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Formation of Embryoid Bodies (EBs) from Mouse Embryonic Stem Cells (mESCs)

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Introduction

Mouse embryonic stem cells (mESCs) are pluripotent stem cells derived from the inner cell mass of the blastocyst, an early stage embryo[1,2]. Two distinctive properties distinguish embryonic stem cells, their pluripotency and their capacity for self-renewal under defined conditions. They are pluripotent in that they are able to differentiate into all derivatives of the primary germ layers, including ectoderm, endoderm, and mesoderm, thus generating every cell type in the body. mESCs also contribute to a wide range of adult tissues in chimeras with intact embryos, including the germ cells.

Embryoid Bodies (EBs) are generated at a normally scheduled passage by plating mESCs into non-tissue culture-treated dishes to prevent attachment. EBs are utilized to test the differential potential of the cells.

Materials Needed

- mESCs growing on MEF feeder culture (Refer to "Feeder-Dependent Culture of Embryonic Stem Cells (mESCs) in KnockOut™ Serum Replacement with Leukemia Inhibitory Factor (LIF)" protocol for growing mESCs)
- KnockOut™ DMEM (Cat. no. 10829-018)
- KnockOut™ Serum Replacement (KSR) (Cat. no. 10828-028)
- MEM Non-Essential Amino Acids Solution, 10 mM (Cat. no. 11140-050)
- GlutaMAX™-I (100X) (Cat. no. 35050-079)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-144)
- StemPro® Accutase® Cell Dissociation Reagent (Cat. no. A11105-01)
- Attachment Factor (Cat. no. S006100)
- Fetal Bovine Serum (FBS), ESC-Qualified (Cat. no. 10439-024)
- 100-mm tissue culture-treated dishes
- 100-mm non-tissue culture-treated dishes
- 37°C water bath
- Appropriate tissue culture supplies

Preparing Media and Materials

Mouse Embryonic Stem Cell (mESC) EB Medium (100 mL of complete medium)

1. To prepare 100 mL of complete mESC EB Medium, aseptically mix the following components:

KnockOut™ DMEM	83 mL
KnockOut™ Serum Replacement (KSR)	15 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
GlutaMAX™-I	1 mL

2. Complete mESC EB Medium can be stored at 2–8°C for up to 1 week.

Mouse Embryonic Stem Cell (mESC) EB Medium with 10% FBS (100 mL of complete medium)

1. To prepare 100 mL of complete mESC EB Medium with 10% FBS, aseptically mix the following components:

mESC EB Medium	90 mL
FBS	10 mL

2. Complete mESC EB Medium with 10% FBS can be stored at 2–8°C for up to 1 week.

Preparing AF-Coated Dishes

Attachment Factor (AF) dishes are required at Day 4 of the EB culture. Prepare one AF-coated plate for each sample of mESC EBs you plan to generate.

1. Cover the whole surface of a 100-mm tissue culture-treated dish with Attachment Factor (AF) solution and incubate the vessels for 30 minutes at 37°C or for 2 hours at room temperature.
2. Using sterile technique in a laminar flow culture hood, completely remove the AF solution from the culture vessel by aspiration just prior to use. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

Note: It is not necessary to wash the culture surface before adding cells or medium.

Passaging mESCs into EBs

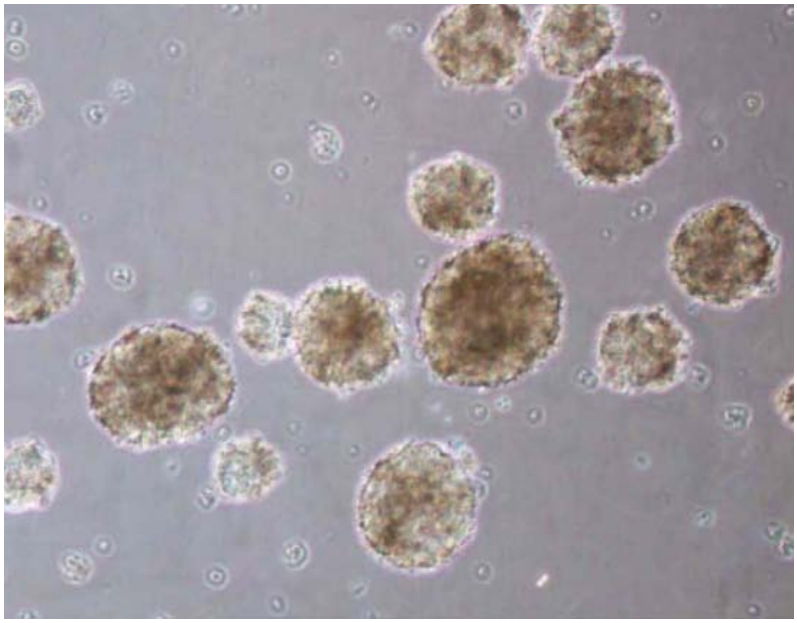
1. Pre-warm the mESC EB Medium, Dulbecco's PBS (DPBS) without Calcium and Magnesium, and the StemPro® Accutase® Solution to 37°C.
2. The EBs will be going into 100-mm tissue culture-treated dishes (i.e., adherent dishes) in order to separate mESC from MEF feeder cells. Prepare dishes by adding 10 mL of mESC EB Medium to each dish.
3. Aspirate the spent medium from the dish containing mESCs, and rinse the dish once with DPBS (e.g., 2 mL for one well of a 6-well plate).
4. Aspirate DPBS and add StemPro® Accutase® Solution to the dish containing mESCs. Adjust the volume of StemPro® Accutase® Solution for various dish sizes (refer to Table 1).

Table 1 Reagent Volumes (in mL per well or per dish)

Culture Vessel	Surface Area	DPBS	StemPro® Accutase® Solution	mESC EB Medium
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm^2/well	1 mL/well	0.5 mL/well	1 mL/well
24-well plate	2 cm^2/well	0.5 mL/well	0.3 mL/well	0.5 mL/well
35-mm dish	10 cm^2	2 mL	1 mL	2 mL
60-mm dish	20 cm^2	4 mL	2 mL	4 mL
100-mm dish	60 cm^2	12 mL	5 mL	12 mL

5. Incubate the dish(es) for 1–2 minutes in a 37°C, 5% CO_2 incubator until individual single cells start to round up.
6. Add an appropriate amount of mESC EB Medium to each dish (refer to Table 1) to stop dissociation reaction. Gently pipet the dissociated cells up and down sufficiently to disperse the colonies into a single-cell suspension. Make sure to pipet gently to minimize the formation of bubbles.
7. Transfer the mESC suspension from each well into a separate 15-mL conical tube and centrifuge the tube(s) at 250 × *g* for 5 minutes to pellet the cells.
8. Carefully aspirate the supernatant(s) from the mESC pellet(s).
9. Resuspend the pellet(s) with an appropriate amount (approximately 2 mL) of mESC EB Medium.
10. Add resuspended cells to each 100-mm prepared adherent dish containing 10 mL mESC EB Medium.
11. Incubate the adherent dish(es) for 40 minutes in a 37°C, 5% CO_2 incubator to separate the mESCs from the MEF feeder layer.
12. Carefully collect the mESCs that are detached from the MEF feeder layer and adjust the cell concentration to 5.5×10^5 cells/mL with mESC EB Medium.
13. Plate 5 mL of the cell suspension (at 5.5×10^5 cells/mL) in one 100-mm non-adherent dish (i.e., not tissue culture–treated).
14. Incubate the cells for 3 days in a 37°C, 5% CO_2 incubator to allow them to form EBs. Replace spent medium daily.

Figure 1 Bright field images (10X) of Embryoid Bodies derived from Gibco® Mouse (C57BL/6) ESCs. Dissociated mouse ESCs were allowed to aggregate for 3 days on non-adherent culture dishes.

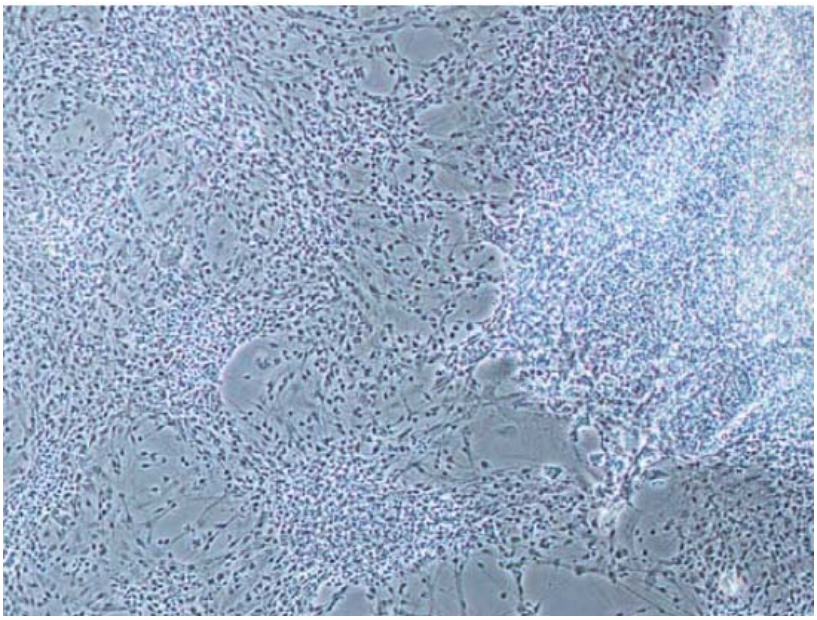


15. Plate EBs in mESC EB Medium supplemented with 10% FBS in an AF-coated dish.
16. Incubate EBs overnight in a 37°C, 5% CO_2 incubator to allow them to attach to the dish.

Note: While cells are attaching, be careful when opening and closing the incubator doors to avoid disturbing the even distribution of cells on the surface of the wells.

17. Once the EBs are attached to the dish, aspirate medium and add mESC EB Medium (**without** FBS) to the dish. Return dish to the incubator and replace spent medium every other day.
18. At Day 14 after EB differentiation, stain the differentiated cells with antibodies against endodermal, mesodermal, and ectodermal markers.

Figure 2 Embryoid Bodies derived from Gibco® Mouse (C57BL/6) ESCs after being transferred to gelatin-coated culture dishes, allowed to attach for one day, and differentiated for 14 days.



Immunocytochemical Staining of mESC Embryoid Bodies

Immunocytochemical staining is used to evaluate the ability of the mESCs to differentiate into the three primary germ layers. Stain the differentiated cells (at Day 14) with antibodies against the mesoderm marker smooth muscle actin, the ectoderm marker βIII-tubulin, and the endoderm marker α-fetoprotein (refer to Table 2).

Table 2 Phenotypic Markers

Germ Layer	Antigen	Source	Dilution Ratio	Antibody Type
Ectoderm	βIII-tubulin	Sigma, Cat. no. T8660	1:1000	Mouse IgG_{2b}
Endoderm	α-fetoprotein(AFP)	R&D Systems Cat. no. AF5369	1:200	Goat IgG
Mesoderm	Smooth muscle actin	Life Technologies Cat. no. 08-0106	1:1	Mouse IgG_1-kappa

Additional Materials Needed

- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-250)
- 4% Paraformaldehyde (PFA) (US Biologicals, Cat. no. 19943)
- Goat serum (Cat. no. 10000C)
- Bovine Serum Albumin (BSA) (Cat. no. 15561-020)
- Dimethyl Sulfoxide (DMSO) (Sigma, Cat. no. D2650)
- 1% Triton X-100 (Cat. no. HFH-10)
- Alexa Fluor® 594 Goat Anti-Rabbit IgG Antibody (Cat. no. A11037)
- DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) (Cat. no. D3571)
- ProLong® Gold Antifade Reagent (Cat. no. P36930) (optional)

Preparing Blocking Buffer (10 mL)

1. To prepare 10 mL of Blocking Buffer, aseptically mix the following components:

DPBS without Calcium and Magnesium	7.2 mL
Goat serum	0.5 mL
1% Triton X-100	0.3 mL
50 mg/mL BSA	2.0 mL

2. Blocking Buffer can be stored at 2–8°C for up to 2 weeks.

DAPI Stock Solution (10 mL)

1. To prepare 10 mL of DAPI Solution, aseptically mix the following components:

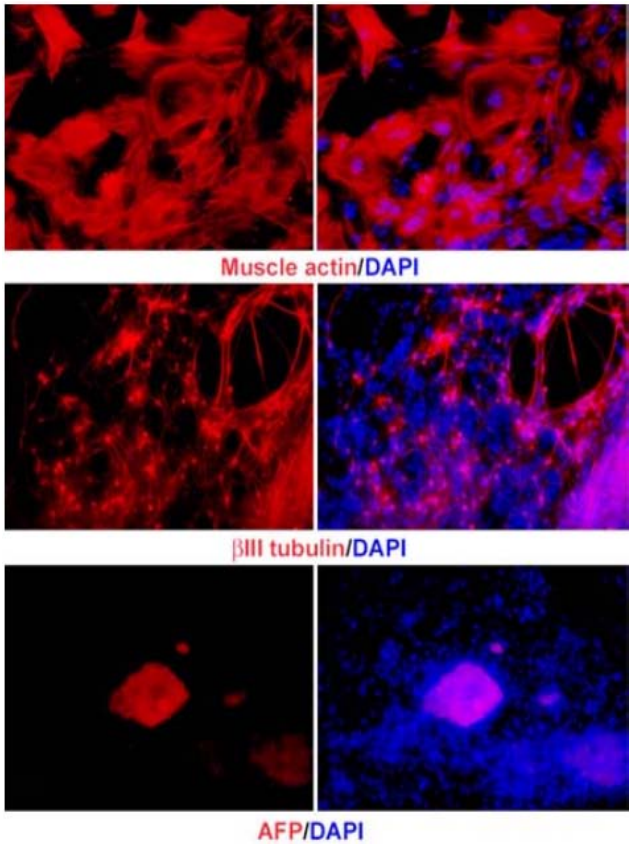
DAPI	10 mg
DMSO	10 mL

2. Aliquot and store at –20°C. Dilute in DPBS without Calcium and Magnesium (1:1000) to make working solution.

Staining Procedure

1. Aspirate medium from the culture vessel.
2. Rinse the cells with DPBS (without Calcium and Magnesium) once.
3. Fix cells with 4% PFA for 15–20 min at room temperature.
4. Rinse cells with DPBS (without Calcium and Magnesium) three times for 5 minutes at room temperature.
5. Block cells in Blocking Buffer for 30 minutes at room temperature.
6. Add each primary antibody (βIII-tubulin, α-fetoprotein, and smooth muscle actin diluted in blocking buffer as indicated above in Table 2) separately to the cells. Use 6 mL (total volume) per 100-mm dish.
7. Incubate cells with primary antibodies at 4°C overnight.
8. Next day, rinse cells with DPBS (without Calcium and Magnesium) three times for 10 minutes at room temperature.
9. Treat the cells with the secondary antibody, Alexa Fluor® 594 Goat Anti-Rabbit IgG, diluted in Blocking Buffer (1:1000). Use 6 mL per 100-mm dish.
10. Incubate cells with secondary antibody for 1 hour at room temperature.
11. Rinse cells with DPBS (without Calcium and Magnesium) twice for 10 minutes at room temperature.
12. Stain cells with DAPI working solution (diluted as instructed above) for 5 minutes.
13. Rinse cells with DPBS (without Calcium and Magnesium) for 10 minutes before visualizing.
14. ProLong® Gold Antifade Reagent can be used at this time in the final wash step.

Figure 3 Fluorescence images (20X) showing differentiating Embryoid Bodies of Gibco® Mouse (C57BL/6) ESCs stained with antibodies against the mesoderm marker smooth muscle actin, the ectoderm marker ßIII-tubulin, and the endoderm marker AFP (red, left panels) at day 14 of differentiation. Cell nuclei were counterstained with DAPI (blue, right panels).



APPENDIX

A. In the mESC EB Medium, KnockOut™ DMEM (Cat. no. 10829-018) can be substituted with the following alternatives**:**

i. KnockOut™ DMEM/F-12 (Cat. no. 12660-012)

To prepare 100 mL of complete mESC EB Medium using KnockOut™ DMEM/F-12, aseptically combine the components listed in the table below.

Component	Stock Concentration	Final Concentration	Volume
KnockOut™ DMEM/F-12 (Cat. no. 12660-012)	—	1X	83 mL
KnockOut™ Serum Replacement (KSR) (Cat. no. 10828-028)	—	15%	15 mL
GlutaMAX™-I (Cat. no. 35050-061)	200 mM	2 mM	1 mL
MEM Non-Essential Amino Acids Solution (Cat. no. 11140-050)	10 mM	0.1 mM	1 mL

B. Dissociation Enzymes/Tools for Harvesting mESC

Dissociation Enzyme /Tools	Application	Suggested concentration
TrypLE™ Express (Cat. no. 12604-021)	Dissociation to single cells	1X ready to use

References

1. Evans, M., Kaufman, M. (1981) Establishment in culture of pluripotent cells from mouse embryos. Nature *292*, 154–156.
2. Martin, G. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA *78*, 7634–7638.

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