DIFFERENTIATION OF INDUCED PLURIPOTENT STEM CELLS INTO CORTICAL NEURONS

(Protocol adapted from Yichen et al., Nat Protoc. 2012[1])

Cellular reprograming of adult somatic cells to a pluripotent state allows the generation of patient-specific induced pluripotent stem cells (iPSC) and holds the potential to overcome the limitations of mouse models of cortical diseases such as autism and schizophrenia.

This protocol describes the generation of deep- and upper-layer excitatory neurons from human iPSCs based on the differentiation toward the anterior neuroectodermal lineage due to dual SMAD signaling inhibition.[1]

MATERIALS

REAGENTS:

TeSR™-E8™ Kit , Stemcell Technologies, cat. no. 05940
DMEM/F-12, GlutaMAX, Life Technologies, cat. no. 31331-028
Neurobasal, Life Technologies, cat. no. 12348-017
Insulin, Sigma, cat. no. 19278
l-Glutamine, Life Technologies, cat. no. 25030-024
B-27 supplement, Life Technologies, cat. no. 17504-044
N-2 supplement, Life Technologies, cat. no. 17502-048
Non-essential amino acid solution, Life Technologies, cat. no. 11140-050
PBS, without calcium and magnesium, Life Technologies,cat. no. 14190-094
Penicillin-streptomycin, Life Technologies, cat. no. 15140-122
Sodium pyruvate, Sigma, cat. no. S8636
2-Mercaptoethanol, Life Technologies, cat. no. 21985-023
Matrigel (growth factor reduced), BD Bioscience, cat. no. 354230
Laminin, Sigma, cat. no. L2020
Poly-l-ornithine solution, Sigma, cat. no. P4957
EDTA Solution 0.5 M, pH 8, AppliChem, cat. no. A4892.0500
Dispase, Corning -BD Bioscience, cat. no. 354235
StemPro® Accutase® Cell Dissociation Reagent, Life Technologies, cat. no. A11105-01
SB431542, Tocris Bioscience, cat. no. 1614
Dorsomorphin, Stemgent, cat. no. 04-0024
ROCK inhibitor (ROCKi) Y27632, Stemgent, cat. no. 04-0012

REAGENT SETUP:

EDTA 0.5 mM Solution

Add 500 µl of the 0.5 M EDTA stock solution (pH 8.0), to 500 ml of Calcium/Magnesium-free PBS, containing 0.45 g NaCl. Sterile filter (0.22um), aliquot and store at 4 °C for 6 months.

Matrigel-coating:

Dilute the 1 ml of matrigel stock solution 1:30 with PBS. Cover the bottom of a 12-well plate with matrigel (500µl/well) of the diluted solution. Incubate at 37 °C for 30 minutes. Aspirate the solution before addition of the medium.

Poly-l-ornithine/laminin-coating:

Cover the bottom of each well of a 6-well plate with 1 ml of 0.01% (wt/vol) poly-l-ornithine solution. Incubate at 37 °C for 4 h. Aspirate the solution and coat with laminin as described.

Laminin-coating:

Dilute the 1 mg/ml laminin stock 1:50 with PBS to a final concentration of 20 µg/ml. Cover the bottom of the dish with 1 ml of the diluted solution. Incubate at 37 °C for 4 h. Aspirate the solution before addition of the medium.

TeSR™-E8™ medium:

Pipette the entire 25 mL of thawed TeSR™-E8™ 20X Supplement and the entire 1 mL of thawed TeSR™-E8™ 500X Supplement to the TeSR™-E8™ Complete Medium Bottle, for a total volume of 500 mL. Complement with 1% Penicillin-streptomycin and mix well.

Neural maintenance medium:

This is a 1:1 mixture of N-2 and B-27-containing media. N-2 medium consists of DMEM/F-12 GlutaMAX, 1× N-2, 5 µg/ml insulin, 1 mM l-glutamine, 100 µM nonessential amino acids, 100 µM 2-mercaptoethanol, 50 U/ml penicillin and 50 mg/ml streptomycin. B-27 medium consists of Neurobasal, 1× B-27, 2 mM l-glutamine, 200µM ascorbic acid, 50 U/ml penicillin and 50 mg/ml streptomycin. Store the medium at 4 °C and use it within 3 weeks.

Neural induction medium:

Neural maintenance medium is supplemented with 1 µM Dorsomorphin and 10 µM SB431542. Store the medium at 4 °C and use it within 5 d.

SAFETY ISSUES

The use of human biological material can be potentially dangerous as a carrier of diseases. Care should still be taken to ensure there is no exposure to the cultured cells. There is also the risk of that pluripotent stem cells are potentially teratoma forming if these cells are accidently delivery in vivo (eg pricking the skin with a syringe needle). Therefore, autologously-derived cells (ie cells derived from people working in the lab) should not be cultured in the lab.

NEURAL INDUCTION

Preparing for neural induction: TIMING ~1 day

1. Coat each well of a 12-well plate with Matrigel (500µl/well) for 30 min at 37 °C.
2. Isolate the PSC colonies with **2 mL** of **0.5mM EDTA**:
    a) Aspirate medium 25 cm ² cell culture flasks
    b) Rinse 2x 2 ml EDTA 0.5 mM
3. Add 2 mL of 0.5mM EDTA and let stand 3 minutes under the bench. Aspirate the EDTA solution and knock the flask 3-5x to promote cell detachment.
4. Re-suspend the colonies in **1 ml of** E8 ™ Medium with ROCKi (**10uM**) careful and thoroughly (no more than 5x)
5. Plate sufficient volume of cell suspension to complete 200 000 cells per well
6. Ensure that the cells are evenly distributed in the wells and dishes. Allow the cells to attach overnight in the incubator

    ***CRITICAL STEP**: Ensure that the plating density is as high as proposed here to ensure that the cells reach 100% confluence 1 day after plating.*

Neural induction: TIMING ~10 days

1. Check the cells on the day after plating. If the cells have reached ~**100% confluence, wash with pre-warmed PBS and add 1 ml of neural induction medium per well**. If the cells are not 100% confluent, incubate in E8 medium until they reach 100% confluence, at which point the medium should be switched to neural induction medium.

2. Continue to incubate cells (10 days), replacing the neural induction medium every day. Closely monitor for morphological changes of the Pluripotent Stem Cells (PSCs) during differentiation. PSCs with large nuclei should gradually be replaced by tightly packed neuroepithelial cells with notably smaller nuclei (Figure1).
3. Between days 8 and 12 after plating, a uniform neuroepithelial sheet should appear. At this point, collect the neuroepithelial cells using dispase. A**dd 100 µl of 37 °C dispase** stock directly into the medium in the well of the 12-well plate.

   CRITICAL STEP: *Ensure that a uniform neuroepithelial cell layer has formed before passaging the cells.*
4. Incubate at **37 °C for 3 min**.
5. Gently break the neuroepithelial sheet into aggregates of 300 to 500 cells by scrapping the cells and slowly pipetting up and down three times with a P1000 pipette.
6. Pellet the cells by centrifugation at **900rpm for 2 min** at room temperature and discard the supernatant. Re-suspend the cells with **10 ml of 37 °C neural maintenance medium**, pellet the cells by centrifugation at **900rpm for 2 min** at room temperature and discard the supernatant. **Repeat this wash.**
7. Gently resuspend the cells in **200 µl of neural induction medium**.
8. Plate the cells from each well of the 12-well plate into individual **laminin+poly-L-ornithine-coated 6-well plate containing 2 ml of neural induction medium**. Allow cells to attach in the incubator overnight and change the medium to neural maintenance medium the next day.

   CRITICAL STEP: *The time required for complete neural induction varies among different PSC lines, but generally occurs between 8 and 12 d. The sizes of the passaged cell aggregates influence the cell fate of the primitive neuroepithelium. Generally, the smaller the cell clumps, the fewer the cortical stem and progenitor cells that are generated.*

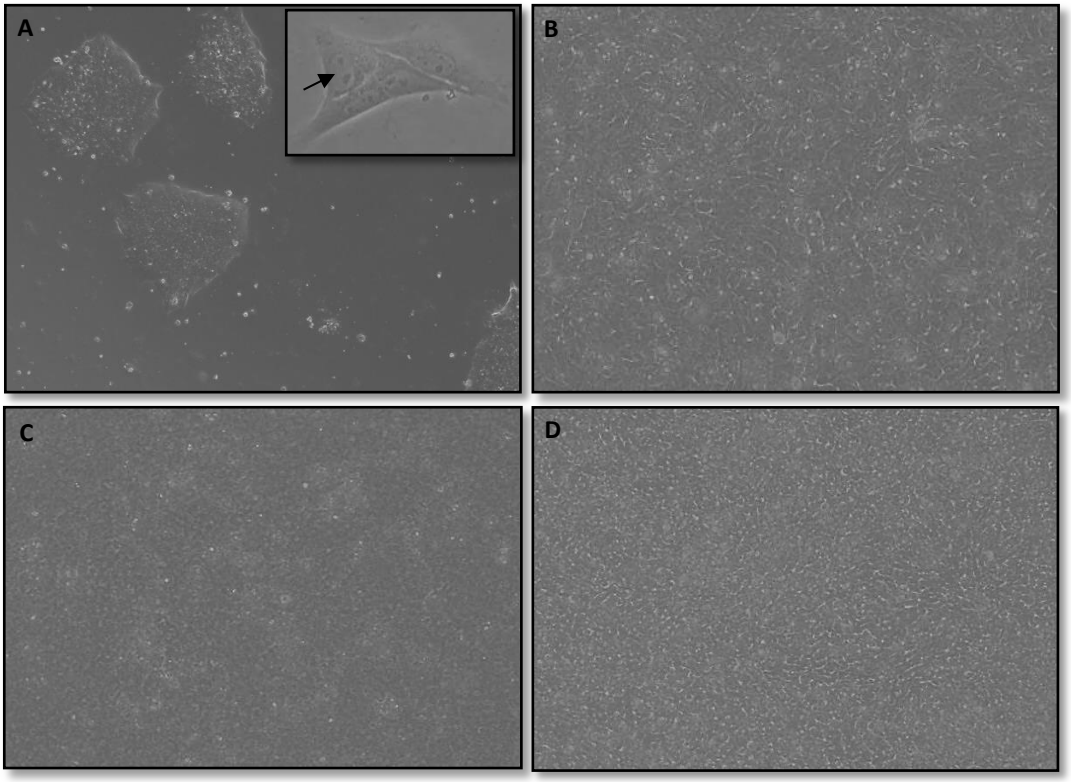

FIGURE 1. PSCs with large nuclei (A) should gradually be replaced by tightly packed neuroepithelial cells with notably smaller nuclei (B- Day 3, 20x; C- Day 5, 20x; D- Day 9, 20x).

NEURAL DIFFERENTIATION

Neural Stem Cells (NSC) expansion and differentiation: TIMING ~ 20 days

1. Neural rosette structures should be obvious when cultures are viewed with an inverted microscope around days 12–17 after neural induction in neural maintenance medium (Figure 2.A). From this point, change the medium every other day.
2. Upon appearance of rosettes, add **20 ng/ml of FGF2 for 2–4 d**, which promotes the expansion of the NSCs but does not block neuronal differentiation.
3. After withdrawal of FGF2, around days 16–20 after neural induction, cells should be split and expanded further by passaging with dispase.

   CRITICAL STEP: *Avoid adding higher concentrations of FGF2 or longer FGF2 treatment. These can caudalize the regional identity of the NSCs, resulting in a loss of cortical identity.*
4. Add **200 µl of dispase** per well and incubate at 37 °C for 3 min.
5. Gently break the detached cell clumps into **aggregates of 300 to 500 cells** by scrapping and slowly pipetting up and down three times with a P1000 pipette.

6. Pellet the cells by centrifugation at **900rpm for 2 min at room temperature** and discard the supernatant.
7. Resuspend the cells with **10 ml of 37 °C neural maintenance medium**, pellet the cells by centrifugation at **900rpm for 2 min at room temperature** and discard the supernatant. Repeat this wash.
8. Gently resuspend the cells in neural maintenance medium.
9. Plate the cells at a ratio of **1:3 laminin-coated wells** and allow the cells to attach in the incubator overnight. **Replace the medium the next day.**
10. Maintain the cells for a further 4–6 d. Cells can be expanded further at this point by repeating the dispase splitting.
11. Between days 20 and 30, substantial neurogenesis should occur. When neurons first begin to accumulate at the outside of the rosettes (Figure 2.B), cells should be passaged using Accutase.
12. Add **500µl of Accutase** per well and incubate at **37 °C for 5 min**.
13. Pipette the cells up and down 3 or 4 times in the Accutase solution to dissociate the cell clumps into a single-cell suspension.
14. Dilute Accutase with **4 volumes of neural maintenance medium**; centrifuge at **900rpm for 5 min at room temperature** to collect the cells.
15. Discard the supernatant and resuspend the cell pellet in neural maintenance medium. Plate cells into laminin-coated wells **at 50,000 cells per cm2** and incubate overnight.
16. Replace the medium on the day after plating and continue to incubate the cells.
17. Change the culture medium every other day.
18. On days 27–31 after initial plating, passage the culture using Accutase at a ratio of 1:4 onto laminin-coated 6-well plates.

    Passaging the cells after day 35, at which point the proportion of neurons is becoming relatively high, is not recommended. As neurons are fragile, particularly mature neurons, their survival rate after passage is low.
19. Continue to culture cells for up to another 50–60 d after the last passage, with medium changes every second day.

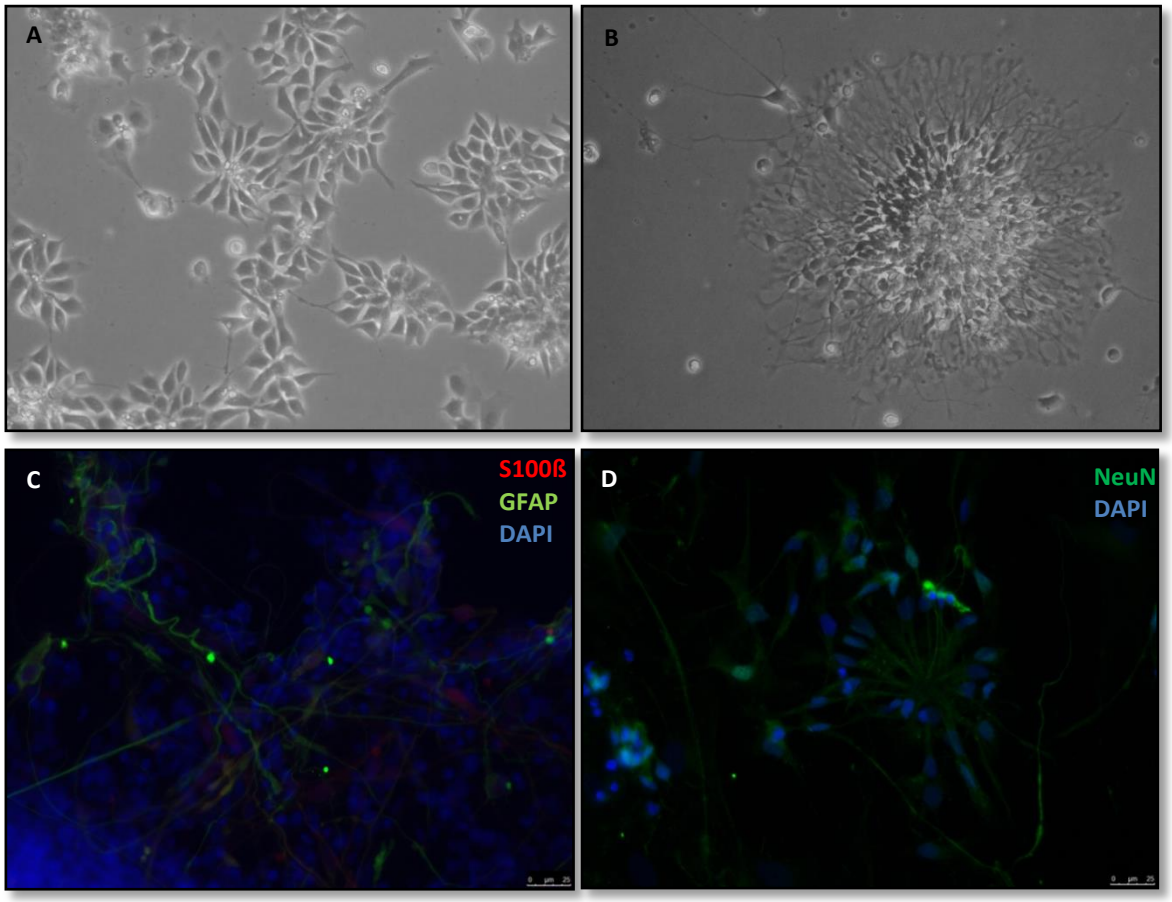

FIGURE 2. A- Neural rosettes 13 days after neural induction; **B-** Neural rosette 21 days after neural induction presenting accumulation of neurons in the periphery; **C**- Immunofluorescence staining of $GFAP^+$ and $S100^+$ astrocytes, 70 days after neural induction; **D-** Immunofluorescence staining of $NeuN^+$ neurons, 70 days after neural induction.

References:

[1] Yichen Shi, Peter Kirwan & Frederick J Livesey. ***Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks.*** Nat Protoc. 2012 Oct;7(10):1836-46.