Supplementary Materials

Mansor, N. I., Azmi, N., Ling, K. H., Rosli, R., Hassan, Z. and Nordin, N. (2019) "Prospective stem cell lines as in vitro neurodegenerative disease models for natural product research", *Neuroscience Research Notes*, 2(1), pp. 16-30. doi: 10.31117/neuroscirn.v2i1.25.

Method S1. Propagation of 46C mouse embryonic stem (mES) cell and Rat amniotic fluid stem cell (R3).
The 46C mES cells were a gift from John Mason (University of Edinburgh, United Kingdom), meanwhile, R3 AFS cells were established in-house from amniotic fluid of full-term pregnancy rats. The cell lines were maintained in GMEM (BHK-21; Gibco) supplemented with 15% (v/v) foetal bovine serum (FBS; Gibco); 1% MEM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco) and 10 ng/mL human recombinant leukaemia inhibitory factor (LIF 1010; Millipore). The cells were seeded at a cell density of 4.0×10^4 cells/cm^2 into 25 cm^2 cell culture flasks (TPP) coated with 0.1% (w/v) gelatin (Sigma). The gelatin-coated flasks or plates were prepared by pre-coating with gelatin for at least 5 min prior to cell seeding. The cells were incubated at 37°C, 5% CO_2 and 90% humidity (AutoFlow IR Water-jacketed CO_2 Incubator, US). The cells were sub-cultured every other day when the cells were 70-80% confluent.

Method S2. Neural differentiation assay
Neural differentiation assay was carried out either through spontaneous formation of multicellular aggregates, known as embryoid bodies (EBs) using 4-/4+ protocols adapted from Bain *et al.* [1] with the addition of all-trans retinoic acid (ATRA) as the neural inducer (46C) or by adherent monoculture (R3) adapted from Ying *et al.* [2].

Method S2a. Neural differentiation assay trough EBs formation
For EBs formation, 5.0×10^6 undifferentiated stem cells were seeded in 100 mm petri dish for 4 days in the absence of LIF, followed by another 4 days in the presence of *all trans* retinoic acid (Sigma) (4-/4+ induction). The medium was changed every two days. At the end of the induction period (8th day), the EBs were dissociated into single cells with 4× trypsin-EDTA, and plated on Poly-D-Lysine (PDL)/laminin (Sigma) coated plates at the density of $2.0 - 3.0 \times 10^4$ cells/cm^2.

Method S2b. Neural differentiation assay trough adherent monoculture
Undifferentiated stem cells were dissociated with 0.25% trypsin/EDTA with 10% chicken serum and neutralized with 1×PBS with 10% FBS. After centrifugation at 115×g for 5 minutes, the cells were plated into 0.1% gelatin-coated tissue culture plates in N2 medium (DMEM/F12 medium supplemented with N2) at 1.1×10^4/cm^2 cell density and incubated in a 5% CO^2 incubator at 37 °C and 90% humidity. The medium was changed to N2B27 on the next day and incubated for another 7 days. N2B27 is a 1:1 mixture of N2 medium and Neurobasal medium supplemented with B27 (B27 medium) (all from Gibco). The medium was changed every two days.

Method S3. Immunocytochemistry
Immunocytochemistry (ICC) was prepared in 24-well plates. The attached neurons were fixed in 4% paraformaldehyde (PFA; 50 mM NaOH, 1×PBS) for 30 min, followed by permeabilization in 1% Triton-X100 for 15 minutes at room temperature (RT). Cells were then incubated in blocking solution (0.3% bovine serum albumin, 1% appropriate serum, 0.1% Tween-20 in 1×PBS) for 30 min prior to incubation with primary antibody at 4°C overnight. After washing with 1×PBS, the cells were then incubated with

fluorochrome-conjugated secondary antibody with for 2 hrs at RT in the dark. After washing, the cells were counterstained with DAPI (Sigma) or TO-PRO3 (Molecular Probe) for 10 - 15 min at RT. Meanwhile, Hoechst 33342 were stained prior to fixation step for10 - 15 min in 37°C. The cells were then left in 1× PBS in the dark until visualization with an inverted florescent microscope (Olympus). List of antibodies were listed in Table 1:

Table S1. List of antibodies used for immunocytochemistry

Primary antibody	Secondary antibodies
Class III Beta-Tubulin (mouse) IgG2b 1:200 (Sigma)	Alexa Fluor 488 Goat anti-mouse IgG2b (+L; Life Tecnologies)
Anti-Glial Fibrillary Acid Protein (GFAP) IgG 1:200 (Sigma)	Alexa Fluor 488 Goat anti-rabbit IgG H+L 1:200 (Life Tecnologies)
Rabbit polyclonal to Choline Acetyltransferase (ChAT) 1:200 (Abcam)	Alexa Fluor 488 Goat anti-rabbit IgG 1:200 (Abcam)
Rabbit polyclonal to Tyrosine Hydroxylase (TH) IgG 1:200 (Millipore)	Alexa Fluor 488 Goat anti-rabbit IgG 1:200 (H+L; Life Tecnologies)

Method S4. Analysis of intercellular reactive oxygen species (ROS)

Intracellular reactive oxygen species (ROS) was measured using Oxiselect Intracellular ROS Assay Kit (Cell Biolabs). The cell permeable 2',7'-dichlorodihydroflourescein diacetate (H2DCF-DA) diffuses and is deacetylated by cellular esterases to non-fluorescent 2',7'-dichlorodihydrofluorescin (DCFH) which reacts with ROS (which includes hydroxyl, peroxyl and other ROS activity within a cell) to form a highly fluorescent 2',7'- dichlorodihydrofluorescein (DCF). The matured neurons were washed twice with 1X PBS followed by pre-incubated with 100 µL of 1× DCFH-DA/ N2B27 medium for 45 min at 37°C. The supernatant was removed and the cells were washed twice with 1× PBS after which they were treated with oxidants and incubated for 24 h. The supernatant was then discarded and the cells were washed twice with PBS prior to trypsinization. The cells were resuspended in 500 µL of 1× PBS prior to DCF analysis using a flow cytometer (LSR Fortessa; BD Biosciences, USA). 10^4 events were acquired and analyzed using FACs Diva Software.

References

1. Bain, G.; Kitchens, D.; Yao, M.; Huettner, J. E.; Gottlieb, D. I. Embryonic Stem Cells Express Neuronal Properties in Vitro. Dev. Biol., **1995**, 168, 342 – 357.
2. Ying, Q.L.; Stavridis, M.; Griffiths, D.; Li, M.; Smith, A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat. Biotechnol., 2003, 21(2), 183 – 186. https://doi:10.1038/nbt780