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Reproducible expansion and characterization of mouse neural stem/progenitor cells in adherent cultures derived from the adult subventricular zone

Michelle H. Theus, Jerome Ricard, and Daniel J. Liebl

The Miami Project to Cure Paralysis and Department of Neurological Surgery, University of Miami, 1095 NW 14th Terrace, Miami, FL, 33136 USA

Abstract

Endogenous neural stem/progenitor cells (NSPCs) residing in the subventricular zone (SVZ) of the adult mouse forebrain have been shown to enhance their neurogenic potential in response to CNS injury. Mechanisms involved in regulating adult neurogenesis under naïve or stressed conditions can be studied using a monolayer cell-culture system of the nestin-expressing NSPC lineage to analyze proliferation, survival and differentiation. Here, we describe a protocol for the expansion of NSPCs for studies aimed at understanding the functional role of NSPCs in maintaining adult neurogenic processes. In this unit, we outline in detail the procedures for: (1) isolation, maintenance and culture of the NSPC component of the SVZ niche from the lateral wall of the lateral ventricle; (2) characterization of NSPC functions by examining proliferation, survival and differentiation; and (3) efficient siRNA transfection methods in 96-well format.

Keywords

Neural progenitor cell; subventricular zone; cultures; proliferation; survival; differentiation; siRNA

INTRODUCTION

Neurogenesis, the generation of new neurons, is an embryonic process that is maintained in the adult subventricular zone (SVZ)-rostral migratory (RMS)-olfactory bulb system and involves coordinated efforts in regulating the cell growth and differentiation of the type-B1, glial fibrillary acidic protein (GFAP)-positive neural stem cells and type-C, epidermal growth factor receptor (EGFR)-positive transit-amplifying intermediate progenitor cell populations (Morshead, Reynolds et al. 1994; Doetsch, Garcia-Verdugo et al. 1997; Doetsch, Caille et al. 1999). These two populations of neural progenitor cells (NSPCs) have typically been grown *in vitro* as neurospheres in serum-free medium containing epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF), which maintains their self-renewal and multi-lineage differentiation potential. This method of culturing NSPCs is restrictive since the cellular dynamics within a compact sphere are affected by position and size of the neurosphere, where limited exposure to oxygen and media supplements, cell-cell contact, and paracrine effects can all influence cell growth, survival and differentiation. These variabilities make it difficult to accurately measure growth rates, cell differentiation, migration potential and survival using standard assays. Adherent cultures, of NSPCs derived

from the SVZ, allow for greater versatility in the analysis of cellular functions as well as the possibility of co-culture systems.

This unit describes a method for the expansion of SVZ-derived mouse NSPCs as a monolayer that can be used subsequently for experiments examining proliferation, survival and differentiation. The adherent cell culture method also allows for efficient gene knockdown using siRNA approaches, a difficult task when cells are grown as neurospheres.

ISOLATION AND EXPANSION OF NSPCS FROM THE ADULT SVZ

This protocol is used to isolate the nestin-positive neural stem and transit-amplifying intermediate progenitor cells (Figure 1, A–C) from the freshly dissected lateral wall of the lateral ventricles. Tissues are enzymatically digested and prepared for monolayer cultures in plastic-culture dishes. Cells are grown to confluency and growth factors added every other day based on previous methods (Scheffler, Walton et al. 2005) with modifications.

Materials

5 adult male mice (2–4m)
Stereomicroscope
Leibovitz L-15 medium (Invitrogen)
100 mm × 20mm Uncoated tissue culture treated dishes (BD biosciences)
40 µm cell strainer (Becton Dickinson)
0.05% Trypsin-EDTA (Invitrogen)
Sterile phosphate buffered saline (PBS) (Invitrogen)
DMSO (Sigma)
Cryovials (Corning)
Mr. Frosty Cryo Freezing Container (Nalgene # 5100-0001)

Methods

Isolation of NSPCs and culture initiation—

1. Prior to use, add EGF and basic FGF (40 ng/ml final) to MCM-E/F. Mix well.
2. Prepare 10 ml dissociation solution (see recipe), filter sterilize through a 0.22 µm syringe filter and pre-warm at 37°C in a water bath.
3. Using sterile technique, carefully dissect the lateral wall of the lateral ventricles from 5 mice in L-15 medium. Then place the dissected lateral wall pieces in 10 ml of dissociation solution (step 2) and incubate in a 37°C water bath for 15 min.
4. Triturate 10 times with a 5 ml pipette and return to 37°C water bath for 15 min.
5. Add 5 ml of MCM-E/F (step 1) and triturate 10 times with a 5 ml pipette
6. Place a 40 µm strainer over a 50 ml conical tube and pipette digested tissue into the strainer to remove tissue debris.
7. Transfer cell suspension that was collected in the 50 ml conical tube to a 15 ml tube and spin at 1000 rpm for 5 min.
8. Resuspend pellet in 10 ml of MCM-E/F and place in a 100 mm untreated culture dish and incubate at 37°C for 24 hours

9. After 24 hours, remove medium with non-adherent cells and transfer to a new 100 mm untreated culture dish.
10. Add EGF and FGF (20 ng/ml final each) to the dish every other day.
11. Change medium after 4 days in culture and continue growing up to 1 week.
12. Confluent cells can now be passaged with 0.05% trypsin/EDTA and replated. The cells are defined to be passage 1 and can be passaged for several generations or frozen and stored at P1 in liquid nitrogen.

Passaging—

1. Once cells become confluent, aspirate medium and rinse dish with 10 ml PBS.
2. Aspirate PBS and add 2 ml 0.05% trypsin/EDTA and incubate at 37°C for 3 minutes.
3. Stop trypsin reaction with 4 ml MCM-E/F and transfer cell suspension to a 15 ml tube.
4. Spin at 1000 rpm for 5 minutes.
5. Aspirate medium and replace with fresh MCM-E/F.
6. Count cells on hemacytometer.
7. Replate 500,000 cells/100 mm dish in 10 ml MCM-E/F. Plate as many dishes as you can during P1 to expand and freeze after they reach confluency.
8. Add fresh EGF and bFGF (20 ng/ml final each) every other day until cells reach confluency, approximately 1 week.
9. After 1 week repeat passage procedure (1–6).
10. Replate in 100 mm dishes (7) to continue passage and begin High-throughput studies if desired by plating 2,000–10,000 cells per well in a 96-well plate in 100 μ l MCM-E/F.

Freezing and thawing—

1. Once cells become confluent during Passage 1, they can be stored in liquid nitrogen using a slow freeze method.
2. Following the passage procedure (steps 1–7 above), spin down the cells and resuspend the pellet in freezing medium (see recipe). Add enough freezing medium to make the cell suspension at a final concentration of 1×10^6 cells/ml.
3. Quickly add 1 ml of cell suspension in freezing medium to a pre-labeled cryovial. Transfer the cryovials to a Mr. Frosty CryoFreezing container (Nalgene # 5100-001) and place at -80°C overnight. The cryovials are then ready to be permanently placed in liquid nitrogen.
4. To thaw cells, add cryovials to a 37°C water bath just until thawed. Quickly add the 1 ml of cell suspension to 10 ml of MCM-E/F in a 15 ml tube, as extended exposure of cells to DMSO is toxic. Mix by inverting tube.
5. Spin cells at 1000 rpm for 5 min. and resuspend in 10 ml fresh MCM-E/F, transfer to a 100 mm culture dish and incubate at 37°C until confluent. Add fresh EGF and bFGF (20 ng/ml final each) every other day. Cells should reach confluency by 5–7 days. Continue to passage primary neural stem/progenitor cells up to 6 passages before thawing a new vial.

ANALYSIS OF PROLIFERATION, SURVIVAL AND DIFFERENTIATION OF MOUSE NSPCs IN 96-WELL FORMAT

Materials

MCM-E/F (see recipe)
Differentiation medium (see recipe)
Bromodeoxyuridine (BrdU, Sigma)
anti-BrdU antibody (Roche)
borate buffer (see recipe)
Live/Dead Assay (Invitrogen)
Hoechst (Invitrogen)
10% buffered formalin (Azer Scientific)
96-well plates
1X PBS
1X PBST
Fluorescence Microscope

Methods

Proliferation—

1. Seed 2,000 cells/well in a 96-well plate in 100 μ l MCM-E/F and incubate overnight at 37°C for 24 hours.
2. Treat cells if desired and incubate up to addition 3 days.
3. Label dividing NSPCs with BrdU (10 μ M; dissolved in 1X PBS) for 1 hour and immediately aspirate medium and fix with 10% buffered formalin for 10 minutes.
4. Wash each well with 1X PBST for 5 minutes (3 washes total)
5. Remove 1X PBS and add 100 μ l of 2N HCL+ 0.2% Triton to each well and incubate at 37°C for 30 minutes. This step is necessary to denature the DNA and to permeabilize the cell membrane.
6. Wash each well with 100 μ l borate buffer. Wash once for 10 minutes.
7. Wash each well once with 1X PBST.
8. Remove last PBS wash and add 50 μ l of anti-BrdU (1:200) in PBST to each well overnight at 4°C.
9. Wash each well 3 times with PBST
10. Apply desired secondary antibody, wash with 1X PBS and counter stain with 1 μ g/ml Hoechst.
11. Analysis of proliferation can be performed up to 4 days after plating when seeding at 2,000 cells without overcrowding under normal or any treated conditions.

Survival—

1. Seed cells as described above.

2. Treat cells if desired 24 hrs. after plating and incubate up to 3 days.
3. Analyze cell death using Live/Dead assay. Label dead NSPCs and counter stain with Hoechst. Add 100 μ l of MCM-E/F containing dissolved Live/Dead solution at 1:1000 and Hoechst at 1 μ g/ml (Mixed well) to each well containing 100 μ l medium with cells.
4. Incubate at 37°C for 10 min. and immediately count the number of labeled cells against the total number of Hoechst-positive cells.

Differentiation—

1. Seed 10,000 cells/well in 1 ml MCM-E/F in 24-well plates (Basic protocol 2).
If high magnification microscopy is required later, place a round glass coverslip at the bottom of the well. The cells grow a little less well on the glass however. This density allows for the cells not to be too close from each other before initiating differentiation.
2. Treat cells if desired and incubate up to 3 days in MCM-E/F medium.
3. To initiate differentiation, switch the medium from MCM-E/F to differentiation medium.
4. When differentiation needs to be assessed, wash the cultures twice in 1X PBS, then fix in 500 μ l 4% paraformaldehyde at room temperature for 20 minutes.
5. Wash with 1X PBS, then proceed with immunostaining.

The development of neurons can be examined using an antibody against β – tubulin III (Millipore #MAB5544 Figure 1D) used at 1:1000. Neurons can be observed as early as two days after initiating differentiation. The longer the differentiation period, the more neurons and the longer the processes they extend.

EFFICIENT siRNA TRANSFECTION OF MOUSE NSPCS USING HiPerfect SYSTEM IN 96-WELL FORMAT

Efficient siRNA transfection of mouse neural stem/progenitor cells has been hindered by the high level of toxicity following standard protocols. We have found that using the HiPerfect reagent solution with a modified transfection protocol results in high transfection efficiency (~60%) and low toxicity. This protocol was designed in 96-well format to minimize reagent use, amount of cells required and to allow set-up of multiple screenings for high-throughput studies if desired. Here we describe the transfection of adherent NSPCs using a fluorescein-labeled siRNA (Figure 1, E–H), which should be used as a positive control alongside any siRNA to be tested. Each siRNA should first be titrated to a final concentration that ranges from 10 nM to 75 nM per well, using the lowest concentration that does not cause any toxicity. We also suggest initially knocking down GAPDH using GAPDH siRNA control and testing efficiency by Western blot analysis at 48 hrs. post-transfection.

Materials

MCM-E/F without FBS

MCM-E/F (See protocol 1)

HiPerfect (Qiagen #301704)

Fluorescein-labeled Signal Silence control siRNA (Cell signaling)

96-well plates

1X Phosphate buffered saline (PBS)

Fluorescence Microscope

Methods

1. Seed 2,000–4,000 cells in 100 μ l MCM-E/F per well in a 96-well plate and incubate at 37°C for 24 hours.
2. Prior to transfection, all reagents should be thoroughly thawed, vortexed and placed on ice.
3. Prepare a batch of MCM-E/F without 5% FBS and heat in a 37°C water bath.
4. Prepare siRNA complexes as follows (Recipe is for transfection of 4 wells in a 96-well plate):
 - a. Add 5 μ l Fluorescein-labeled siRNA (stock= 10 μ M; final 50 nM/well) to 250 μ l MCM-E/F w/o FBS and vortex for 10 seconds.
 - b. Add 1.9 μ l of HiPerfect reagent, vortex and incubate for 15 minutes at room temperature.
 - c. Mix solution with gentle pipetting and add 50 μ l of solution to each well and rotate the 96-well plate to mix.
 - d. Incubate at 37°C for 24 hours. Wash transfected wells with MCM-E/F medium to visualize transfected cells (a fluorescence microscope is used to visualize the fluorescein-labeled siRNA in transfected cells).
5. It is not necessary to change the medium during the 24–72 hr. transfection incubation.
6. Test efficiency of transfection using GAPDH control siRNA (Dharmacon) after 48–72 hours by Western blot or immunofluorescence analysis using anti-GAPDH antibody.
7. Treat cells as needed after efficient transfection is established, usually after 24 hours the medium can be replaced with the one containing drugs or treatments containing MCM-E/F. Proliferation and survival can be subsequently tested after another 24–48 hours.

REAGENTS AND SOLUTIONS

Monolayer cell culture medium (MCM-E/F)

DMEM/F12 (InVitrogen) containing:

5% (v/v) fetal bovine serum (HyClone)

1% (v/v) N2 supplement (InVitrogen)

35 μ g/ml bovine pituitary extract (BPE) (InVitrogen)

1X penicillin-streptomycin solution (from 100X stock, InVitrogen)

20 ng/ml epidermal growth factor (EGF) (Millipore)

20 ng/ml basic fibroblast growth factor (bFGF) (Millipore)

Store at –80°C.

Solution 1

50 ml 10X HBSS (InVitrogen)
2.7 g D-glucose (Sigma)
7.5 ml HEPES 1M (InVitrogen)
Adjust pH to 7.5 and bring the volume up to 500 ml with water.
Sterilize by filtering through 0.22 μ m filters and distribute as 5 ml aliquots.
Store at -80°C .

Dissociation solution

10 ml of solution 1 (see recipe above) containing:
13.3 mg trypsin (Sigma #T4665)
7 mg hyaluronidase (Sigma #H3884)
2.0 mg kynurenic acid (Sigma #K3375)

Differentiation medium

DMEM/F12 medium (InVitrogen) containing:
5% (v/v) fetal bovine serum (HyClone)
1% (v/v) N2 supplement (InVitrogen)
1X penicillin-streptomycin solution (from 100X stock, InVitrogen)

Freezing medium

85% (v/v) DMEM/F12 (InVitrogen)
5% (v/v) fetal bovine serum (HyClone)
10 % (v/v) dimethylsulfoxide (DMSO) (Sigma)

Borate buffer

Dissolve 3.81 g sodium tetraborate decahydrate in 50 ml of water
Adjust the pH to 8.5 and add water up to 100 ml.

1X PBST

Mix 200 μ l Triton-X100 to 100 ml PBS.

COMMENTARY

Neural stem/progenitors cells within the adult CNS are restricted to neurogenic regions or niches. Growth within these highly populated zones, like the SVZ, are regulated by local environmental cues such as cell-cell contact proteins, diffusible molecules, extracellular matrix molecules and neighboring blood vessels (Alvarez-Buylla and Garcia-Verdugo 2002; Jin, Mao et al. 2002; Ricard, Salinas et al. 2006; Shen, Wang et al. 2008). These interactions work in concert to regulate proliferation, survival and migration during normal homeostasis and under stress conditions such as brain injury. Providing an efficient method for analyzing cellular function within the heterogeneous environment of the SVZ is critical, but employing *in vitro* models of SVZ-derived cells is also important for examining mechanism. Reproducible expansion of adherent monolayer cultures can now be used in replacement of

the neurosphere assay to address such issues, and can be derived from either the neonatal or adult SVZ (Del Valle, Theus et al. 2011; Theus, Ricard et al. 2010).

Troubleshooting

One advantage of SVZ-derived cells is the flexibility to culture cells as both adherent monolayers and neurospheres, and the flexibility to interchange between each method. Although adherent cultures can be initiated immediately from cells extracted from the lateral wall of the ventricle, it is also possible to begin with neurospheres then transfer to monolayer medium. This can sometimes yield a purer population of nestin-positive cells, which can be followed by trypsinization and re-suspension in monolayer medium. Inversely, undifferentiated adherent cultures may also be grown as neurospheres at any passage by re-suspension in neurosphere media containing EGF and bFGF. Adherent cultures should always be checked for nestin expression using immunostaining methods prior to further passaging and freezing. However, to limit the growth of cells that may accumulate chromosomal aberrations, frozen stocks are re-derived after 6–7 passages when assessing proliferation, survival and migration.

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Figure 1.

Adherent cultures of NSPCs during normal growth, differentiation and siRNA knockdown. (A and B) Bright field images at 10× magnification of NSPCs grown on 100 mm plastic dishes at 24 hr (A) and 5 days (B) after plating. (C) Double immunofluorescence labeling using anti-nestin (green) and anti-glial fibrillary acidic protein (GFAP; red) is used to identify neural progenitor cells and visualized under fluorescence at 10× magnification. (D) Differentiation of NSPCs into β -tubulin III-positive neurons (green) and GFAP-positive astrocytes (red) following 7 days growth factor removal is visualized under fluorescence at 40× magnification (Rola, Mizumatsu et al. 2006). Representative images at 20× magnification of adherent NSPCs that were cultured for 24 hr in the presence of fluorescein-

labeled control siRNA (F;green) at 50 nM. Cells were co-labeled with Hoechst nuclear stain (H; blue). Scales bars=200 μ M.