Chapter 2

A New Chemical Approach to the Efficient Generation of Mouse Embryonic Stem Cells

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Abstract

Here, we present a highly efficient and reproducible method for the establishment of mouse embryonic stem cells (mESCs) from embryonic day 3.5 (E3.5) whole blastocysts. This protocol involves the use of small molecules SB431542 and PD0325901, which inhibit transforming growth factor-β (TGF-β) and extracellular signal-regulated kinases (ERK1/2), respectively. This protocol is universal in the derivation of mESC lines from NMRI, C57BL/6, BALB/c, DBA/2, and FVB/N strains, which have previously been considered refractory or non-permissive for ESC establishment. The efficiency of mESC lines generation is 100%, regardless of genetic background.

Key words Mouse, Embryonic stem cells derivation, Small molecule, ERK, TGF-β

1 Introduction

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass (ICM) of murine embryos and have the long term capability to self-renew in culture conditions as well as differentiate into derivatives of three embryonic germ layers by chimera or teratoma formation (1, 2). These potential capabilities make mESCs the best model for studying early mammalian developmental events, particularly by knocking out various genes and making germ-line chimeras (3). Furthermore, in recent years, extensive efforts have been undertaken with mESCs to develop efficient differentiation protocols for the derivatives of the three embryonic germ layers to be potentially used in regenerative medicine.

Another area of research interest in the field of mESCs involves understanding the molecular basis of pluripotency, particularly because of the considerable properties these cells have as naïve pluripotent stem cells (4). Naïve mESCs by having a highly efficient clonogenicity from single cells, lower rate of doubling time, efficient contribution to chimeric embryos, and the absence of differentiation

Uma Lakshmipathy and Mohan C. Vemuri (eds.), *Pluripotent Stem Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 997, DOI 10.1007/978-1-62703-348-0_2,

bias have demonstrated the ground state of pluripotency (4, 5). Some reports have been published that converted human ESCs (hESCs) as primed stem cells into their naïve state in order to resolve their sensitivity to single cell dissociation or to prevail over their developmental propensity among different lines. These initial attempts, however, have been unable to produce long-term maintenance of naïve hESCs without the expression of ectopic transgenes (6). Therefore, more research is necessary to recognize the mechanisms of naïve pluripotency.

We have recently reported a new, highly efficient route for the generation of mESCs from different mouse strains such as NMRI, BALB/c, C57BL/6, DBA/2, and FVB/N which have previously been considered as refractory or non-permissive (7). We have shown that the dual inhibition of ERK and TGF-β signaling pathways by using a combination of PD0325901 (PD03) and SB431542 (SB43) small molecules, respectively, during the line derivation procedure could overcome observed differences between various strains. Recent publications show that the use of PD032 with GSK3 inhibitor CHIR99021 (CHIR) could support the derivation and maintenance of the pluripotency ground state in different strains (8). However, CHIR or other GSK3 inhibitors induce chromosomal instability (9, 10). It seems that inhibition of TGF-β which induce differentiation in mESCs by an autocrine process has preference over the inhibition of the more complicated GSK3. When compared to the other available protocols for the derivation of mESCs, our protocol differs as follows: a new signaling inhibition by small molecules, technical simplicity, high efficiency and reproducibility, and universality for various genetic backgrounds.

2 Materials

2.1 Chemicals

All reagents and materials should be sterile.

1. EGTA.
2. L-glutamine, 100× (i.e., 200 mM), Store aliquots at −20°C. Use 1:100.
3. Mineral oil.
4. Ethanol 70% (v/v).
5. Mouse fetus, embryonic day 12.5 (E12.5) or E13.5 mouse embryos from NMRI mouse strain.
6. Sodium bicarbonate.
7. Ultrapure deionized water.

2.2 Disposables

1. 15 and 50 ml conical tubes.
2. Plastic disposable pipettes (5, 10, 25 ml).

3. 0.22-μm vacuum filtration (500 ml).
4. 0.22-μm pore size filter.
5. Glass Pasteur pipettes, 9 in. Sterilize by autoclave.
6. Center-well plate.
7. 24-well plates.
8. 12-well plates.
9. 6-well plates.
10. Flask T-25.
11. Flask T-75.
12. 60 mm non-adhesive bacterial plate.
13. Syringes (1, 5, 20, 50 ml).
14. Filter pipette tips (0.5–10, 5–100, 50–1,000 μl).
15. Cryovials.

2.3 Equipment

1. Stereomicroscope.
2. Inverted phase contrast microscope (4, 10, 20, and 40× objectives).
3. Micropipette (1–10, 10–100, 100–1,000 μl)
4. Pipettor.
5. Laminar flow hood (Class I & II).
6. Hemocytometer.
7. Tissue culture incubator, with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO2 in air.
8. Water bath.
9. Liquid nitrogen tank.
10. Foot-operated Venturi pump.
11. Cryovial storage rack.
12. Freezers –20°C and –70°C.

2.4 Reagent Setup

1. *LIF solution*: Dilute 10^7°U/ml Leukemia inhibitory factor (LIF) ten times Phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+} (PBS^-). Store in 1 ml aliquots at –20°C (even though Chemicon recommends storage at 4°C).
2. *β-Mercaptoethanol*: Add 70 μl β-mercaptoethanol in 10 ml PBS– to 100 mM (1,000×). Sterilize through a 0.22-mm filter. Solution should be maintained in a tube with a dark cover. b-Mercaptoethanol is toxic; avoid inhalation, ingestion, or contact with eyes, skin, or mucous membranes.
3. Mouse *ES cell culture medium*: To one, 500 ml bottle of Knockout DMEM, add 75 ml fetal bovine serum ES-qualified

(ES-FBS), 5 ml nonessential amino acid solution (NEAAs), 5 ml penicillin/streptomycin, and 50 µl β-mercaptoethanol. Store in 50 ml aliquots at 4°C. Prior to use, add 500 µl L-glu and 500 µl LIF to give a final concentration of 1,000 U/ml LIF.

4. *MEF c*ulture medium: Dissolve one pack of DMEM in 800 ml deionized water and add 10 ml Pen/Strep, 7 µl b-mercaptoethanol (original stock, undiluted), and 3.4 g sodium bicarbonate. Add 2.5–3 ml HCl (1 N) and then water to reach 850 ml. Sterilize through a 0.22-mm vacuum filter. Add 150 ml fetal bovine serum to the medium.
5. Mitomycin C: Prepare a 2 mg/ml stock solution by dissolving 2 mg of Mitomycin C in 1 ml of deionized water and store at 4°C. Solution should be maintained in a tube with a dark cover. Mitomycin C is very toxic if inhaled or swallowed, and poses a danger of cumulative effects.
6. Freezing medium: Mix 10% dimethyl sulfoxide, 50% ES-FBS, and 40% mESC medium. Always prepare fresh on ice. Keep DMSO away from sources of ignition and electrostatic charge.
7. EGTA: To make 100× (50 mM) EGTA solution, dissolve 951 mg EGTA in 49.4 ml deionized water. Add 600 µl of 10 M NaOH (pH = 8) to solute. Sterilize through a 0.22-µm filter. Store at room temperature. For a working solution, add 500 µl of 100× stock in 49.5 ml PBS^-.
8. Acid Tyrode's solution: Mix 8 µg/ml NaCl, 0.2 µg/ml KCl, 0.24 µg/ml $CaCl_2 \cdot 2H_2O$, 0.1 µg/ml $MgCl_2 \cdot 6H_2O$, 1 µg/ml glucose, 4 µg/ml polyvinylpyrrolidone, pH 2.5. Alternatively, the solution can be purchased as acidic Tyrode's solution.
9. Trypsin: 10× trypsin (2.5%) containing 2.5 g/l of trypsin (1:250), 8.5 g/l NaCl, and 9 µl/ml 1 M NaOH in Dulbecco's $PBS/MgSO_4$.
10. Trypsin/EDTA: (0.05%/0.53 mM). Store 10 ml aliquots at –20°C and, after thawing at 4°C, use within 2 weeks. Do not leave at room temperature.
11. PD0325901 solution: For a 10 mM concentrated stock solution, reconstitute 1 mg of PD0325901 by adding 207.4 µl of DMSO. Store aliquots at –20°C. For making media, add 1 µl of stock solution to 10 ml of prewarmed media (1 µM final concentration).
12. SB431542 solution: For a 10 mM concentrated stock solution, reconstitute 1 mg of SB431542 by adding 1.3 ml of DMSO. Store aliquots at –20°C. For making media, add 1 µl of stock solution to 1 ml of prewarmed media (10 µM final concentration).

13. *0.1%* gelatin: Dissolve 1 g of gelatin in deionized water and sterilize by autoclaving 30 min at 120°C. Store at room temperature.
14. Gelatin-coated plates: Cover the bottoms of the dishes with a 0.1% gelatin solution and incubate for at least 5 min. Remove excess gelatin solution. Coated plates can be stored at 4°C for up to 6 months.

3 Methods

All tissue culture reagents (medium, trypsin, etc.) should always be at least hand warmed and, ideally, prewarmed to 37°C.

3.1 Preparation of Mouse Embryonic Fibroblasts

1. Prepare mouse embryonic fibroblasts (MEFs), and generate and inactivate feeder cells following standard procedures. In general, we prepare MEFs as previously described (11–13) from a 12.5–13.5 day post-coitum outbred NMRI mouse strain in accordance with relevant guidelines and regulations (see Note 1). All media are prewarmed at 37°C. We inactivate confluent MEFs with mitomycin C for 1.5–3 h (10 mg/ml).
2. Prepare gelatin-coated center-well plates.
3. After mitomycin C inactivation, wash MEFs twice with PBS, trypsinize, and replate onto gelatinized center-well plates. We split the cells based on the area of the original and desired dishes. For example, mitotically inactivated MEFs in a small T flask which has a 25 cm^2 area is split into one 12-well plate (see Note 2). Alternatively, the MEFs are counted and replated 30,000–35,000 cells/cm^2 (60,000–70,000 cells/center-well).

3.2 Blastocyst Recovery and ICM Expansion

1. Replace MEF medium with mESC medium, including 1 μM PD03/10 μM SB43, 1–3 h before blastocyst recovery.
2. Prepare mESC medium drops under mineral oil in a 60-mm plate and place at 37°C, 5% CO_2.
3. Kill time-mated females at E3.5 and immediately dissect their uteri into 5 ml of mESC in 60-mm plate preheated to 37°C in the incubator (see Note 3).
4. Transfer the uteri into 2 ml of fresh preheated mESC in a 60-mm plate.
5. Flush the blastocysts out of the uterine horn under a stereomicroscope using a 1-ml syringe with a 26-G needle.
6. Collect blastocysts under a stereomicroscope using mouth-controlled pipette, transfer to a new culture dish containing microdrops of pre-equilibrated mESC medium and wash.

7. Remove zona pellucida from the blastocysts by subjecting them to pre-incubated acid Tyrode's solution as described before (14) (Fig. 1). Monitor until the zona pellucida disappear; this will take only seconds to a few minutes, depending on how much medium is carried over (see Note 4).
8. Under a dissecting microscope (ideally within a tissue culture hood) transfer the zona free-blastocysts onto mitotically arrested MEFs in the center-well plates (maximum ten blastocysts per plate) which have been prepared one day earlier and contain 700 µl mESC medium that consists of PD03/SB43 pre-equilibrated at 37°C and 5% CO_2.
9. Incubate at 37°C and 5% CO_2. Do not disturb for ~48 h to allow the blastocysts to partially attach to the culture dish. mESC medium can be then renewed every other day while the ICM expands.
10. Prepare and label appropriate numbers of 24-well plates with confluent layers of mitotically inactivated MEFs 1 day in advance.
11. After 7–9 days, under a stereomicroscope, make cuts around the outgrowth by a thin Pasteur pipette (mechanical passage, passage 0) to remove the surrounding trophoectoderm and pick up the individual full-grown ICMs (see Note 5) (Fig. 1). If the outgrowth is large (more than 200 mm in diameter) cut into pieces before pick up.
12. Culture the isolated outgrowths individually on a fresh 24-well dish covered with a mitotically arrested MEF. Make sure that the blastocyst has been successfully transferred by monitoring for its presence under a stereomicroscope.

3.3 First Trypsinization and mESC Proliferation

1. After 2 days, aspirate the mESC medium including SMs from the wells containing expanded secondary outgrowths on MEFs, wash with PBS^- and add 100 µl of EGTA to the wells for 3 min (see Note 6).
2. Remove EGTA and add 100 µl 2.5× trypsin (0.625% w/v) solution to the well and incubate for 3–5 min in an incubator (see Note 7).
3. Remove the trypsin with a pipette and add 500 µl of mESC medium without SMs to submerge the outgrowth.
4. Dissociate the outgrowths into individual cells and small cell clumps, by pipetting up and down 10–20 times with a 1,000 µl pipette tip.
5. Transfer all the cell suspensions into a well of a 24-well plate that contains fresh MEF feeder and mESC medium. After this step, the SM is removed.
6. Renew the medium every day.

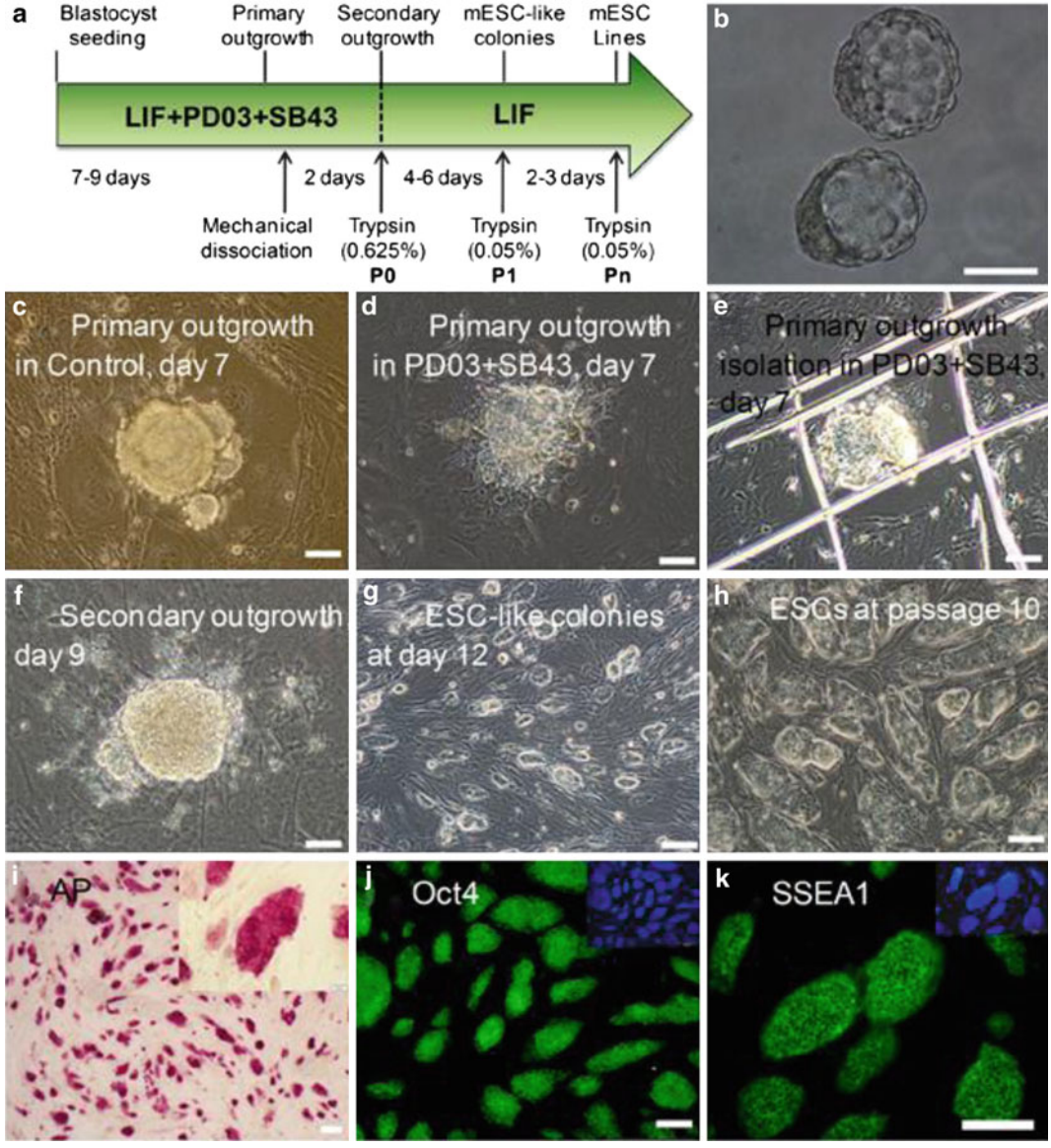

Fig. 1 The procedure of mESC line generation in the presence of PD03 + SB43 and the characterization of a derived line. (**a**) Schematic of the mESC line derivation procedure. (**b**) Zona-free blastocysts from DBA/2 mouse strain. (**c**) A 7-day outgrowth of a whole blastocyst without small molecules (control). In this condition, the outgrowths were large with numerous surrounded trophectodermal and differentiated cells. (**d**) A 7-day outgrowth of a whole blastocyst in presence of PD03 + SB43. The ICM outgrowths surrounded by a few trophectodermal cells. (**e**) Mechanical cutting of primary outgrowth by a thin Pasteur pipette (Passage 0), followed by replating on fresh MEF to make secondary outgrowth (**f**) after 48 h. (G) The secondary outgrowths were treated with 1× EGTA, and subsequently trypsinized (0.625% w/v) (Passage 1). (**h**) A representative picture of passage 10. The expression of (**i**) Alkaline phosphatase, (**j**) Oct4, and (**k**) SSEA1 in generated mESC line. Scale bar: 100 μm

7. 4–6 days after trypsinization, typically packed dome mESC colonies will be detected in all wells (passage 1) (see Notes 8 and 9) (Fig. 1).
8. Dissociate colonies with trypsin/EDTA (0.05% w/v) and passage them into 12-well dishes, followed by 6-well and then a small T flask, respectively (see Note 10).

3.4 Expansion and Freezing/Thawing of mESCs

Passage mESCs when they reach confluency. Usually, they are subcultured every 2–3 days. Passage the mESCs with EGTA and then trypsin/EDTA (0.05% w/v). Mix the resulting single-cell suspension with mESC medium and split the cells 1:3 to 1:6. The mESC medium should be changed daily. mESCs are frozen/thawed as previously described (12, 13).

3.5 Anticipated Results

The above described protocol was applied to different mouse strains of various genetic backgrounds, which were previously considered refractory or non-permissive for ESC establishment. We observed that dual inhibition of ERK1/2 and TGF-β hijacked the strain type in mice during mESC line derivation. The established mESC lines showed dome-shaped colony morphologies, high nuclear-cytoplasmic ratios, the ability to propagate following trypsin digestion and clonal growth from single cells. They expanded in mESC medium over multiple passages and maintained their pluripotency over long-term culture as determined by the expression of alkaline phosphatase (AP) activity and SSEA-l, as well as Oct4 (Fig. 1). The established lines had the ability to differentiate into lineages of all three germ layers in vitro by embryoid body formation. The evaluated lines exhibited high rates of chimerism when injected into blastocysts.

We believe that the protocol presented here offers a simple and efficient derivation of mESCs from strains of various genetic backgrounds which may be valuable for the generation of ESCs in various mammalian species.

4 Notes

1. NMRI-derived MEFs support mouse ESCs better than Balb/c-derived MEFs.
2. We usually use MEF passages 2–4, because the later-passage MEFs do not support ESC derivation to the same extent.
3. Blastocysts from NMRI strain were recovered by superovulation. For the other strains (C57BL/6, BALB/c, DBA/2, and FVB/N), embryos were obtained by natural mating.
4. The culturing of zona-free blastocysts increases their attachment to MEF. Zona-free blastocysts are very sticky. It is helpful

to pipette with an up and down motion and to always have some medium in the pipette before collecting the zona-free embryos.

5. In the presence of PD03/SB43 the occurrence of trophoblast-derived cells is significantly reduced and the initial outgrowth typically looks more like a mESC colony.
6. We found this procedure increased the efficiency of mESC derivation compared to trypsinization of whole ICM and trophectoderm outgrowths as reported by Meissner and colleagues (12).
7. We used different concentrations of trypsin and observed that 2.5× trypsin (0.625% w/v), has the most effective single cell dissociation of outgrowths with the lowest death rates.
8. In the presence of PD03/SB43, the mESC derivation efficiency decreased when 4.5 dpc blastocysts (based on Batlle-Morera et al. (15)) were used instead of 3.5 dpc blastocysts.
9. FBS functions as a trypsin inhibitor. One milliliter of mESC medium can inactivate a maximum of 20 μl of 2.5% trypsin. Make sure that the ratio of 2.5% trypsin and mESC medium is 1:50 or lower (16).
10. The dual inhibition of Erk1/2 and TGF-β by PD03/SB43 promotes the derivation of mESC lines from NMRI, C57BL/6, BALB/c, DBA/2, and FVB/N strains, which previously have been considered refractory or non-permissive for ESC establishment (17–20).

Acknowledgments

This study was funded by grants provided from Royan Institute, the Iranian Council of Stem Cell Technology, and the Iran National Science Foundation (INSF).

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http://www.springer.com/978-1-62703-347-3

Pluripotent Stem Cells
Methods and Protocols
Lakshmipathy, U.; Vemuri, M.C. (Eds.)
2013, X, 292 p., Hardcover
ISBN: 978-1-62703-347-3
A product of Humana Press