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Effective Derivation and Manipulation of Mouse Embryonic Stem Cells

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1. Introduction

Mouse embryonic stem (ES) cells were first isolated in 1981 from the inner cell mass of the blastocyst before implantation within the uterine wall. ES cells have the unique property of pluripotency and also of capable of infinite self-renewal. They can be maintained in undifferentiated state in culture or be differentiated to multilineage cell types from all three embryonic layers, both in vivo and in vitro (Evans & Kaufman, 1981; Martin, 1981; Brook & Gardner 1997). These properties of ES cells have made them an extremely interesting and important tool for basic and applied research, especially for the studies of embryogenesis, gene function, and development. However, current protocols for mouse ES cell derivation are often very inefficient and require a great deal of specialized training and expertise, the potential of mouse ES cells has not yet been fully and systematically exploited.

There are numerous protocols available for mouse ES cell line derivation from blastocysts, and interestingly the effectiveness of derivation of ES cells is largely based on the mouse strain. In practice the efficiency of derivation in strains other than 129 does not usually exceed 10% (Bryja et al., 2006; Battle-Morera et al., 2008). Moreover, some protocols require the use of sophisticated techniques, such as isolation of inner cell mass via immunosurgery of intact blastocysts, isolation of epiblast cells from implanted the egg cylinderstage embryos, or selective ablation of differentiated cells. Other variations could include derivation on feeder layers, the absence of supporting feeders all together, or the use of conditioned media or the use of serum replacement (McWhir et al., 1996; Schoonjans et al., 2003; Cheng et al., 2004; Tesar, 2005; Bryja et al., 2006; Doungpunta, et al., 2009). Rho kinase inhibitor Y-27632 and the dissociation reagent Accutase were reported to significantly inhibit apoptosis of human ES cells during passaging (Watanabe, et al., 2007; Ruchi, et al., 2008), and since then we have adapted these methods in our mouse ES cell derivation protocol. Our data demonstrates that Y-27632 and Accutase increase the efficiency of mouse ES cell derivation, and the resultant ES cells retain developmental pluripotency (including stable karyotype, surface markers, teratoma formation, and the ability to undergo germline transmission). In this chapter, we describe a simple and efficient protocol for derivation of mouse ES cells and provide details on how to culture and manipulate the resultant cells. As compared to other available protocols, this method does not require special equipment, genetic modification, or advanced training other than regular tissue culture and animal handling skills. It is our hope that this protocol will allow investigators new to the ES field

to efficiently derive mouse ES cell lines even if they do not have previous experience in this area.

2. Materials

2.1 Mice

Mice were purchased from Beijing Vitalriver Laboratory Animal Technology (Beijing, China). The mice were housed at 25°C under 50%~60% relative humidity with a 12h light:12h dark photoperiod (lights on at 06:00) until they were required. Mice were fed with commercial pelleted food and water *ad libitum*. All experimental protocols and animal handling procedures were reviewed and approved by the Laboratory Animal Care and Use Committee of Hebei Province.

2.2 Equipment

Special care should be taken to assure that the following equipment is decontaminated before use and periodically check that the equipment is functioning properly.

1. Autoclaves (Boxun Apparatus YXQ-LS-30II, Shanghai)
2. Dissecting microscope (Nikon SMZ645)
3. Freezers and refrigerators (Xinfei BCD-213KA, Henan)
4. Inverted microscope with 4×, 10× and 20× objectives (Olympus IX71)
5. Liquid nitrogen storage tanks (Dongya YDS-50B-125, Sichuan)
6. Micro fusion chamber (gap between electrodes: 0.2mm) (Eppendorf, 4308 030.003)
7. Microforge (Narishige MF-900)
8. Micromanipulator set (Narishige MM-89)
9. Multiporator (Eppendorf AG 22331)
10. Piezo impact drive system (Prime Tech, PMM-150FU)
11. Pipette puller (PN-30) and pipette (B100-75-10) (Sutter Instrument)
12. Sterile horizontal flow hood with UV light (Boxun Apparatus VS-840-1, Shanghai)
13. Table-top centrifuge (Guohua TGL-16, Changzhou)
14. Tissue culture incubator: settings: 37°C, 5% CO₂, normal atmospheric concentration, and a saturated aqueous atmosphere (Sanyo MCO-15AC)
15. Water bath: Set at 37°C for regular use but can also be used at 56°C to heat inactivate serum (BHW2, GB11241-89, Beijing)
16. Water purification equipment and medium filtration devices (Pall PL 5123, America)

2.3 Plasticware and other materials

It is recommended that disposable plastic material should be used for all tissue culture work.

1. Cell counter
2. Centrifuge tubes (Nunc, 15ml and 50ml)
3. Cryovials (Nalgene, 1.8ml)
4. Embryo handling pipette
5. Eppendorf tubes (1ml)
6. Equipment for dissection (razor blades, scissors, micro dissecting scissors, straight and curved forceps, tweezers)
7. Four-well plates (Nunc)
8. Gas burner

9. High-quality CO₂ (Beijing Oxygen Plant Specialty Gases Institute Company, Beijing)
10. Microcapillaries harvard GC100T-10 (Harvard Apparatus LTD)
11. Pasteur pipettes
12. Petri dishes (Nunc, 35, 60 and 90mm diameter)
13. Repeat pipettman (Eppendorf)
14. Sterile filter with 0.22µm membrane with low protein binding (Millipore)
15. Syringes (Weigao, Shandong, 1ml, 10ml, and 20ml)

2.4 Reagents and solutions

All chemicals, reagents and solutions should be cell culture tested or of analytical grade.

1. 2, 2, 2-tribromoethyl alcohol (Sigma, T48402)
2. 2-Mercaptoethanol (Invitrogen 21985-023)
3. 70% Ethanol (Hengxing Chemical Preparation Company, Tianjin)
4. Acetic acid (Zhongliante Chemical Preparation Company, Beijing)
5. Agar (Sigma, A1296)
6. Albumin from bovine serum (BSA) (Sigma, A3311)
7. D-Glucose (Sigma, G8769)
8. Dimethyl sulfoxide (DMSO) (Sigma, D2650)
9. Distilled water, embryo tested (Invitrogen, 15230-162)
10. Dulbecco's Modified Eagle's Medium (DMEM), (1×), liquid, with L-glutamine, 4500 mg/L D-glucose, without sodium pyruvate (Invitrogen, 12430-054)
11. Dulbecco's phosphate buffered saline without Ca²⁺ and Mg²⁺ (DPBS) 1×, liquid (Invitrogen 14190-144)
12. ES cell medium: DMEM supplemented with 15% fetal bovine serum, 1% non-essential amino acids, 1% penicillin/streptomycin, 1% L-glutamine, 0.1mM 2-mercaptoethanol, 1000 IU/ml LIF, Filter sterilize and store at 4°C up to 3-4 weeks.
13. ESGRO Complete Accutase (Millipore, SF006)
14. Ethylenediaminetetraacetic acid disodium (EDTA) (Sigma, ED2SS)
15. Fetal bovine serum (FBS) (Invitrogen, 12483-020)
16. Fluorinert FC-77 (Sigma, F4758)
17. Freezing medium: 90% serum plus 10% DMSO
18. Fusion buffer: 0.3M mannitol (Sigma, M-9546) containing 0.1mM MgCl₂, 0.05mM CaCl₂, 0.5mM HEPES and 0.1% BSA
19. Giemsa stain (SSS Reagent Company, Shanghai)
20. GlutaMAX-I Supplement (Invitrogen, 35050-061)
21. HEPES sodium salt (Sigma, H3784)
22. Human chorionic gonadotrophin (HCG) (Sansheng, Ningbo, 10000 IU): add 200ml 0.9% NaCl for 50 IU/ml aliquots and store at -20°C
23. KaryoMAX® colcemid® solution, liquid (10µg/ml) in DPBS (Invitrogen, 15212-012)
24. KSOM with / 1/2 amino acids, glucose and phenol red (Millipore, MR-121-D)
25. Leukaemia inhibitory factor (LIF) (Millipore, LIF2010)
26. M2 media (Sigma, M7167)
27. Methanol (Hengxing Chemical Preparation Company, Tianjin)
28. Mineral oil (Sigma, M8410)
29. Mitomycin C (Sigma, M-4287)
30. Mouse embryonic fibroblast (MEF) medium: DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin/streptomycin, 1%

L-glutamine, 0.1mM 2-mercaptoethanol. Filter sterilize and store at 4°C for up to 3-4 weeks.

31. Paraformaldehyde (Kermel, Tianjin)
32. Penicillin/Streptomycin (Invitrogen, 15140-122)
33. Pregnant mare serum gonadotrophin (PMSG) (Sansheng, Ningbo, and 5000 IU): add 100 ml 0.9% NaCl for 50 IU/ml aliquots and store at -20°C.
34. Protease (Sigma, P8811)
35. Tert-amyl alcohol (Sigma, 240486)
36. Triton X-100 (Sigma, T9284)
37. Trypsin-EDTA (0.05% with EDTA·4Na) (Invitrogen, 25300-054)
38. Y-27632 dihydrochloride monohydrate (Sigma, Y0503)

3. Methods

3.1 Isolation of mouse embryonic fibroblasts

1. Quick and humane sacrifice of pregnant female CD1 mice (E13.5) by cervical dislocation.
2. Dissect out uterine horns and transfer into 90mm dish.
3. Carefully remove fetuses from the uterus using forceps and scissors and rinse in DPBS (see Fig.1A).

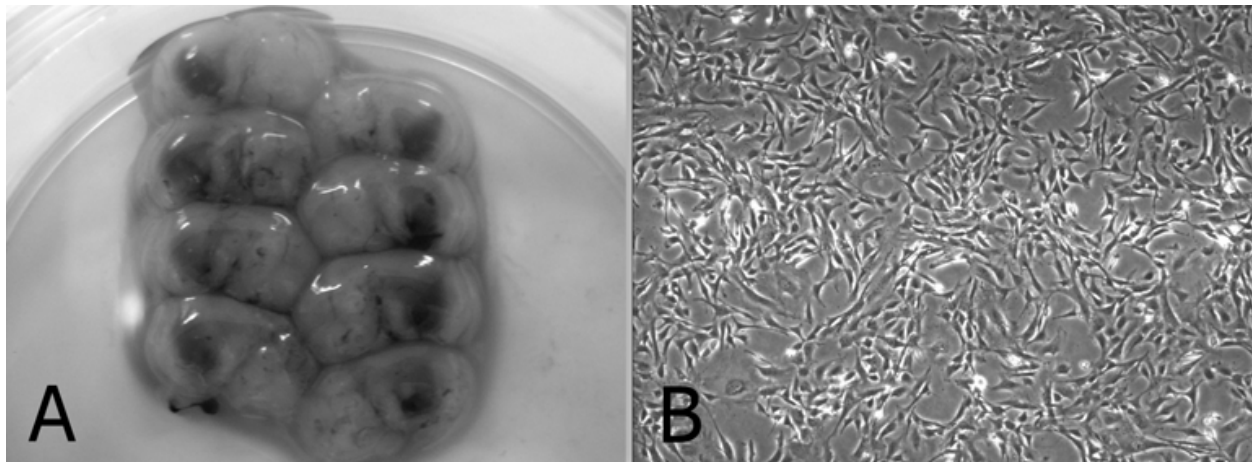


Fig. 1. E13.5 mouse fetuses (A) and primary mouse embryonic fibroblasts (B)

4. Cut away brain and dark red organs, wash with fresh DPBS, and remove as much blood clots as possible.
5. Place tissue into a 60mm dish. Using an elbow iris scissors finely mince the fetuses into 1mm³ pieces.
6. Wash with fresh DPBS three times.
7. Add 2ml of 0.05% trypsin/EDTA and incubate at 37°C for 5-10min.
8. Inactivate trypsin/EDTA by adding equal volume of MEF medium. Using a syringe pipette up and down several times to dissociate the tissue clumps into single cells.
9. Collect the cell suspensions in a fresh 15ml centrifuge tube and pellet cells by centrifugation at 200g for 5-10min.
10. Carefully take off the supernatant and resuspend cells in 1ml MEF medium.
11. Seed cells into approximately two 60mm dishes containing 6ml MEF medium. Incubate dishes in a 37°C incubator with 5% CO₂ for approximately 3h.

12. Change medium after cells attach.
13. Allow cells to grow until confluence (repeat step 6-10). Split the cells 1:3-4 (label these cells as P1) (see Fig.1B).
14. When cells reach confluence, repeat step 6-10. Resuspend cells in freezing medium and freeze each plate in three cryovials.
15. Transfer the cryovials to -80°C freezer, the next day transfer cells to a liquid nitrogen storage tank.

3.2 Mouse embryonic fibroblast feeder layer preparation

1. Remove cells from liquid nitrogen and thaw quickly in 37°C H_2O bath.
2. Transfer cells to a fresh 15ml centrifuge tube and pellet cells by centrifugation at 200g for 5-10min.
3. Carefully take off the supernatant and resuspend cells in 1ml MEF medium.
4. Seed cells into 60mm dish containing 6ml MEF medium.
5. Allow cells to grow until confluence, discard the medium and add 2ml MEF medium containing $10\mu\text{g}/\text{ml}$ mitomycin C.
6. Incubate dishes in a 37°C incubator with 5% CO_2 for 3h.
7. Carefully take off the medium and wash four times with DPBS (see Note 1).
8. Add 0.05% Trypsin/EDTA for 1min and then add an equal volume of MEF medium for inactivation.
9. Using mechanical force through pipetting, recover the cells from the dish, centrifuge and resuspend the cell pellet in MEF medium.
10. Seed 5×10^5 cells into a 35mm dish containing 2ml MEF medium.
11. Put dishes in a 37°C incubator with 5% CO_2 until use.

3.3 Isolation of blastocysts

1. Mouse embryonic fibroblast feeder layers should be changed into ES medium 2h prior to blastocyst isolation.
2. Quick and humane sacrifice of pregnant female C57BL/6 mice (E3.5) mated to 129/Sv by cervical dislocation.
3. Open the abdominal cavity, the uteri are removed by cutting across the cervix and are cut below the junction with the oviducts.
4. Place the uteri in a small volume of M2 medium in a 35mm dish and flush each horn with M2 medium.
5. Collect and wash blastocysts three times in M2 medium.
6. Transfer the blastocysts onto the prepared MEF feeder layer and culture at 37°C within a CO_2 incubator.

3.4 Isolation and dissociation of ICM outgrowth

1. After 4 to 5 days, gently circle the ICM outgrowths with a finely drawn glass probe, removing the ICM from the surrounding trophoblast cells.
2. Take a sterile non-coated Petri dish and add several small drops (30 μl) of DPBS and Accutase (see Note 2).
3. Transfer the ICM outgrowths to the drops of DPBS, and then repeat this procedure in the Accutase drops and incubate at 37°C for 15-20 min.

- Using a P200 pipette and yellow tip, transfer the ICM outgrowths to a small drop of ES cell medium and pipet outgrowths into small cell clumps of 5-10 cells using mechanical force (see Note 3).
- Transfer the cell clumps into 4-well plates with fresh MEF feeders and ES cell medium supplemented with 10 μ M Y-27632 (see Note 4).
- After 24h of culture, change replace the medium with normal ES cell medium without Y-27632.
- Change ES medium every other day, and observe the cultures. After 4 to 6 days ES cell colonies will appear (see Fig. 2).
- Definitive ES cell colonies are collected and dissociated into individual cells using the method above for ICM outgrowth dissociation. The cells are seeded again in 35mm dishes with fresh feeder layers. This is considered passage 1.

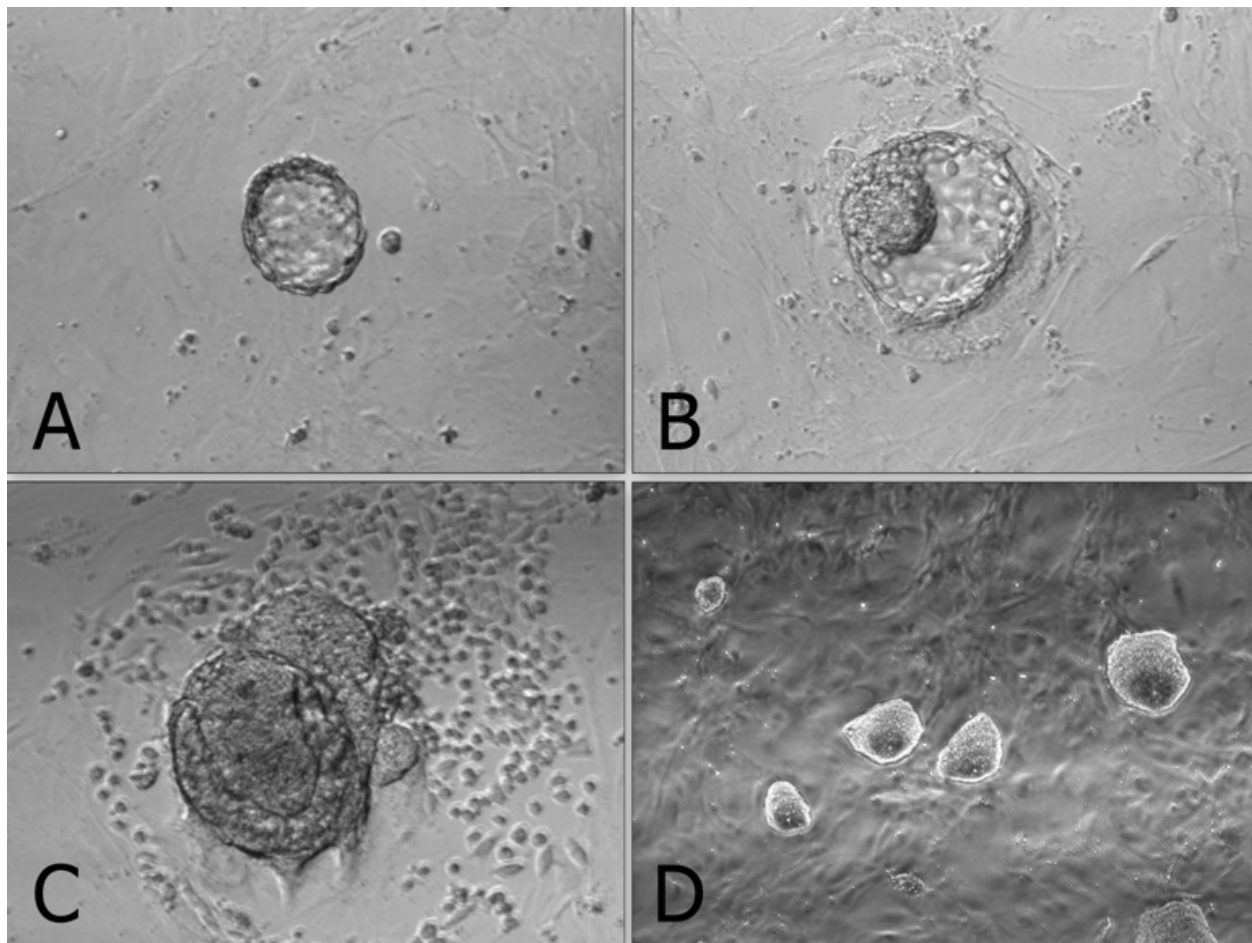


Fig. 2. Derivation of ES cells from mouse blastocysts. A. Blastocyst (E3.5). B. Blastocyst attach and spread on MEF feeder layer. C. Expanded ICM outgrowth, ready for the first dissociation. D. ES cell colonies.

3.5 Culture and passage of ES Cells

- When ES cell colonies become large and within proximity of other colonies it is necessary to passage
- Change fresh ES cell medium 2h prior to passaging.

3. Aspirate the old medium and wash the cells twice with DPBS.
4. Add appropriate amount of 0.05% trypsin/EDTA solution, and incubate for 1-2min at room temperature.
5. Once the cell colonies begin to detach, carefully remove trypsin/EDTA solution, and add 1ml ES cell medium, and further dissociate the detached ES colonies into single cell suspension by pipetting several times.
6. Adjust the concentration of ES cells, and transfer the suspensions into 35mm dishes at a rate of 1:5.

3.6 Freezing of ES Cells

1. Change fresh ES cell medium 2h prior to freezing.
2. Trypsinize cells and harvest as described earlier.
3. Collect the cells by centrifugation at 200g for 5min.
4. Remove the supernatant and resuspend the pellet in freezing medium.
5. Aseptically aliquot the suspension into sterile freezing vials, label each vial with the date and cell type/clone number, and place the vials into a thermos cup.
6. Freeze the cells overnight at -80°C , then transfer to the liquid nitrogen.

3.7 Thawing of ES cells

1. Remove a vial of frozen cells from the liquid nitrogen, and transfer to 37°C water bath.
2. Transfer cell suspension to a 15ml centrifuge tube, and add ES cell medium to 5ml.
3. Pellet cells by centrifugation at 200g for 5min.
4. Carefully take off the supernatant and resuspend cells in 1ml ES cell medium.
5. Plate ES cell suspension onto a prepared feeder layer in a 35mm dish at a suitable density.

3.8 Karyotyping of ES Cells

1. Add fresh ES cell media containing colcemid at a final concentration of $0.1\mu\text{g}/\text{ml}$ to an exponentially growing ES cell cultures. Return to the incubator for 40min.
2. Wash slides in fresh fixation solution (3:1 methanol : acetic acid) and then soak them in ice cold water until ready to use (distilled water plus some ice). It is important for the slides to be both cold and wet when ready for use.
3. Wash cells twice with DPBS. Completely dissociate colonies into single cell with trypsin. Add MEF media and resuspend the cells in a 15ml tube. Spin down at 200g for 10min. Remove the supernatant.
4. Add 1ml 0.56% KCl dropwise. Flick the tube to loose the pellet again to a single cell suspension (no big chunks). Add 9ml of 0.56% KCl and incubate for 15min.
5. Add 2-3 drops of fresh fixation solution to the tube, and invert it several times, Spin down the cells at 200g for 5min.
6. Remove the supernatant. Add 1ml fixation solution. Flick tube to resuspend pellet, add 9ml fixation solution. Spin down the cells at 200g for 5min.
7. Repeat step 6 three times and resuspend cells in appropriate fixation solution. Adjust cell density to $1 \times 10^6/\text{ml}$.
8. Remove slide from the water, blot edges to remove excess liquid and drop the cell suspension (dropwise) from at least one foot above the surface of the slide (2-3 drops/slide). Allow the cells spread and then place slides on heat stage (60°C) to speed

up drying). Prepare 3 or 4 slides for each sample. The remaining cells can be stored at -20°C for several years.

9. Stain slides with Giemsa solution for 15min. Rinse slides with running water.
10. Photograph chromosome spreads and count (see Fig. 3A)

3.9 Alkaline phosphatase stain

Staining for alkaline phosphatase should be performed at room temperature using an alkaline phosphatase detection kit (Boster Bio-Tech AR1023, Wuhan).

1. Aspirate the medium and wash the ES cell cultures twice with DPBS for 1min each.
2. Fix ES cells with 4% paraformaldehyde in DPBS at 4°C for 15min.
3. Remove paraformaldehyde and add freshly prepared alkaline phosphatase staining solution.
4. Keep reactions in the dark, and incubate at room temperature until desired degree of color development has occurred (This process usually takes 20-40min).
5. Terminate the color reaction by washing with running water.
6. Observe the images under an inverted microscope (see Fig. 3B).

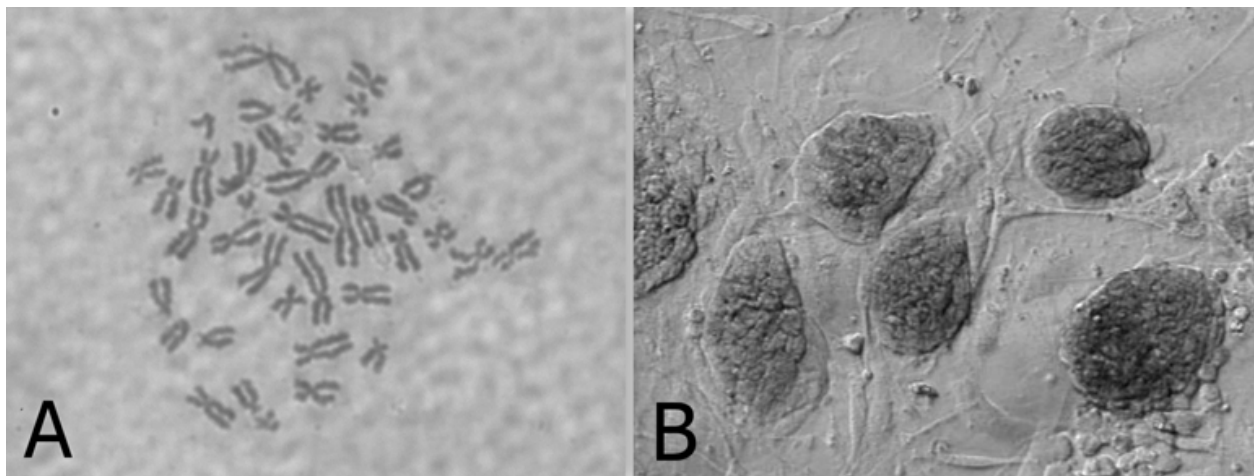


Fig. 3. A. Karyotype of ES cells. B. ES cell colonies with positive alkaline phosphatase activity.

3.10 Immunostaining of ES Cells

1. Seed ES cells on feeder layers in 4- well plate until near confluence.
2. Remove media and fix cells with 4% paraformaldehyde for 10min at room temperature.
3. Wash with DPBS for three times.
4. Add 0.2% Triton X-100 in DPBS for 5min, and then wash with DPBS for three times.
5. Add DPBS with 10% FBS for 30min.
6. Dilute primary antibodies (rabbit anti-oct-4 polyclonal IgG, rabbit anti-nanog polyclonal IgG, rabbit anti-sox-2 polyclonal IgG, mouse anti-ssea-1 monoclonal IgG) (Santa Cruz, sc-9081, sc-33760, sc-20088, sc-21702) at 1:200 dilution rate in DPBS with 10% FBS.
7. Add primary antibodies and incubate cells overnight at 4°C refrigerator.
8. Wash with DPBS three times.

9. Dilute second antibodies (donkey anti rabbit IgG-FITC, donkey anti mouse IgG-FITC) (Santa Cruz, sc-2090, sc-2099) at 1:500 dilution rate in DPBS with 10% FBS.
10. Add appropriate second antibodies into cells and incubate 2h at room temperature.
11. Wash with DPBS three times and observe under an inverted fluorescent microscope (see Fig. 4).

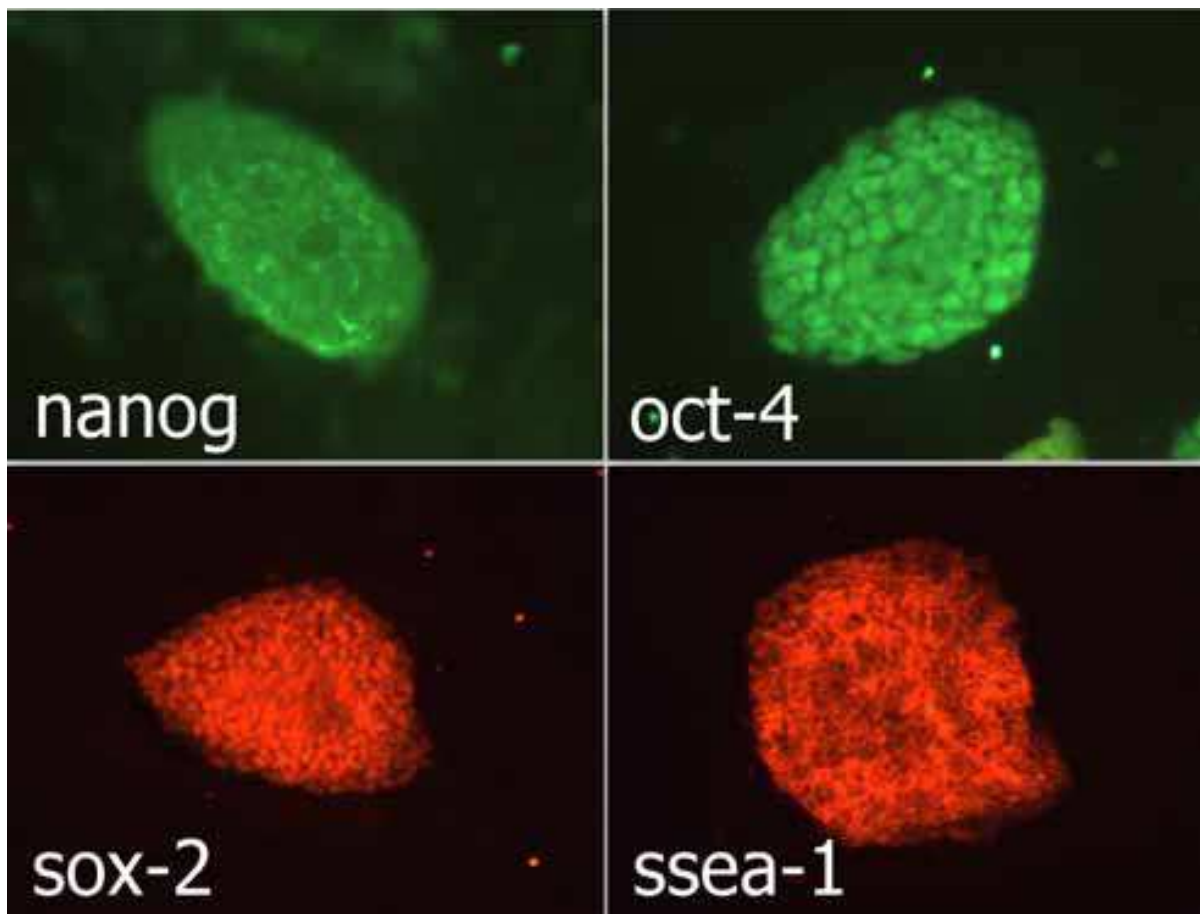


Fig. 4. Immunostaining of ES cell pluripotent markers (nanog, oct-4, sox-2, ssea-1).

3.11 Formation of embryoid bodies

1. Trypsinize and dissociate ES cell colonies into single cells.
2. Collect by centrifugation and resuspend the cells at a density of 5×10^5 cells/ml in MEF medium supplemented with $10 \mu\text{M}$ Y-27632.
3. Transfer 2ml cell suspension into a 35mm dish coated with 1% agar (see Note 5).
4. Culture overnight at incubator, carefully collect cell aggregates and transfer them into a new 35mm dish coated agar. Add 2ml MEF medium without Y-27632 for further culture.
5. After 3-5 days, many cystic embryoid bodies appear (see Fig.5).

3.12 Formation of teratoma

1. Pretreat ES colonies with $10 \mu\text{M}$ Y-27632 for 1h prior to dissociation.
2. Dissociate with Accutase, harvest the cells by centrifuge at 200g for 10min and remove the supernatant.

3. Add DPBS and resuspend cells to a final density of 2×10^6 cells/ml.
4. Subcutaneously inject 0.2 ml cell suspension into the CD1 mouse groin (see Note 6).
5. Teratoma will be observed approximately 6 weeks later (see Fig. 6).

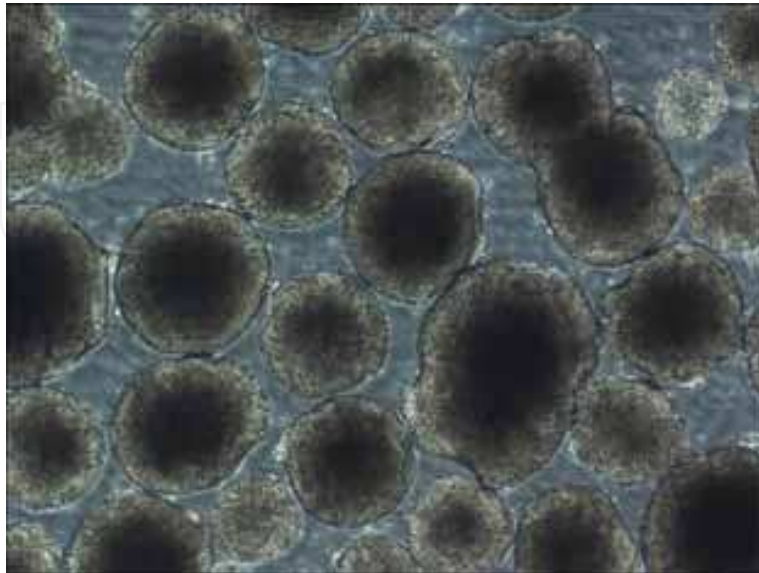


Fig. 5. Cystic embryoid bodies by ES cell suspension culture



Fig. 6. Formation of teratoma by injecting ES cells into CD1 mouse groin

3.13 Production of chimeric mice by aggregation with eight-cell stage embryos

1. Prepare several drops of KSOM culture medium containing $10 \mu\text{M}$ Y-27632 in a 35mm Petri dish and cover with mineral oil.
2. Sterilize a darning needle by dipping in ethanol. Make a series of 10 small depressions in each microdrop by pressing a darning needle into the Petri dish surface (see Note 7). Keep the plate in a 37°C incubator at 5% CO_2 .
3. Oviducts from pregnant female mice (E2.5) are flushed with M2 to collect eight-cell embryos.
4. Place several drops of M2, 0.5% protease and KSOM in 35mm Petri dish (do not mix).
5. Wash the embryos with three drops of protease medium and transfer them into drops of protease. Incubate embryos at 37°C for 5-7min.

6. Wash embryos three times with KSOM and when zona pellucida begins to disappear transfer them into drops of KSOM.
7. Transfer embryos into an aggregation plate (one embryo in each depression). Return plate to the incubator.
8. Pull a glass capillary tube using a pipette puller, this will be used for ES cell clump preparation.
9. Pick ES cell colonies from Petri dish and wash them three times with fresh DPBS.
10. Transfer colonies to trypsin/EDTA and incubate at 37°C for 3-5min.
11. Immediately transfer the colonies into a drop of KSOM and wash them three times.
12. Colonies should be dissociated into 15-20 cell clumps using a glass capillary tube and mechanical force.
13. Using a pipette, place one clump of ES cells into each depression.
14. Return the plate to incubator and culture overnight (see Fig. 7).
15. Transfer 8-10 aggregation blastocysts to each uterine horn of pseudopregnant CD1 females (2.5dpc). The female CD1 mouse will deliver approximately 17 days later.

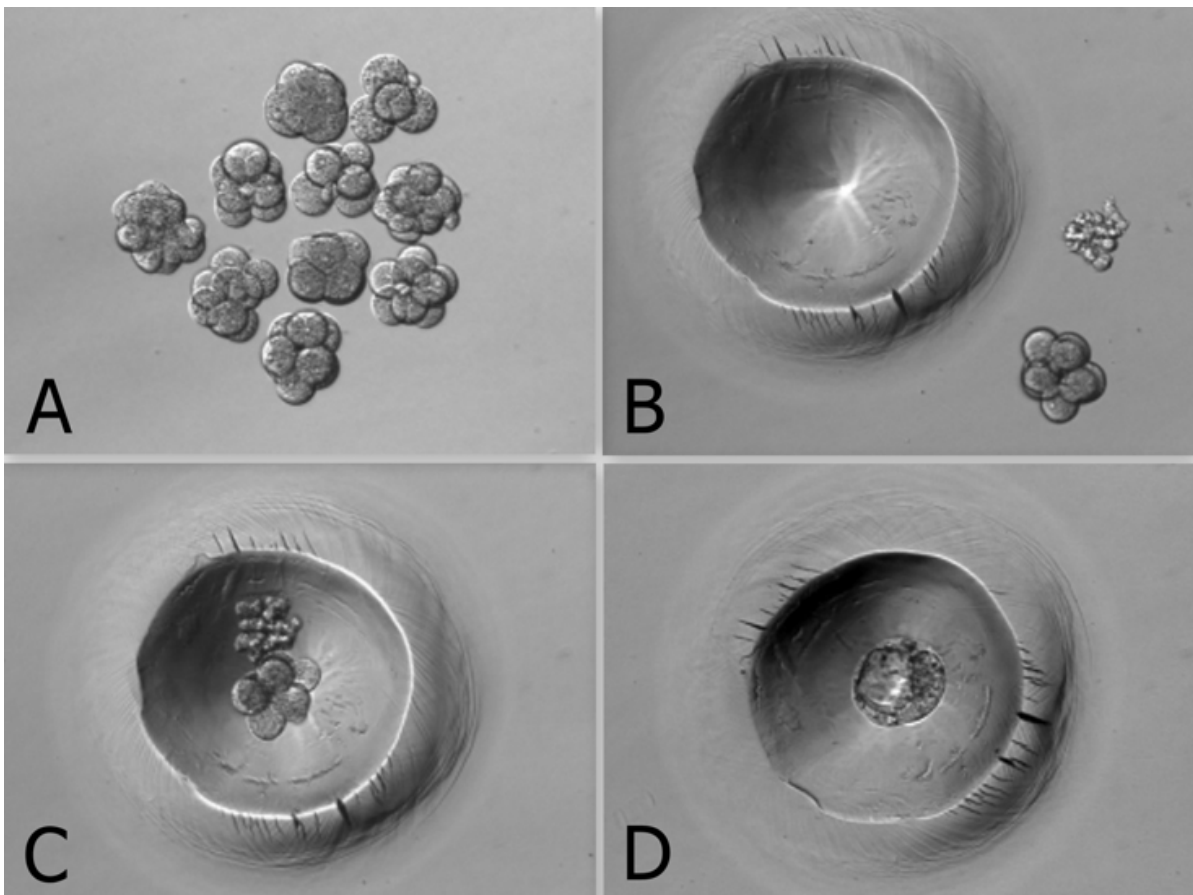


Fig. 7. Production of chimeras by aggregation. A. 8-cell embryos without zona pellucida. B, C. Aggregation depression, ES cells and embryos. D. Aggregate of ES cells and embryo

3.14 Production of chimeric mice by tetraploid blastocyst injection

Production of tetraploid mouse embryos

1. Collect 2-cell embryos (E1.5) of the CD1 outbred strain by flushing the oviducts and wash them using several drops of M2 medium.

2. Transfer them into KSOM drops and keep at incubator until electrofusion.
3. Wash 2-cell embryos through several drops of fusion buffer and place 20-30 embryos into the fusion buffer between the electrodes of the 0.2mm micro fusion chamber (see Fig. 8A).
4. Set Eppendorf Multiporator mode to electrofusion: 2V voltage and 15s duration for embryo prealignment; 20V voltage, 50 μ s duration and two pulses for fusion.
5. Transfer the embryos to the M2 drop and wash them through several drops of M2 medium.
6. Transfer the embryos into KSOM drops under mineral oil and culture at 37°C incubator.
7. In the next 30-60min, choose all properly fused embryos and incubate them in KSOM medium under mineral oil at 37°C, 5 % CO₂ (see Fig. 8B).
8. After 24h culture, most of embryos should develop to blastocyst stage and are ready to be injected with ES cells.

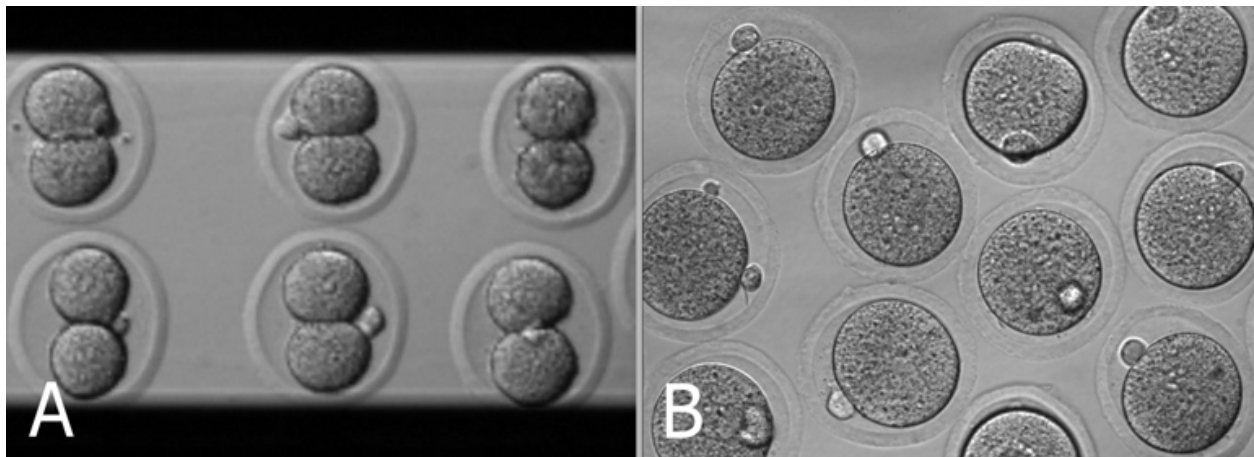


Fig. 8. A. 2-cell stage embryos aligned in fusion chamber. B. Fused embryos.

Preparation of ES cells for blastocyst injection

1. Replace fresh ES medium 3h prior to injection.
2. Pick ES cell colonies and rinse them with DPBS.
3. Transfer the colonies into Accutase drops and incubate at 37°C for 5 to 7min.
4. Using a P200 pipette, transfer the colonies to a small drop of ES medium with 10 μ M Y-27632, and break up the colonies into single cell suspension.
5. Incubate the single cell suspension with 10 μ M Y-27632 for 1h at room temperature prior to injection to remove cell membrane blebs (see Fig. 9) (see Note 8).

Microinjection needles preparation using a PN-30 micropipette puller and a MF-900 microforge

1. Turn on the machine and choose appropriate settings. For the PN-30 puller, use magnet (main) 90, magnet (sub) 30, and heater 80, as the starting values.
2. Fix one glass capillary (100mm) on the needle puller.
3. Start the pulling process of the needle.
4. Remove the needle carefully and check the tip under a stereomicroscope.
5. Mount a capillary in the microforge and break this at the required diameter by fusing the glass onto the glass bead on the microforge and turning off the heat while drawing it away.

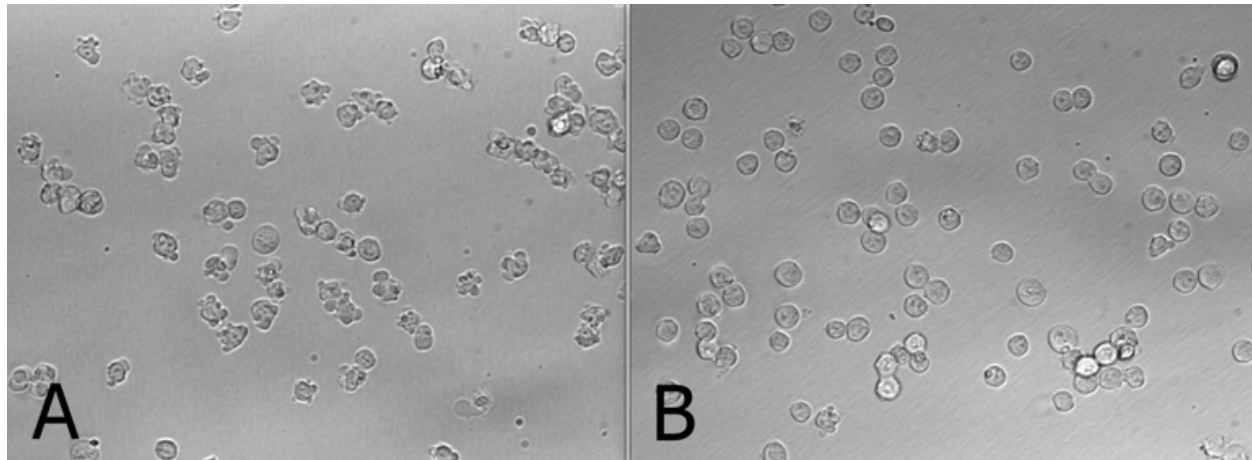


Fig. 9. Compare to the control cells without Y-27632 treatment (A), the treatment of Y-27632 make ES cells smooth and soft (B) so that they can easily be picked up by injection pipette.

6. For making holding pipets: break the holding pipet at a point where the outside diameter is approx $80\mu\text{m}$ and the inside diameter approx $60\mu\text{m}$, and then move the holding pipet toward the glowing glass bead, and the cut will start to melt inward creating a smoother, narrower tip.
7. For making injection pipets: break the injection pipet at a point where the outside diameter is approx $15\mu\text{m}$ and the inside diameter approx $12\mu\text{m}$.
8. Hold the pipet above the glass bead and heat a point about 5mm from the pipet tip. The pipet will bend under gravity as the glass melts. Allow the pipet to bend approx 60° .

Piezo-supported microinjection

1. Prepare dishes for injection using the lids of standard 35mm plastic disposable Petri dishes.
2. Place a $400\mu\text{l}$ drop of M2 medium on the lid and cover it with mineral oil.
3. Fluorinert FC-77 in a 15mm length was back-loaded into an injection pipette. Push Fluorinert FC-77 through the shoulder to near the tip to empty the air in the pipette
4. Attach the injection pipette to the pipette holder of the piezo unit, and hang the piezo unit on the micromanipulator, and aspirate 1mm M2 into the pipette to keep ES cells away from Fluorinert FC-77.
5. Attach the holding pipette on the other side of the micromanipulator.
6. Transfer embryos and ES cells into the drop of M2 under mineral oil.
7. Aspirate several ES cells, and use several piezo pulses (e.g., intensity=3, frequency=3) to penetrate the zona and trophectoderm layer (see Note 9).
8. Advance the tip of the pipette near the opposite side of the blastocysts from where it is held by the holding pipette.
9. Inject 15-20 ES cells into the blastocoel cavity (see Fig. 10A, B).
10. Culture injected blastocysts in KSOM medium at 37°C , 5 % CO_2 for 2h, and allow them recovery.
11. Transfer 8-10 injected blastocysts to each uterine horn of 2.5dpc pseudopregnant CD1 femals.
12. Recipient mice will give birth after 17 days (see Fig. 10C, D).

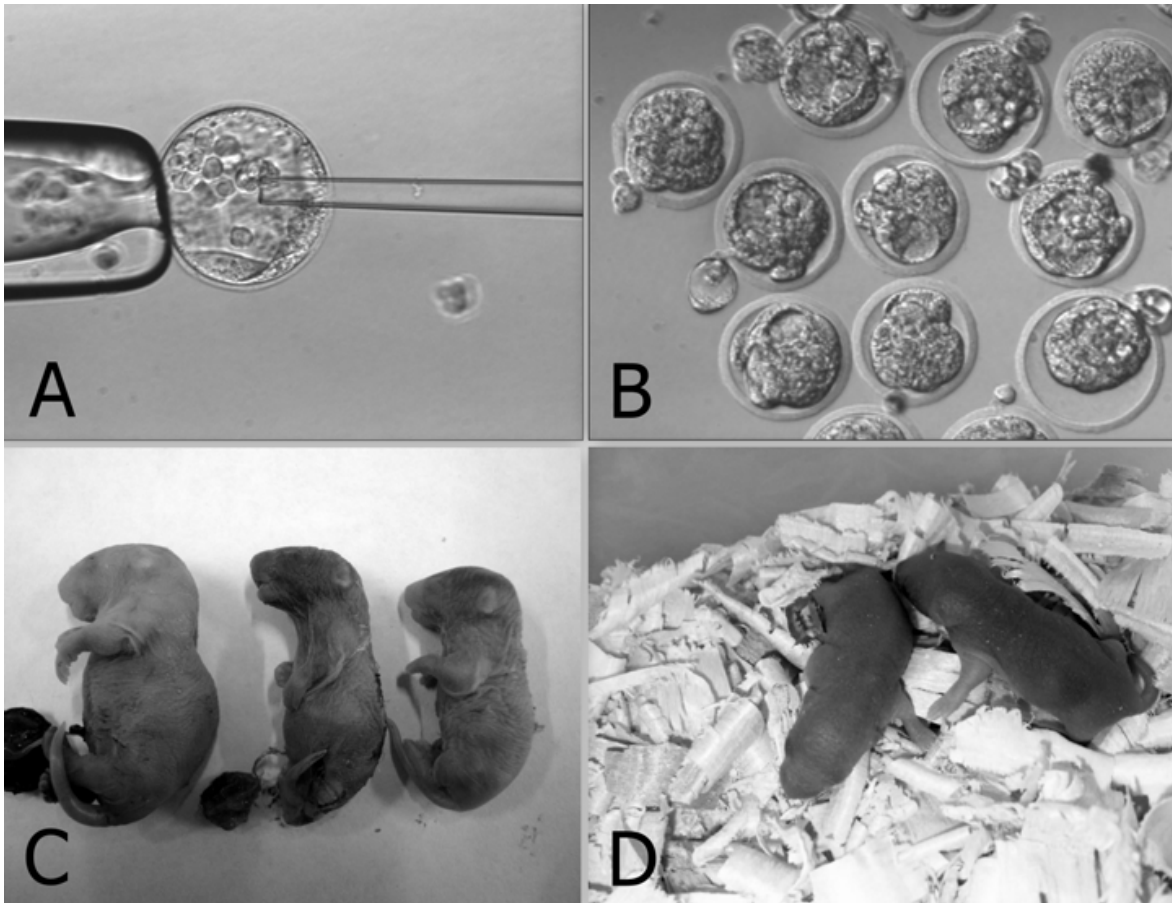


Fig. 10. A. Piezo-actuated tetraploid blastocyst injection. B. Injected tetraploid blastocysts. C. Dead ES mouse pups. D. Survival ES mouse pups.

3.15 Embryo transfer and Caesarean sections

1. Select a pseudopregnant CD1 female (2.5dpc) as an embryo recipient.
2. Prepare the transfer pipette, forceps and scissors etc.
3. Anesthetized mice by intraperitoneal injection of Avertin, after drug delivery quickly put the mouse back into the cage (see Note 10).
4. After the mouse is fully anesthetized place the mouse in the prone position on a lid of a Petri dish. Using an absorbent ball with 75% ethanol sterilize the area and shave the animal.
5. Make a 0.5-1cm transverse dorsal incision at the below of the last ribs.
6. Uncover incision to find ovarian fat pad, pull it out using forceps and hold it using clamps.
7. Collect the embryos carefully one by one lined up in the transfer pipette with as little medium in between as possible.
8. Make a hole through the uterine wall into the lumen with a 25G needle. The site of this hole should be near the last loop of the oviduct where it descends into the top of the uterine horn at the UT junction.
9. Insert the transfer pipette into a hole and carefully expel the embryos. Check the transfer pipette to ensure that all embryos have been transferred. 8-10 embryos are usually transferred into each horn.
10. When embryos are to be transferred into uteri, put the ovary back into the abdominal cavity and suture the incision.

11. Place the mouse in clean cage. Lighting to heat until it regains consciousness.
12. After 17 days, the pups will be born.

4. Notes

1. Mitomycin C (Sigma, M4287): Dissolve the powder into sterile distilled DMEM and adjust the concentration to 10 μ g/ml just before use. Wash layers several times with DPBS to completely remove any mitomycin C residue from the feeder layers.
2. Accutase is a ready to use cell detachment solution of proteolytic and collagenolytic enzymes, and it does not contain mammalian or bacterial derived products. Cell lines tested for Accutase application includes fibroblasts, keratinocytes, vascular smooth muscle cells, primary chick embryo neuronal cells, bone marrow stem cells, 293 cells, 3T3 cells, HeLa cells, insect cells, human embryonic stem cells and human neural stem cells. Moreover, Accutase is quite gentle on cells and will not induce cell death if cells are treated a little longer than the optimal time period. Thus, we use Acutase instead of trypsin for enzymatic dissociation of ICM.
3. This is a key step for ES cell isolation. Watch the whole dissociation process under a microscope. Careful not to pipette with too much force, as single cells will not survive. Compared to trypsin, Accutase treated ICM outgrowths are easier to dissociate into small cell clumps.
4. The ROCK inhibitor Y-27632 permits survival of dissociated human embryonic stem cells. Similar to human ES cells, we find that mouse ICM cells show sensitivity to trypsin. They undergo cell death after dissociation, and the cloning efficiency of dissociated ICM cells is generally very low. Our data indicates that increased cellular adhesion induced by Y-27632 enhances the survival of dissociated ICM cells. With more ES-like colonies appearing when the dissociated ICM cells are seeded in medium supplemented with 10 μ M Y-27632 for 24h. Moreover, we have not observed adverse effects of Y-27632 treatment on pluripotency in maintenance culture even after a number of passages.
5. Dishes are coated with agar to completely prevent cell attachment. This can increase cell aggregation and embryonic body formation. In brief, add 1g agar into 100ml DPBS and autoclave for 30min. Cool to 50°C at room temperature. Rapidly cover the internal wall and bottom of 35mm dishes with agar solution. Remove the extra solution and place dishes in hood until the remaining agar solidification. In addition, adding Y-27632 in the embryonic body medium can also enhance suspending cells to aggregate and embryonic body formation.
6. Typically, severe combined immunodeficient mice are used for making teratoma. This strain of mouse is expensive and not easily raised. Our data indicates that most mouse strains could be recipient for teratoma production by inguinal subcutaneous injection, such as CD1, C57BL/6 \times 129/Sv.
7. Carefully make small depressions using needle and appropriate force in the Petri dish surface. Too much force may break the dish and insufficient force will not produce the required depressions, which can easily lead to the loss of embryos and ES cells.
8. Prior to blastocyst injection, the pretreatment of 10 μ M Y-27632 for 1h at room temperature can alter the membrane architecture of ES cells so that they can be easily collected by injection pipet. Otherwise, the ES cell surface is covered with blebs (see Fig. 10).
9. Within the microinjection pipette, ES cells should be kept approximately 100 μ m away from the pipette tip. Damage to ES cells may occur if the cells are very close to the tip when the piezo pulses are applied.

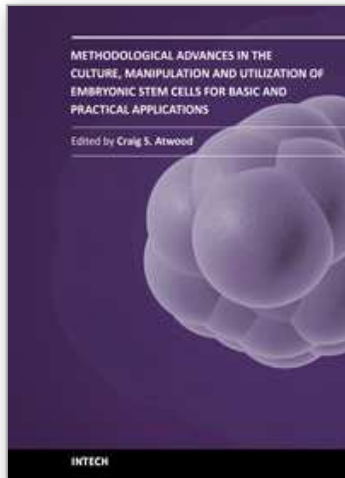
10. Avertin anesthetic: 1.25% stocks avertin is prepared by mixing 2.5g of 2, 2, 2-tribromoethyl alcohol (Sigma), 5ml of tert-amyl alcohol (Sigma) with 200ml water. Avertin is intraperitoneally injected at a dose of 0.02ml per gram of body weight for anesthetization.

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