It Takes Two—Or Three: Comparing Human Pluripotent Stem Cell-Derived Glia-Neuron Co-Cultures to Neuron Monoculture Under Basal and Injury Conditions

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INTRODUCTION

There are established methods to generate high-purity neuron, astrocyte, and microglia monocultures from human pluripotent stem cells (hPSCs). Previous work has demonstrated that glial cells play an important role in neuronal functions including synaptogenesis and homeostasis. However, neuronal monocultures lack these physiologically important glia-neuron interactions. We created a protocol to co-culture hPSC-derived forebrain neurons with astrocytes to assess the impact of glia co-culture on neuronal morphology. We then developed a tri-culture model by adding hPSC-derived microglia to the neurons and astrocytes. We performed a wound injury assay on the neurons in mono- and tri-cultures. Our results demonstrate that pure populations of astrocytes, neurons, and microglia can be cultured together to display functional properties compared to neuron monocultures. This system can be used to further study the functional impact of glia-neuron interactions.

METHODS

Forebrain Neuron Differentiation: hPSCs maintained in mTeSR™ Plus were differentiated into neural progenitor cells (NPCs) following the embryoid body (EB) AggreWell™ method using STEMdiff™ SMADi Neural Induction Kit. NPCs were further differentiated into forebrain neurons after 1 week of culture in STEMdiff™ Forebrain Neuron Differentiation Medium, and matured for 7 days using STEMdiff™ Forebrain Neuron Maturation Medium before co- and tri-culture. Cell identity was confirmed using immunocytochemistry (ICC) for > 90% expression of beta-III-tubulin (βIII-Tub; clone TUJ1).

Astrocyte Differentiation: hPSCs maintained in mTeSR™ Plus were differentiated into NPCs following the EB AggreWell™ method using STEMdiff™ SMADi Neural Induction Kit. NPCs were further differentiated into astrocytes after 3 weeks of culture in STEMdiff™ Astrocyte Differentiation Medium, and matured for 3 additional weeks using STEMdiff™ Astrocyte Maturation Medium before co-culture. Cell identity was confirmed by ICC analysis for > 70% S100β and glial fibrillary acidic protein (GFAP)-expression.

Co-Cultures of Forebrain Neurons and Astrocytes: Forebrain neuron precursor cells were seeded at 2.6 x 10^4 cells/cm^2 onto tissue culture plates coated sequentially with 15 µg/mL poly-L-ornithine (PLO) and 10 µg/mL laminin for 7 days of maturation. hPSC-derived astrocytes were seeded on top of the neuronal precursors at an astrocyte-to-neuron ratio of 2:1 in STEMdiff™ Astrocyte Maturation Medium for 24 hours, then in STEMdiff™ Forebrain Neuron Maturation Medium thereafter.

Microglia Differentiation: hPSCs maintained in mTeSR™ Plus were differentiated into CD43-expressing hematopoietic progenitor cells (HPCs) for 12 days using STEMdiff™ Hematopoietic Kit. HPCs were differentiated for 24 days using STEMdiff™ Microglia Differentiation Kit. Microglia purity was confirmed by flow cytometry for > 80% co-expression of CD45 and CD11b. For the tri-culture injury assay in figures 4 and 5, the microglia were differentiated from a hPSC line stably transfected for constitutive green fluorescence protein (GFP) expression.

Tri-Cultures of Forebrain Neurons, Astrocytes, and Microglia: Forebrain neuron precursor cells were seeded onto tissue culture plates coated sequentially with 15 µg/mL PLO and 10 µg/mL laminin for 7 days of maturation. For long-term culture and the ICC image in figure 3, the forebrain neuron precursor cells were seeded at 1.5 x 10^4 cells/cm^2. For the tri-culture injury assay, the forebrain neuron precursor cells were seeded at either 4.0 x 10^4 or 6.0 x 10^4 cells/cm^2. Astrocytes were seeded on top of the matured forebrain neurons at a 1:1 ratio. The next day, microglia were seeded at a 2:2:1 or 1:1:1 neuron-astrocyte-microglia ratio in Tri-Culture Medium (BrainPhys™ Complete hPSC Neuron Kit with STEMdiff™ Microglia Supplement 2). The culture was analyzed by ICC for βIII-Tub, GFAP, and ionized calcium binding adaptor molecule 1 (IBA1) expression after 7, 21, and 30 days. A scratch injury was applied to the cultures using the Incucyte® Woundmaker Tool on the 7th day of tri-culture and cultures were fixed for ICC analysis 48 hours later.

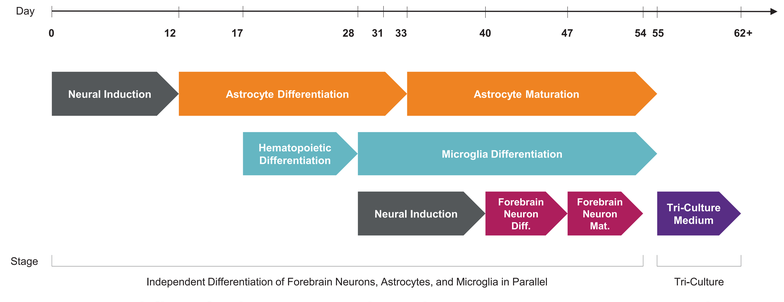

FIGURE 1. Workflow for hPSC-Derived Forebrain Neuron, Astrocyte, and Microglia Tri-Culture

RESULTS

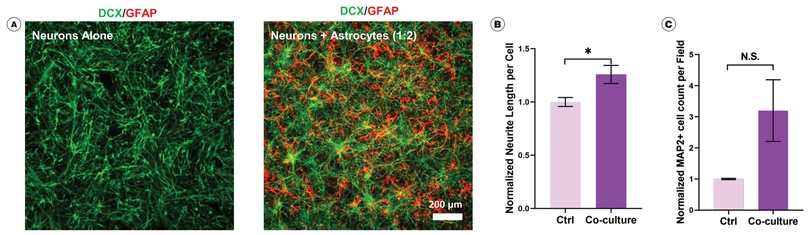

FIGURE 2: hPSC-derived Forebrain Neurons Co-cultured with Astrocytes Display Increased Neurite Length and a Trend to Increased Survival

(A) Representative images of forebrain neurons cultured alone (left) or in a co-culture with astrocytes (right) stained with doublecortin (DCX) for neurons (green) and GFAP for astrocytes (red). Scale bar = 200 µm. Statistical data of **(B)** neurite length per cell and **(C)** MAP2-positive (mature neuron) cell count in the control (neuron only) and co-culture group. The bars show the mean and SEM. Statistics calculated with unpaired t-test. * = $p < 0.05$; N.S. = not significant; Ctrl: n = 4; Co-culture: n = 2 (2 cell lines per experiment).

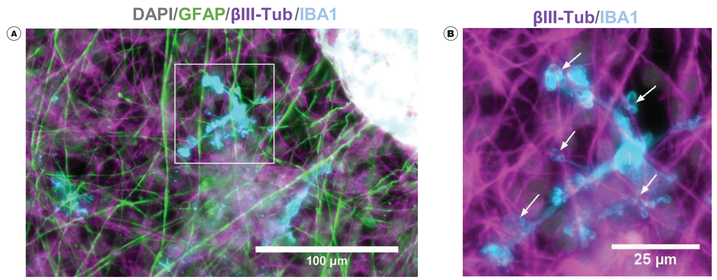

FIGURE 3: hPSC-derived Microglia Integrate Among Neurons and Astrocytes in Tri-Culture

(A) Representative ICC image of a day 21 tri-culture stained for IBA1 (cyan), GFAP (green), and βIII-Tub (magenta). This tri-culture was seeded at a 2:2:1 ratio of neurons-to-astrocytes-to-microglia. **(B)** The magnified image shows multiple somatic junctions between a IBA1+ microglial cell and neighboring βIII-Tub+ neurons labeled with white arrows. Scale bars = (A) 100 µm and (B) 25 µm.

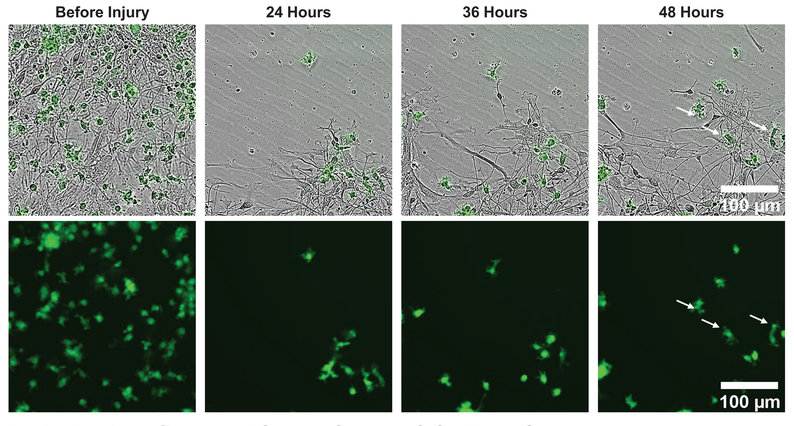

FIGURE 4: Microglia were Observed Around the Scratch Area

Representative brightfield (top) and fluorescent (bottom) time-lapse image series of the tri-culture with forebrain neurons, astrocytes, and GFP-expressing microglia before and 24, 36, and 48 hours after injury. This tri-culture was seeded at a 2:2:1 ratio of neurons-to-astrocytes-to-microglia. The microglial processes overlap with the adhered astrocytes and neurons 48 hours after injury (white arrows). Scale bars = 100 µm.

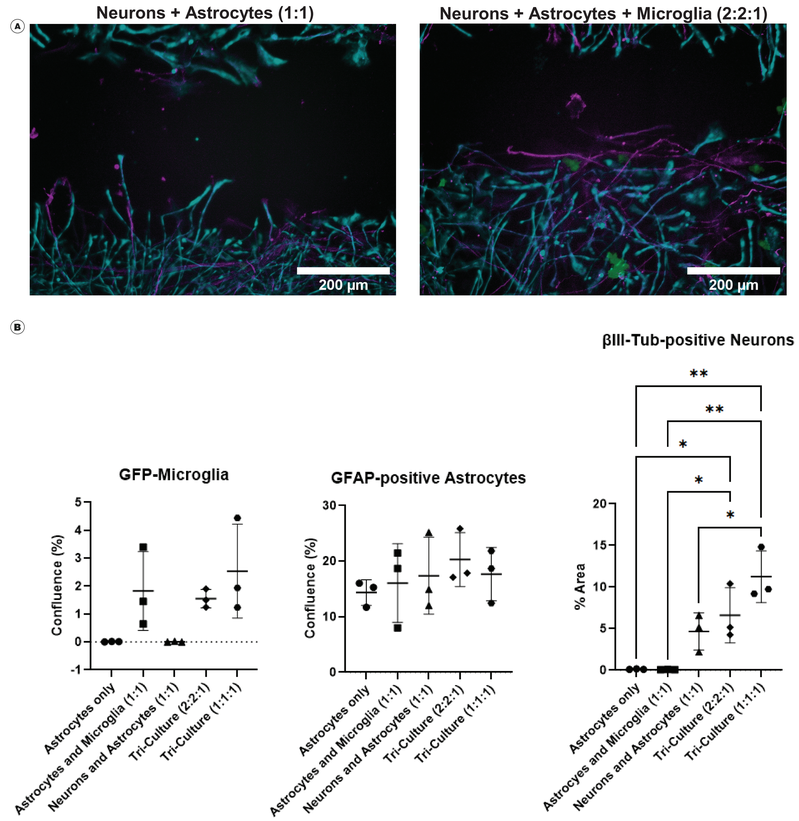

FIGURE 5: Forebrain Neurons, Astrocytes, and Microglia are Present in Scratched Area 48 Hours After Injury

(A) Representative ICC images of the co- and tri-cultures 48 hours after injury with microglia stably expressing GFP (green) and stained with GFAP (cyan) and βIII-Tub (magenta). Scale bar = 100 µm. **(B)** The tri-culture displayed a positive correlation as the addition of microglia led to an increase in the number of βIII-Tub+ neuronal axons in the area of injury. The ratios for the tri-culture indicate the amounts of neurons-to-astrocytes-to-microglia. Data represents mean ± SD. Each dot represents a technical replicate. Normality was determined by the Shapiro-Wilk test and statistics calculated with the ordinary one-way ANOVA test. * = $p < 0.05$, ** = $p < 0.01$.

Summary

- Astrocytes, forebrain neurons, and microglia generated by STEMdiff™ kits can be cultured together to establish physiologically relevant co- and tri-culture models that feature glia-neuron interactions.
- Co-culturing forebrain neurons with astrocytes increases neurite length and promotes increased neuronal survival compared to monocultured forebrain neurons.
- Increasing the amount of microglia included in the tri-culture model exhibits a trend of increased recovery to wound injury.