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Nuclear Reprogramming of Somatic Nucleus Hybridized With Embryonic Stem Cells by Electrofusion

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Summary

Cell fusion is a powerful tool for understanding the molecular mechanisms of epigenetic reprogramming. In hybrid cells of somatic cells and pluripotential stem cells, including embryonic stem (ES) and embryonic germ cells, somatic nuclei acquire pluripotential competence. ES and embryonic germ cells retain intrinsic *trans* activity to induce epigenetic reprogramming. For generating hybrid cells, we have used the technique of electrofusion. Electrofusion is a highly effective, reproducible, and biomedically safe in vitro system. For successful cell fusion, two sequential steps of electric pulse stimulation are required for the alignment (pearl chain formation) of two different types of cells between electrodes in response to alternating current stimulation and for the fusion of cytoplasmic membranes by direct current stimulation. Optimal conditions for electrofusion with a pulse generator are introduced for ES and somatic cell fusion. Topics in the field of stem cell research include the successful production of cloned animals via the epigenetic reprogramming of somatic cells and contribution of spontaneous cell fusion to generating intrinsic plasticity of tissue stem cells. Cell fusion technology may make important contributions to the fields of epigenetic reprogramming and regenerative medicine.

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

1. Introduction

Cell fusion is a phenomenon combining genetic and epigenetic information between two different types of cells, resulting in the creation of a new cellular phenotype and function. Fused cells can grow as polynuclear cells, called heterokaryons. In other cases, one combined nucleus is formed through cell division after cytoplasmic membrane fusion. The phenotype of hybrid cells varies depending on the combination of parental cells for fusion. Specialized gene products from one of the parental cells are maintained; others are often extinguished via a tissue-specific repressive mechanism, a phenomenon called *extinction* (*1*). Thus, it has been believed that epigenetic information

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derived from parental cells coordinately regulates the new phenotypes and functions of somatic hybrid cells.

One well-known cell fusion phenomenon occurring in vivo is fertilization. The union of two pronuclei from an MII oocyte and sperm creates the next generation with genetic diversity. Soon after fertilization, sperm chromatin is reprogrammed by nucleoplasmin because of the exchange of sperm-specific basic proteins to histones *(2)*, almost resulting in the equalization of paternal and maternal chromatin through subsequent cell divisions. Cell fusion is also involved in the technique of animal cloning. The somatic donor nucleus is introduced into a recipient MII oocyte by cell fusion or direct injection. In the cloned embryos, the somatic type of cell phenotype and function is reprogrammed to the pluripotential type as a result of genomewide epigenetic modifications via the activity of *trans*-acting factors preserved in unfertilized oocytes. This phenomenon has been called *nuclear reprogramming*.

Using electrofusion between ES cells and adult somatic cells, it has been demonstrated that ES cells have an intrinsic capacity for the nuclear reprogramming of specialized somatic genomes *(3,4)*. In hybrid cells of ES cells and thymocytes, epigenetic modifications specific to somatic cells are fully reprogrammed to the ES epigenotype, as 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) tissue-specific gene expression from the reprogrammed somatic genomes after in vivo and in vitro differentiation, and (5) de-condensed chromatin formation in the reprogrammed somatic nuclei as marked by histone-tail modifications of H3 and H4 hyperacetylation and H3 lysine 4 hypermethylation *(3,5,6)*. Moreover, embryonic germ (EG) cells derived from gonadal primordial germ cells of mouse E11.5 and 12.5 embryos, in which the majority of parental imprints are erased *(7)*, possess additional activity for inducing reprogramming of parental imprints on somatic genomes accompanied by DNA demethylation in the EG hybrid cells *(8)*. Therefore, electrofusion between pluripotential stem cells and somatic cells will contribute to our understanding of the mechanisms of nuclear reprogramming involved in epigenetic modifications on DNA and chromatin.

Cell fusion was first induced by the Sendai virus (HVJ) among Ehrlich's ascites tumor cells, resulting in the formation of giant polynuclear cells *(9)*. Since Harris's group reported cell fusion between two different species, mouse and human, in 1965 *(10)*, the technique has proven to be a powerful approach to analyzing biological interactions between differentiated cell types. Various techniques lead to cell fusion mediated by other viruses such as Paramyxoviruses, including Oncornavirus, Coronavirus, Herpesvirus, and Poxvirus and by treatment with various chemical agents such as calcium ions, lysolecithin, and polyethylene glycol (PEG). However, virus- and chemical-mediated cell fusion has difficulties with respect to the efficiency of inducing cell fusion and reproducibility and biomedical safety for clinical applications. Since the 1980s, efforts have been made to optimize many parameters of electrofusion, resulting in improvements in fusion efficiency, which is now significantly better than that of cell

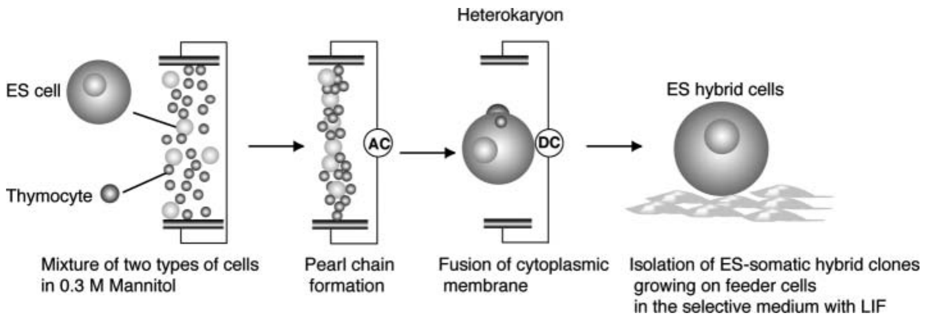

Fig. 1. Scheme of electrofusion system. The cell mixture of embryonic stem (ES) cells and somatic cells suspended in nonelectrolyte 0.3 *M* mannitol is applied into the 1-mm gap between two electrodes fixed on a Microslide. AC application initiates cell alignment and sequential DC electroporation pulse induces reversible breakage of cytoplasmic membranes, leading to the fusion of cell membranes between adjacent cells. After cell fusion treatment, only ES-somatic hybrid cells survive in the selection medium.

fusion with polyethylene glycol ***(11)***. The electrofusion parameters, however, were optimized for making hybridomas for the stable production of antibodies.

In this chapter, we introduce an electrofusion procedure to create hybrid cells from pluripotential stem cells and adult lymphocytes. The overall procedure is summarized (*see* **Fig. 1**). The electrofusion procedure is subdivided into several parts: (1) cell culture of ES and fused cells; (2) pretreatment of somatic cells; (3) setup of the Electro Cell Manipulator (ECM) 2001 (BTX), AC (alternating current)/DC (direct current) pulse generator with a Microslide chamber; (4) operation of the electrofusion generator; (5) selection of postfusion cells; and (6) isolation and cloning of fused cells. In the process of electrofusion, AC pulses induce the alignment and compression of the cells, and DC pulses transiently make revertible pores in cytoplasmic membranes to initiate the process of fusion among cells. The alignment voltage, pulse length, and electroporation voltage and the number of DC pulses should be precisely controlled.

After cell fusion, ES hybrid cells with somatic cells should be isolated with selection medium. Thus, genetically marked ES cells or somatic cells are needed for cell fusion experiments. For example, normal ES cells were hybridized with thymocytes derived from ROSA26 transgenic mice, which carry the ubiquitously expressed *neo/lacZ* transgene ***(12)***. Consequently, ES cells hybridized with the thymocytes can survive and grow only in medium supplemented with a protein synthesis inhibitor, G418. The ES hybrid cells and their derivatives are visualized as cells positive for X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining, which allows one to observe their contribution in chimeric embryos and tissues ***(3,5,8)***.

In another selection system, male ES cells deficient in the *Hprt* gene on the X chromosome are used for isolating ES hybrid cells with wild-type somatic cells. The somatic cell-derived *Hprt* gene rescues the HPRT activity in the hybrid cells. In DNA synthesis, the purine nucleotide can be synthesized by the *de novo* pathway and recycled by the salvage pathway. When electrofusion-treated cells are cultured in medium

with HAT (hypoxantine, aminopterin, and thymidine), the *de novo* pathway is interfered with, and only the salvage pathway functions. As a consequence, the *Hprt*-deficient ES cells die; ES hybrid cells are able to survive and grow (*5,6*).

To succeed in obtaining ES hybrid cells, we recommend that (1) genetic markers highly expressed in the undifferentiated state are used for isolating hybrid clones, (2) the origin of somatic cells is carefully chosen for creating undifferentiated hybrid cells, and (3) the culture condition is optimized for stably maintaining the pluripotential state of ES cells and ES hybrid cells.

2. Materials

2.1. Cells and Animals

1. Normal or *Hprt*-deficient ES cells.
2. Adult mice (6–8 wk old).
3. Neo^r primary embryonic fibroblasts (PEFs) produced from E12.5 embryos of ROSA26 transgenic mice carrying the ubiquitously expressed *neo/lacZ* gene.

2.2. Instruments

2.2.1. Cell Fusion

1. Electro Cell Manipulator ECM 2001 (BTX, Holliston, MA).
2. Microslide chambers with a 1-mm electrode gap (BTX, cat. no. P/N450-1).
3. Micrograbber cables (BTX, cat. no. 464).
4. 100-mm bacterial dishes.
5. Inverted microscope with ×10 and ×20 objectives.

2.2.2. Cell Culture

1. Humidified incubator at 37°C with 5% CO_2 and 95% air.
2. 60-mm plastic tissue culture dishes.
3. 10- and 30-mm well plastic tissue culture plates.
4. 15-mL conical tubes.
5. 0.2-µm microfilter.
6. 200- and 1000-µL disposable pipets with autoclaved tips.

2.2.3. Single-Cell Isolation of Adult Thymic Lymphocytes

1. Syringe (2.5-mL).
2. 18-gage needle.
3. 15- and 50-mL conical tubes.
4. 60-mm bacterial dishes.

2.3. Solutions

1. PEF medium: prepare the culture medium for PEFs in a 500-mL Dulbecco's modified Eagle's medium (DMEM) bottle and store it at 4°C. To 500 mL DMEM (Sigma, St. Louis, MO; cat. no. D5796), add 50 mL fetal bovine serum (FBS); 5 mL 200 m*M* glutamine (Sigma, cat. no. G7513); and 5 mL 10,000 IU/mL penicillin and 10 mg/mL streptomycin (100X penicillin-streptomycin) (Invitrogen, Carlsbad, CA; cat. no. 10378-016).
2. ES medium: prepare the culture medium for ES cells in a 500-mL DMEM/nutrient mixture F12 HAM (F12) bottle and store it at 4°C. To 500 mL DMEM/F12 (Sigma, cat. no. D6421),

add 75 mL FBS; 5 mL 200 m*M* glutamine; 5 mL 100X penicillin-streptomycin; 5 mL 100 m*M* sodium pyruvate (Sigma, cat. no. S8636); 8 mL 7.5% sodium bicarbonate (Sigma, cat. no. S8761); 4 µL 10^{-4} *M* 2-mercaptoethanol (Sigma, cat. no. M7522); and 50 µL 10^7 U/mL recombinant leukemia inhibitory factor (LIF; final 1000 U/mL) (Chemicon, Temecula, CA; cat. no. ESG1107).

3. 0.25% trypsin/1 m*M* ethylenediaminetetraacetic acid·4Na. Dispense 0.25% trypsin/1 mM ethylenediaminetetraacetic acid·4Na (Invitrogen, cat. no. 15090-046) into aliquots and store them at −20°C.
4. Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; Sigma; cat. no. D8537). Store at 4°C.
5. Mitomycin C (MMC; Sigma, cat. no. M4287). Prepare a 0.2-mg/mL MMC solution in PBS (Sigma, cat. no. D8537). Dispense aliquots to 1.5-mL tubes. Store at −20°C.
6. 0.1% gelatin: dissolve 0.1 g gelatin from porcine skin, type A (Sigma, cat. no. G1890) in 100 mL distilled water. Sterilize by autoclaving. Store at 4°C.
7. Fresh nonelectrolyte solution (0.3 *M* mannitol): dissolve 2.74 g D-mannitol (Sigma, cat. no. M9546) in 50 mL distilled water. Filter through a 0.2-µm filter. Keep at 4°C.
8. ES medium with G418: dissolve antibiotic G418 (Geneticin; Sigma, cat. no. A1720) in distilled water at 50 mg/mL. Sterilize through a 0.2-µm filter. Store at 4°C or −20°C. Add 50 µL of the G418 solution to 10 mL ES medium to obtain a final concentration of 250 µg/mL.
9. ES medium with HAT: dissolve HAT media supplement (Sigma, cat. no. H0262) supplied in a vial with 10 mL DMEM (50X stock solution) (*see* **Note 1**). Store at −20°C. Add 200 µL stock solution in 10 mL ES medium.

3. Methods

3.1. Electrofusion

3.1.1. Feeder Cells for ES Cells

Inactivated *neo*^{r} PEFs are routinely used as feeder cells for culture of ES and hybrid cells and for selection of hybrid cell colonies with G418. Use 4×10^5 feeder cells/30-mm culture dish, 1×10^6 feeder cells/60-mm culture dish, and 2.5×10^6 feeder cells/100-mm culture dish.

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 the CO_2 incubator.
3. Prepare the frozen stock of MMC-treated feeder cells at a cell concentration of 5×10^6 cells/mL for each cryotube.
4. Store in liquid nitrogen.

3.1.2. 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 ES medium.

3.1.3. ES Cells

One of the most important factors for cell fusion experiments is that the culture conditions 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 (*see* **Note 2**).

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.

3.1.4. 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 the half lobe of thymus in the barrel of a sterile 2.5-mL syringe with a sterile 18-gage needle.
5. Expel and draw up the thymus gently through the needle via the tip of the needle several times in a 50-mL conical tube with 2 mL DMEM to dissociate the thymus into a single-cell suspension.
6. Keep for several min at room temperature.
7. Transfer the supernatant excluding cell clumps to a 15-mL conical tube and add 10 mL DMEM.
8. Spin down the thymocytes in 15-mL conical tubes at 300*g* for 5 min.
9. Resuspend the thymocytes in 10 mL DMEM.

3.1.5. Purification of ES Cells

1. Coat 60-mm culture dishes with 0.1% gelatin for at least 30 min at 37°C.
2. Trypsinize the ES cells and remove excess trypsin quickly.
3. Add 3 mL ES medium to inactivate the trypsin and dissociate the cells into a single-cell suspension by gentle pipetting.
4. Plate the cells on a fresh gelatin-coated 60-mm culture dish.
5. Incubate the ES cells in the CO_2 incubator for 30 min to separate feeder cells from ES cells.
6. Collect unattached ES cells and harvest them by centrifugation at 300*g* for 5 min.
7. Resuspend the cell pellet in 10 mL DMEM and transfer the cell suspension into a 15-mL conical tube.

3.1.6. ES-Somatic Cell Mixture in 0.3 M Mannitol

1. Spin down the ES cells and the thymocytes in 15-mL conical tubes at 300*g* for 5 min, separately.
2. Wash the cells with 10 mL DMEM and spin them down at 300*g* for 5 min and repeat to remove FBS completely.
3. Add 5–10 mL DMEM and adjust the density of ES cells and thymocytes to 1×10^6 cells/mL.
4. Pellet a 1:5 mixture of ES cells and thymocytes (1 mL ES cell suspension and 5 mL 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 density of 6×10^6 cells/mL (*see* **Note 3**). Use them for electrofusion immediately.

3.2. Setup of Electro Cell Manipulator ECM 2001

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

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

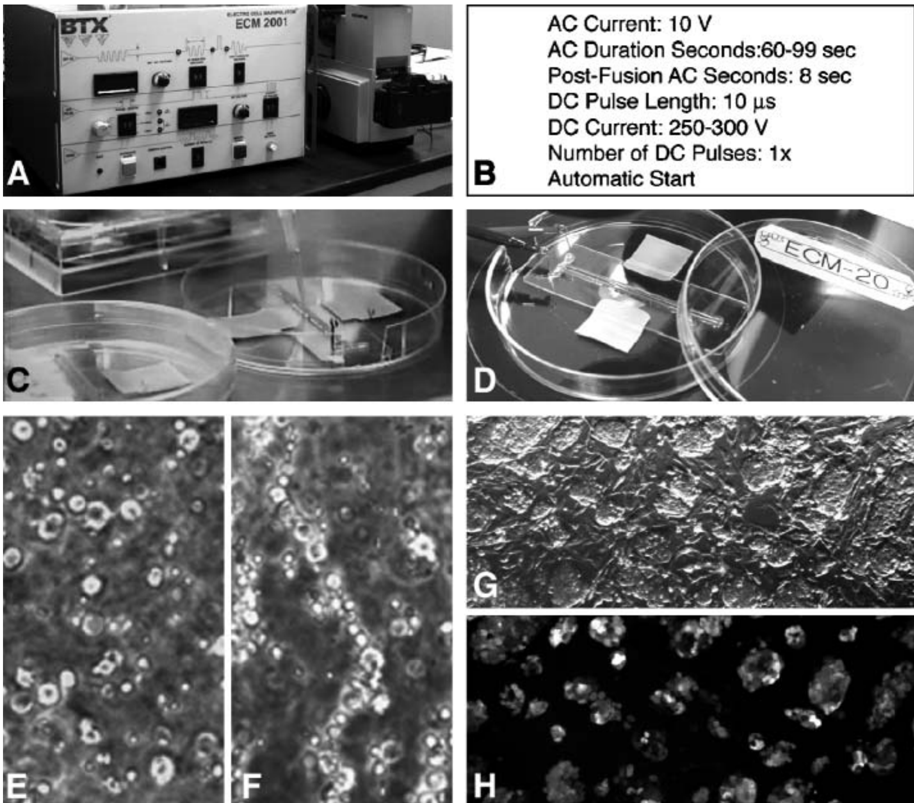

Fig. 2. (**A**) Setup of AC/DC pulse generator Electro Cell Manipulator 2001 (BTX). (**B**) The fusion parameters recommended. (**C**) Cell mixture of embryonic stem (ES) cells and thymocytes applied between electrodes using a micropipet. (**D**) Microslide chamber in the 100-mm plastic dish made with the bacterial dish and Micrograbber cable. Cell mixture (**E**) before AC application and (**F**) during AC pulse stimulation. (**G**) ES hybrid cells cultured on inactivated PEFs expressing the *Oct4-GFP* transgene derived from thymocytes (**H**).

3.2.2. Automatic Operating Parameters

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

1. 10 V AC.
2. 60- to 90-s AC duration.
3. 250–300 V (280 V) DC. Adjust DC voltage according to the gap distance between electrodes. Appropriate electric field strength is 2.5–3.0 kV/cm.
4. 10-µs DC pulse length.
5. Number of DC pulses: one.
6. AC postfusion for 8 s.

3.2.3. 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. Apply 40 µL cell mixture into the 1-mm electrode gap on the Microslide at room temperature (*see* **Fig. 2C**).
4. Connect the Microslide with the Micrograbber cable to the ECM 2001 (*see* **Fig. 2D**).
5. Place the chamber on an inverted microscope to allow observation of cell alignment (*see* **Note 4**).

3.3. Operation of ECM 2001 With Microslides

The automatic operation switch is used to initiate AC followed by DC. AC is utilized to induce a nonhomogeneous electric field, resulting in cell alignment and pearl chain formation (*see* **Fig. 2E,F**) (*see* **Note 5**). DC is utilized 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 the compression of cells, which helps the process of fusion between the cell membranes.

1. Press the automatic operation switch of the ECM 2001.
2. Add 40 µL DMEM to the fusion mixture between electrodes to induce recovery of the membrane reformation immediately after electroporation.
3. Leave the cell mixture for 10 min at room temperature.
4. Transfer the cell mixture directly to a 30-mm culture dish containing inactivated PEFs with 3 mL ES medium with LIF.
5. Repeat the cell fusion procedure sequentially using several Microslides. Usually, the cell suspension recovered from three Microslides (80 µL × 3) is plated into one 30-mm culture dish. As a control, plate the untreated cell mixture and culture it under the same conditions.
6. Incubate the cells at 37°C in the CO_2 incubator for 24 h.
7. Change the medium to ES medium with the proper supplement to select ES hybrid cells 24 h after cell fusion.
8. Change the selection medium once a day.

As a result of the 7-d treatment, nonfused ES cells and hybrid cells derived from ES cells are killed, and thymocytes are nonadherent. Thus, the hybrid cells of ES cells and somatic cells survive and form colonies. Several colonies of hybrid cells per 10^4 host ES cells appear using the previously mentioned procedures for electrofusion.

3.4. Isolation and Cloning of Hybrid Cells

3.4.1. Selection With G418

1. Perform the automatic operation of electrofusion of the mixture of normal ES cells and thymocytes collected from the 6- to 8-wk-old ROSA26 transgenic mice carrying the ubiquitously expressed *neo/lacZ* transgene.
2. Culture the electrofusion-treated cells in ES medium for 24 h.
3. Change the medium to the ES medium supplemented with G418. ES hybrid cell colonies can be detected at 7–10 d.

4. Prepare a 24-well culture plate containing 1×10^5 inactivated neo^r PEFs per 10-mm well and 0.8 mL ES medium with G418 supplement for selection.
5. Pick up the colonies with a micropipet and transfer each colony into the 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- and 60-mm culture dishes with ES medium on inactivated PEFs (*see* **Fig. 2G,H**) (*see* **Note 6**).
7. Analyze the karyotypes of the hybrid cells before conducting further studies.

3.4.2. Selection With HAT

1. Use ES cells deficient in *Hprt* and normal thymocytes.
2. Culture the electrofusion-treated cells in ES medium for 24 h.
3. Change the medium to the ES medium with HAT supplement. ES hybrid cell colonies can be detected at 7–10 d.

4. Notes

1. Each vial of HAT media supplement contains 5×10^{-3} *M* hypoxantine, 2×10^{-5} *M* aminopterin, and 8×10^{-4} *M* thymidine.
2. It is strongly recommended that one identify a satisfactory production lot of FBS that can supply supplements to support more effective cell growth without inducing differentiation.
3. Usually, a 1 mL mixture of ES cells and thymocytes is sufficient for the fusion experiment.
4. It is important to determine optimal fusion conditions under the microscope each time.
5. The successful formation of pearl chains during the AC pulse stimulation is extremely important for the efficiency of cell fusion. The pearl chain formation is mainly influenced by cell density and contamination from cell debris, serum, or salts in mannitol buffer. Do the following to improve the conditions:
   a. Pellet the mixed cells by centrifugation and resuspend the cells in a suitable amount of fresh 0.3 *M* mannitol.
   b. Increase the cell density if pearl chains are poorly formed.
   c. Decrease the cell density if cell movement is disturbed.
   d. 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 in size to ES cells are used as a fusion partner, prepare a 1:1 cell mixture at 2×10^6 cells/mL.
6. When cells become nearly confluent in the 60-mm culture dish, we determine that a hybrid cell line is established as the first passage.

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