
11. When the mouse is fully anesthetized, prepare the animal by shaving the surgical area.
12. With a 70% alcohol swab, wipe away all loose fur, and then swab the skin with a betadine swab. Repeat again with another alcohol and betadine swab in the same order.
13. Next make a 5-mm incision in the skin approximately 1 cm away from the thigh area of the hind limb on the back of the mouse, holding the skin with a #7 curved forceps.
14. Once an incision has been made through the skin, grasp the underlying muscle with a pair of Foerster forceps, and cut through until reaching the peritoneal cavity.
15. Look for the fat pad and the ovary and using Foerster forceps, gently pull out the ovary by the fat pad.

Note: The ovary and fat pad may be seen through the dorsal muscle if there is a low amount of fat on the mouse. The ovary will have a bright red color due to the formation of the corpus luteum. Do not use mice that lack a corpus luteum on the ovary. This would indicate that the mouse is not pseudopregnant. The ovarian fat pad will appear bright white in color with a smooth texture. Avoid the rough-looking, beige tissue that is attached to the spleen, which is most likely the pancreas.

16. Grab the fat pad with a Dieffenbach clamp and gently lay it over the other side of the back onto the 2 × 2 sterile gauze. The mesometrium structure should be on the bottom of the field of view.

17. Orient the mouse in such a way that the ovary and fat pad are in the 9 o'clock position. Gently grasp the uterus with Foerster forceps, and with a 25-ga needle attached to a 1-cc syringe make a hole in the uterus under the forceps into the lumen.

Note: If the needle is inside the lumen, it should move freely with no resistance.

18. Insert the transfer pipette, loaded with injected blastocysts, inside the hole in the uterus just enough so the transfer pipette is inside the hole. Gently blow the embryos inside the uterus by mouth pipette, using the bubbles as a marker to see if the blastocysts are inside the lumen.

Note: If the bubbles inside the transfer pipette do not move when blowing them into the lumen of the uterus, the transfer pipette maybe touching the inside uterine wall. Slightly pull back the transfer pipette and try again. If this does not work, take out the transfer pipette and place in a dish containing a small amount of injection media under the dissecting microscope. If a blood clot is seen, dislodge it and reload the transfer pipette again with the injected blastocysts. Repeat step 18.

19. Under an embryo dissecting microscope, check to see if there are any embryos left in the transfer pipette. Repeat step 18 if blastocysts are left inside the transfer pipette

Note: The hole for the transfer pipette should be made near the tip of the uterine horn just as the taper from utero-tubal junction ends and becomes full size. This will allow for a higher rate of implantation than transferring near the cervix. There is also the added advantage of being easier than transferring at the very tip of the uterine horn especially since there is a much bigger lumen to put the 25-gauge needle and transfer pipette into.

20. Release the Dieffenbach clamp, and hold the fat pad with the Foerster forceps. Place the uterus and ovary back inside the peritoneal cavity. Do this while holding the incision open by the skin with the curved #7 forceps.

21. Gently swab the lateral dorsal muscle incision with a sterile cotton swab saturated with 0.5% Marcaine or 0.5% Naropin analgesic.

22. Hold together the skin incision with a #7 curved forceps, and close the wound with a 9-mm wound clip.

23. Place the mouse on a 37 °C slide warmer that has gauze placed on top of it. Allow the mouse to stay on the slide warmer until the mouse wakes up.

24. Place the mouse in a cage for further recovery. When the mouse appears fully recovered from the Avertin, transfer it back into the animal rack.

Note: Enough embryos should be transferred to allow the embryos to be born healthy. If too few embryos are born, the pups will tend to be large in size, causing trauma to the pups and mother, as well as decreased lactation. If too many pups are transferred, the pups could be severely runted and die. Based on the authors' experience using B6D2F1 and B6CBAF1 recipient mice, only 6 mice will be implanted per uterine horn; none of the blastocysts will migrate to the other uterine horn. At least 5 embryos should be transferred into each uterine horn with a maximum of 12 embryos transferred if transferring into both horns. If new to this surgical procedure, allow for a greater number of embryos when transferring to allow for enough embryos to implant.

SUPEROVULATION OF EMBRYO DONOR FEMALES

C57BL/6 females at 3 weeks of age are considered to be the best choice for superovulation in the production of blastocysts. If not available, then 4-week-old females would be the next best choice. Mice 5 to 6 weeks of age or older are not considered the best choice for superovulation

because of the fact that the females would be producing these comparable hormones themselves. If using C57BL/6 females at 5 to 6 weeks of age, you should not use hormones for superovulation and let the mice naturally ovulate instead. An advantage of superovulation is that fewer mice are needed to produce the number of blastocysts required for ES cell injections. The drawback with superovulation is the tendency to produce abnormal embryos. However, once at the blastocyst stage, most abnormal or defective embryos should have died or have stopped at an earlier developmental time point. C57BL/6 blastocysts are generally considered the standard in blastocyst injection to generate chimeric mice. However, other groups have used different strains for this purpose. The strain of mice chosen would depend on the ES cell line being used.

One hormone that we use in our lab is pregnant mare serum gonadotropin (PMSG), which is a follicle-stimulating hormone (FSH) involved with the formation of the egg follicles. Another hormone we use is human chorionic gonadotropin (HCG), which is a luteinizing hormone (LH) that releases the formed eggs and also acts as an attractant to the stud male. Ten C57BL/6 females are used for each injection day, and 1 female is mated to each stud male. C57BL/6 males are only used once per week to ensure plugging and fertility. A light/dark cycle of 14 hours of light 10 hours of dark is a good choice for the rooms where the animals will be held. It is thought to be more conducive for mating.

Materials

10 C57BL/6NCR females or equivalent strain (3 to 4 weeks of age) per injection day

10 C57BL/6NCR males (7 weeks up to 10 months of age)

PMSG 5 IU per ml (see Reagents and Solutions)

HCG 5 IU per ml (see Reagents and Solutions)

Needle 30-ga, 1/2 in. (Becton Dickson #305106 or equivalent)

Tuberculin syringe (1 cc)

1. 6 days before the planned day of blastocyst injections, intraperitoneally inject 0.1 cc of PMSG at 1300 hours into each C57BL/6NCR female mouse.
2. 2 days (47 hours) later intraperitoneally inject 0.1 cc of HCG at 1200 hours into each C57BL/6NCR female mouse.
3. Mate C57BL/6NCR females with C57BL/6NCR males soon after all females are injected with HCG.
4. The next morning, before 0900 hours, check females for copulation plugs.

Note: Actual times may vary depending on your animal facility and other variables. The plugging by C57BL/6 males becomes variable after 10 months of age with a reduced number of blastocysts and a greater number of earlier stage embryos. At about 11 months to 12 months of age, mice that are superovulated may produce only zygote-stage embryos at E3.5 p.c. At one year of age the C57BL/6 males will become sterile. The C57BL/6 stud males are replaced at 9 to 10 months of age.

GENERATION OF PSEUDOPREGNANT RECIPIENT FEMALES

Various strains of mice can be used for the purpose of pseudopregnant foster mothers. Outbred mice tend to be the best mothers, and 2 notable examples are the Swiss Webster and CD1 strains. C57BL/6 hybrid strains are another alternative, as well as B6D2 and B6CBA. The use of inbred strains is generally not recommended, but if it is unavoidable, the FVB/N strain is

the best choice. The strain of mouse chosen should be proven to have good mothers that take care of their offspring.

Vasectomized males of just about any strain could be used as long as they are known for their ability to plug females. Balb/c males seem to be the most commonly used for this purpose. Using strains of a different coat color can be useful to detect leakiness of the vasectomized male, allowing one to distinguish truly chimeric offspring from those of the leaky males.

Even if the vasectomized mice are from a reliable supplier, the males should be tested for sterility. Before the vasectomized males become too old and lose their plugging ability, new ones should be ordered and tested prior to use. The males could be mated for a 2-month period with females, or if time is a factor, there is the option of superovulating the females with PMSG and HCG.

Materials

50 B6D2F1 (C57Bl/6NCr $\times$ DBA/2NCr) females or equivalent strain (10 to 12 weeks of age)

25 Balb/cAnNCr vasectomized males or equivalent strain (at least 6 to 7 weeks to 18 months of age)

1. Mate 1 female to one vasectomized male 4 days prior to the harvest date for collection and injection of blastocysts.
2. The next morning, before 0900 hours, check the mated females for copulation plugs. The plugged females should be separated into another cage and marked with the date they were plugged.

Note: The authors initially mate 25 females 4 days before the start of ES cell injections. An additional 25 females are used to replace any plugged females. Unplugged females are kept with the males until 2 days prior to the last injection day for the week. Any unused females will then be separated out from the males and then can be reused for future matings. A period of at least 5 days should elapse before the mice are mated again. This will allow the mice to reestablish the estrus cycle.

Note: The time window from when the female is plugged by the male to when the plug can no longer be seen is thought to be 12 hours. If needed, females can be mated with the males in the morning to generate pseudopregnant recipients in the afternoon.

FABRICATION OF GLASS PIPETTES

Some of the pipettes used in the making of chimeric mice require a lot more skill and practice than others. The pipette that is the most difficult to make is the injection pipette followed by the transfer pipette and then the holding pipette. Three variables that will affect the quality of pipettes produced are the melting of the glass tubing, the length of time the glass tubing is pulled, and how much force is applied to the glass tubing during the pull. When first learning to pull the transfer pipette under a microflame, these variables are harder to control. Experience is required to control these variables in being able to pull a good transfer pipette. For the pipette puller that the authors use in the lab, time and force of the glass tubing is controlled by the solenoid, and temperature is set by the heater control knob. How fast the ends of the pipettes are melted by the glass bead on the microforge will depend upon how close the pipette is to the glass bead, the heat level setting of the filament, and the size of the glass bead.

Both injection and holding pipettes can be purchased from vendors, but the expense of these premade pipettes can be costly if a lot of them will be used. After the initial expenditure of

buying the equipment, such as a microforge and pipette puller, the real expense is that of the glass tubing.

Materials

Pipette puller (Kopf 1720 vertical pipette puller with nichrome heater coil or equivalent)

Microforge (Technical Products International Defonbrune style microforge with 10× eye pieces and reticle, 4× and 10× objectives or equivalent)

Instrument holder to hold pipettes (Leica #520145 or equivalent)

C-flex tubing: I.D. 1/32 in. × O.D. 3/32 in. × wall 1/32 in. × approximately 4 mm long (Cole Parmer #6424-60 or equivalent) to hold pipettes inside instrument holder

Microflame assembly (For pulling transfer pipettes and polishing ends to pipettes) rectangular support stand plus rod (PGC Scientific #78-9400-12 or equivalent)

Alumaloy tri-grip utility buret clamps with micro clamp holder (PGC Scientific #78-9400-12 or equivalent)

9-in. pasteur pipette shortened to 7 in.

Tygon tubing Formulation R-3603: I.D. 1/4 in. × O.D. 3/8 in. × wall 1/16 in.

Glass tubing (Drummond Scientific #N-51A: I.D. 0.8 mm × O.D. 1.0 mm × length 150 mm or equivalent)

Plasticene (Fisher Scientific # P148-1LB or equivalent)

1-cc tuberculin syringe

Needle 30-ga, 1/2 in. (Becton Dickson #305106 or equivalent)

Top of 10-cc tissue culture dish (Falcon 3010 tissue culture plate or equivalent)

1.25% Tween 80 solution (see Reagents and Solutions)

15-cm tissue culture dish (Falcon 3015 tissue culture plate or equivalent)

Diamond pencil

Dissecting microscope (10×/23 eye pieces, 0.6–6.6× zoom, with base illumination)

Syringe pipette assembly (To coat injection pipettes)

Tuberculin syringe (12 cc)

Female luer 1/8 in. (Ark-Plas Products #10-15FL012N or equivalent) to connect syringe with tubing

Pipette insert and reservoir (Drummond microcaps #1-000-0300 or equivalent) A modified 1000-μl pipette tip may also be used as a reservoir.

Natural latex tubing (I.D. 1/8 in. × wall 1/32 in. × approximately 8 in. long)

Transfer pipettes

1. While holding a piece of glass tubing in each hand, place this tubing into a microflame.
2. Leave the glass tubing in the upper part of the flame until it becomes soft and can be easily moved up and down.
3. Quickly, take the glass tubing out of the flame, and pull it apart in one continuous motion.
4. Position the glass tubing between the thumbnail and forefinger and break apart.

Note: The pipettes can be broken right after the pipettes have been pulled and allowed to cool for a few seconds. A diamond pencil can be used to score the glass first before breaking it. Another alternative is to break the pipettes between the thumbnail and forefinger.

5. Look at the pulled pipette to see if it is the right size.

Note: Viewing the pulled transfer pipette takes practice. The only way to gauge the size of the transfer needle is to use a microforge that has a reticle in the eyepiece.

6. Insert a previously pulled transfer pipette into a pipette holder on a microforge.
7. Orient the transfer pipette so that the one can see it in the microforge optics.
8. Under 40 $\times$ magnification, adjust the focusing knob so that the outer diameter of the transfer pipette is in focus.
9. Move the filament so that the outer diameter of the glass bead is also in focus, especially the area that will touch the transfer pipette.
10. Turn on the power for the filament that will heat the glass bead, and touch an area near the tip of the pipette.

Note: Avoid any areas that are chipped, broken, or cracked.

11. When the holding pipette has melted enough, turn off the power to the filament and glass bead and allow the glass bead to contract, breaking the transfer pipette.
12. Repeat steps 10 and 11 if the pipette becomes distorted and there is not a clean break.
13. Turn on the power to the filament to reheat the glass bead, and bring the glass bead next to the opening of the transfer pipette. Polish the opening, but not so much that the polishing actually narrows the opening of the transfer pipette.
14. If needed, shorten the nontip side of the transfer needle with a diamond pencil by scoring the glass tubing and breaking at the score.
15. Store in a 15-cm tissue culture dish containing Plasticene to hold the pipettes.

Note: The inner diameter size of the transfer pipette should be big enough so the fully expanded blastocysts are not constricted, which could possibly damage them. A pipette too small is more susceptible to blockage by blood clotting. The pipette should also not be too big that the blastocysts are bunched up together side by side instead of being lined up in a row. A general range for the inner diameter would be approximately 8 to 10 units. If using a reticle to make different types of pipettes for ES cell injection, the measurement scale for the reticle at 40 $\times$ magnification is 1 unit = 2.5 μ m, and at 100 $\times$, 1 unit = 10 μ m.

Holding pipettes

1. Insert glass tubing into a pipette puller, adjust settings, and pull pipette.
2. Insert a previously pulled holding pipette into a pipette holder on a microforge.
3. Looking through the optics, move the pipette until it is the correct outer diameter on the reticle scale. At 40 $\times$ magnification, this would be approximately 5 units and at 100 $\times$ magnification this would be approximately 12 to 13 units.
4. Make sure that the holding pipette is in focus, and then orient the glass bead on the filament so that it is also in focus, especially the edge of the glass bead that will touch the holding pipette.

5. With the edge of the holding pipette and the edge of the glass in the same plane, turn on the power of the filament that will heat the glass bead and gently touch the holding pipette. Allow the edge of the holding pipette to melt and bind to the glass bead.
6. When the holding pipette has melted enough, turn off the power to the filament and glass bead. Allow the glass bead to contract, breaking the holding pipette.
7. Repeat step 5 if the pipette becomes distorted and there is no clean break.
8. Turn on the power to the filament and glass bead, and bring the glass bead up next to the opening of the holding pipette.
9. Melt the inner diameter of the holding pipette to 20 μm in size. At 100 $\times$ magnification, this would be 2 units on the reticle scale.
10. Turn on the power to the filament and glass bead, and at some distance from the end of the holding pipette, bring the glass bead next to the holding pipette and make a 30 degree bend in the holding needle.
11. Polish the back end of the holding pipette with a microflame by briefly putting the end into the flame. If the pipette is allowed to stay too long in the flame, it will be sealed closed.
12. Store in a 15-cm tissue culture dish containing Plasticene to hold the pipettes.

Note: For the inner diameter of the holding pipette, it is necessary to use a higher magnification than 40 $\times$ to make the holding pipette inner diameter of 20 μm . Magnification of at least 100 $\times$ will work well for this purpose. If 40 $\times$ magnification is used, the size of the inner diameter will be inconsistent and most likely will be larger than 20 μm . Inconsistency in size will lead to control problems with the holding pipette. This is especially important if the blastocyst is held by the trophoblasts and not the inner cell mass during injections. This can make injections very difficult, and the blastocyst could be suctioned into the holding pipette. The authors use a heat setting of 12.0 units and a solenoid setting of 1.0 A for pulling holding pipettes.

Injection pipettes

1. Insert glass tubing into a pipette puller, adjust settings, and pull pipette.
2. Place a previously pulled injection pipette on top of a 10-cm lid from a plastic tissue culture dish under a dissection microscope.
3. With a 30-ga needle attached to a 1-cc syringe, gently tap the glass pipette at a perpendicular angle with the edge of the needle.

Note: When the bevel is facing toward you to the side, it is easier to see the edge of the 30-ga needle.

4. Under a dissecting microscope, check the tip of the broken injection pipette. If the tip of the injection pipette needle appears to be in good shape overall, with no barbs, breaks, or jagged edges, save the pipette.

Note: With the settings that authors use for pulling injection pipettes, the pipette can be broken a few times to get a proper broken pipette. After several attempts, it is best to use another pulled pipette.

5. Place previously broken injection pipette into a pipette holder on a microforge.
6. Orient the injection pipette so that it can be seen in the microforge optics.
7. Under 100 $\times$ magnification, check again if the broken injection pipette appears to be free of defects and is of good size and shape. If defects are seen, discard pipette.

Note: For the inner diameter of the injection pipette, we prefer a size of ~20 μm .

8. If the pipette appears to still be of good quality, orient the pipette such that the bevel opening is either facing directly toward or away from you under the optics.
9. Focus the tip of the injection pipette and then orient the glass bead on the filament so that it is also in focus.
10. Turn on the power to the filament that will heat the glass bead and gently touch the tip of the pipette to the glass bead and allow it to melt just a little.
11. Pull away the glass bead only very slightly, and turn off the power to the filament, allowing the tip of the needle to contract from the glass bead.
12. Take the sharpened injection pipette out of the holder and set it aside.
13. Pour 1.25% Tween 80 solution into a sterile 3-cm dish.
14. Insert the injection pipette into a pipetting device with a 12-cc syringe attached to it.
15. Withdraw the Tween 80 solution inside the injection pipette to the hub and expel it back into the dish.
16. Lift out the injection pipette and continue expelling the Tween 80 solution out of the injection pipette to remove any excess.
17. Repeat steps 14 and 15 if necessary to ensure that the inside of the injection pipette is coated.
18. Polish the back end of the injection pipette with a microflame by briefly putting the nontip end into the flame. If the pipette is allowed to stay too long in the flame, it will be sealed closed.
19. Store in a 15-cm tissue culture dish containing Plasticene to hold the pipettes.

Note: The temperature setting should not be too high or the inner diameter will become smaller. The temperature of the glass bead should be high enough to actually melt the pipette tip. Melting and pulling the pipette tip too much will create a bevel that is too long, which would make ES cell injections more difficult. If the tip is too long or there is a filament like structure on the tip, the tip can be gently broken again and the melting of the tip can be repeated. If the tip is still too short after the first melting it can be repeated. If the inner diameter becomes too narrow or has a bevel too long, it is best to start over with another broken injection pipette. The authors prefer an injection pipette with an inner diameter of approximately 20 μm , which can be successfully obtained by using a heat setting of 14.2 units and a solenoid setting of 4.2 A when pulling injection pipettes.

GENERATION OF GERMLINE MICE

Various strains can, in theory, be used as the host blastocyst and as the ES cell line. Generally the most common strain of mouse used for the host blastocyst is C57BL/6 mice and 129 strain ES cells. It is best to use strains of mice that are different in coat color for the blastocyst and the ES cell line. The mating scheme will be determined by what strains of mice are used in the generation of chimeric mice.

If one is using a C57BL/6 donor strain for the blastocyst, the chimeras could be mated with either a C57BL/6 or a Black Swiss to generate germline mice. Each of these mice has disadvantages and advantages. The Black Swiss mice are better breeders, and the mothers take better care of their offspring and, unlike C57BL/6 females, are not disturbed easily by noise and vibration. However, if C57BL/6 mice are used, the genetic background is conserved, and if Black Swiss mice are used, this might be lost since they are outbred mice. This could have

possible implications on the mouse phenotype for the knockout mouse created. Also, if using either the C57BL/6 or Black Swiss strains of mice to mate with the chimeric mice, the germline mice will be on a mixed strain background, and in order to achieve an isogenic C57BL/6 or 129 strain background, the mice will have to be backcrossed at least 10 times. It is possible to use speed congenics and PCR methodology to limit the amount of backcrosses needed for a deleted gene of interest on a particular isogenic strain background.

Another option is the mating of the C57BL/6 x 129 chimera with an isogenic strain of 129 mice that has been used to generate an ES cell line. The 129 isogenic strain can be mated to the chimera to obtain F1 founder mice on the isogenic strain of the ES cell line in only one backcross generation. One drawback to this scheme is that some strains of 129 mice have low fecundity and may result in breeding problems in the analysis of the mouse knockout phenotype. If ES cells have contamination from other strains in the genetic background, then the mice will still have to be backcrossed at least 10 times in order to obtain isogenic purity.

If using a color marker to determine the chimeric composition of the ES cells, it is best to choose the chimeras with the highest contribution possible. They should be robust and preferably male, and they should not be dwarfs. Female chimeras could be used if needed, but are not preferred in that ES cells are generally male. Any female chimera would likely have lost the Y chromosome, becoming XO, and this is thought to be more unstable.

Female chimeras have been known to generate germline mice even with the loss of the Y chromosome. Another advantage of using male chimeras over females is that a large number of offspring can be generated in a short period of time, ensuring the transfer of the knockout allele to the F1 generation offspring.

When mating the chimeras, the most efficient mating scheme would be the mating of 2 females to 1 male chimera. Another approach is the so-called “shotgun” approach, the mating of 4 females to 1 male chimera. One drawback to this approach is that there could be a delay in the generation of offspring because of the fact that the males may become distracted by so many females and delay plugging. Furthermore, there could be a lot of mice generated at one time, which would take up a lot of cage space.

The mating scheme chosen to generate germline mice must conform to the ACUC regulations of the institution where the animal work is taking place.

REAGENTS AND SOLUTIONS

Use tissue culture-grade reagents when working with cells and embryos. For common stock solutions see **APPENDIX**; for suppliers see **SUPPLIERS APPENDIX**.

Avertin, 2.5%

To make 100% Avertin stock solution, add 50 ml of tertiary amyl alcohol (Fisher Scientific A730-1) to 50 g of 2,2,2-tribromoethanol (Fluka catalogue # 90710). Add the smallest stirring bar possible to the mixture and place on a stirring plate. Not all 50 ml of tertiary amyl alcohol may fit inside the 2,2,2-tribromoethanol bottle due to the stirring bar inside. Allow the mixture to stir at room temperature until the stirring bar is heard. Keep stirring the mixture overnight at 4 °C to make sure the 2,2,2-tribromoethanol is dissolved into solution. The next day with a 1 ml pipette, check to see if the 2,2,2-tribromoethanol is completely dissolved. If there are no solid crystals in solution, the stock solution is ready. The color of the Avertin stock solution should have a clear to very pale yellow color. If the color of the solution has a dark brown color, discard solution. For the 2.5% working solution, allow sterile 1× DPBS to warm up to room temperature. To 10 ml of DPBS add 250 µl of Avertin stock solution, and shake and

vortex until fully dissolved. Filter this solution through a 0.22- μ m, nonpolystyrene filter unit. Store in a polypropylene tube covered with foil.

There will be some variation in each batch of stock Avertin, so it is essential that it be tested on mice prior to using in surgery for efficacy and toxicity. Both solutions should be kept to a minimum at room temperature and shielded from light, which will cause the breakdown of Avertin into toxic products. If a glass container is unavailable, use plastic containers such as polypropylene. Avoid using polystyrene, as it will chemically react with the Avertin stock solution and become toxic to the mouse and may lead to peritonitis. The recommended dosage of Avertin is between 0.015 and 0.017 ml/g body weight. If the working solution is being reused, it is suggested that the solution be refiltered soon after use to reduce the risk of possible bacterial contamination. If it is thought that the working solution is contaminated, discard it.

Human chorionic gonadotropin (hCG) 5 I.U

Under sterile conditions, add 10 ml DPBS to a vial containing 10,000 units of chorionic gonadotropin (Sigma #CG10). Take 2 ml of this solution and dilute into 40 ml of PBS. Aliquot this solution into tubes and store in a -20°C freezer.

Injection media (10% DMEM)

81 ml Dulbecco's Modified Eagle's Medium (DMEM) containing 4,500 mg/L D-glucose, L-glutamate, and 25 mM Hepes (Invitrogen #12430054)

8 ml of fetal bovine serum (preferably already ES cell tested)

0.5 ml penicillin/streptomycin containing 10,000 units per ml penicillin and 10,000 $\mu\text{g/ml}$ streptomycin (Gibco #15140-122)

Filter the prepared media with a 0.22- μ m filter unit. Divide the injection media into three 50-ml tubes. Use one tube for each injection day.

Microdrop cultures

Place 30 μl of injection media on a 3-ml tissue culture plate. Overlay the drop of media with prefiltered, embryo-tested quality mineral oil (Sigma #M-3516 or equivalent).

Microinjection setup

An inverted microscope is generally used, and it should have 10 $\times$ and 20 $\times$ objectives, with the 40 $\times$ objective being optional. The 20 $\times$ objective is more useful for those who are first learning ES cell injections, but the 10 $\times$ objective is used more often by more experienced researchers. There are 3 optic systems most commonly used for microinjections. Hoffman generally works best for viewing items through plastic, Nomarski or differential interference contrast (DIC) is better suited for looking through glass, and phase contrast can be used for either plastic or glass. In the authors' lab, DIC optics are used in the microinjection microscope. General injection and holding line systems would consist of a syringe-type unit, line tubing, and pipette holder and may or may not have a stopcock attached to an oil-filled syringe. Luer locking or other types of connection systems should be used whenever possible to minimize or eliminate possible leaks that might occur. For the injection and holding line systems, one that is filled with oil, rather than air, will tend to give better control. Silicone oil would be a better choice than mineral oil in that mineral oil will tend to shrink, causing air bubbles that affect control. An injection line system with a stopcock and a tuberculin-type syringe with an oil-filled reservoir will allow the flushing out of debris and air bubbles from the injection and holding lines and both pipettes. A 12-cc oil-filled syringe reservoir will allow the microsyringe units to be aligned back to the midpoint, as opposed to one that does not.

For a microsyringe controlling unit, the authors prefer a device made by Minnetonka Instruments, which has an internal Hamilton syringe and an external Mitutoyo syringe. The injection line tubing and Hamilton syringe should be a smaller volume than the holding system because the injection pipette is much smaller than the holding one. As for micromanipulators, the authors have had successful experience with Narishige and Leica manual manipulators. There are multiple options available, and users should decide which model will work best for their use. It should include fine as well as coarse movement. A manual model is preferred for the actual injection of the blastocyst, because it allows for more precise movement and control, unlike some powered systems. To avoid any vibration problems that could affect microinjections, an air suspension table using N₂ or CO₂ gas cylinders should be considered.

Pregnant mares serum gonadotropin (PMSG) 5 I.U

Under sterile conditions, add 3 ml of sterile PBS to a vial of 2000 units of PMSG (Sigma #G 4527). Add this solution to a tube containing 40 ml of PBS, aliquot it into tubes, and store the tubes in a -20 °C freezer.

1.25% Tween 80 solution

Add 1.25 ml of Tween 80 solution (Fluka #93780) into 98.75 ml of sterile H₂O. Filter through a 0.22-μm filter unit.

COMMENTARY

Background Information

Early cancer studies in mice initially done by (Stevens 1970, 1973) led to the generation of the strain of mice known as the 129. Subsequent developmental studies in these mice made possible the creation of 2 main mouse cell lines, the embryonal carcinoma (EC) cells and the embryonal stem (ES) cells. EC cells were originally derived from teratomas that came from Stevens's mice (Kahan et al. 1970; Rosenthal et al. 1970). ES cells were initially derived from the inner cell mass (ICM) of mouse blastocyst embryos from 129 mice (Evans and Kaufman 1981; Martin 1981).

With the development of the ES cell injection technique into blastocyst mouse embryos (Gardner 1968), the true potential of these cell lines to be able to contribute to the developing mouse embryo could then be assessed. The EC cells initially showed promise with pluripotency in the formation of chimeric mice and the contribution to various tissues (Brinster 1974). However, EC cells had problems with developmental abnormalities, tumors, and a lower germline transmission efficiency compared to ES cells (Papaioannou and Rossant 1983). ES cells, on the other hand, showed this pluripotency in chimeric formation and totipotency in germline transmission to the offspring without the problems associated with EC cells (Bradley et al. 1984).

Upon understanding the mechanisms of homologous recombination, the possibility of generating knockout mice became possible. Folger et al. (1982) were the first to demonstrate that through homologous recombination, nonreplicating DNA could be transferred into a mammalian cell. This would later lead to "gene targeting" of a plasmid into the β -globin gene of mammalian cells (Smithies et al. 1985). The first gene to be targeted in ES cells was the hypoxanthine phosphoribosyltransferase (*Hprt*) gene (Thomas and Capecchi 1987; Doetschman et al. 1988). This had the benefit of it being X-linked, and only one copy was needed to show deletion in male ES cells. Later, ES cells with the *Hprt* mutation would possess the ability for germline transmission (Hooper et al. 1987; Kuehn et al. 1987; Koller et al. 1989; Thompson et al. 1989). While the generation of a mutant mouse with this gene showed initial promise as a model for Lesch-Nyhan syndrome, the phenotype did not correlate with

the human disease (Samuel et al. 1993). The *Hprt* knockout mouse phenotype highlights what may happen when one attempts to delete a human gene in the mouse.

An alternative approach to generate chimeric mice is the ES embryo aggregation technique. This technique can be further divided into 2 basic methods. One of them is the diploid aggregation technique, which involves ES cells cultured with morula stage embryos. This can further be divided into 2 techniques. One technique developed by (Wood et al. 1993) involves culturing the morula on a layer of ES cells. The other method, devised by (Khillan and Bao 1997), also uses morula and ES cells but with a defined microwell and a given number of cells. A second morula aggregation method, in contrast, uses a tetraploid developed by (Nagy et al. 1993): 2-cell embryos are electrically fused together and cultured until the 4-cell stage. These 4-cell tetraploid embryos are then used to “sandwich” the ES cells, allowing integration (fusion) to occur. This is done in a well on a tissue culture dish. All 3 methods have the advantage of not requiring injection skills or the expensive equipment needed to perform the ES injection procedure.

The last key component to generating chimeric mice and subsequent knockout mice is the uterine surgical transfer technique. This technique was initially developed by (McLaren and Michie 1956). They determined that a critical parameter for success in the uterine transfer technique is the surgical transfer of E3.5 blastocyst embryos into E2.5-aged recipient females. Based on this work, (McLaren and Biggers 1958) cultured morula-stage embryos in vitro to the blastocyst stage and surgically transferred them by the uterine technique to successfully generate live offspring.

A recent development suggests the possibility of using other types of stem cells instead of ES cells to generate chimeric and knockout mice. Guan et al. (2006) isolated spermatogonia stem cells (SSC) and maintained them under ES cell growth conditions. As a result, these cells retain characteristics of both SSC and ES cells, and they were subsequently named multipotent adult germline stem cells (maGSCs). This technique may be another method to derive cells that are ES cell capable, without having to obtain them from the inner cell mass of a blastocyst. This may make it easier to acquire ES-like cells from a strain of mouse where currently none is available.

Critical Parameters and Troubleshooting

There are 3 main areas where problems may be encountered while creating chimeric mice: harvesting blastocyst embryos, injecting ES cells into the blastocyst embryo, and surgically reimplanting embryos into pseudopregnant female mice. The ES injection technique can largely be controlled by conditions in the laboratory, which can make problems in this area easier to solve. The other 2 areas of harvesting the blastocyst embryos and surgically reimplanting them depend on conditions in the lab, the in vivo variability of the mice themselves, and conditions in the animal mouse facility. Thus, solving problems in these last 2 areas can be quite complex. It is probably best to look at laboratory conditions first, which are more easily controlled, then proceed to looking at the mice themselves and to the animal facility conditions. It is important that detailed notes be taken, which can greatly help to pinpoint problems. Aspects of all related procedures should be assessed periodically so that potential problems can be addressed early.

Low number of blastocysts collected—The number of blastocysts obtained through the process of superovulation and harvesting of blastocyst embryos can depend on several factors. The age of the mice and the conditions in the animal facility are 2 areas affecting blastocyst yield. Females that are 3 weeks old are preferable because they are more easily induced by the PMSG and HCG hormones. If not available, 4-week-old females are the next best choice. Female mice 6 weeks or older should be producing their own reproductive hormones, which

could interfere with any hormone injected intraperitoneally. For the breeding stud males, a range of 7 weeks to ~ 10 months of age has worked well in our experience. Around 10 months of age, plugging by the stud males becomes variable. Another sign that the stud males are too old is that only very early stage embryos are recovered. Replacing the stud males will solve both of these problems. In the animal room where the mice are being held, the most important factor is the timing of the light and dark cycle. A light and dark cycle of 14 hours light and 10 hours dark is considered to be conducive for the mice to mate. The light cycle should be checked if there is any doubt that it is working correctly.

Hormone-related issues can also influence blastocyst yield. These would include the source where the hormones came from, a possible bad lot, or how the hormones were prepared. A reputable source that others have had success with should be the first choice. Another lot number of the same hormones should be tried to see if similar results are obtained. If the hormones have not been diluted to the proper concentration they should be discarded. Freezing and thawing of hormones is to be avoided as this will decrease the activity and effectiveness. The proper dosage of hormone being injected intraperitoneally and the timing of the hormone injections are also important.

What day and time the blastocysts are to be harvested will determine the timing of the hormone injections. If at the desired time of day only morula or earlier stage embryos are obtained, both of the hormone injections should be done at earlier times. If only hatched blastocysts are collected, hormone injections should be done at later times.

When new to the technique of harvesting and collecting blastocysts, it is not uncommon to obtain a lower embryo yield than that of someone who is experienced. Blastocysts can be lost while harvesting the uterine horns. Since the blastocysts are free-floating inside the uterine horns, care should be taken to not cut holes in the uterine wall when cutting off the mesometrium structure. Letting the uterine horns snap like a rubber band should also be avoided when extracting out the uterine horn as this can also contribute to losing blastocysts. After all of the uterine horns have been taken out of the original holding dish, check to see if there are any blastocysts left on the dish. If there are a lot of them on the dish, then there is likely a problem with the harvesting technique. Practice is needed until the technique is mastered. The way the uterine horns are flushed out can also affect blastocyst yield. The opening of the cervix should be oriented slightly up and to the side. This will allow one to see the injection media being flushed out of the cervix while keeping the flushed media inside the 3-cm dish. In our experience, a 30-ga needle works best for flushing the blastocysts out of the uterine horns. A good amount of pressure can be applied to the syringe with less chance of media coming out of the dish, unlike that of larger gauge needles. It should be noted that even under optimal conditions the process of superovulation and harvesting the blastocysts doesn't always work well all the time. There will be days when very few embryos are recovered.

Blastocyst injection procedure—Blastocysts are very hardy embryos and can withstand injections quite well, unlike embryos at earlier developmental stages, such as zygotes. There are 3 main areas where problems might arise during ES cell injections of blastocysts: the injection and holding line systems, the injection pipette, and the holding pipette.

In the injection and holding line systems, problems that may commonly occur are that the oil does not move inside the pipette, there is a lack of control or the movement is very slow, or the cells are continuously taken up by the holding and injection pipette when the line is open. These problems could be due to air in the system, there maybe a leaky or worn-out stopcock, or there could be a leaky joint or connection. If air is found in the system, a 19-ga needle and syringe filled with oil (or the oil-filled syringe reservoir) is a good way to purge this out. A

leaky stopcock due to wear must be replaced with a new one. Reconnect the loose joint or connection and replace any fittings or pieces of tubing if necessary.

Another area where problems might arise could be the injection pipette. Debris or oil can become stuck to the inside and outside of the injection pipette. There maybe blockage or air bubbles that form inside the injection pipette. Cells may lyse inside the injection pipette. The injection pipette may not penetrate inside the blastocyst and collapse before penetrating the blastocoel cavity. Debris, silicone oil, air bubbles, or other types of blockage may be flushed out with the syringe reservoir filled with silicone oil. If this method does not remove the debris or oil attached to the inside of the pipette, a smaller MEF cell maybe used much like a pipe cleaning device. Debris on the outside of the injection pipette may be removed by moving up the injection pipette through the media/oil and oil/air interfaces. The holding pipette could be used to pull debris off the injection needle, but care should be taken not to block the inside of the holding pipette. The reason for lysis of ES cells inside the injection pipette could be due to the fact some of the ES cells inside the pipette are too large, of poor quality, or have come in contact with oil. ES cells should be well trypsinized to remove cell surface proteins and kept cold to impede them from reforming. If not, this can cause “stickiness” inside and outside the injection pipette, impeding injections. If the inner diameter of the injection pipette is too small, then the cells could lyse. Failure to inject into the blastocoel cavity can be the result of a dull or badly damaged injection pipette. It is best to inject between the junction of the trophoblast cells, which will make injections easier. The injection pipette should be at approximately the midpoint of the blastocyst height so that it will be in the center of the blastocoel cavity. If it is on the bottom of the blastocoel cavity, it will be harder to inject. The injection pipette should be raised up to avoid this from happening. It is very important to have really good injection needles when new to the injection procedure as this will make injections much easier and can help avoid much of the frustration that is often encountered when learning the injection technique. If all other attempts fail to correct problems encountered in injections of the blastocyst, the injection pipette needs to be replaced.

The third and last area where problems can occur is that of the holding pipette. It could become blocked with debris or have air bubbles forming inside. Erratic holding control may be experienced or there is no holding suction at all. If bubbles form or the inside of the holding pipette becomes blocked, the holding pipette may be flushed out with the syringe reservoir filled with silicone oil. Usually this method will not work with a large blockage. If this happens, the holding pipette will need to be replaced. In our experience, a holding pipette with an inner diameter of approximately 20 μm works best for the holding control of the holding pipette. A smaller diameter may not have enough holding power, while a larger diameter tends to give more erratic control and the possibility of the blastocyst ending up inside the holding pipette. If the blastocyst moves when attempting to inject, this could be due to the fact that the holding pipette is too low to the surface of the well of the injection dish and there is not enough room between the holding pipette and the surface of the well. It also may mean that the holding pipette is not properly aligned or is too close to the shoulder of the injection dish. Touching the blastocyst to the bottom of the well adds much-needed stability for holding.

Low birth and survival rate—There could be a number of reasons for a small litter size or for why pseudopregnant females are not taking care of the pups. The best place to start is to do in utero analysis of uninjected blastocysts by extracting out the uterine horns of pseudopregnant recipient females and looking at E9.5 embryos. If there is a low percentage of embryos being implanted, this could most likely be due to factors related to the surgical technique.

Surgical technique can be influenced by several factors. Only perform the uterine surgical transfer procedure on mice that have the corpus luteum (red spots) present on the ovary.

Absence of the corpus luteum suggests the female was not plugged or was not in estrus when plugged by the vasectomized male. Avoid touching the uterine horns and ovary when possible, except to hold the uterus to make the hole inside the uterus to the lumen and inserting the transfer pipette. When making the surgical incision in the dorsal muscle wall, avoid cutting the blood vessels, which can cause excessive bleeding making it hard to find the ovary and fat pad. Like the surgical incision, avoid the blood vessels in the uterine wall when inserting the 25-ga needle in the uterine wall to make the hole in the lumen. If a blood vessel is damaged during this procedure, bleeding might clog the inside of the transfer pipette. Mouth pipetting the embryos will be difficult if not impossible. Reload the transfer pipette, and make another attempt to transfer the blastocysts. Both the 25-ga needle and transfer pipette should pass freely inside the hole in the uterine wall. This indicates that both have entered inside the lumen. The attempt to transfer the blastocysts will not be successful if there is any obstruction. The procedure of mouth pipetting, the quality of the transfer pipette, and how the transfer pipette is loaded can influence the outcome of the surgical transfer procedure. As the investigator becomes skilled in using the mouth pipette, this factor can largely be ruled out. If the inner diameter of the transfer pipette is too small, there is a tendency for the transfer pipette to become easily clogged by bleeding that can happen during the procedure, and if the inner diameter is too big, this may affect the control in that it will be too sensitive and the blastocyst will migrate up the transfer pipette. Also, a transfer pipette that is too small in diameter may make it difficult to blow the blastocysts inside the lumen of the uterus. The way a transfer pipette is loaded with blastocysts can make a difference in the transfer results. There is a lot of variation in the way a transfer pipette can be loaded with blastocysts. Air bubbles and oil are factors that can help to stabilize the embryos inside the transfer pipette, but will move when blown out with the mouth pipette. After the transfer of the blastocysts has taken place, examine the transfer pipette under a dissecting microscope to see if there are any left inside. If any blastocysts are left, they should be transferred again until they are no longer in the transfer pipette.

If the rate of implantation of E9.5 embryos is high, then there may be other factors other than the surgical technique, one of these being related to the culture conditions of the ES cells and blastocyst embryos. Reagents that are used to grow the ES cells or that may come into contact with the blastocysts could be the culprit. Serum, DMEM media, and oil should be tested on the parental cell line first before they are used on targeted ES cell clones. Reagents used more specifically with the in vitro conditions of the blastocyst should be tested before they are to be injected with ES cells. If these reagents have been tested before, they are most likely not the cause. However, if a new lot is now being used, it is worthwhile to investigate. Another lot should be tried to see if similar results are obtained. If a new lot of a particular reagent has been determined as the cause, it should be discarded and replaced as soon as possible. Haploid insufficiency of the targeted gene or the way the construct is designed may influence embryo lethality and pup death; if other targeted ES cell clones are generally doing well, then this could be the cause.

The other area that may affect the gestation of the pseudopregnant recipient litters is the conditions in the animal room where the mice are being housed. Care should be exercised not to stress the recipient mother because it could cause physiological conditions leading to reabsorption of the mouse embryos in utero and the death of the newborn pups. Probably the key embryonic developmental time points where this stress would likely be the most detrimental are at E4.5 to E5, when the embryos implant, and shortly before birth. The first week of birth is the most critical time for the newborn pups. If they survive this week, there generally should be no complications. Stress factors to avoid include the Bruce effect, ammonia levels, noise, vibration, animal handling, animal food nutrition, ventilation, and the light/dark cycle. These conditions in the room should be monitored to determine which of them might be the cause. Infectious agents such as viruses, which may be present as a latent infection in the animal colonies, can affect knockout mouse production.

Finding the exact cause of embryo lethality can be difficult and time-consuming as numerous factors affecting pregnancy need to be ruled out. Even the simplest of factors might be the cause of the problem and should not be overlooked. For example, we determined that a defective wheel on one of the animal racks was the possible cause for the pups being reabsorbed in utero and the pseudopregnant mothers not taking care of the pups after birth. When the rack was replaced, these problems went away. Lastly, one factor that is often overlooked is the effect of lighting on embryos, whether it is in the lab or surgical room. This could cause apoptosis to occur in the ICM of the blastocyst embryo, which would have detrimental effects on injected embryos and the potential for live pups being born.

Anticipated Results

With practice, this surgical transfer technique should result in at least 70 to 80 percent or better of the surgical recipients giving birth. Even uninjected blastocysts, when surgically transferred and implanted, may end up being reabsorbed during gestation. This can happen as early as E9.5. The number of pups born will be influenced by the strain of female mouse used for the pseudopregnant recipient. In our experience, the B6D2 and B6CBA strains of mice have only given birth to no more than 6 pups per uterine horn, regardless of how many were surgically transferred. If more than 6 embryos are surgically transferred, these extra embryos most likely will not implant.

Blastocysts are very hardy embryos, especially when compared to early stage ones. They tolerate changes in temperature, pH, and the technique of ES injection. Unless there is severe trauma of the embryos, like that of damaging the ICM, the embryos should not be negatively impacted. In the hands of a skilled injectionist, the ES cell injection procedure should not adversely affect the birth rate of pups being born.

There are numerous ES cell lines available for the purpose of making chimeras to generate knockout mice. Testing of the parental ES cell lines the investigator plans to use should be first evaluated for any possible ill effects from these cells. Their efficiency in generating chimeric mice needs to be evaluated before carrying out any gene targeting related experiments on them. Some markers to look at in evaluating the effectiveness of the parental cell include the percentage of chimerism, the ratio of the number of male chimeras born to females, and the ability to generate germline offspring. In a good parental ES cell line, the chimerism should be high, the male-to-female ratio high, and the number of pups needed to see germline transmission low. It is possible to see chimerism or germline transmission as early as 5 days with a small patch of fur becoming visible. This is much easier to see if an "albino" ES cell line is used, in that these white areas would appear nude before the fur starts to grow in. Germline transmission on other types of 129 ES cell lines used with a C57BL/6 host blastocyst will have either a medium brown or agouti (golden brown) appearance.

Even if good parental lines are used, there can be great variability in a targeted clone's ability to create a high percentage of chimeras and in the germline potential of these chimeric mice. This is most likely due to trauma the ES cells have to undergo in the electroporation of the gene targeting construct and selection process to determine a correctly targeted clone. In theory it should take only 2 clones to generate 2 different germline chimeric lines leading to the establishment of 2 independent knockout mouse lines. This would confirm that the phenotype is due to the gene targeting construct and not to the cells themselves. Several targeted clones should be available to ensure that there is germline transmission from 2 independent clones, should some of the clones give low chimerism and are not germline.

A situation may arise where lethality is most likely related to the clones being injected or the construct that is electroporated inside the ES cells. This can result in no chimeric pups being born at all or only a few surviving to birth and then dying shortly thereafter. If everything is

generally going well with other constructs and ES cell clones, then other factors affecting gestation can be ruled out. If the ES clones in question have been injected additional times and large numbers of blastocysts have been injected with similar results, then this further suggests a construct-related problem. Further injections will most likely be of little use. There is also the possibility of embryonic and perinatal lethality occurring in the germline F1 mice. As previously mentioned with chimeras, this could be due to targeting construct-related issues. The construct should be reevaluated and any problems corrected. Additional clones can be generated and injections performed. Lastly, lethality could be due to the function of the gene deleted and its essential role in embryonic development. This haploid insufficiency may be the reason for embryonic and pup lethality. If this occurs, the only kinds of phenotypic analyses that may be carried out would be in utero analysis of the developing embryos or in vitro tissue culture analysis.

Time Considerations

Plan to start the initial intraperitoneal PMSG hormone injections 1 week before the day when the blastocysts will be needed. The entire process of harvesting the blastocysts, injecting them, and surgically transferring them back to a pseudopregnant recipient female is generally done the same day. Even for the experienced injectionist this can make for a long day, especially when problems with the injection system and bad cell morphology of the ES cell clones happen on the same day. The surgical procedure should be practiced before attempting the injection procedure since it generally takes longer to master. Having a video display for all the procedures aids greatly in the visualization of these techniques and can shorten the time required to learn them, which is expected to take 6 months or less. How long it takes will ultimately depend on previous experience, the person instructing the procedures, and the desire of the investigator to learn the procedures.

LITERATURE CITED

- Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 1984;309:255–256. [PubMed: 6717601]
- Bradley, A. Production and Analysis of Chimeric Mice. In: Robertson, EJ., editor. *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. Oxford University Press; New York: 1993. p. 113-151.
- Brayton, C.; Mähler, M.; Nicklas, W. Viral Infections. In: Hedrich, H., editor. *The Laboratory Mouse*. Elsevier Academic Press; New York: 2004. p. 357-391.
- Brinster RL. The effects of cells transferred into the mouse blastocyst on subsequent development. *Journal of Experimental Medicine* 1974;140:1049–1056. [PubMed: 4610074]
- Chia R, Achilli F, Festing MFW, Fisher EMC. The origins and uses of mouse outbred stocks. *Nature Genetics* 2005;37:1181–1186. [PubMed: 16254564]
- Conner DA. Chimeric Mouse Production by microinjection. *Current Protocols in Molecular Biology* 2001;23.7.1–23.7.20. [PubMed: 18265159]
- Doetschman T, Maeda N, Smithies O. Targeted mutation of the *Hprt* gene in mouse embryonic stem cells. *PNAS* 1988;85:8583–8587. [PubMed: 3186749]
- Doetschman T. Interpretation of phenotype in genetically engineered mice. *Laboratory Animal Science* 1999;49:137–143. [PubMed: 10331542]
- Eango R, Riba L, Housman D, Hunter K. Generation and mapping of *Mus spretus* strain-specific markers for rapid genomic scanning. *Mammalian Genome* 7:340–343.
- Folger KR, Wong EA, Wahl G, Capecchi MR. Patterns of integration of DNA microinjection into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. *Molecular and Cellular Biology* 1982;2:1372–1387. [PubMed: 6298598]
- Gardner RL. Mouse chimeras obtained by the injection of cells into the blastocyst. *Nature* 1968;220:596–597. [PubMed: 5686740]

- Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 2006;440 :1199–1203. [PubMed: 16565704]
- Hogan, B.; Beddington, R.; Constantini, F.; Lacy, E. *Manipulating the Mouse Embryo: A Laboratory Manual*. Vol. 2. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 1994.
- Hooper M, Hardy K, Handyside A, Hunter S, Monk M. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* 1987;326:292–295. [PubMed: 3821905]
- Kahan BW, Ephrussi B. Developmental potentialities of clonal in vitro cultures of mouse testicular teratoma. *Journal of the National Cancer Institute* 1970;44 :1015–1036. [PubMed: 5514468]
- Khillan JS, Bao Y. Preparation of animals with a high degree of chimerism by one-step coculture of embryonic stem cells ad preimplanation embryos. *Biotechniques* 1997;22:544–549. [PubMed: 9067035]
- Koller BH, Hagemann LJ, Doetschman T, Hagaman JR, Huang S, Williams PJ, First NL, Maeda N, Smithies O. Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. *PNAS* 1989;86:8927–8931. [PubMed: 2573070]
- Kuehn MR, Bradley A, Robertson EJ, Evans MJ. A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* 1987;326:295–298. [PubMed: 3029599]
- Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *PNAS* 1981;78:7634–7638. [PubMed: 6950406]
- McLaren A, Michie D. Studies on the transfer of fertilized mouse eggs to uterine foster-mothers: I. factors affecting the implantation and survival of native and transferred eggs. *Journal of Experimental Biology* 1956;33:394–416.
- McLaren A, Biggers JD. Successful development and birth of mice cultivated in vitro as early embryos. *Nature* 1958;877–878. [PubMed: 13590153]
- Matouk C, Gosselin D, Malo D, Radzioch D. PCR-analyzed microsatellites for the inbred mouse strain 129/Sv, the strain most commonly used in gene knockout technology. *Mammalian Genome* 1996;7:603–605. [PubMed: 8678982]
- Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *PNAS* 1993;90:8424–8428. [PubMed: 8378314]
- Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. Vol. 3. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 2003. *Manipulating the Mouse Embryo: A Laboratory Manual*.
- Papaioannou VE, Rossant J. Effects of the embryonic environment on proliferation and differentiation of embryonal carcinoma cells. *Cancer Surveys* 1983;2:165–183.
- Papaioannou, V.; Johnson, R. Production of Chimeras by Blastocyst and Morula Injection of Targeted ES Cells. In: Joyner, AL., editor. *Gene Targeting: A Practical Approach*. Vol. 2. Oxford University Press; New York: 2002. p. 133-175.
- Papaioannou, VE.; Behringer, RR. *Mouse Phenotypes: A Handbook of Mutation Analysis*. Cold Spring Harbour Laboratory Press; Cold Spring Harbour, N.Y.: 2005.
- Rosenthal MD, Wishnow RM, Sato GH. In vitro differentiation of clonal populations of multipotent mouse cells derived from a transplantable testicular teratocarcinoma. *Journal of the National Cancer Institute* 1970;44:1001–1014. [PubMed: 4936569]
- Samuel K, Clarke AR, Ansell JD, Hooper ML. Age dependent selection against phosphoribosyl transferase-deficient cells in mouse haematopoiesis. *Development* 1993;118:859–863. [PubMed: 8076522]
- Silver, LM. *Mouse Genetics: Concepts and Application*. Oxford University Press; New York: 1995.
- Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten, Sharp LE. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nature Genetics* 1997;16:19–27. [PubMed: 9140391]
- Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. Insertion of DNA sequences into the human chromosomal B-globin locus by homologous recombination. *Nature* 1985;317:230–234. [PubMed: 2995814]

- Stevens LC. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Developmental Biology* 1970;21:364–382. [PubMed: 5436899]
- Stevens LC. A new inbred subline of mice (129/terSv) with a high incidence of spontaneous congenital testicular teratomas. *J Natl Cancer Inst* 1973;50:235–242. [PubMed: 4692863]
- Stewart, CS. Production of Chimeras between Embryonic Stem Cells and Embryos. In: Wasserman, PM.; DePamphilis, ML., editors. *Guide to Techniques in Mouse Development. Methods in Enzymology*. Vol. 225. Academic Press; New York: 1993.
- Takenaka M, Horiuchi T, Yanagimachi R. Effects of light on development of mammalian zygotes. *PNAS* 2007;104:14289–14293. [PubMed: 17709739]
- Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 1987;51:503–512. [PubMed: 2822260]
- Thompson S, Clarke AR, Pow AM, Hooper ML, Melton DW. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. *Cell* 1989;56:313–321. [PubMed: 2912572]
- Wells. Production of Chimeras Derived from Murine Embryonic Stem Cells. In: Clarke, AR., editor. *Transgenesis Techniques. Methods in Molecular Biology*. Vol. 180. Humana Press; Totowa, New Jersey: 2002. p. 127-149.
- Wood SA, Pascoe WS, Schmidt C, Kemler R, Evans MJ, Allen ND. Simple and efficient production of embryonic stem cell-embryo chimeras by coculture. *PNAS* 1993;90:4582–4585. [PubMed: 8506303]
- Wong GT. Speed congenics: applications for transgenic and knock-out mouse strains. *Neuropeptides* 2002;36:230–236. [PubMed: 12359513]

VIDEO REFERENCES

- Pederson, RA.; Papaioannou, V.; Joyner, A.; Rossant, J. Targeted mutagenesis in mice: A video guide. Cold Spring Harbor Laboratory Press; 1989.
- Pederson, RA.; Rossant, J. Transgenic techniques in mouse development: A video guide. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1989.

CD REFERENCE

- Training in survival rodent surgery [CD-ROM]. Bethesda, MD: Subcommittee of the NIH Animal Research Advisory Committee; 2001.

KEY REFERENCES

- Hogan, B.; Beddington, R.; Constantini, F.; Lacy, E. *Manipulating the Mouse Embryo: A Laboratory Manual*. Vol. 2. Cold Spring Harbour Laboratory Press; Cold Spring Harbour, N.Y: 1994. Good overall reference on mouse surgeries and injections. A useful companion to the third edition.
- Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. *Manipulating the Mouse Embryo: A Laboratory Manual*. Vol. 3. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y: 2003. The best overall source on procedures needed to generate chimera and knockout mice and other related procedures.
- Robertson, EJ. *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. Oxford University Press; New York: 1993. This reference source is especially useful for working with ES cells and troubleshooting problems encountered during ES cell injections. See Bradley et al. 1987
- Joyner, AL. *Gene Targeting: A Practical Approach*. Vol. 2. Oxford University Press; New York: 2000. Another good reference for the procedures related to the production of chimeric mice. Especially useful for information in construct design. See Papaioannou and Johnson 2002
- Wasserman, PM.; DePamphilis, ML. *Guide to Techniques in Mouse Development*. Vol. 225. Academic Press; New York: 1993. *Methods in Enzymology*. This reference is good in characterizing the best morphology of ES cells to inject. It also describes multiple versions of the same procedures. See Stewart 1993

INTERNET RESOURCES

Brown, Gary, editor. The Microinjection Workshop [Internet]. 1996. Available from:
<http://www.microinjectionworkshop.net>

This site is useful for links to other transgenic-related Internet sites. There are also transgenic-related protocols as well as images of these techniques.

BioSupplyNet [Internet]. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2005.
Available from: <http://www.biosupplynet.com/>

This is a use full website for finding products and reagent required for procedures in the generation of chimeric mice and related procedures.

Figure 1. Collection of blastocyst embryos. A. Holding of skin and fur prior to removal. B. Removal of skin and fur. C. Removal of peritoneal membrane. D. Uterine horns exposed. E. Removal of mesometrium tissue from the uterine horns. F. Drawing showing the harvesting and flushing of uterine horns.

Figure 2.

ES cell morphology. ES and MEF cell suspension and the different types of cells and morphology seen: A. 100 $\times$. Larger MEFs cells are denoted by black arrows and differentiated ES cells by white arrows. B. 400 $\times$. Various types of cells seen in suspension. MEF cells are marked by black arrows. Differentiated ES cell is marked by a white arrow. Good morphology, medium-sized ES cells are marked by a gray arrows. Good morphology, large, undifferentiated ES cells are marked by a black diamond. Small ES cells are marked by a white diamond. Translucent ES cells are marked by a gray diamond. It is suggested that medium, good morphology ES are the cells to inject into the blastocysts for optimal results.

Figure 3.

Blastocysts and sequential ES injection. A. Blastocysts at various stages of development: top left, early stage blastocyst; top middle, middle to late stage blastocyst; top right, oval-shaped blastocyst; bottom left, blastocyst in the process of hatching (arrow) showing early bud structure; bottom middle, blastocyst on the verge of hatching with pronounced trophoblast morphology; and bottom right, hatched blastocyst. B-G. Panels showing sequential steps in blastocyst injection. H. Injected blastocysts in various stages of collapse.

Figure 4.

Diagram of injection dish. The dish has an outer diameter of 4 inches, an inner diameter of approximately 1 inch, and a height of half an inch. The dimensions of the inflow and outflow ports are approximately 1.5 inches long with an inner diameter of 3/8 of an inch.

Figure 5.

Diagram of blastocyst injection chamber. The blastocyst is held by the holding pipette and is touching the bottom of the injection well for stability. The injection pipette is unbent and is inside the blastocoel cavity at a 30° angle.

Figure 6. Diagram of transfer pipette with loaded blastocysts. Mineral oil is first loaded inside the pipette. This is followed by the first bubble, injection media, another bubble, and the blastocysts.

Figure 7.

Diagram of uterine transfer of blastocysts. A. The uterine horn has been extracted from the peritoneal cavity. B. The surgical incision closed by wound suture. C. Hole made in the uterus by 25-ga needle into the lumen. D. Transfer pipette placed inside the hole of the uterus into the lumen, showing air bubbles present inside the pipette.

Figure 8.

Fabrication of pipettes with microforge. A. Breaking pulled injection pipette with a 30-ga needle and sharpening tip with the microforge. A broken injection pipette is moved to the glass bead so that the tip is touching the glass bead. The injection pipette is allowed to fuse and pulled slightly. The power is turned off and the glass allowed to contract. B. Breaking and polishing holding pipette using glass bead of the microforge. A pulled holding pipette is moved to the glass bead so that the edge of the tubing is touching the top and allowed to fuse. The power is turned off and the tubing broken by contraction. The inner diameter is melted to approximately 20 microns. C. Bending holding pipette at 30 degrees using glass bead. The holding pipette is brought near the glass bead of the microforge and allowed to bend at approximately 30 degrees.

Making transfer pipette would be the same as that used in panel B, except the edges of the pipette are only polished and they are not bent at a 30 degree angle.

Figure 9. ES microinjection setup. A. Picture of microinjection setup. B. Picture of holding system detailing micrometer syringe assembly, syringe reservoir, stopcock, the tubing, and fittings. C. Picture of injection system detailing micrometer syringe assembly, syringe reservoir, stopcock, the tubing, and fittings.

Table 1
Embryo Collection and Recipient Schedule

Day	Collect embryos	PMSG (1:00 p.m.)	HCG (12:00 p.m.)	Separate embryo donor females	Pseudopregnant recipient females
Monday	C		B		Separate
Tuesday		C		B	Separate
Wednesday	A				Separate
Thursday		A	C		No matings, rest day
Friday	B			C	Mate (25 B6D2 F1)
Saturday		B	A		Separate
Sunday				A	Separate

- Each letter denotes a group of (10) C57B16 females used for a certain injection day.
- Recipient females that are plugged are separated and replaced with another B6D2 female for a total of (50) B6D2 females for the week of injections.
- Group A females could be separated on Monday instead of Sunday if it is not necessary to know the number of plugged donor females.

Table 2

Timeline for the Generation of Knockout Mice

Injection of targeted ES cells into C57BL6 host blastocyst	• Day 1
Transfer of injected blastocyst into foster mother	• Day 1
Chimeric pups born	~ 3 weeks
Chimerism seen in chimeric mice	~ 1 month
Mating of chimeric mice to C57BL6 mice	~1.5 months
Birth of potential F1 heterozygote founders	~ 2 months, 1 week
Determine germline transmission by coat color	~ 2.5 months
Mating of F1 heterozygous pair	~ 3 months, 3 weeks
F2 knockouts born	~ 4.5 months

(This is a suggested timeline and may vary due to animal reproduction.)