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Formation of Embryoid Bodies (EBs) from Mouse Pluripotent Stem Cells (mPSCs)

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Introduction

Mouse embryonic stem cells (mESCs) are pluripotent stem cells derived from the inner cell mass of the blastocyst while mouse induced pluripotent stem cells (miPSCs) are reprogrammed from somatic cells[1,2]. Two distinctive properties distinguish these two types of mouse pluripotent stem cells (mPSCs): their pluripotency and their capacity for self-renewal under defined conditions. Because they are pluripotent, mPSCs can be manipulated in vitro for differentiation into many cell types. *In vivo*, mouse ESCs contribute to a wide range of adult tissues including the germ cells and are often used as vehicles for modifying the mouse genome or for chimera studies. Media containing KnockOut™ Serum Replacement have been shown to increase the efficiency of ESC establishment[3,4], and KnockOut™ Serum Replacement – Multi-Species uses that same KnockOut™ Serum Replacement formulation.

After mPSCs are generated, either by derivation or reprogramming, embryoid bodies (EBs) are utilized to test their differentiation potential. EBs are generated by plating mPSCs with LIF-depleted media in non-tissue culture-treated dishes that prevent attachment. This protocol describes how EBs are generated and differentiated from mPSCs.

Materials needed

- mPSCs growing on MEF feeder culture (Refer to MAN0007198 "Feeder-Dependent Culture of Mouse Pluripotent Stem Cells (mPSCs) with Leukemia Inhibitory Factor (LIF)" for a protocol on growing mPSCs.)
- KnockOut™ DMEM (Cat. No. 10829018)
- KnockOut™ Serum Replacement Multi-Species (KnockOut™ SR – Multi-Species) (Cat. No. A3181501 or A3181502)
- MEM Non-Essential Amino Acids Solution, 10 mM (Cat. No. 11140050)
- GlutaMAX™-I (100X) (Cat. No. 35050079)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. No. 14190144)
- StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- TrypLE™ Express Enzyme (1X), no phenol red (Cat. No. 12604013)
- Attachment Factor (Cat. No. S006100) (Refer to MAN0007198 "Feeder-Dependent Culture of Mouse Pluripotent Stem Cells (mPSCs) with Leukemia Inhibitory Factor (LIF)" for a protocol on how to prepare Attachment Factor-coated vessels.)
- Fetal Bovine Serum (FBS), ESC-Qualified (Cat. No. 10439024 or 16141061)
- Nuclon Sphera plate for EB formation (Cat. No. 174932, 174944 or 174945)
- 37°C water bath
- Appropriate tissue culture supplies

Prepare media and materials

Mouse Pluripotent Stem Cell (mPSC) EB Medium (100 mL of complete medium)

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

KnockOut™ DMEM	83 mL
KnockOut™ Serum Replacement – Multi-Species or FBS	15 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
GlutaMAX™-I, 100X	1 mL
2-mercaptoethanol, 1000X	100 µL

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

Note: KnockOut™ DMEM/F12 (Cat. No. 12660012) can substitute for KnockOut™ DMEM. KnockOut™ Serum Replacement (Cat. No. 10828028 or 10828010) can substitute for KnockOut™ SR – Multi-Species.

Passage mPSCs into EBs

1. Pre-warm the mPSC EB Medium, Dulbecco's PBS (DPBS) without Calcium and Magnesium, and Enzyme solution of either StemPro™ Accutase™ Solution or TrypLE™ Express Enzyme to 37°C.
2. Aspirate spent medium and feed mPSCs with pre-warmed mPSC culture medium 2–4 hours before passaging.
3. Aspirate the spent medium from the dish with a Pasteur pipette, and rinse the dish twice with Dulbecco's PBS (DPBS) without Calcium and Magnesium. Refer to Table 3 for recommended volumes.
4. Aspirate DPBS and add Enzyme solution to the dish containing mPSCs. Adjust the volume of Enzyme Solution for various dish sizes (refer to Table 1).

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

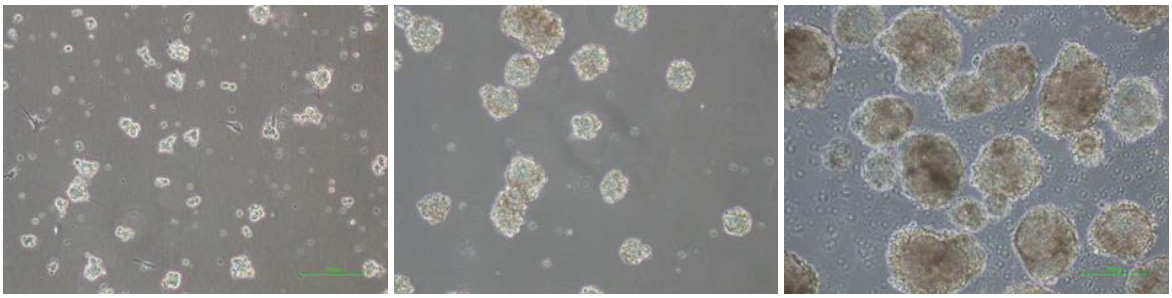
Culture vessel	Surface area	DPBS	Enzyme solution	mPSC EB Medium
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm^2/well	1 mL/well	0.5 mL/well	1 mL/well
24-well plate	2 cm^2/well	0.5 mL/well	0.3 mL/well	0.5 mL/well
35-mm dish	10 cm^2	2 mL	1 mL	2 mL
60-mm dish	20 cm^2	4 mL	2 mL	4 mL
100-mm dish	60 cm^2	12 mL	5 mL	12 mL

5. Incubate the dish for 3–5 minutes in a 37°C, 5% CO_2 incubator until individual single cells start to round up.
6. Gently pipet MEF and mPSC sheet up and down sufficiently to disperse the colonies away from the feeder layers and into a single-cell suspension using a p1000 pipet. Make sure to pipet gently to minimize the formation of bubbles. Add an appropriate amount of mPSC Culture Medium to the dish to stop the dissociation reaction.
7. Transfer the mPSC suspension into a 15-mL conical tube and centrifuge the tube at 200 × *g* for 5 minutes to pellet the cells.
8. Carefully aspirate the supernatant from the mPSC pellet.
9. Rinse mPSCs one more time by resuspending the cell pellet with an appropriate amount (refer to Table 1) of mPSC EB Medium and centrifuge down.
10. Carefully aspirate the supernatant from the mPSC pellet then resuspend cell pellet in mPSC EB Medium to obtain a cell concentration of 2–3 × 10^5 cells/mL.

Optional: Mouse feeder cells can be depleted from the cell pellet using the differential attachment propensity between mPSCs and mouse fibroblasts. Plate the cells on a tissue culture-treated plate in mPSC EB medium and let the fibroblasts adhere for 30–60 minutes. MEFs will adhere loosely to the plastic while mPSCs will stay in suspension. Collect the cell suspension, which will contain mostly ES cells and only a negligible fraction of MEFs.

11. Plate the mPSCs into Petri dishes or Petri flasks in mPSC EB medium at 2–3 × 10^5 cells/mL (i.e. 2–3 × 10^6 cells per 100-mm dish). The seeding density can be adjusted for different mPSC lines.
12. Incubate the cells in a 37°C, 5% CO_2 incubator to allow them to form EBs.
13. On the next day, feed EBs with fresh mPSC EB medium by transferring EBs into a 15-ml conical tube and spin the tube at 200 x *g* for 1 minute.
14. Resuspend EBs in fresh mPSC EB medium and replate them in a new Petri dish or Petri flask.
15. The medium can be changed every 2–3 days thereafter directly in the dish or flask. This can be accomplished by tilting the plate or flask and removing as much of the medium as possible before adding fresh medium.
16. Culture the EBs until they reach the optimal size for subsequent differentiation protocols.

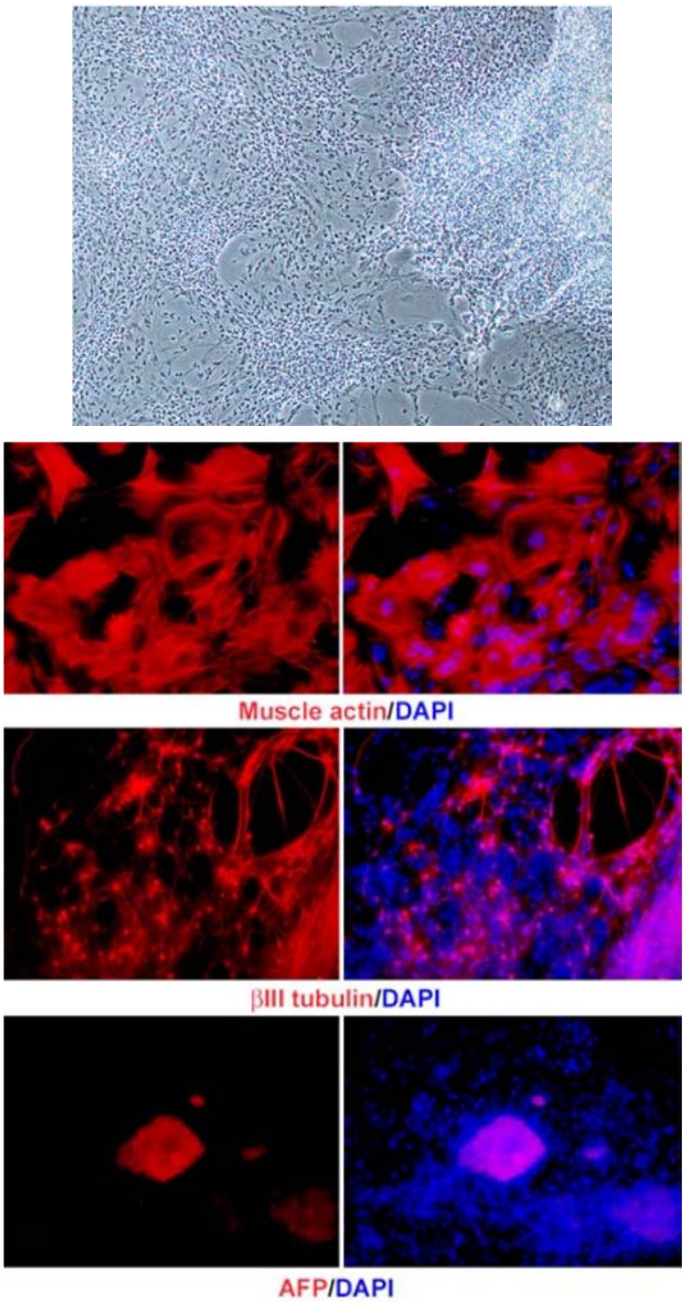
Figure 1. Bright field images (10X) of embryoid bodies derived from 129S2/SvPas ESC line. Dissociated mouse ESCs were allowed to aggregate for 1, 3, 7 days in a non-adherent culture flask.



17. Plate the EBs in mPSC EB Medium supplemented with 10% FBS in an Attachment Factor-coated dish.
18. 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 the EBs in the dish.
19. Once the EBs are attached to the dish, aspirate medium and add mPSC EB Medium (**without** FBS) to the dish. Return the dish to the incubator and replace spent medium every other day.
20. At Day 14 after EB differentiation, stain the differentiated cells with antibodies against endodermal, mesodermal, and ectodermal markers.

Figure 2. EBs after attachment and differentiation. EBs derived from C57BL/6 mESCs were transferred to gelatin-coated culture dishes, allowed to attach for one day, and differentiated for 14 days. They were imaged under phase contrast (top), then stained using antibodies for trilineage markers (bottom): Smooth muscle actin (mesoderm), ßIII-tubulin (ectoderm), and Alpha fetoprotein (AFP, endoderm).



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