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Uniform Embryoid Body Production and Enhanced Mesendoderm Differentiation with Murine Embryonic Stem Cells in a Rotary Suspension Bioreactor

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Abstract

Embryonic stem cells (ESCs) are capable of differentiating into almost all cell types in vitro and hold great promise for drug screening, developmental studies and have a huge potential in many therapeutic areas. ESCs can aggregate to form embryoid body (EB) in static suspension culture by spontaneous differentiation, which resembles an intact embryo; while static suspension culture cannot prevent agglomeration of cells and offers little control over the size and shape of EBs, it results in aggregation of EBs into large, irregular masses, which prejudice the efficiency of differentiation of cells. Recently, bioreactor-based platforms have been shown to not only offer a beneficial effect on increasing diffusion of nutrients and oxygen which promotes cell viability and proliferation but also display local biomechanical properties (e.g., low fluid shear stresses and hydrodynamic force) in tissue development and organogenesis. This chapter describes a protocol for using a rotary suspension bioreactor to produce embryoid bodies and process the differentiation of mouse embryonic stem cells (mESCs), and to assess the efficiency of EB differentiation in the bioreactor by real-time PCR and immunostaining.

Keywords: Mouse embryonic stem cell, Rotary cell culture system, EB formation, Differentiation

1 Introduction

Embryonic stem cells (ESCs) provide an elegant model for studying cell fate determination and are promising cell sources for biomedical applications including regenerative medicine, cell therapy, and drug discovery [1, 2]. Differentiated ESCs possess a potentially unlimited source of transplantable cells. Thus, it is a prerequisite and very important for achieving clinical application to acquire specific types of differentiated cells with high purity in highly efficient and cost-effective ways. Though the commonly used embryoid body (EB) formation method can generate 3D cell aggregates in static suspension condition, which can be achieved by spontaneous differentiation, static suspension culture offers little control over the size and shape of EBs and often results in agglomeration of EBs into large, irregular masses, which will affect the efficiency of the differentiation of cells [3, 4]. Unlike traditional static culture methods, bioreactor systems have the ability to achieve large scale

and can be integrated with chemical induced cell differentiation, both of which are critical for potential clinical applications [5–7].

Rotary cell culture system (RCCS) was originally designed by National Aeronautics and Space Administration (NASA) as an effective tool to simulate microgravity in space for cell growth. This 3D dynamic culture system has been recommended by researchers, which offers distinct advantages over classic static cultures in large-scale mammalian cell cultures, such as prevention of sedimentations, increased mass transfer and diffusion of oxygen [8]. In addition, the RCCS creates a low fluid shear and hydrodynamic force, which might strengthen tissue development and organogenesis [9, 10]. Therefore, RCCS has been used for expansion and differentiation of various stem cell types. Li et al. reported that human mesenchymal stem cells (MSCs) could be expanded about a 29-fold after 8 days of culture in RCCS bioreactor [11]. In keeping with these reports, we recently reported that RCCS can stimulate the proliferation of human epidermal stem cells and subsequent formation of 3D structure [12]. Moreover, we also demonstrated that RCCS can increase the efficiency of EB formation and augment mesendoderm differentiation of mouse embryonic stem cells (mESCs) by modification of Wnt/β-catenin signaling [13]. In the following section, we describe a protocol for using the rotary cell culture system to produce embryoid bodies and process differentiation of mouse embryonic stem cells (mESCs). We analyze the efficiency of EB differentiation in the rotary suspension bioreactor by real-time PCR and immunohistochemistry.

2 Materials

2.1 Cell and Cell Lines

1. Inactivated MEFs: Mouse embryonic fibroblasts (MEFs) are treated with mitomycin C and stored in liquid nitrogen (2×10^6 cells/vial).
2. ORG1: a mouse embryonic stem cell line that expresses GFP under promoter Oct4 [14].

2.2 Medium and Supplements for Cell Culture

1. PBS: Ca^{2+}, Mg^{2+} free, PBS (–) (Cat# 14190-144, Gibco), store at 4 °C.
2. Medium for inactivated MEF: High-glucose Dulbecco's modified Eagle's medium with GlutaMAX 1× (DMEM-GlutaMAX, Cat# 11960-044, Invitrogen), 10 % heat-inactivated fetal bovine serum (FBS, Cat# 11960-044, Gibco), 1× penicillin–streptomycin (Cat# 10378-016, Gibco), store at 4 °C.
3. Medium for mESC growth: DMEM-GlutaMAX, 15 % heat-inactivated FBS (Cat# 11960-044, Gibco) (*see* **Note 1**), 1 %

nonessential amino acid (NEAA, Cat# 11140-050, Gibco), 1× penicillin–streptomycin (Cat# 10378-016, Gibco), 0.1 mM beta-mercaptoethanol (Cat# 21985-023, Gibco), and 1000 U/ml recombinant LIF (Cat# ESG1107; Millipore). Store at 4 °C.

4. EB induction and differentiation medium: DMEM-GlutaMAX with 20 % heat-inactivated FBS (Cat# 11960-044, Gibco), 1 % NEAA (Cat# 11140-050, Gibco), 0.1 mM 2-mercaptoethanol (Cat# 21985-023, Gibco). Stable for 2 weeks at 4 °C.
5. Solution of gelatin (0.2 %): gelatin from porcine skin (Cat#G1890 Sigma) is dissolved at 0.2 % solution in water, autoclaved and stored at 4 °C.
6. Collagenase IV solution: Collagenase IV (Cat# 17104-019, Gibco) power is dissolved at 1 mg/ml in PBS and stored at 4 °C.
7. 0.05 % solution of trypsin–EDTA (Cat# 25300-054, Gibco), stored at 4 °C.

2.3 Bioreactor Equipment

Rotary Cell Culture System (RCCS-4D, SYNTHECON), the RCCS is composed of the following three parts.

1. Culture vessel (10 ml), sterilized clear plastic circular units with a half-inch diameter fill port (Fig. 1a) and two sampling or injection syringe ports (Fig. 1b) (*see* **Note 2**).

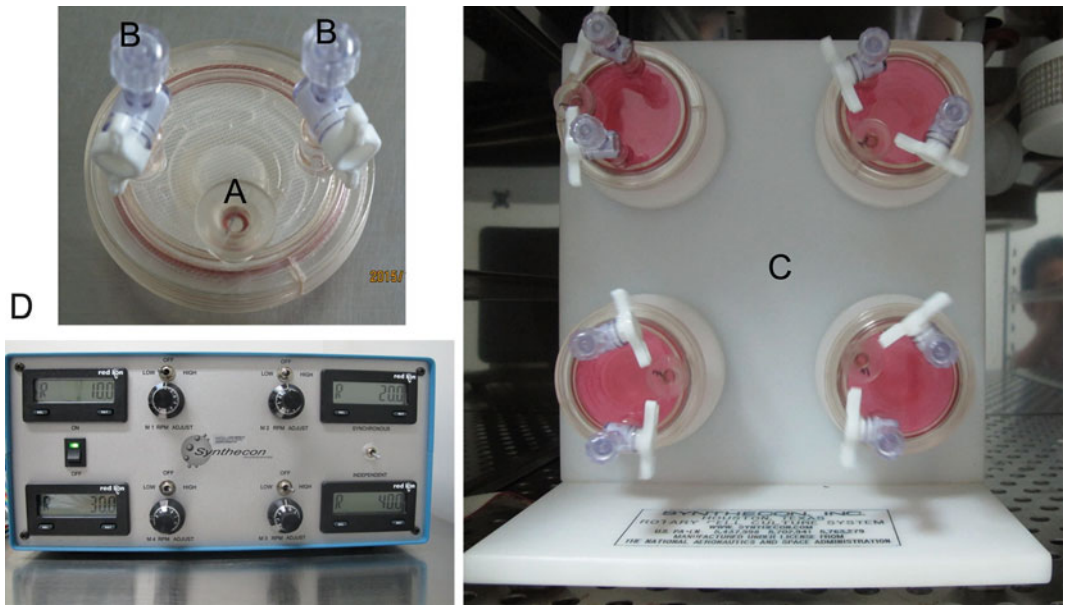

Fig. 1 Rotary bioreactor used in the described methodology. (**a**, **b**) 10 ml disposable vessel, including fill port (**a**), and two syringe ports (**b**). (**c**) Rotator base, aims to support and rotate the culture vessel. (**d**) Power supply, containing the electronic motor speed controls

2. Rotator Base, aims to support and rotate the culture vessel (Fig. 1c).
3. Power supply, containing the electronic motor speed controls. This equipment is used to adjust vessel rotation speed (Fig. 1d).

2.4 Real-Time PCR

1. TRIzol® Reagent (Cat# 15596018, Invitrogen, USA).
2. RQ1 RNase-free DNase I (Cat# M6101, Promega, USA).
3. M-MuLV reverse transcriptase (Cat# M0253L, New England Biolabs, China).
4. GoTaq® qPCR Master Mix (SYBR Green) (Cat# A6001, Promega, USA).
5. LightCycler® 480 II System thermocycler platform (Roche, USA).

2.5 Immunostaining

1. Fixative buffer: solubilization of 4 g paraformaldehyde (PFA, Cat# P-6148, Sigma) powder in 100 ml PBS (*see* **Note 3**). Store at −20 °C.
2. 1.5 % aqueous agar solution: 1.5 g agar power (Cat# A1296, Sigma) mixing with 100 ml water, heating to dissolve and at approx 60 °C.
3. Permeabilization buffer: 0.2 % Triton X-100 (Cat# 0694, Amresco, USA) in PBS. Store at −20 °C.
4. Blocking solution: 5 % bovine serum albumin (BSA, Cat# A1933, Sigma) in PBS or 5 % normal rabbit serum (Cat# 10510, Invitrogen) in PBS. Store at −20 °C.
5. Primary antibody: goat anti-brachyury polyclonal antibody (N-19) (Cat# sc-17743, Santa Cruz), rabbit anti-Gata6 monoclonal antibody (clone D61E4) (Cat# 5851S, Cell Signaling Technology), and rabbit anti-Sox1 polyclonal antibody (Cat# 4194S, Cell Signaling Technology).
6. Secondary antibodies: goat anti-rabbit TRITC-conjugated secondary antibodies (ZSGB BIO, Cat# ZF-0316 China) and rabbit anti-goat TRITC-conjugated secondary antibodies (ZSGB BIO, Cat# ZF-0317 China).
7. Nuclear staining solution: 0.1–1 μg/ml of Hoechst (bisBenzimide H33342 trihydrochloride) (Cat# B2261, Sigma) in PBS.
8. Nikon Ti microscope (Nikon, Japan).

3 Methods

3.1 Cultivation of mESCs on Inactivated MEF

1. Add 3 ml 0.2 % gelatin solution to 60 mm tissue culture dish. Incubate for 1 h at 37 °C incubator.
2. Remove the gelatin solution and wash twice with PBS for inactivated MEF seeding.
3. Thaw a vial of frozen inactivated MEFs (treated with mitomycin C and stored in liquid nitrogen, 2×10^6 cells/vial) quickly in a 37 °C water bath. Carefully transfer the cells into a 15 ml centrifuge tube. Add 10 ml of warm MEF medium, while gently shaking the tube.
4. Centrifuge at $200 \times g$ for 5 min, aspirate supernatant, and resuspend the pellet in 4 ml of MEF medium.
5. Seed onto the 0.2 % gelatin-coated 60 mm dish in MEF medium and place in a 37 °C, 5 % CO_2 incubator for 12–24 h.
6. Remove the MEF medium and wash cells once with sterile PBS (3 ml/dish) before plating of mESCs.
7. Thaw mESCs from liquid nitrogen by placing cryovials in a 37 °C water bath, gently move the contents from cryovials to 15 ml centrifuge tubes, add 6 ml prewarmed mESC growth medium, and centrifuge at $200 \times g$ for 5 min.
8. Remove supernatant, gently resuspend cells in 4 ml mESC growth medium, transfer them into the 60 mm dish coated with inactivated MEFs, place in a 37 °C incubator and shake the plate in quick side to side, forward to back motions to distribute the cells uniformly. Culture the cells at 37 °C incubator, with 5 % CO_2 and 95 % humidity.
9. Perform daily medium changes. Check for undifferentiated colonies (Fig. 2) that are ready for subculture (dense centered) or EB formation approximately 3–4 days after thawing.

3.2 RCCS Preparation Before Cell Culture Initiation

1. Unscrew the fill port and place fill port cap on a sterile 100 mm dish.
2. Fill the vessel by pipetting the 5 ml EB induction and differentiation medium through the open fill port.
3. Close the fill port with the cap and place the vessel to incubate for 10–30 min in the rinsing solution at 37 °C incubator and prepare for cell inoculation and cultivation.
4. Attach the multicolored ribbon cable of the power supply and the rotator base.

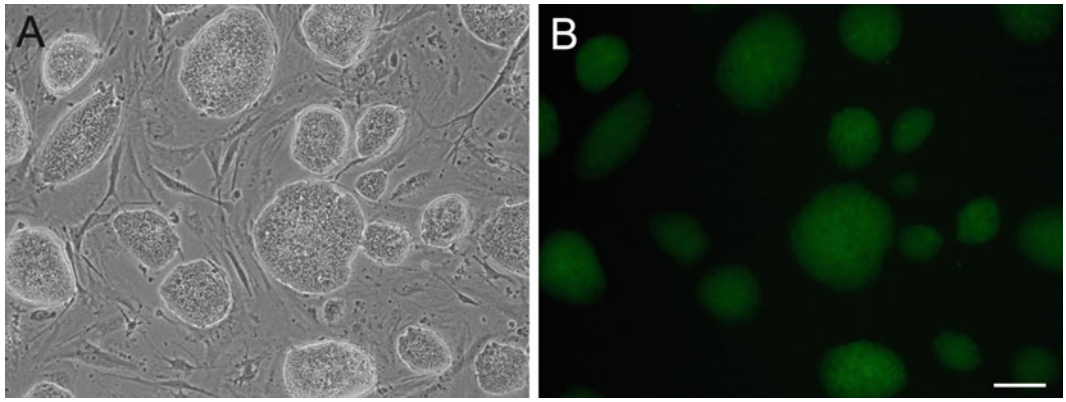

Fig. 2 Mouse embryonic stem (mES) cell colony appearance on MEF. When single cell suspensions were plated on MEF feeder, colonies showed up and remained undifferentiated. (**a**) Phase contrast of mES colony after 3 day cultured on MEF. (**b**) Oct4-GFP reporter fluorescence of mES colony after cultured on MEF. Bars: 50 μm

3.3 Single Cell Suspension Preparation for EB Induction in RCCS

1. Prior to EB induction, prepare single cell suspension from mESC colonies that have been cultured on inactivated MEFs as described above (*see* Subheading 3.1, **steps 8** and **9**).
2. Aspirate the mESC growth medium and rinse the cells once gently with 3 ml PBS.
3. Then, remove the PBS and add 2 ml solution of Collagenase IV (1 mg/ml, Invitrogen) to the 60 mm dish and incubate at room temperature for 2–3 min.
4. Add 2 ml EB induction and differentiation medium to the 60 mm dish and flush the dish gently to make mESCs detach as colonies.
5. Transfer dissociated mESC colonies to 15 ml centrifuge tubes, add 4 ml prewarmed EB induction and differentiation medium, and centrifuge at 50 × *g* for 3 min.
6. Remove the supernatant and add 2 ml solution of trypsin–EDTA (0.05 %) to the 15 ml centrifuge tube, and incubate at 37 °C for 2–3 min.
7. Add 4 ml EB induction and differentiation medium to the tube, and gently pipette the cell suspensions 5–10 times to ensure any remaining clumps are fully dissociated.
8. Centrifuge the cells at 200 × *g* for 5 min at room temperature (15–25 °C).
9. Aspirate the supernatant and resuspend the pellet in 10 ml of EB induction and differentiation medium.
10. Transfer the cell suspension to a 100 mm dish (Cat# 430166 Corning).

11. Incubate the cells at 37 °C with 5 % CO_2 and 95 % humidity for 30–40 min (*see* **Note 4**).
12. Harvest the non-adherent mESCs by drawing up the liquid from the dish. And transfer the suspension with non-adherent mESCs to a 15 ml centrifuge tube.
13. Centrifuge the harvested mESCs at 200 × *g* for 5 min at room temperature (15–25 °C).
14. Remove and discard the supernatant of 15 ml centrifuge tube. Resuspend the cell pellet in 2 ml of EB induction medium.
15. Count cell number using the Scepter 2.0 Handheld Automated Cell Counter (Cat# PHCC20040 EMD Millipore) and Scepter with 40 μm Scepter Sensors according to the product instruction. For more information, please refer to the Product Information Sheet for the Scepter cell counter available on www.merckmillipore.com.

3.4 EB Induction and Differentiation in RCCS Bioreactor

1. Adjust the desired cell concentration suspension at 1–2 × 10^6/ml with EB induction medium.
2. Remove the rinsing solution in 10 ml vessel that is prepared as described above (*see* Subheading 3.2).
3. Open the two syringe ports and the fill port. Transfer 1 ml cell suspension into the 10 ml vessel through the fill port.
4. Add 6–8 ml EB induction medium until the vessel is nearly full and close the fill port with cap.
5. To remove the bubbles in vessel, place two syringes (5 ml) on each syringe port, one is a media-filled syringe which has 2–3 ml of EB induction medium and the other is an empty syringe.
6. Inject the medium in the media-filled syringe through the syringe port and make sure the bubbles are removed into the empty syringe.
7. Close the syringe valves and remove the syringes. Place the syringe caps on the syringe ports and close it (*see* **Note 5**).
8. Attach the 10 ml cell suspension-filled vessel onto the rotator base by slowly turning in clockwise direction.
9. Place the vessel and rotator base into the cell culture incubator at 37 °C with 5 % CO_2 and 95 % humidity.
10. Turn on the power supply and set the initial rotation speed to 15–20 rpm (*see* **Note 6**).
11. After 24–48 h, ES cells are allowed to aggregate spontaneously in RCCS bioreactor and EBs are formed (Fig. 3a, b). Media change should be performed every 2 days.

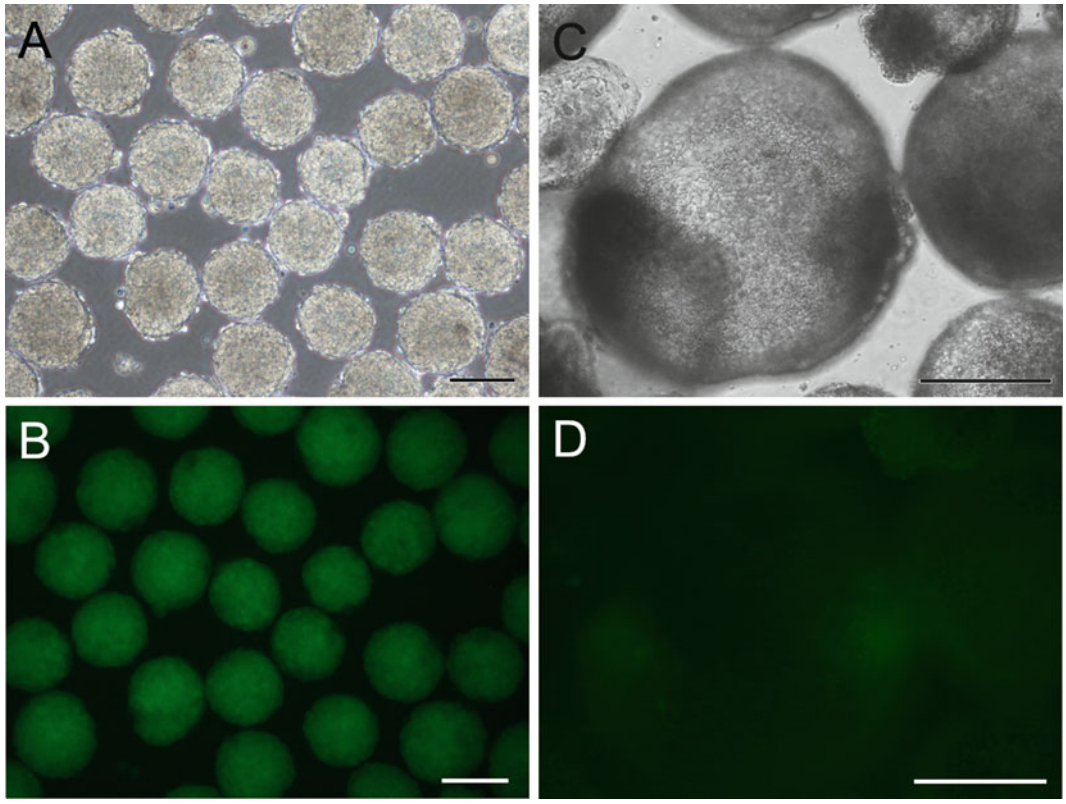

Fig. 3 Mouse embryoid body (mEB) appearance in rotary suspension culture. When mES cells are dissociated into single cell suspension and plated on RCCS bioreactor, they will form aggregates or embryoid bodies. These mEBs should appear homogeneous and as rounded spheres and increasingly cystic structures. (**a**, **b**) Phase contrast (**a**) and Oct4-GFP reporter fluorescence (**b**) of mEB after 24–48 h cultured in RCCS. (**c**, **d**) Phase contrast (**c**) and Oct4-GFP reporter fluorescence (**d**) of mEB cultured in RCCS for 8 days. Bars: 100 μm

12. For the medium change, turn off power and remove the vessel from the rotator base and take it to a biological hood. Let the cell aggregates settle to the bottom in static.
13. Open the valves and place two syringes (10 ml) on each syringe port; one is media-filled syringe which has 5–10 ml of EB induction medium and the other is empty syringe
14. Very carefully use the media-filled syringe to withdraw medium from the vessel as described above (*see* Subheading 3.2, **steps 6–8**). Usually, about 1/2 of the conditioned medium is left in the vessel and 1/2 of fresh medium will be added into the vessel (*see* **Note** 7).
15. Attach the vessel to the rotator base again and place them in the CO_2 incubator.
16. For 8 days of culture, EBs in RCCS exhibit a cystic structure (Fig. 3c, d).

3.5 Analysis of EBs Formed in RCCS: Real-Time PCR Detection

1. During EB differentiation, the EBs are collected at different time points.
2. For EBs collection, turn off power and remove the vessel from the rotator base and transfer the EBs from RCCS to a 15 ml centrifuge tube.
3. Perform the total RNA extraction, purification, reverse transcription, and real-time PCR according to the Product Information Sheet [15]. Briefly, EBs in different days of culture are collected by centrifugation, and the total RNA is purified with TRIzol reagent (Cat# 15596018 Life Technologies) (*see* **Notes 8** and **9**). The genomic DNA is eliminated using RQ1 RNase-free DNase I (Cat# M6101 Promega), and the cDNA is then synthesized with M-MuLV reverse transcriptase (Cat# M0253S, New England Biolabs). qPCR is performed using GoTaq® qPCR Master Mix (SYBR Green) (Cat# A6001 Promega) in the LightCycler® 480 II System thermocycler platform (Roche, USA).
4. The real-time PCR procedure is 94 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 68 °C for 25 s. Forward and reverse primers used in qPCR reactions (*see* Table 1). Primers are verified by melting curve examination, and their amplification efficiencies are calculated using a relative standard curve method [16, 17]. mRNA expression of each gene relative to that of GAPDH is calculated using the ΔCt method with efficiency correction (Fig. 4).

Table 1
Primers used for qPCR experiments

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Gapdh	CATGTTCCAGTATGACTCCACTC	GGCCTCACCCCATTTGATGT
Sox2	GGTTACCTCTTCCTCCCACTCCAG	TCACATGTG CGACAG GGGCAG
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
Gata4	CACCCCAATCTCGATATGTTTGA	GCACAGGTAGTGTCCCGTC
Sox17	GATGCGGGATACGCCAGTG	CCACCTCGCCTTTCACCTTTA
Sox1	AGATGCACAACTCGGAGATCAG	GAGTACTTGTCCTTCTTGAGCAGC
neuroD	AACCGCATGCACGGGCTGAA	GTTGGCAGATGCGGGGGCAT
Mixl1	GTCTTCCGACAGACCATGTACC	CCCGCCTTGAGGATAAGGG
Brachyury	GCTTCAAGGAGCTAACTAACGAG	CGTCACGAAGTCCAGCAAGA

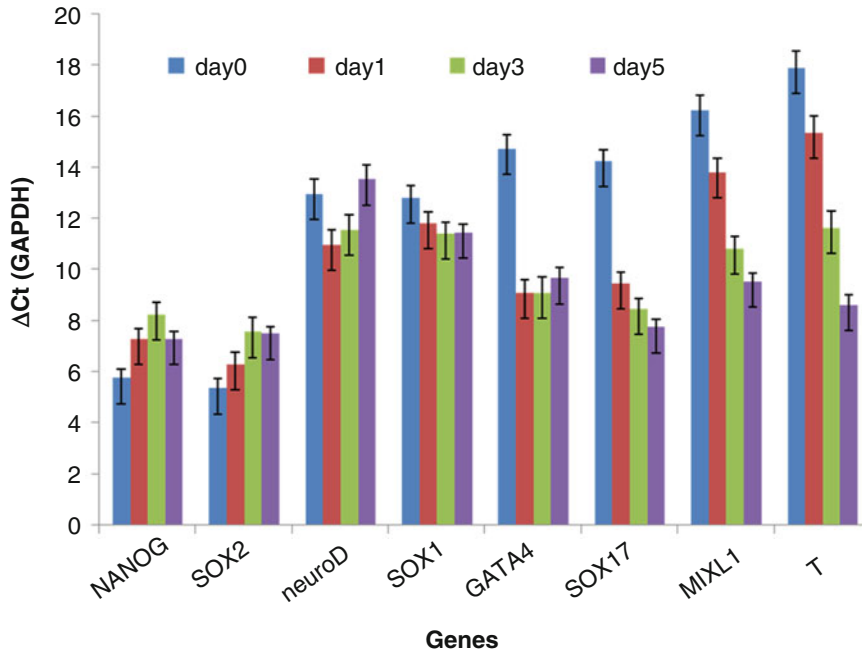

Fig. 4 The mRNA levels of pluripotent genes (NANOG, SOX2), ectoderm genes (neuroD, Sox1), endoderm genes (Gata4, Sox17), and mesoderm genes (Mixl1, T) in RCCS formed EBs were assessed by real-time PCR at day 0, 1, 3, and 5. mRNA expression of each gene relative to that of GAPDH was calculated using the ΔCt method. Mean and SEM from three separate experiments are shown

3.6 Analysis of EBs Formed in RCCS: Preparation of Slides and Immunostaining

1. After 6 days of culture, the EBs in RCCS are collected and prepared for immunostaining.
2. Transfer EBs from RCCS to 1.5 ml microcentrifuge tube and place the tube at static condition for 10 min to allow the EBs to sink to the bottom.
3. Remove medium from the tube and wash the EBs with PBS once.
4. Then fix with 4 % PFA at room temperature for 30 min and wash the EBs once with PBS.
5. Prepare 1.5 % aqueous agar solution and place the fixed and washed EBs into the tube containing the agar solution (1.5 % aqueous agar solution). The EBs are then centrifuged at 200 × *g* for 5 min. After discarding the supernatant, 1.5 % aqueous agar solution is added and let harden in a refrigerator (4 °C) for 30 min.
6. After 30 min, the EBs are enclosed as a pellet in hardened agar blocks.
7. The agar blocks are embedded and frozen in OCT (Tissue Tek). Frozen tissue sections are cut at 10 μm on Leica cryostat and mounted on SuperFrost Plus slides (Fisher) as previously described [18].

8. Let the slides dry for 10–20 min at room temperature and fix the slides with 4 % PFA at room temperature for 15 min.
9. Wash the slides twice with PBS for 5 min and permeabilize for 15 min with 0.2 % Triton X-100 at room temperature.
10. Block the slides for 1 h at 37 °C with blocking solution (*see* **Note 10**).
11. Incubate the slides with primary antibody at 4 °C (or few hours at RT with less antibody) in blocking solution.
12. Wash the slides twice for at least 15 min with 0.2 % Triton X-100 in order to reduce the background and wash once for 15 min in PBS.
13. Incubate the slides with secondary antibody in blocking solution for 1 h at room temperature.
14. Wash the slides twice for at least 15 min with 0.2 % Triton X-100 and wash once for 5 min in PBS.
15. Add nuclear stain solution to the slides and incubate for 5 min at room temperature.
16. Wash the slides twice for 5 min in PBS.
17. Put one drop of mounting medium, add coverslip, and seal with nail polish.
18. Observe the slides and acquire images under Nikon Ti microscope (Fig. 5).

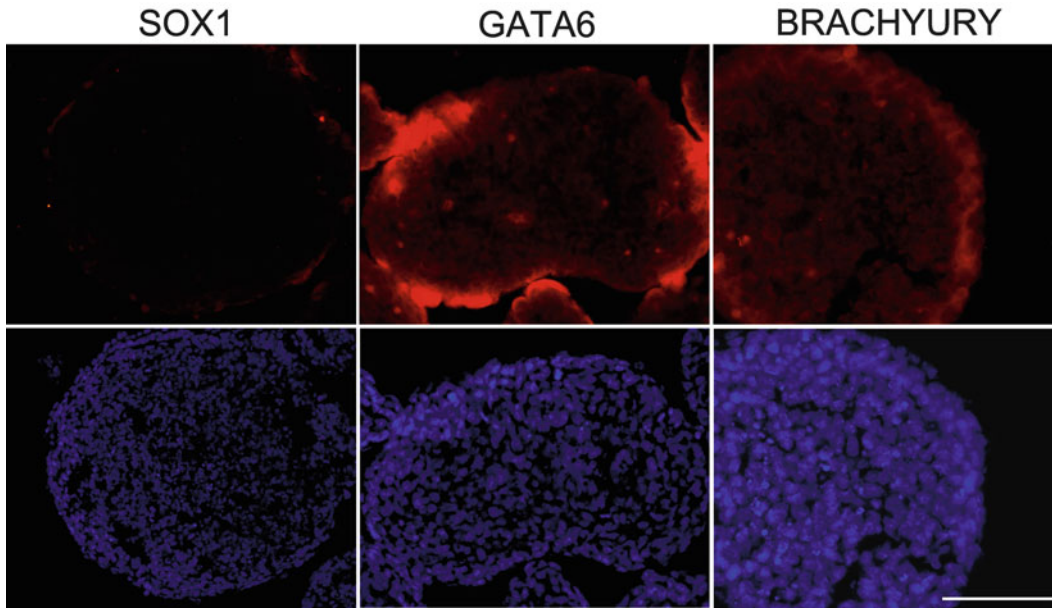

Fig. 5 Immunocytochemical staining of ectoderm marker (SOX1), endoderm marker (GATA6), and mesoderm marker (brachyury) in RCCS formed EBs at culture of day 4. *Blue*, nuclei. Bars: 100 μm

4 Notes

1. It is very important to use high-quality FBS and keep the same FBS lot throughout the cell culture. The quality of FBS should be tested before being used in large scale to culture mESCs. Different lots have differential effects on cell growth rate which in turn affects the EB formation and differentiation.
2. All disposable vessels are for single use; avoid reusing or resterilizing them.
3. Solubilization of paraformaldehyde powder needs low heat (52–56 °C), so do not heat the solution above 56 °C for a prolonged period of time, and adjust pH of 4 % paraformaldehyde solution (pH 7.2–7.4) with 1 N sodium hydroxide solution.
4. Importantly, to purify more mESCs separated from inactivated MEF, it is very important to control the attachment time of cell suspensions (no less than 30 min and no more than 1 h), and the cells should be observed every 5 min under microscope after 30 min attachment.
5. Check the vessel and be certain that valves and fill port are closed properly, ensuring that culture medium is not leaking from the vessel.
6. To get the higher EB concentration and homogenous EBs, we suggest the rotation speed of bioreactor in the range of 15–20 rpm.
7. After completion of medium change, make sure bubbles are removed.
8. In order to preserve RNA samples, which are very vulnerable to degradation at room temperature, do not wash cells before addition of TRIzol® Reagent to avoid increased chance of mRNA degradation.
9. Homogenize RNA samples using power homogenizer at room temperature to permit complete dissociation of the nucleoprotein complex.
10. Attention: do not let the frozen sections dry out in 37 °C and it should be carried out in a humidified chamber all the time to avoid drying of the sample after the slides have been fixed by 4 % PFA.

Acknowledgments

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