Poster: #412.23/B13

Generation, Maintenance, and Cryopreservation of Neural Progenitor Cells derived from Human Pluripotent Stem Cells using the STEMdiff™ Neural System

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1. Introduction

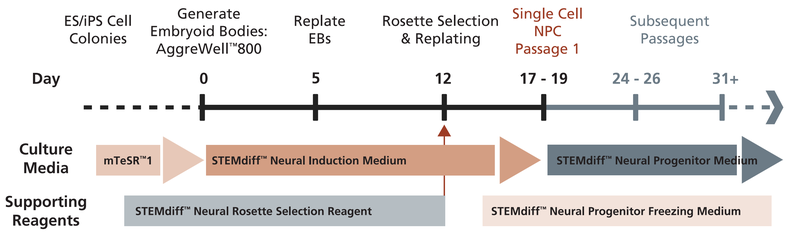
Neural progenitor cells (NPCs) generated from human pluripotent stem cells (hPSC), including human embryonic stem (hES) cells and induced pluripotent stem (hiPS) cells are extensively used for studying human nervous system development, modeling neurological disorders, and screening for therapeutic molecules. We have reported that CNS-specific NPCs can be produced efficiently using STEMdiff™ Neural Induction Medium (NIM) in conjunction with AggreWell™800. Recent publications have shown that neural induction from hPSCs can also be achieved in a single-step monolayer culture system, which does not require aggregate formation and therefore simplifies the process of neural induction. Here we describe: 1) the use of STEMdiff™ NIM in a monolayer-based neural induction system and 2) the use of two new defined media for propagating [STEMdiff™ Neural Progenitor Medium (NPM)] and cryopreserving [STEMdiff™ Neural Progenitor Freezing Medium (NPFM)] NPCs, respectively. Toward the first objective, hPSCs maintained in mTeSR™1 were seeded at 250,000 cells/cm^2 and cultured as monolayers in NIM for up to 10 days. Cells were assessed at different time points for neural induction by the appearance of PAX6 with concomitant down regulation of OCT4. In the hES cell lines tested, approximately 25 - 30% of the cells were PAX6$^+$OCT4$^-$ by day 3 and all cells were PAX6$^+$OCT4$^-$ by day 6 (n = 5). In the hiPS cell lines tested, 30 - 40% of cells were PAX6$^+$OCT4$^-$ on day 5 and induction to PAX6$^+$OCT4$^-$ cells was complete by day 9 (n = 4). These data show that CNS-specific NPCs can be efficiently produced in a single-step from hPSC using STEMdiff™ NIM with a monolayer protocol.

For the second objective, we generated NPCs using STEMdiff™ NIM in either the standard AggreWell™800 system or the monolayer system. NPCs were then dissociated into single-cell suspensions and either cultured further in NPM or cryopreserved in NPFM. Cells were passaged in STEMdiff™ NPM every 7 days and cell expansion was calculated at each passage. Overall NPCs propagated in NPM exhibited 3 - 5 fold expansion (n = 8) over the first 3 passages. NPCs were maintained in NPM with low spontaneous neuronal differentiation (<10%) for at least 10 passages. Lastly, we showed that NPCs generated using NIM could be cryopreserved in NPFM, with high viability and recovery rate upon thawing. Thawed NPCs could be further expanded or used for differentiation experiments.

We have now expanded the application of STEMdiff™ NIM in a monolayer-culture based protocol for neural induction. With this, our STEMdiff™ Neural is an integrated system for the generation, maintenance, and cryopreservation of hPSC-derived NPCs.

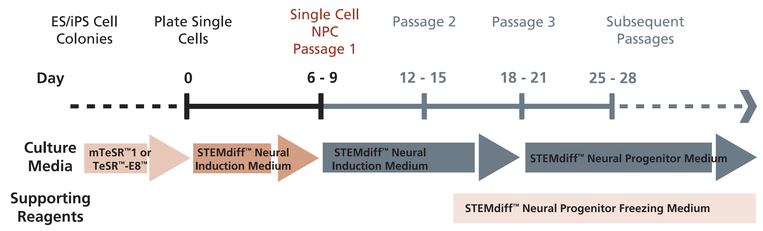
2. Methods

A. STEMdiff™ Neural: EB Protocol



Embryoid bodies (EBs) are formed from hPSCs previously maintained in mTeSR™1 or TeSR™2, in AggreWell™800 with STEMdiff™ Neural Induction Medium (NIM) and maintained over 5 days. EBs are harvested and plated (day 5), and colonies with abundant neural rosettes are formed. Colonies containing rosettes can be isolated (day 12) using STEMdiff™ Neural Rosette Selection Reagent (NRSR) and replated in STEMdiff™ NIM. Once this NPC-enriched culture is established (days 17 - 19), single cells can be isolated and further cultured in STEMdiff™ Neural Progenitor Medium (NPM) every 7 days.

B. STEMdiff™ Neural: Monolayer Protocol

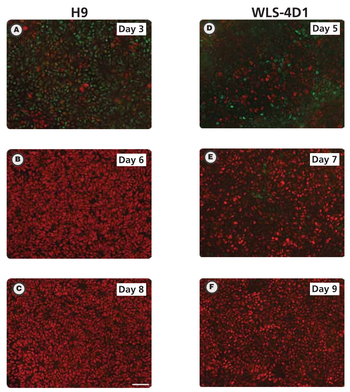


Human ES or iPS cells are harvested from mTeSR™1 or TeSR™-E8™ cultures and plated at 200,000 - 250,000 cells/cm^2 in STEMdiff™ NIM onto poly-L-ornithine/laminin or Matrigel-coated plates. After 6 (for hES) or 9 (for hiPS) days, NPCs are generated, which are then sub-cultured in STEMdiff™ NIM for an additional 2 passages. Starting at passage 3 (days 18 - 21), NPCs are isolated as single-cell suspensions and further expanded in STEMdiff™ Neural Progenitor Medium (NPM) every 7 days.

Results

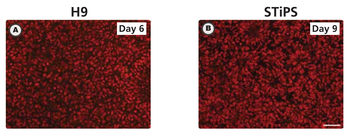
3. STEMdiff™ NIM can be efficiently used in a monolayer neural induction protocol

FIGURE 1. Induction of human ES and iPS cells into neural progenitor cells using a monolayer protocol



(A) After 3 days of induction, in hES cells tested (H9), roughly 25% of the cells were PAX6$^+$ (red), with many cells still OCT4$^+$ (green). **(B)** By 6 days most, if not all the cells, were PAX6$^+$/OCT4$^-$. At day 8, virtually all cells were PAX6$^+$/OCT4$^-$. For iPS cells (WLS-4D1), the timing of neural induction was slightly longer **(D - F)**. **(D)** After 5 days of induction, about 25 - 30% of the cells were PAX6$^+$ (red), with many cells still OCT4$^+$ (green). **(F)** By 7 days, many more cells in culture were PAX6$^+$/OCT4$^-$. **(F)** At day 9, all cells were PAX6$^+$/OCT4$^-$. Scale bar = 50 µm; A - F taken at same magnification.

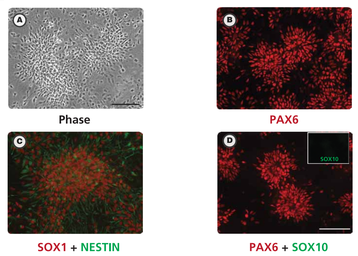
FIGURE 2. Neural induction of TeSR™-E8™-maintained hPSC cells using a monolayer protocol



Human ES and iPS cells that were previously maintained in TeSR™-E8™ on Vitronectin-XF™ substrate were subjected to the monolayer culture neural induction method. **(A, B)** Cells are PAX6$^+$ (red) and OCT4$^-$ (green) by day 6 for hES cells (**A**; H9), and day 9 for hiPS cells (**B**; STiPS, derived using TeSR™-E7™ reprogramming medium). Scale bar = 50 µm; A, B taken at same magnification.

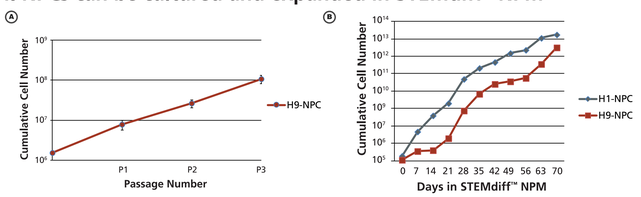
4. STEMdiff™ Neural Progenitor Medium

FIGURE 3. NPCs cultured in STEMdiff™ NPM expressed CNS-type neural progenitor markers



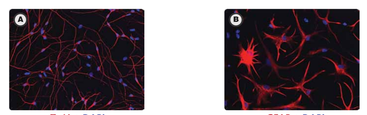
(A) Typical NPC morphology observed in cultures (shown: day 6, passage 1; scale bar = 100 µm). NPCs were processed for immunocytochemistry to detect PAX6, SOX1 and NESTIN expression. **(B - D)** NPCs maintained in STEMdiff™ NPM express CNS-type NPC markers PAX6 (**B, D**; red), SOX1 (**C**; red) and NESTIN (**C**; green), but not the neural crest marker SOX10 (**D**; green, single channel shown in inset). Photomicrographs in B - D were taken at the same magnification; scale bar = 100 µm.

FIGURE 4. NPCs can be cultured and expanded in STEMdiff™ NPM



Neural progenitor cells were seeded at 125,000 cells/cm^2 and passaged on average every 7 days. **(A)** NPCs cultured in STEMdiff™ Neural Progenitor Medium expanded 3 - 5-fold at each passage. **(B)** NPCs can be maintained for >10 passages in NPM, generating large numbers of NPCs for downstream experiments.

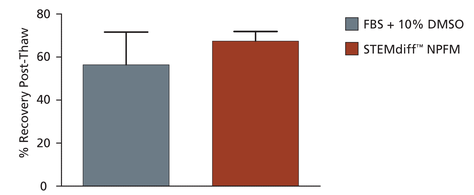
FIGURE 5. NPCs maintained in STEMdiff™ NPM can differentiate into neurons and astrocytes



NPCs were passaged for multiple passages in STEMdiff™ NPM and then seeded at low density (30,000 cells/cm^2), in differentiation media to induce differentiation. Under specific conditions, NPCs can differentiate into different CNS cell types, including neurons (**A**; class III β-tubulin detected using TuJ-1 clone) and GFAP-positive astrocytes **(B)**. DAPI used to stain nuclei.

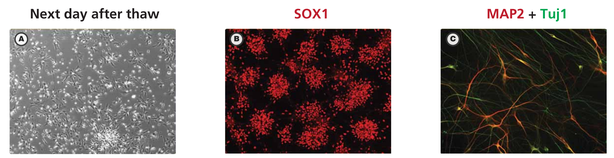
5. STEMdiff™ Neural Progenitor Freezing Medium

FIGURE 6. NPCs cryopreserved in STEMdiff™ Neural Progenitor Freezing Medium show reproducibly high recovery after thawing



Recovery of NPCs frozen in STEMdiff™ NPFM (red bar) was comparable to recovery in cryopreservation with serum-containing medium (90%FBS/10%DMSO, grey bar, mean ± SEM, n = 6). Percent recovery = # viable cells/ # input cells during cryopreservation.

FIGURE 7. NPCs cryopreserved in STEMdiff™ NPFM can be used for downstream differentiation experiments



NPCs frozen with STEMdiff™ Neural Progenitor Freezing Medium displayed healthy morphology one day after thaw **(A)**, expressed NPC marker, SOX1 **(B),** and can be differentiated into MAP2 and class III β-tubulin-immunoreactive neurons **(C)**.

Summary

- STEMdiff™ Neural Induction Medium (NIM) can be used to generate CNS-type NPCs using either aggregate or monolayer protocols
- STEMdiff™ Neural Progenitor Medium (NPM) supports culture and expansion of NPCs and these NPCs:
  - Express CNS-type neural progenitor markers
  - Are multipotent and can differentiate into neurons and astrocytes
- STEMdiff™ Neural Progenitor Freezing Medium (NPFM) supports cryopreservation of NPCs with high recovery rates post-thaw
- Together, the STEMdiff™ Neural system consisting of NIM, NPM, and NPFM form a complete system that supports generation, expansion, and cryopreservation of hPSC derived NPCs