Neural precursor generation from the mouse embryonic stem cell line D3

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I. Media Preparation

HepG2 medium (1L)

875 ml DMEM, High Glucose (Invitrogen, Cat# 12100-061)
100 ml Fetal Bovine Serum, ES-grade (HyClone, Cat# SH30070.03)
10 ml Sodium Pyruvate, 100 mM Solution (Cellgro, Cat# 25-000-CI)
10 ml Penicillin-Streptomycin Solution, 10000 U/ml, 10000 µg/ml (Cellgro, Cat# 30-002-CI)
5 ml L-Glutamine, 200 mM Solution (Cellgro, Cat# 25-005-CI)

MEDII conditioned medium

1. Seed $1.125x10^7$ HepG2 (ATCC, Cat# HB-8065) cells in 64 ml HepG2 medium in a T225 flask and incubate at 37°C and 5% CO_2 for 96 hours.
2. Collect conditioned medium into 250 ml centrifuge tubes and spin at 3000 rpm and room temperature for 5 min.
3. Filter conditioned medium (22 µm) and store at 4°C for up to 30 days.
4. To repeat steps 1-3, detach HepG2 cells from the T225 with 5ml Trypsin-EDTA (Invitrogen, Cat# 25200) and forceful banging against the T225. Neutralize Trypsin with 10 ml HepG2 medium and count cells prior to reseeding. Conditioned medium can be collected from the same HepG2 cell population for up to 3 months.

50% MEDII medium (200 mL)

100 ml HepG2 medium
100 ml MEDII conditioned medium
1 ml Penicillin-Streptomycin Solution, 10000 U/ml, 10000 µg/ml (Cellgro, Cat# 30-002-CI)
0.5 ml L-Glutamine, 200 mM Solution (Cellgro, Cat# 25-005-CI)
0.4 ml β-mercaptoethanol, 50 mM Solution*

*50 mM working stock is made by adding 100 µl 14.3M β-mercaptoethanol (Sigma, Cat# 60-24-2) to 28.42 ml Phosphate Buffered Saline (Cellgro, Cat# 21-040-CM).

Chemically defined medium (100 mL)

50 ml DMEM, High Glucose (Invitrogen, Cat# 12100-061)
50 ml Hams F-12 (Invitrogen, Cat# 11765-054)
1 ml Penicillin-Streptomycin Solution, 10000 U/ml, 10000 µg/ml (Cellgro, Cat# 30-002-CI)
0.5 ml Sodium Pyruvate, 100 mM Solution (Cellgro, Cat# 25-000-CI)
0.5 ml L-Glutamine, 200 mM Solution (Cellgro, Cat# 25-005-CI)
0.1 ml 1000x ITSS*
0.1 ml 10 µg/mL FGF2**

*1000x ITSS is 5 mg/ml Insulin, 5 mg/ml Transferrin, 5 µg/ml Sodium Selenite. This can be made by adding 2.5 ml of 10 mg/ml Insulin, 2.5 ml of 10 mg/ml Transferrin, and 25 µl of 1 mg/ml Sodium Selenite. For making 10 mg/ml Insulin, 100 mg of Insulin (Sigma I-1882, 100 mg) is dissolved in 9.9 ml ddH_2O + 0.1 ml glacial acetic acid (and stored at -20^oC). For making 10 mg/ml Transferrin, 100 mg Transferrin (Sigma 90190, 100 mg) is dissolved in 10 ml ddH_2O (and stored at -20^oC). For Sodium Selenite, 0.150 g of Sodium Selenite (Sigma, S-5261) is dissolved in 15.0 ml ddH_2O to first make 10 mg/ml, which is then diluted 10-fold to make 1 mg/ml.
**50 µg of FGF2 (Human Fibroblast Growth Factor-basic, Unconjugated, Peprotech#F100-18B) is diluted in 5 ml ddH_2O to make 10 µg/ml.

II. Differentiation Protocol

Day 0 – Seed
1. Suspend $1.0x10^6$ D3 ES (ATCC, Cat# CRL 1934) cells in 10 ml 50% MEDII medium and add to 100 mm bacterial-grade petri dish (BD Biosciences, Cat# D1990-4). Incubate at 37°C and 5% CO_2 for 48 hours.

Day 2 – Split 1:4
2. Transfer ~10 ml cells into a 50 ml tube and wait ~2 min to let the cells settle at the bottom. Use the supernatant to rinse the dish and collect any remaining cells. Transfer them to the same 50 ml tube.
3. Centrifuge at 1200 rpm for 30 sec. Aspirate medium and gently resuspend in 40 ml 50% MEDII medium. Add 10 ml to each of four 100 mm petri dishes. Incubate at 37°C and 5% CO_2 for 48 hours.

Day 3 – Collect
4. Collect cells from 2 dishes for analysis (EBM3/EPL stage).

Day 4 – Split 1:2
5. Swirl each dish with wide circles to gather cell aggregates (embryoid bodies) at the center of the dish and transfer all the aggregates to a 50 ml tube.
6. Let the aggregates settle for ~3 min. Aspirate medium and gently resuspend in 40 ml 50% MEDII medium. Add 10 mL to each of four 100 mm petri dishes. Incubate at 37°C and 5% CO_2 for 24 hours.

Day 5 – Feed
7. Swirl each dish with wide circles to gather the aggregates at the center of the dish and aspirate medium from one side. As the bodies start to move toward that side of the dish, slowly move the aspiration pipette to the other side of the dish until all medium is removed. Add 10 ml 50% MEDII medium. Incubate at 37°C and 5% CO_2 for 24 hours.

Day 6 – Collect and Feed
8. Collect cells from 2 dishes for analysis (EBM6 stage).
9. Feed remaining cells by repeating step 7.

Day 7 – Switch to Chemically defined medium
10. Feed cells by repeating step 7, except use 10 ml Chemically defined medium.

Day 8 – Feed
11. Feed cells again by repeating step 10.

Day 9 – Collect
12. Collect remaining cells for analysis (EBM9/NPC stage).