STEMdiff™ Neural Induction System for the Generation and Propagation of Rosette-Forming Neural Progenitor Cells from Human Pluripotent Stem Cells

Alexandra Blak[1], Eileen Yoshida[1], Jennifer Antonchuk[1], Terry E, Thomas[1], Allen C. Eaves[1,2] and Sharon A. Louis[1]

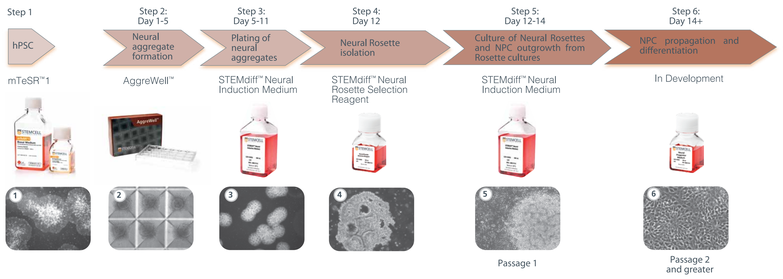
[1] STEMCELL Technologies Inc., Vancouver, BC, Canada [2] Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada

Introduction

We have developed the STEMdiff™ Neural Induction System for the generation of neural progenitor cells (NPCs) from human pluripotent stem cells (hPSCs) consisting of: 1) STEMdiff™ Neural Induction Medium (STEMdiff™ NIM), which rapidly and efficiently induces "neural rosettes" indicating initial neural differentiation; and 2) STEMdiff™ Neural Rosette Selection Reagent (STEMdiff™ NRSR), which provides selective detachment of "neural rosettes". We also provide optimized protocols which combine the use of STEMdiff™ NIM with AggreWell™800, a tool to generate uniformly sized aggregates, to standardize the first step of the neural induction protocol. Neural aggregates were formed from hPSCs in STEMdiff™ NIM using the AggreWell™800 procedure and the aggregates were maintained within the AggreWell™ plates for 5 days. The aggregates were then harvested from AggreWell™800 plates and plated onto Poly-L-Ornithine/laminin (PLO/L) coated plates in STEMdiff™ NIM for a total of 7 days. Cultures were treated with STEMdiff™ NRSR to selectively detach rosette clusters followed by re-plating the rosette clusters onto PLO/L coated plates in STEMdiff™ NIM. Upon reaching ~80-90% confluence, the cultures were enzymatically dissociated and the resulting single-cell suspension either sub-cultured multiple times in STEMdiff™ NPC medium (in development) or subjected to differentiation conditions. Our results showed that neural aggregates generated in STEMdiff™ NIM formed colonies containing up to 100% neural rosettes upon attachment. Characterization of neural rosette structures by immunocytochemistry revealed that the cells within these structures co-expressed the NPC markers, Pax6, Sox1 and Nestin, confirming the induction of bona fide NPCs. Those NPCs were isolated from neural rosettes and cultured in STEMdiff™ NPC medium where they re-formed secondary neural rosettes for up to 3 passages. Furthermore, NPCs could be differentiated into neurons at each passage. This work demonstrated that the new STEMdiff™ Neural Induction System is a highly efficient system for the production of pure populations of NPCs from hPSCs. In addition, the STEMdiff™ NPC medium, currently in development, allows the propagation of these NPCs and maintainance of their neurogenic potential. Work is on-going to further characterize these NPCs, and to examine their potential to maintain functional properties of the developing embryonic neural tube as well as their ability to differentiate into the three mature cell types of the CNS.

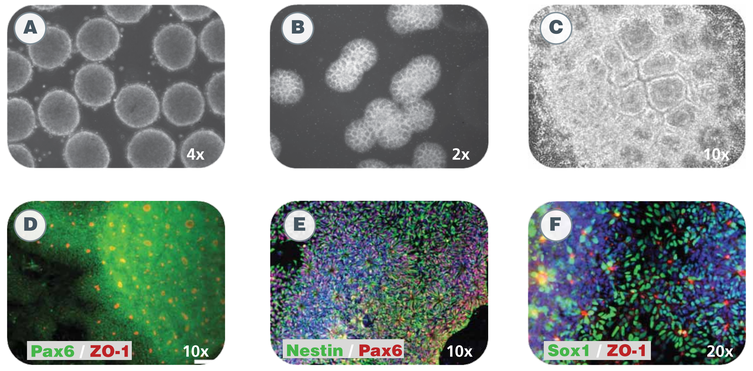
Methods

FIGURE 1: STEMdiff™ Neural – A complete induction and differentiation system



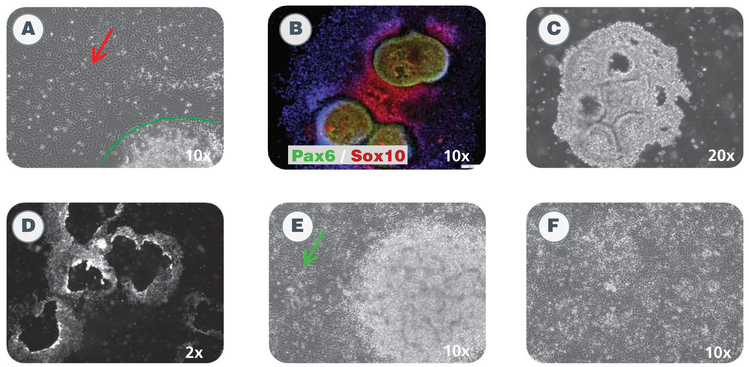
Step 1: hPSCs are maintained in mTeSR™1 according to standard protocols.
Step 2: AggreWell™800 plates are used to form uniform, size-controlled neural aggregates in combination with STEMdiff™ NIM.
Step 3: After 5 days, neural aggregates are harvested from the AggreWell™800 plate and seeded onto PLO/L coated plates in STEMdiff™ NIM. Colonies containing neural rosette structures form within 24 hours.
Step 4: Neural rosettes containing putative NPCs are selectively detached from the adherent culture after 7 days, using STEMdiff™ NRSR. After gentle trituration, these rosette-containing "clusters" are re-plated and cultured on PLO/L coated plates in STEMdiff™ NIM. This is denoted as Passage 1.
Step 5: During the following 2-5 day culture period, NPCs migrate out from neural rosette clusters. When cultures reach 80-90% confluence, they are dissociated using ACCUTASE™ to obtain a single cell suspension of NPCs, which is then re-plated onto PLO/L coated plates in Neural Progenitor Cell Medium (in development) (Passage 2).
Step 6: From day 14 on, NPCs can be propagated in Neural Progenitor Cell Medium for multiple passages or differentiated into neurons and glial cells.

FIGURE 2: Neural aggregate formation, attachment and characterization of NPCs within neural rosettes



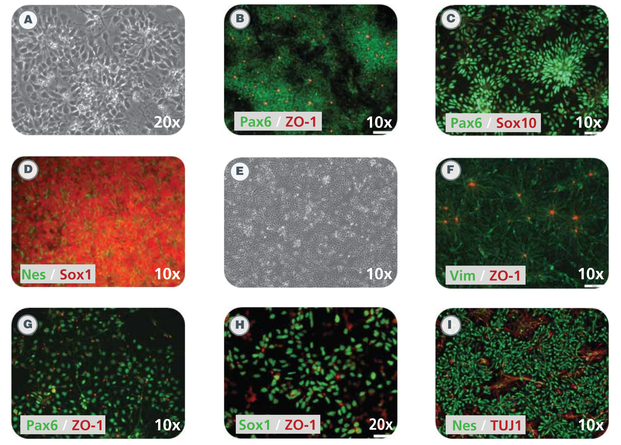
(A) Neural aggregates each containing 10,000 cells after harvest from AggreWell™800 plates shortly before plating onto PLO/L coated dishes. (B) Neural aggregates from (A), 1 day after attachment on PLO/L coated dishes. Neural rosette structures are present in 100% of colonies derived from neural aggregates generated in AggreWell™800 and STEMdiff™ NIM. (C) Neural rosettes in the center of a neural aggregate shown in higher magnification. (D) Neural aggregates 2 days after plating onto PLO/L coated dishes were stained using immunocytochemistry (ICC) with antibodies targeting NPCs. Neural rosettes within neural aggregates express Pax6 (green), identifying the presence of early-stage NPCs within the rosettes. ZO-1 (red), marks the lumen of each neural rosette. (E) Neural rosettes co-express pan-neural marker Nestin (green) and Pax6 (red) confirming the presence of bona fide NPCs within neural rosettes. (F) Most cells within neural rosettes express Sox1, a typical later-stage NPC marker, whereas ZO-1 marks the lumen of each individual rosette. Cells are counterstained using DAPI (E and F).

FIGURE 3: Using the STEMdiff™ NRSR on day 7-neural aggregate cultures – Neural progenitor cell populations can be selectively enriched from rosette structures



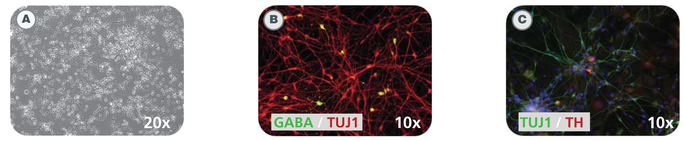
(A) Neural rosettes structures were observed in attached neural aggregates at day 7 after plating (marked by green half-circle). "Flat" cells are seen (described in Zhang *et al.*, 2001; Curchoe *et al.*, 2010) emerging at the periphery of attached neural aggregates (marked with red arrow). (B) "Flat" cells in (A) stained positive for Sox10, a marker for neural crest progenitor cells (NCPCs). The presence of these NCPCs is thought to interfere with downstream differentiation of NPCs towards mature CNS cell types, therefore selective detachment of the NPC containing neural rosettes was performed with STEMdiff™ NRSR. (C) A detached neural rosette clusters after treatment with STEMdiff™ NRSR. (D) Remaining "Flat" cells attached to the cell culture dish after treatment with STEMdiff™ NRSR. (E) Isolated neural rosette clusters from (C) after attachment on day 2 of Passage 1. The green arrow marks putative NPCs based on morphology, growing out from the rosette cluster. (F) When cultures from (E) reach 80-90% confluence they were dissociated into a single cell suspension and sub-cultured in Neural Progenitor Cell Medium (in development). NPCs are shown at day 3 of Passage 2.

FIGURE 4: Culture and characterization of NPCs after the NRSR procedure



NPCs can be propagated in Neural Progenitor Cell Medium (in development) for multiple passages and retain NPC marker expression (A) Brightfield image of NPC culture at day 2 Passage 2. NPCs re-form neural rosette structures. (B) NPCs from (A) maintained varying levels of expression of early-NPC marker Pax6 (green). ZO-1 expression is shown in red. (C) Passage 3 NPCs maintained varying expression of Pax6 (green). However, none of the cells expressed the NCPC marker Sox10 (red). (D) NPCs from (A) co-expressed Sox1 (red) and Nestin (green). (E) Brightfield image of NPC culture at day 3 Passage 3. (F) Passage 3 NPCs expressed the neural marker Vimentin (green) and ZO-1 (green). (G) NPCs at passage 5 day 3 expressed Pax6 at varying levels (green) with ZO-1 marking the lumen of individual rosettes (red). (H) Passage 5-NPCs also expressed Sox1 (green) and ZO-1 (red). (I) Signs of differentiation could be observed within passage 5 cultures. Neurons, expressing the neuronal marker TUJ-1 (red), are detected in patches however, the majority of cells express Nestin (green), demonstrating NPC characteristics.

FIGURE 5: NPCs can differentiate into various subtypes of neurons



NPCs were seeded in Differentiation Medium (in development) to induce terminal neuronal differentiation. (A) Brightfield image of passage 5-NPCs which have been cultured in Differentiation Medium for 10 days. (B) Cells from (A) expressed TUJ1 (red) identifying them as mature neurons. A proportion of cells expressed the marker GABA, thus demonstrating that these are GABAergic neurons (green). (C) Passage 3-NPCs seeded in Differentiation Medium spontaneously gave rise to dopaminergic neurons expressing tyrosine hydroxylase (TH) expression (red). TUJ1 expression marks mature neurons (green). Expression of GABA and TH indicate that NPCs have the potential to differentiate into subtypes of neurons which are found in various regions of the brain.

Summary

We have developed a novel system aimed at standardizing neural induction and differentiation of hPSCs: This system includes using AggreWell™800 in combination with STEMdiff™ Neural Induction Medium* for the highly efficient induction of neural rosettes and for the subsequent selection of NPC-containing neural rosettes with STEMdiff™ Neural Rosette Selection Reagent*. NPCs derived in this system can be propagated and maintain typical NPC marker expression for several passages. Furthermore, NPCs respond to developmental cues and can differentiate into neurons and astrocytes. Development is on-going to develop a NPC Medium for the maintenance of NPCs over several passages and a Differentiation Medium which will allow differentiation of NPCs into subtypes of neurons and glial cells.

* Patent pending