

Epigenetic Reprogramming of Somatic Genomes by Electrofusion With Embryonic Stem Cells

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Summary

Cell fusion is an approach for combining genetic and epigenetic information between two different types of cells. Electrofusion for generating hybrid cells between mouse embryonic stem cells and somatic cells, which is a type of nonchemically induced and nonvirus-mediated cell fusion, is introduced here as a highly effective, reproducible, and biomedically safe *in vitro* system. Under optimized electrofusion conditions, cells are aligned and form pearl chains between electrodes in response to AC pulse stimulation, and subsequently adjacent cytoplasmic membranes are fused by DC pulse stimulation. Hybrid cells survive as drug-resistant colonies in selection medium. Cell fusion is a technique that is applied widely in the life sciences. A recent topic of great interest in the field of stem cell research is the successful production of cloned animals via epigenetic reprogramming of somatic nuclei. Interestingly, nuclear reprogramming for conferring pluripotency on somatic nuclei also occurs via cell fusion between pluripotential stem cells and somatic cells. Furthermore, it has been shown that spontaneous cell fusion contributes to generating the intrinsic plasticity of tissue stem cells. Cell fusion technology may make important contributions to the fields of regenerative medicine and epigenetic reprogramming.

Key Words: Cell fusion; hybrid cell; cell alignment; electrofusion; stem cell; ES cell; EG cell; reprogramming; epigenetics; cloning.

1. Introduction

Fertilization is one of the well-known cell fusion phenomena occurring *in vivo*. The union of two pronuclei from an MII oocyte and a sperm creates the next generation and endows it with genetic and epigenetic diversity. Genetically programmed spontaneous cell fusion also is known to occur in the formation of polykaryones, such as myotubes, osteoclasts, and syntrophoblasts *in vivo*. Remarkably, recent developments in the field of stem cell research have

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revealed that spontaneous cell fusion plays an important role in maintaining homeostasis of many tissues and organs via degeneration during defined processes of self-renewal and after tissue damage. The capability of spontaneous cell fusion has been shown by the generation of hybrid cells via in vitro co-culturing of mouse bone marrow cells and embryonic stem (ES) cells (1) and of mouse brain cells and ES cells (2,3). The in vivo contribution of spontaneous cell fusion to regeneration of tissues has been proven by the transdifferentiation of bone-marrow-derived hybrid cells into Purkinje neurons, cardiomyocytes, and hepatocytes (4–7).

A fascinating breakthrough in the area of stem cell research is the successful production of cloned animals via transplantation of committed somatic cell nuclei into enucleated unfertilized oocytes (8). This technique recently has been applied to generate human ES cells derived from cloned blastocysts (9). Nuclear reprogramming from the “somatic type” to the “pluripotential type” is induced by genome-wide epigenetic changes via the activity of *trans*-acting factors in unfertilized oocytes. Interestingly, using electrofusion between ES cells and adult somatic cells, Tada et al. (10) demonstrated that ES cells have an intrinsic capacity for epigenetic reprogramming of somatic genomes. In hybrid cells between ES cells and thymocytes, nuclear reprogramming of somatic genomes has been shown by (1) the successful contribution of the ES hybrid cells to the normal embryogenesis of chimeric embryos, (2) reactivation of the inactivated X chromosome derived from female thymocytes, (3) reactivation of pluripotential cell-specific genes (*Oct4* and *Tsix*) derived from somatic cells, (4) redifferentiation of a variety of cell types independent of the origin of thymocytes in teratomas, (5) tissue-specific gene expression from the reprogrammed somatic genomes after in vivo and in vitro differentiation, and (6) decondensed chromatin formation in the reprogrammed somatic nuclei as marked by histone-tail modifications of H3 and H4 hyperacetylation and H3 lysine 4 hypermethylation (10–13). Moreover, embryonic germ (EG) cells derived from gonadal primordial germ cells of mouse E11.5 and 12.5 embryos, in which most parental imprints are erased (14), possess additional potential for inducing the reprogramming of somatic cell-derived parental imprints accompanied by the demethylation of allele-specific DNA methylation in the EG hybrid cells (15). Therefore, electrofusion between pluripotential stem cells and somatic cells will contribute to elucidating the mechanisms of epigenetic reprogramming involved in DNA and chromatin modifications.

The technique of cell fusion pioneered in 1965 by Harris (16) has proven to be a powerful approach for analyzing biological interactions between differentiated cell types and for studying genomic plasticity (17–19). It has been shown that cell fusion caused by membrane fusion between two different cells is induced by treatment with various chemical agents, such as calcium ions, lyso-

lecithin, and polyethylene glycol and also by mediation with viruses such as paramyxoviruses, including Sendai virus (i.e., HVJ), oncornavirus, coronavirus, herpesvirus, and poxvirus. However, chemical and virus-mediated cell fusion has difficulties with respect to the efficiency of inducing cell fusion and also with respect to the reproducibility and biomedical safety when used for clinical applications. Since the 1980s, efforts have been made to optimize many parameters of electrofusion, resulting in the improvement of the fusion efficiency, which is higher than that of cell fusion with polyethylene glycol (20). However, the electrofusion parameters were optimized for making hybridomas for the purpose of stable production of antibodies.

In this chapter, we introduce an experimental procedure to create hybrid cells by electrofusion between pluripotential stem cells and somatic cells. Electrofusion is nonchemically induced and nonvirus-mediated cell fusion that presents many advantages in making hybrid cells: (1) the optimized conditions are reproducible in independent cell fusion experiments, (2) the electrofused cells are biomedically safer than the chemically or virus-induced fused cells, and (3) the microscale production of hybrid cells can be manipulated by using a Microslide chamber under a microscope without possible contamination with other cells. A description of the electrofusion procedure can be subdivided into several sections: (1) The setup of the Electro Cell Manipulator (ECM) 2001 (BTX), AC/DC Pulse Generator with a Microslide chamber; (2) cell culture conditions of ES and fused cells; (3) pretreatment of somatic cells; (4) operation of the electrofusion generator; (5) cell selection of cells after fusion; and (6) the isolation and cloning of fused cells. In the process of electrofusion, an alternative current (AC) induces alignment and compression of the cells, and a direct current (DC) transiently makes reversible pores in cytoplasmic membranes to initiate the process of fusing adjacent cytoplasmic membranes. The alignment voltage, pulse length, and electroporation voltage and number of DC pulses should be precisely controlled. The overall procedures are summarized in [Fig. 1](#). The following optimized conditions are suitable for electrofusion experiments between ES cells and thymic lymphocytes with the AC/DC pulse generator ECM 2001 (BTX).

2. Materials

2.1. Instruments

2.1.1. Cell Fusion

1. Electro Cell Manipulator ECM 2001 (BTX).
2. Microslide chambers with 1-mm electrode gap (BTX P/N450-1).
3. Micrograbber cables (BTX 464).
4. Inverted microscope with 10× and 20× objectives.

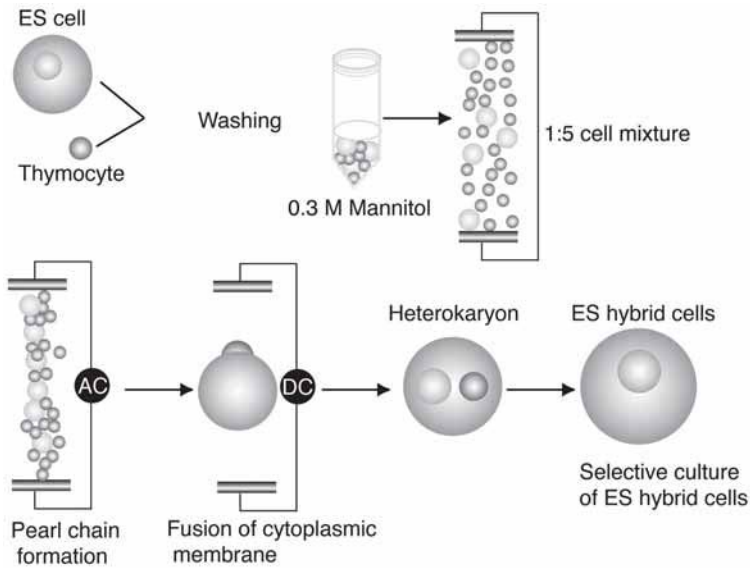


Fig 1. Scheme of electrofusion system. The cell mixture of ES cells and somatic cells suspended in nonelectrolyte 0.3 M mannitol is placed into the 1-mm gap between two electrodes fixed on a Microslide. Presettings of optimized AC voltage, AC duration, DC pulse voltage, and DC pulse number are required. The function of AUTOMATIC START in ECM 2001 initiates cell alignment through AC application and reversible breakage of cytoplasmic membranes by sequential DC electroporation pulse, leading to the fusion of cell membranes between two different cells. After the cell fusion procedure, ES cells and ES–ES hybrid cells are selectively killed using selection medium, while thymocytes are nonadherent. ES–somatic hybrid cells are selectively grown in the selection medium and isolated in independent culture dishes.

2.1.2. Cell Culture

1. Humidified incubator at 37°C, 5% CO₂ and 95% air.
2. 60-mm Plastic tissue culture dishes
3. 60-mm and 100-mm Bacterial dishes.
4. 10-mm and 30-mm Well plastic tissue culture plates.
5. 15-mL and 50-mL Conical tubes.
6. 0.2- μ m Microfilter.
7. 200- μ L and 1000- μ L Dispensable pipets with autoclaved tips.

2.1.3. Single-Cell Isolation of Adult Thymocytic Lymphocytes

For this procedure, a 2.5-mL syringe, 18-gage needle, and 50-mL conical tube are required.

2.2. Reagents

1. Dulbecco's modified Eagle's medium/nutrient mixture F12 HAM (DMEM/F12; cat. no. D6421, Sigma).
2. Dulbecco's modified Eagle's medium (DMEM; cat. no. D5796, Sigma)
3. Fetal bovine serum (FBS; cat. no. 12003-78P, JRH Biosciences).
4. Recombinant leukemia inhibitory factor (LIF; cat. no. ESG1107, Chemicon).
5. 200 mM Glutamine (cat. no. 320-5030A G, Gibco).
6. 2-Mercaptoethanol (cat. no. M7520, Sigma).
7. 10,000 U/mL Penicillin and 10 mg/mL streptomycin (penicillin–streptomycin 100X; cat. no. P-0781, Sigma).
8. 100 mM Sodium pyruvate (cat. no. S8636, Sigma).
9. 7.5% Sodium bicarbonate (cat. no. S8761, Sigma).
10. Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS; cat. no. 10010-023, Gibco).
11. 0.25% Trypsin/1 mM ethylene diaminetetraacetic acid · 4Na (cat. no. 25200-056, Gibco).
12. Mitomycin C (cat. no. M0503, Sigma).
13. Gelatin from porcine skin, Type A (cat. no. G-1890, Sigma).
14. D-mannitol (cat. no. M-9546, Sigma).
15. G418 (Geneticin; cat. no. G-9516, Sigma).
16. Hypoxanthine–aminopterin–thymine media supplement, 50X (HAT; cat. no. H0262, Sigma).
17. 70% Ethanol.

2.3. Solutions

1. PEFs medium: mix 500 mL of DMEM, 50 mL of FBS, 5 mL of 200 mM glutamine, 5 mL of 10,000 U/mL penicillin, and 10 mg/mL streptomycin. Store at 4°C.
2. ES medium: mix 500 mL of DMEM/F12, 75 mL of FBS, 5 mL of 200 mM glutamine, 5 mL of 100X penicillin–streptomycin, 5 mL of 100 mM sodium pyruvate, 8 mL of 7.5% sodium bicarbonate, 4 µL of 10⁻⁴ M 2-mercaptoethanol, and 50 µL of 10⁷ U/mL LIF (final 1000 U/mL). Store at 4°C.
3. 0.25% Trypsin/1 mM ethylenediaminetetraacetic acid · 4Na. Dispense into aliquots and store at –20°C.
4. Mitomycin C (MMC) Prepare solution at 0.2 mg/mL in PBS, dispense into aliquots and store at –20°C.
5. 0.1% Gelatin: dissolve 0.1 g of gelatin in 100 mL of distilled water. Sterilize by autoclaving and store at 4°C.
6. Fresh nonelectrolyte solution: 0.3 M mannitol. Dissolve 2.74 g of mannitol in 50 mL of distilled water. Filter through a 0.2-µm filter. Keep at 4°C.
7. ES medium with G418: dissolve antibiotic G418 in distilled water at 50 mg/mL. Sterilize through a 0.2-µm filter and store at 4°C. Add 50 µL of the G418 solution to 10 mL of ES medium to obtain a final concentration of 250 µg/mL.

8. ES medium with HAT: dissolve HAT media supplement supplied in a vial in 10 mL of 50X DMEM stock solution and store at -20°C . Each vial contains $5 \times 10^{-3} M$ hypoxanthine, $2 \times 10^{-5} M$ aminopterin, $8 \times 10^{-4} M$ thymidine. Add 200 μL of the stock solution to 10 mL of ES medium.

2.4. Cells and Animals

Adult mice, ES cells, and *neo^r* primary embryonic fibroblasts (PEFs) produced from E12.5 embryos of ROSA26 transgenic mice carrying the ubiquitously expressed *neo/lacZ* gene (21).

3. Methods

3.1. Setup of the Electro Cell Manipulator ECM 2001

3.1.1. Setup of AC/DC Pulse Generator, ECM 2001, and the Accessories

1. Place the ECM 2001 beside the inverted microscope (Fig. 2A).
2. Connect the Micrograbber cable to the output jacks on the back of the ECM2001.
3. Switch power on at the back.

3.1.2. Automatic Operating Parameters

Set the optimized electrical parameters to fuse ES cells and thymocytes (Fig. 2A,G).

1. AC: 10 V.
2. AC duration: approx 60 to 99 s.
3. DC: approx 250 to 300 V (280 V). Adjust DC voltage according to gap distance between electrodes. The appropriate electric field strength is approx 2.5 to 3.0 kV/cm. When 2-mm gap Microslides are used, the DC should be around 600 V.
4. DC pulse length: 10 μs .
5. Number of DC pulses: 1.
6. Post-Fusion AC: 8 s.

3.1.3. Setup of Microslide Chambers

1. Sterilize the Microslides by immersion in 70% ethanol followed by flaming.
2. Set a Microslide in a 100-mm plastic dish chamber made from a bacterial dish.
3. For each electrofusion, apply 40 μL of cell mixture into the 1-mm electrode gap on the Microslide.
4. Connect the Microslide with the Micrograbber cable to the ECM 2001 (Fig. 2B).
5. Place the chamber on an inverted microscope to check cell alignment and compression. It is important to determine the optimal fusion conditions under the microscope each time. The fusion conditions may vary depending on cell density and cell size.

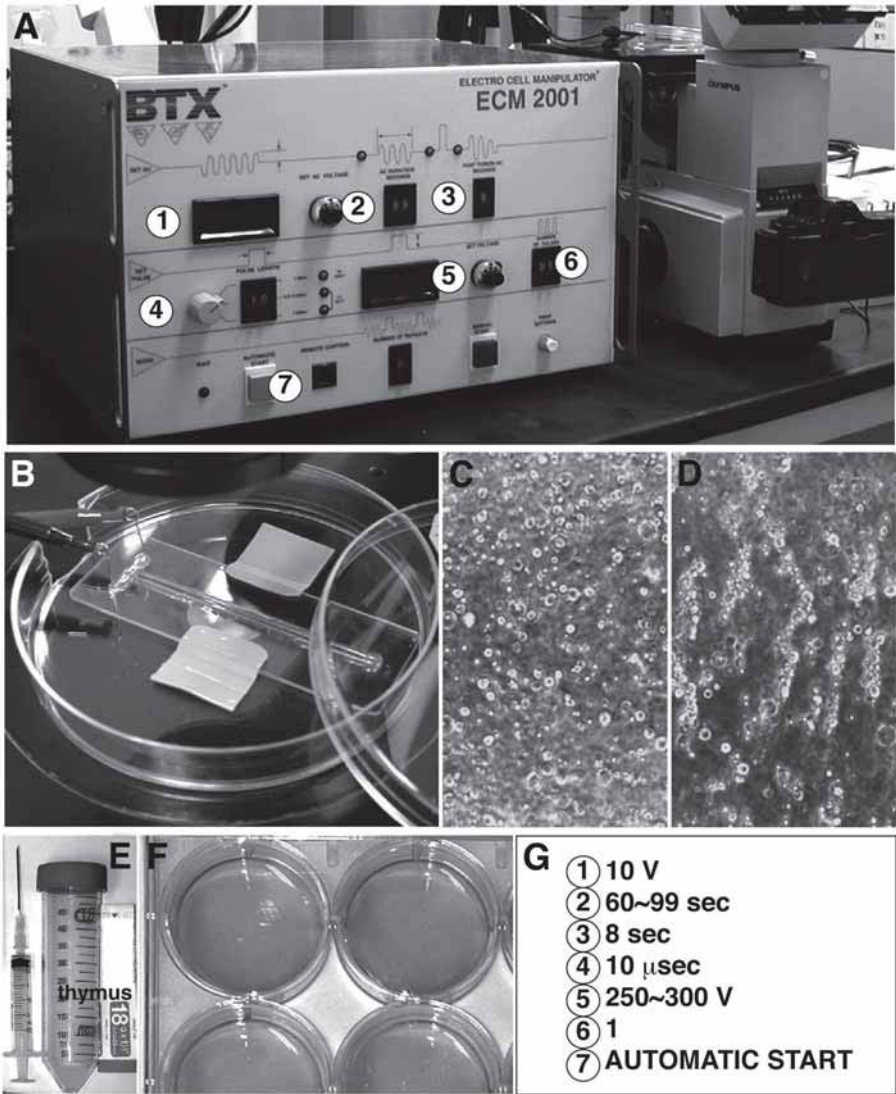


Fig 2. (A) Setup of AC/DC pulse generator ECM2001 (BTX) (B) Microslide chamber in a 100-mm plastic dish made using a bacterial dish and Micrograbber cable. (C) Cell mixture of ES cells and thymocytes applied between electrodes. (D) Pearl chain formation during AC application. (E) Instruments for dissociating mouse thymus into single cells. (F) Culture dishes with inactivated PEFs prepared 1 d before cell fusion. (G) The recommended fusion parameters.

3.2. Electroporation Protocol

3.2.1. Feeder Cells for ES Cells

1. Coat 60-mm culture dishes with 0.1% gelatin for at least 30 min at 37°C.
2. Incubate *neo^r* PEFs with 10 µg/mL MMC for 2 h at 37°C in a CO₂ incubator to produce mitotically inactivated feeder cells. Frozen stocks of MMC-treated PEFs are prepared at a cell concentration of 5×10^6 cells/mL/cryotube and are stored in liquid nitrogen. Inactivated *neo^r* PEFs are routinely used as feeder cells (1×10^6 cells/60-mm culture dish and 2.5×10^6 cells/100-mm culture dish) for culture of ES and hybrid cells, and also for selection of hybrid cell colonies with G418.

3.2.2 PEFs for Fused Cells

1. One day before cell fusion, coat 30-mm culture wells (6-well culture plate) with 0.1% gelatin for at least 30 min at 37°C.
2. Prepare inactivated PEFs (4×10^5 cells/ 30-mm well) in 3 mL of ES medium (Fig. 2F).

3.2.3. ES Cells

One of the most important requirements for cell fusion experiments is that the culture conditions have to be optimized for maintaining the pluripotential competence and full set of chromosomes (80 chromosomes) derived from mouse ES cells and somatic cells through numerous cell divisions. It is strongly recommended that one identify a satisfactory production lot of FBS that can supply supplements to support effective cell growth without differentiation induction.

1. Prepare exponentially growing ES cells cultured on the inactivated PEFs with changes of culture medium once or twice a day.
2. Ascertain that complete sets of chromosomes are maintained in the ES cells before cell fusion.

3.2.4. Somatic Cells

1. Sterilize all dissection instruments (scissors and forceps) by immersion in 70% ethanol, followed by flaming.
2. Sacrifice a 6- to 8-wk-old adult mouse humanely and dissect out the thymus in a clean room if a clean bench is not available.
3. Wash the tissues with sterilized PBS twice in 60-mm Petri dishes.
4. Place a half lobe of a thymus in the barrel of a sterile 2.5-mL syringe fitted with a sterile 18-gauge needle (Fig. 2E).
5. Expel and draw up the thymus gently through the needle via the tip of the needle several times into a 50-mL conical tube with 2 mL of DMEM to dissociate the thymus into a single-cell suspension.
6. Place for several minutes at room temperature.
7. Transfer the supernatant excluding cell clumps to a 15-mL conical tube and add 10 mL of DMEM.

8. Spin down the thymocytes in 15-mL conical tubes at $> 400g$ (1500 rpm) for 5 min.
9. Resuspend them in 10 mL of DMEM.

3.2.5 Purification of ES Cells

1. Coat a 60-mm culture dish with 0.1% gelatin for at least 30 min at 37°C.
2. Trypsinize the ES cells and remove excess trypsin quickly. Add 3.0 mL of ES medium to inactivate the trypsin and dissociate the cells into a single-cell suspension by gentle pipetting.
3. Plate them on a fresh gelatin-coated 60-mm culture dish.
4. Incubate the ES cells in a CO₂ incubator for 30 min to separate feeder cells from ES cells.
5. Collect unattached ES cells and harvest them by centrifugation at $> 400g$ (1500 rpm) for 5 min.
6. Resuspend the cell pellet in 10 mL of DMEM and transfer the cell suspension into a 15-mL conical tube.

3.2.6. ES–Somatic Cell Mixture in 0.3 M Mannitol

1. Spin down the ES cells and the thymocytes in 15-mL conical tubes at 1500 rpm for 5 min, separately.
2. Wash them with 10 mL of DMEM and spin down at 1500 rpm for 5 min and repeat again to remove FBS completely.
3. Add 5 to 10 mL of DMEM and adjust the density of ES cells and thymocytes to 1×10^6 cells/mL each.
4. Pellet a 1:5 mixture of ES cells and thymocytes (1 mL of ES cell suspension and 5 mL of thymocyte suspension). Keep the remaining cells for control experiments.
5. Spin down and resuspend the cell pellet in 0.3 M mannitol to the appropriate cell density of 6×10^6 cells/mL. Usually, 1 mL of the mixture of ES cells and thymocytes is sufficient for the following fusion experiment.
6. Use the cells for electrofusion immediately.

3.2.7. Operation of ECM2001 With Microslides

The automatic operation switch is used to initiate AC followed by DC. AC is used to induce a nonhomogeneous (or divergent) electric field, resulting in cell alignment and pearl chain formation. DC is used to produce reversible temporary pores in the cytoplasmic membranes. When juxtaposed pores in the physically associated cells reseal, cells have a chance to be hybridized by cytoplasmic membrane fusion. AC application after the DC pulse induces compression of the cells, which helps the process of fusion between the cell membranes.

1. Apply 40 μ L of cell mixture between 1-mm gap electrodes on the Microslide at room temperature (Fig. 2C).
2. Place the Microslide in the 100-mm plastic dish chamber and connect the cable.

3. Place the chamber on an inverted microscope to allow for observation of cell alignment (**Fig. 2D**).
4. Press the automatic operation switch of the ECM 2001.
5. Add 40 μL of DMEM to the fusion mixture between the electrodes to induce membrane reformation immediately after electroporation.
6. Leave the cell mixture for 10 min at room temperature.
7. Transfer the cell mixture directly to a 30-mm culture dish containing inactivated PEFs with 3 mL of ES medium supplemented with LIF (**Fig. 2F**).
8. Repeat the cell fusion procedure sequentially by using several Microslides. Usually, cell suspensions recovered from three Microslides (80 μL \times 3) are plated into one 30-mm culture dish. As a control, plate the untreated cell mixture and culture under the same conditions.
9. Incubate the cells at 37°C in a CO₂ incubator for 24 h.
10. Change the medium to ES medium with the proper supplement to select for ES hybrid cells 24 h after cell fusion.
11. Change the selection medium once a day.

As a result of the 7-d drug treatment, nonfused ES cells and hybrid cells derived from ES cells are killed, and hybrid cells derived from thymocytes are nonadherent. Thus, the hybrid cells derived from an ES cell and a somatic cell survive and form colonies. Several colonies of hybrid cells per 10⁴ host ES cells appear using the aforementioned procedures for electrofusion. *See Subheading 4.*

3.3. Isolation and Cloning of Hybrid Cells

We describe two independent chemical selection systems to select for hybrid cells between ES cells and somatic cells. Normal ES cells are hybridized with thymocytes containing the bacterial neomycin resistance (*neo*^r) gene (**10,15**). Thymocytes are derived from ROSA26 transgenic mice, which carry the ubiquitously expressed *neo/lacZ* transgene (**21**). Only ES–thymocyte hybrid cells can survive and grow in ES medium supplemented with the protein synthesis inhibitor G418. In this case, the ES hybrid cells and their derivatives are visualized by positive reaction with X-gal through β -galactosidase activity, which allows us to analyze their contribution to the development of chimeric embryos and tissues (**10,11,15**). In another selection system, male ES cells deficient for the *Hprt* gene on the X chromosome have been used for selecting hybrids with wild-type somatic cells. Electrofusion-treated cells are cultured in ES medium supplemented with HAT. The HAT medium is fatal to the *Hprt*-deficient ES cells, whereas ES hybrid cells, which are rescued by the thymocyte-derived wild-type *Hprt* gene, are able to survive and grow (**11–13**). In the synthesis of DNA, purine nucleotides can be synthesized by the *de novo* pathway and recycled by the salvage pathway. *Hprt* is a purine salvage enzyme

responsible for converting the purine degradation product, hypoxanthine, to inosine monophosphate, a precursor of ATP and GTP. In the presence of aminopterin, the *de novo* pathway is inhibited and only the salvage pathway functions. Consequently, dysfunction of *Hprt* induces cell death in culture with the HAT medium.

3.3.1. Selection With G418

1. Perform the automatic procedure for electrofusion of the mixture of normal ES cells and thymocytes collected from 6- to 8-wk-old ROSA26 transgenic mice carrying the *neo/lacZ* transgene.
2. Culture the electrofusion-treated cells in ES medium for 24 h.
3. Change the medium to ES medium supplemented with G418. ES hybrid cell colonies can be detected by 7 to 10 d.
4. Prepare a 24-well culture plate containing 1×10^5 inactivated *neo^r* PEFs per 10-mm well and 0.8 mL of ES medium supplemented with G418 for selection.
5. Pick up the colonies with a micropipette and transfer each colony into a 10-mm well of the 24-well culture plate on inactivated PEFs.
6. Subculture the colonies every 2 or 3 d and gradually expand the number of cells in 30-mm and 60-mm culture dishes with inactivated PEFs and ES medium. When the cells become nearly confluent in a 60-mm culture dish, we determine that a hybrid cell line is established at passage 1.
7. Analyze the karyotypes of the hybrid cells before using for further studies.

3.3.2. Selection With HAT

1. Use ES cells (XY) deficient for *Hprt* and normal thymocytes collected from 6- to 8-wk-old female (XX) or male (XY) mice.
2. Culture the electrofusion-treated cells in ES medium for 24 h.
3. Change the medium to ES medium supplemented with HAT. ES hybrid cell colonies can be detected by 7 to 10 d.
4. Analyze the karyotypes of the ES hybrid cells, which should be 80, XXXY or 80, XXYY.

4. Note

1. Successful formation of pearl chains during AC duration is extremely important for the efficiency of cell fusion. The pearl chain formation is influenced mainly by the following factors: (1) cell density in mannitol, (2) contamination by cell debris, and (3) contamination by serum or salts from cultured medium. To improve the conditions:
 - Pellet the mixed cells by centrifugation and resuspend the cells in a suitable amount of fresh 0.3 M mannitol.
 - Increase the cell density if pearl chains are formed poorly.
 - Decrease the cell density if cell movement is disturbed.

- The total cell volumes in 0.3 M mannitol have to be carefully controlled to obtain a smooth electric current. When other somatic cells larger than thymocytes and similar-sized with ES cells are used as a fusion partner, prepare a 1/1 cell mixture at 2×10^6 cells/mL.

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