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Isolation and Differentiation of Self-Renewable Neural Stem Cells from Marmoset-Induced Pluripotent Stem Cells

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Abstract

Neural stem cells (NSCs) are multipotent and self-renewing precursor cells that give rise to all cell types of the central nervous system (CNS). They can be used for modeling CNS in vitro, for developmental studies and for cell replacement therapies. NSCs can be derived from pluripotent stem cells through differentiation using specific growth factors. Nonhuman primates (NHP) are critical preclinical models for translational research. Induced pluripotent stem cells (iPSCs) can be generated from NHP for the purposes of allogenic or autologous cell replacement studies. Here, we describe the derivation of NSCs from NHP iPSCs.

Keywords

Neural stem cells; Nonhuman primate; Induced pluripotent stem cells; Marmosets

1 Introduction

Animal cell lines and models have been used to provide significant advances in our understanding of human diseases, development, and evolution. However, there are intrinsic limitations to using smaller animal models as they are not completely representative of human biology. This limitation is mitigated with the use of nonhuman primates (NHP). NHP share similar physiology to that of humans and are an important and necessary intermediary model between, for instance, humans and rodents. In the context of cell replacement therapy, having an animal model with similar physiology, size, and complexity of the human immune system is relevant in preclinical studies. Understanding the subtle intricacies between the host immune system and graft is important prior to initiation of any clinical studies using pluripotent stem-based cell therapies. iPSCs derived from NHP offer the advantage of testing novel therapeutic approaches, such as autologous cell therapy. However, reliable methods for isolating and differentiating specific cell types from iPSCs are needed to understand the similarities and differences between nonhuman and human primates. In this chapter, we describe a method for the derivation of NSCs from NHP iPSCs. These NHP NSCs are able to grow in suspension culture and display a similar morphology to that of NSCs derived from human iPSCs.

2 Materials

2.1 Equipment

1. Cell culture incubator (NuAire, Plymouth, MN, USA).
2. Phase contrast microscope (Zeiss, Oberkochen, Germany).
3. Centrifuge (Eppendorf, Hamburg, Germany).
4. T25 tissue culture flask (Corning, Oneonta, NY, USA).
5. T75 tissue culture flask (Corning, Oneonta, NY, USA).
6. Cell lifter (Fisher Scientific, Pittsburgh, PA, USA).
7. Glass pipettes (Fisher Scientific, Pittsburgh, PA, USA).
8. Centrifuge tubes (15 mL and 50 mL) (Corning, Oneonta, NY, USA).
9. Syringe filters (Corning, Oneonta, NY, USA).
10. Syringes (10, 20, and 60 mL) (BD, Franklin Lakes, NJ, USA).
11. Pipettes (2–25 mL) (Fisher Scientific, Pittsburgh, PA, USA).
12. Pipette aids (10–1000 μ L) (Eppendorf, Hamburg, Germany).
13. Pipette tips (10–1000 μ L) (Accuflo, E&K Scientific, Santa Clara, CA, USA).
14. Water bath (Fisher Scientific, Pittsburgh, PA, USA).
15. Hemocytometer (Hausser Scientific, Horsham, PA, USA) or automated cell counter (Countess, Invitrogen, Carlsbad, CA, USA).

2.2 Reagents

1. NN1 media (NeoNeuron, San Antonio, TX, USA).
2. Basic fibroblast growth factor (Stemgent, Cambridge, MA, USA).
3. Epidermal growth factor (EGF, EMD Millipore, Burlington, MA, USA).
4. Retinoic acid (Sigma-Aldrich, St. Louis, MO, USA).
5. Fetal bovine serum (FBS, GE, Logan, UT, USA).
6. Poly-L-ornithine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA).
7. Accutase (Gibco, Life Technologies, NY, USA).
8. Trypsin neutralizer (Gibco, Life Technologies, NY, USA).
9. Tris base (Fisher Bioreagents, Pittsburgh, PA, USA).
10. Phosphate-buffered saline (PBS, Gibco, Life Technologies, NY, USA).
11. Double-distilled water (Gibco, Life Technologies, NY, USA).

3 Methods

3.1 Preparation of Reagents and Media

Prepare all the reagents under sterile conditions in a horizontal laminar flow hood

1. *Preparation of 10 mM Tris (25 mL):* Dissolve 30.35 mg of Tris base (F.Wt: 121.4) in 15 mL of double-distilled water and adjust the pH to 7.6. Increase the volume to 25 mL and filter sterilize. Store at 4 °C (see Note 1).
2. *Preparation of basic fibroblast growth factor stock solution:* Briefly centrifuge the tube, and reconstitute the bFGF (50 µg) in 2.5 mL of 10 mM Tris solution (pH 7.6) to prepare a 20 µg/mL stock solution. Aliquot and store at −20 °C.
3. *Preparation of epidermal growth factor stock solution:* Briefly centrifuge the tube, and reconstitute the EGF (500 µg) in 5 mL of double-distilled water to prepare a stock solution of 100 µg/mL. Aliquot and store at −20 °C.
4. *Preparation of retinoic acid (RA) stock solution:* Dissolve 3 mg of RA (F.Wt: 300.44) in 1 mL of distilled water to prepare 10 mM stock solution. Aliquot and store at −20 °C.
5. *Poly-L-ornithine hydrobromide (PLO):* Dissolve poly-L-ornithine hydrobromide (PLO) in D.D water under sterile conditions to prepare a 10× stock solution of 0.16 mg/mL. Aliquot and store at −20 °C.
6. *Preparation of neural stem cell (NSC) media (50 mL):* To 50 mL of NN1 media (NeoNeuron), add 50 µL of bFGF (20 µg/mL) and 10 µL of EGF (100 µg/mL) and 10 µL of RA (10 mM) to prepare NSC media with final concentration of 2 µM RA, 20 ng/mL of bFGF and EGF. Filter sterilize and store at 4 °C.
7. *Preparation of differentiation media (50 mL):* To 50 mL of NN1 media, add 500 µL of FBS (final concentration 1%) and 10 µL of RA (10 mM). Filter sterilize and store at 4 °C (see Note 2).

3.2 Isolation of NSCs from iPSCs

1. Culture and expand marmoset iPSCs (on feeder or feeder-free) in a 6-well plate until they are 80–90% confluent.
2. On the day of the experiment, pre-warm the NSC media in a 37 °C water bath.
3. Nonenzymatically detach the iPSC colonies using a cell lifter. Take care not to dissociate colonies into single cells.
4. Carefully collect the colonies from the 6-well plate using a 5 mL pipette, and transfer them to a 15 mL centrifuge tube. Do not triturate the cells.
5. Centrifuge at 1000 rpm ($200 \times g$) for 5 min.

¹Prepare all the solutions and reagents in double-distilled water or ultrapure water (18 MΩ cm at 25 °C).

²Calculate total amount of differentiation media required for the experiment and prepare the media in advance. This will help to maintain constant concentration of all the factors during differentiation.

6. Using vacuum-aided suction, carefully remove the media without disturbing the pellet using a glass pipette.
7. Resuspend the pellet in 5 mL of NSC media.
8. Centrifuge at 1000 rpm ($200 \times g$) for 5 min.
9. Resuspend in 8 mL of NSC media and plate in a T-25 flask.
10. Three days after plating or when the media turns yellow, replace old media with fresh NSC media (NN1 + 20 ng/mL bFGF + 2 ng/mL EGF + 2 μ M RA) (*see* Note 2).
11. To change media, gently decant the media from culture flask into a 50 mL centrifuge tube.
12. Centrifuge the cells at 1000 rpm ($200 \times g$) for 5 min.
13. Carefully remove the supernatant by using vacuum suction without disturbing the pellet.
14. Resuspend the pellet with fresh NSC media and replate them in a T-25 flask.
15. Change the media every 3–4 days or as required.
16. By the end of 2 weeks, the NSCs can be seen to form neurospheres.

3.3 Coating Culture Plates with PLO

1. Under sterile conditions, prepare working solution of PLO (16 μ g/mL) by diluting 1 mL of 10 $\times$ stock solution (0.16 mg/mL) in 9 mL of D.D water.
2. Add PLO working solution to each well of the desired plate type (6–96 well) to cover the entire surface. We typically use the following volumes for each well plate type:

Plate type	Volume (μ L)
96	25–30
48	80
24	250
12	500
6	1000

3. Incubate the plate in the cell culture incubator for a minimum of 2 h.
4. After incubation, wash the plate twice with sterile PBS and plate the cells. The plates can be prepared a day in advance and stored 4 °C under sterile culture conditions.

3.4 Differentiation of iPSC-Derived NSCs

Pre-warm the differentiation media in a 37 °C water bath (Fig. 1).

1. Collect the floating neurospheres from the T-25 flask (*see* Fig. 1) into a 15 mL tube, and centrifuge at 1000 rpm ($200 \times g$) for 5 min.
2. Carefully remove the supernatant by using vacuum suction without disturbing the pellet.
3. Gently resuspend the pellet in fresh 10 mL of fresh NN1 media and centrifuge again at 1000 rpm ($200 \times g$) for 5 min.
4. Resuspend the neurospheres in differentiation media (NN1 + 1% FBS).
5. Plate the neurospheres in PLO-coated wells. We typically use the following volumes for each well plate type:

Plate type	Volume (μ L)
96	100
48	250
24	500
12	1000
6	2000

6. Day 0: The day of plating the neurospheres with differentiation media.
7. Change media every 24 h with fresh differentiation media until the completion of the experiment or transplantation.

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Fig. 1.

Differentiation of NSCs isolated from marmoset iPSCs. (a) Representative phase-contrast image of neurospheres isolated from marmoset iPSCs. NSCs isolated from marmoset iPSCs with or without retinoic acid (RA) were differentiated for 7 days in NN1 media containing 1% FBS. Immunofluorescence staining results showed that NSCs isolated with (b) control media (without RA in NSC media) contained less number of cells positive for neuronal marker β -III-tubulin (TuJ1) compared to (c) those isolated with RA-containing NSC media