Neural induction - Dual SMAD inhibition

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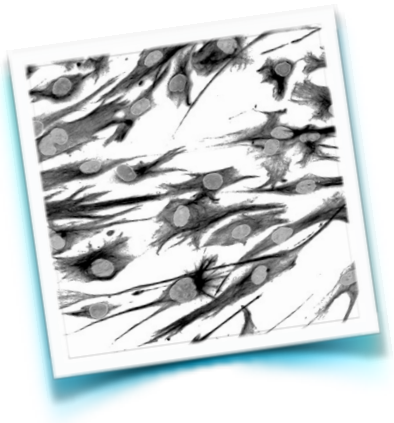

This protocol is based on Chambers et al. 2009.

[Plating pluripotent stem cells]

1. Aspirate ESC Media (hESCM) and add minimal Accutase to coat the dish.
   [1 ml for a 6 well dish, 2 ml for a 6 cm dish, or 5 ml for a 10 cm dish. There is no need for a PBS wash.]
2. Place in the incubator (37˚C) for about 30 minutes - colonies will become single cells.
3. Pipette cells a few times to make a single cell suspension. Filter through a 45 µm strainer to remove any remaining clumps.
4. Wash and centrifuge cells (200xg for 5 min) two times in hESCM to remove Accutase.
5. While washing, prepare Matrigel-coated dishes. Add 19 ml of hESCM to a nearly-thawed Matrigel aliquot and pipette until completely thawed and homogeneous.
   Work quickly and do not let the Matrigel warm up or it will polymerize.
6. After washing hPSCs, resuspend the cells in hESCM with 10 µM Y-27632 and place on a gelatin-coated dish of the same size as used to grow the hPSCs in step 1.
7. Place dish in incubator (37˚C) for 30 min.
8. Collect the non-adherent cells and gently wash the dish with PSCM with 10 µM Y-27632 and centrifuge the cells (200xg for 5 min).
   hPSCs are rounded and therefore non-adherent. Contaminating MEFs are flat and more adherent. This step eliminates many MEFs by leaving them stuck to the dish.
9. Resuspend the cells in complete conditioned media (cCM) with 10 µM Y-27632.
10. Determine the cell concentration using a hemocytometer and add cCM with 10 µM Y-27632 to the appropriate cell concentration for desired density (see chart at end).
11. Aspirate the Matrigel solution and plate cells directly on dish.
    [There is no need to wash the dish though some do.]
12. Twenty-four hours after plating, aspirate media and add fresh cCM with 10 µM Y-27632.
13. Forty-eight hours after plating, aspirate the media and add fresh cCM - from this point on, Y-27632 is no longer necessary.
14. Cells can be maintained for additional days in cCM until the desired density is reached (~90-95% for CNS or about 60% for a mixture of neural crest and CNS).

[Neural induction]

1. To initiate differentiation, add KSR containing 10 µM SB431542 and 200 ng/ml Noggin.
   Defined as Day 0 of the induction. 100 nM LDN 193189 can be used instead of Noggin for

the protocol as well, although we do not currently understand possible downstream changes in cell fate.

2. On day 1 of differentiation, aspirate the KSR and add fresh KSR with 10μM SB431542 and 200 ng/ml Noggin.
3. On day 2 of differentiation, aspirate the KSR and add fresh KSR with 10μM SB431542 and 200 ng/ml Noggin.
4. On day 4, aspirate the KSR and add a mixture of KSR/N2 media (3:1) with 10μM SB431542 and 200 ng/ml Noggin (final concentration).
5. On day 6, aspirate the KSR and add a mixture of KSR/N2 (1:1) with 10μM SB431542 and 200 ng/ml Noggin (final concentration).

6. On day 8, aspirate the KSR and add a mixture of KSR/N2 (1:3) with 10μM SB431542 and 200 ng/ml Noggin (final concentration).
7. On day 10, cells can be passaged en bloc or as single cells onto Matrigel-coated dishes.

[Variation 1: Passage of neural cells en bloc]

1. Mechanically dissociate thickened neurectoderm using a 200 μL pipette into small pieces, or cut monolayer of cells using a StemProEZ Passage tool (Invitrogen)
2. Plate the blocks of tissue onto Matrigel-coated dishes in N2 containing the appropriate growth factors at 2:1 or 3:1.

    Note that this is 2:1, not 1:2 - you want less surface area because not all of the pieces will attach and you need very high cell density to get good progression of CNS neural cells.

[Variation 2: Passage of neural cells as single cells]

1. Aspirate differentiation media and add minimal Accutase to coat the dish and let sit at 37°C until all cells are rendered to single cells (approximately 30 minutes).
2. Avoiding bubbles, triturate the cells in the dish using a pipette with additional N2 media until the cells are in single cell suspension and filter using a 45 μm cell strainer.
3. Wash and centrifuge cells (200 x g for 5 minutes) twice in N2 media.
4. Resuspend the cells in N2 media.
5. Determine the cell concentration using a hemocytometer.
6. Resuspend the cells in N2 media to a cell concentration of 5 x 106/mL.
7. Prepare Matrigel-coated dish by carefully aspirating all liquid from the dish, taking care not to touch the surface. Let dish dry for 15 minutes before plating drops.
8. Spot plate with 20 μL drops of the cell suspension (1x105 cells) and let stand in the hood for 20 minutes before slowly adding N2 containing the appropriate growth factors. Move dish carefully to the incubator.

[Differentiation into neurons]

1. Minimal supportive media for differentiating neural cells into neurons is N2 containing 20 ng/ mL of BDNF and 200 μM ascorbic acid. Differentiations are usually carried out for at least one(and sometimes two) weeks with media changes every two to three days.

2. To enrich for motor neurons, 20 ng/mL BDNF, 200 µM ascorbic acid, 50 ng/mL SHH (C25II) and 1 µM retinoic acid are added to the N2 base medium.
3. To enrich for dopamine neurons, 20 ng/mL BDNF, 200 µM ascorbic acid, 50 ng/mL SHH (C25II) and 100 ng/mL FGF8 are added to the base N2 media for one week, followed by 20 ng/ mL BDNF, 200 µM ascorbic acid, 20 ng/mL GDNF, 1 ng/mL TGF-β3, and 500 µM cAMP for all subsequent weeks.
4. To enrich for rosettes, use high density replating, 50 ng/ml SHH (C25II) and 100 ng/ml FGF8 and 20 ng/ml BDNF and 200 µM ascorbic acid.

[Materials]

N2 media *contains DMEM/ F12 powder (Gibco/Invitrogen, cat no. 12500-062) in 550 mL of distilled water. 1.55 g of glucose (Sigma, cat. no. G7021), 2.00 g of sodium bicarbonate (Sigma, cat. no. S5761), putrescine (1 ml aliquot of 1.61 g dissolved in 100 mL of distilled water; Sigma, cat. no. P5780), progesterone (20 uL aliquot of 0.032g dissolved in 100 mL 100% ethanol; Sigma, cat. no. P8783), sodium selenite (60 uL aliquot of 0.5 mM solution in distilled water; Bioshop Canada, cat. no. SEL888), and 100 mg of transferrin (Celliance/Millipore, cat. no. 4452-01) are added. 25 mg of powdered insulin (Sigma, cat. no. I6634) is added to 10 mL of 5 mM NaOH and is shaken until completely dissolved. The solubilized insulin is added to the media, and double-distilled water (with a resistance of 18.2 MΩ) is added to a final volume of 1000 mL before sterile filtration.*

Recently, the Studer and Tomishima labs have had problems with the supply chain for this media. This has caused us to explore new sources, and many are now using Neurobasal with N2 supplements and B27 (without retinoic acid) and L-glutamine.

MEF media
900 ml DMEM (Invitrogen cat.# 11965-118) 100 ml FBS (Invitrogen, cat.# 26140-095)
version 3.1 - 7/12/2011 4
Filter sterilize.

Pluripotent Stem Cell Media
790 ml DMEM:F12 (Invitrogen, cat.# 11330-032)
200 ml Knockout serum replacement (KSR; Invitrogen, cat. no. 10828-028)
5 ml L-glutamine (200 mM, Invitrogen, cat.# 25030-081)
10 mL MEM non-essential amino acids (MEM NEAA; Invitrogen, cat.# 11140-050)
1 mL of 2-mercaptoethanol (Invitrogen, cat.# 21985-023)
The medium is sterile filtered in the hood and FGF2 is added after filtration to a final concentration of 6 ng/ml.

KSR media *contains 820 mL of Knockout DMEM (Invitrogen; cat. no. 10829-018), 150 mL Knockout Serum Replacement (Invitrogen, cat. no. 10828-028), 10 mL L-glutamine (200 mM, Invitrogen, cat. no. 25030-081), 10 mL MEM NEAA (Invitrogen, cat. no. 11140-050), and 1 mL of 2-mercaptoethanol (Invitrogen, cat. no. 21985-023).*

MEF mouse embryonic fibroblasts *(MEFs, Applied Stem Cell, Inc., ASF1213)*
MEF conditioned media (CM) is harvested from MEF coated flasks. MEFs are plated at a density of 50,000 cells/cm2 in a T225 flask in MEF media. The next day, the cells are washed once with PBS before adding 100 mL of hESC media. Incubate media with MEFs for 24 hours before removal. The medium is now known as "conditioned media" (CM) and can be directly used or stored at 4˚C for less than two weeks. Additional

hESC media can be conditioned daily for up to ten days on the same flask of feeders. Just before using, FGF2 is added to CM to a final concentration of 10 ng/mL, hereafter called complete CM (cCM).

Geltrex Basement Membrane Matrix (*Life Technologies, A1413201). Thaw the frozen vial of Geltrex on ice overnight in a 4˚C refrigerator. Prepare 1 mL aliquots in a 50 mL centrifuge tube using chilled pipettes and freeze at -20˚C. Geltrex must be thawed slowly to prevent gelatinization. Chilled pipettes and 50 mL centrifuge tubes should be used when making aliquots of the Geltrex.*

Sterile 1xDPBS *(Gibco/Invitrogen, cat. no. 14190)*

0.1% gelatin in PBS*, Chemicon/Millipore, cat. no. ES-006-B*

Dispase *(StemCell Technologies, 07913) is thawed, mixed and stored in working aliquots at -20˚C. Do not warm to 37˚C for too long or you will inactivate the enzyme.*

Recombinant FGF2 *(R&D Systems, cat.# 233-FB-001MG/CF) dissolved in PBS with 0.1% BSA to 100 µg/ml.*

Recombinant Mouse Noggin / Fc Chimera *(R&D, cat. no. 719-NG) dissolved in 1x DPBS containing 0.1% BSA to 100 µg/ml (500x stock if using at 200 ng/ml).[We usually use LDN193189 now]*

Recombinant Human BDNF *(R&D Systems, cat. no. 248-BD) dissolved in 1x DPBS containing 0.1% BSA to 10 µg/ml (500x stock if using at 20 ng/ml).*

Recombinant Mouse Sonic Hedgehog (C25II) N-Terminus *(R&D Systems, cat. no. 464-SH; see Note 4) dissolved in 1x DPBS containing 0.1% BSA to 100 µg/ml (2000x stock if using at 50 ng/ml).*

SHH can be purchased that is either mouse or human (although they are 92% identical at the amino acid level). Furthermore, it can be purchased with engineered isoleucines on the N- terminus: these modifications make the protein more hydrophobic and likely act as a membrane tether and intercellular transport mechanism. The engineered modifications phenocopy the cholesterol and palmitate modifications that occur in mammalian cells and are necessary since bacterial expression of the protein does not provide these mammalian modifications. Most of our previous work uses conventional SHH (such as R&D Systems cat. # 461-SH) but we have found that it is more economical to use 10-fold less of the isoleucine-modified version.

Recombinant mouse Fgf-8b *(FGF8; R&D Systems, cat. No. 423-F8) dissolved in 1x DPBS containing 0.1% BSA to 100 µg/ml (500x stock if using at 200 ng/ml).*

Recombinant human GDNF *(PeproTech, cat. no. 450-10) dissolved in 1x DPBS containing 0.1% BSA to 10 µg/ml (500x stock if using at 20 ng/ml).*

Recombinant human TGF-β3 *(R&D Systems, cat. no. 243-B3) dissolved in 4 mM HCl containing 0.1% BSA to 20 µg/ml (20,000x stock if using at 1 ng/ml).*

Recombinant Delta-like 4 *(R&D Systems, cat. no. 1506-D4-050) dissolved in 1x DPBS containing 0.1% BSA to 200 µg/ml (400x stock if using at 500 ng/ml).*
[We rarely use or need this - rosettes should form fine without it.]

Dibutyryl cAMP sodium salt *(cAMP; Sigma, cat. no. D0260) dissolved in sterile water to 100 mM (200x stock if using at 500 µM).*

Retinoic acid *(Sigma, cat. no. R2625) dissolved in DMSO to 10 mM. Do not filter sterilize: DMSO will degrade membrane. Primary storage should be at -80˚C while working stocks can be kept at -20˚C. Protect from light, and only freeze-thaw 3 times before discarding.*

Ascorbic acid *(Sigma, cat. no. A5960). Add 1.76g/100 ml sterile water and filter sterilize.*

SB431542 *(Tocris Bioscience, cat. no. 1614) dissolved in 100% ethanol to 10 mM (1000x stock).*

Y-27632 dihydrochloride *(Tocris Bioscience, cat. no. 1254) dissolved in filtered water to 10 mM (1000x stock).*