

## Abstract

Human induced pluripotent stem cells (iPSC)-derived neural progenitor cells (NPCs) and neurons are an attractive *in vitro* model to study neurological development, neurotoxicity and to model diseases. However, there is a lack of validated normal and Parkinson's NPCs and media that support differentiation into multiple types of neurons for disease modeling, drug screening, and toxicity screening. Here, we investigated the expression of genes associated with the differentiation of NPCs during three weeks in dopaminergic differentiation media. Expression of both TuJ1 early neuron and TH dopaminergic neuronal genes was significantly increased in a time-dependent manner ( $p < 0.05$ ) in three types of NPCs tested. Furthermore, expression of genes associated with glutamatergic (vGLUT2 and GLS2) and GABAergic (GABA) neurons was also increased in a time-dependent manner ( $p < 0.05$ ) in two types of normal NPCs (ATCC<sup>®</sup> ACS-5003 and ATCC<sup>®</sup> ACS-5007) while expression of these genes was induced and peaked at the end of two weeks in Parkinson's NPCs (ATCC<sup>®</sup> ACS-5001). This shows that our NPCs and dopaminergic differentiation media are capable of producing GABAergic and glutamatergic neurons, in addition to dopaminergic neurons. Noticeably, there was significant induction of motor neuron gene (LIM3) expression in ACS-5003 and ACS-5001 NPCs, but not in ACS-5007 NPCs while there was significant induction of cholinergic (ChAT) neuron gene expression in ACS-5007 and ACS-5001 NPCs, but not in ACS-5003 NPCs during dopaminergic differentiation. To validate that our NPCs and dopaminergic neuron differentiation media are suitable for drug screening, we conducted neurotoxicity screenings in three types of NPCs and NPCs-derived neurons using ReliBlue™ cell viability reagent (ATCC<sup>®</sup> 30-1014) assay and high content imaging analysis. We found that paclitaxel and vincristine significantly induced neurotoxicity ( $p < 0.001$ ) in both ACS-5003 and ACS-5001 NPCs while piperine didn't induce any significant neurotoxicity in all types of NPCs tested. Furthermore, amiodarone and chlorhexidine at 10 or 100  $\mu\text{M}$  significantly decreased cell viability ( $p < 0.001$ ) in both ACS-5003 and ACS-5001 NPCs. Cisplatin and hydroxyurea at 100  $\mu\text{M}$  significantly induced neurotoxicity in ACS-5003 NPCs, but not in ACS-5001 NPCs. Furthermore, paclitaxel didn't induce any significant neurotoxicity in both ACS-5003 and ACS-5007 NPCs-derived neurons by high content imaging analysis although it was toxic to NPCs with a  $\text{IC}_{50}$  of  $\sim 1 \mu\text{M}$ . This study demonstrates that our iPSC-derived NPCs and dopaminergic differentiation media are suitable for studying neurological development and neurotoxicity screening.

## Methods

**Dopaminergic neuron differentiation:** Normal human NPCs (ATCC<sup>®</sup> ACS-5003™; a non-reporter line) and normal MAP2-Nanoluc<sup>®</sup> HaloTag<sup>®</sup> reporter NPCs (ATCC<sup>®</sup> ACS-5007™) along with Parkinson's NPCs (ATCC<sup>®</sup> ACS-5001™) were seeded in CellMatrix Basement Membrane Gel (ATCC<sup>®</sup> ACS-3035™)-coated 12-well plates at a seeding density of 10,000 cells/cm<sup>2</sup> and cultured in NPC expansion media (ATCC<sup>®</sup> ACS-3003™) overnight prior to treating NPCs with dopaminergic differentiation media (ATCC<sup>®</sup> ACS-3004™) for up to three weeks.

**qRT-PCR:** RNA was extracted from ACS-5003, ACS-5007, and ACS-5001 NPCs treated with the dopaminergic differentiation media for 0, 1, 2, or 3 weeks and cDNA was synthesized for qRT-PCR analysis of neuronal gene expression by using TaqMan<sup>®</sup> probes (Thermo Fisher Scientific, Inc.) shown in Table 1.

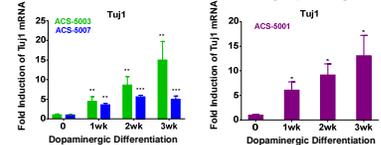
**Table 1. TaqMan<sup>®</sup> probes used for gene expression analysis**

Type of neurons	Name of TaqMan probes
Early neuron	TuJ1 (Neuron-specific class III beta-tubulin)
Dopaminergic neuron	TH (Tyrosine hydroxylase)
Glutamatergic neuron	GLS2 (Glutaminase 2), vGLUT2 (Vesicular glutamate transporter 2)
GABAergic neuron	GABA (Gamma-aminobutyric acid receptor subunit beta-3)
Motor neuron	LIM3 (LIM homeobox protein 3)
Cholinergic neuron	ChAT (Choline acetyltransferase)

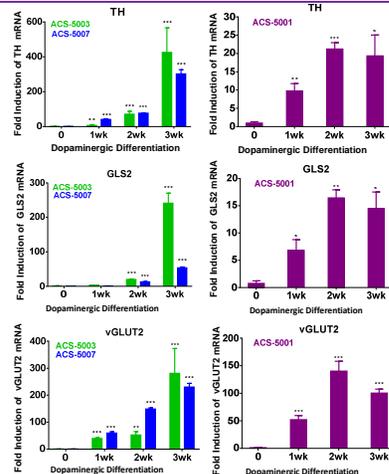
**Neurotoxicity assay:** ACS-5003 and ACS-5001 NPCs were seeded in CellMatrix Basement Membrane Gel-coated 96-well plates at a seeding density of 40,000 cells/cm<sup>2</sup> and cultured in NPC expansion media for two days prior to adding multiple neurotoxicants at 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , or 100  $\mu\text{M}$  for two days. Neurotoxicity was assessed by using ReliBlue™ cell viability reagent (ATCC<sup>®</sup> 30-1014). To examine neurotoxicity of neurons using high content imaging analysis, ACS-5003 and ACS-5007 NPCs were seeded in Cell Matrix Basement Membrane Gel-coated 96-well and 24-well plates, respectively, at a seeding density of 10,000 cells/cm<sup>2</sup> and treated with dopaminergic differentiation media for three weeks prior to adding 10  $\mu\text{M}$  of paclitaxel, cisplatin, or chlorhexidine for two days.

## Results

**Figures 1-5. Fold induction of different neuronal markers mRNA during three weeks of neuronal differentiation of ACS-5003, ACS-5007, ACS-5001 NPCs using dopaminergic differentiation media (ATCC<sup>®</sup> ACS-3004).**

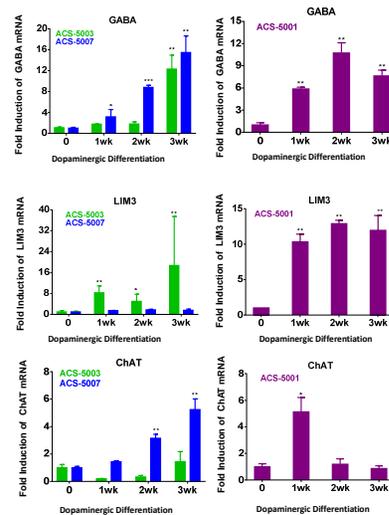


**Figure 1. Early neuronal marker.** TuJ1 mRNA increased significantly in ACS-5003, ACS-5007, ACS-5001 cells within a week (wk) of differentiation down the neuronal pathway ( $n=1$ ,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$ , Two-Way ANOVA).



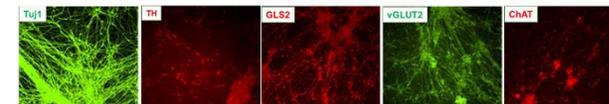
**Figure 2. Dopaminergic neuronal marker.** TH increased significantly and reached maximum level by the end of three weeks of differentiation in three types of NPCs ( $n=1$ ,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. day 0, Two-Way ANOVA).

**Figure 3. Glutamatergic neuronal markers.** Markers GLS2 and vGLUT2 mRNAs increased significantly in a time dependent manner during neuronal differentiation in ACS-5003 and ACS-5007 NPCs. However, expression of GLS2 and vGLUT2 reached a peak at week 2 in ACS-5001 Parkinson's NPCs ( $n=1$ ,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. day 0, Two-Way ANOVA).



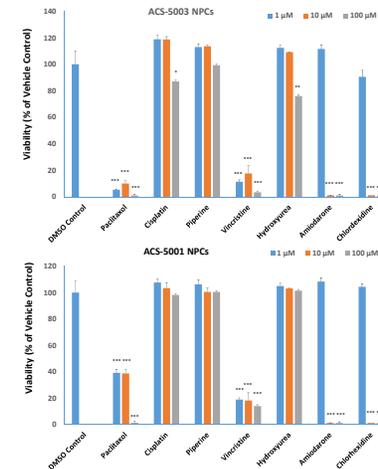
**Figure 4. GABAergic neuronal marker.** GABA mRNA increased significantly in ACS-5003 and ACS-5007 NPCs in a time dependent manner during neuronal differentiation. However, expression of GABA mRNA reached a peak at week 2 in ACS-5001 Parkinson's NPCs ( $n=1$ ,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. day 0, Two-Way ANOVA).

**Figure 5. Motor and cholinergic neuronal markers.** There was a significant increase in LIM3 mRNA in ACS-5003 and ACS-5001 NPCs while ChAT mRNA increased significantly in ACS-5007 and ACS-5001 NPCs during dopaminergic differentiation ( $n=1$ ,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. day 0, Two-Way ANOVA).

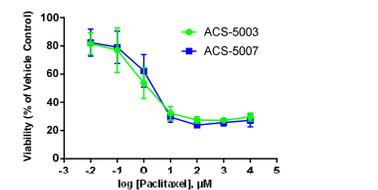


**Figure 6. Immunocytochemistry using TuJ1, TH, GLS2, vGLUT2, and ChAT antibodies in ACS-5007 NPCs differentiated into neuronal cells using ACS-3004 dopaminergic media for three weeks.** Consistent with qRT-PCR data, there was a marked increase in expression of TuJ1, TH, GLS2, vGLUT2, and ChAT neuronal markers in both ACS-5007 and ACS-5003 NPCs tested. Original magnification, x20. Similar results were observed in ACS-5003 cells (data are not shown).

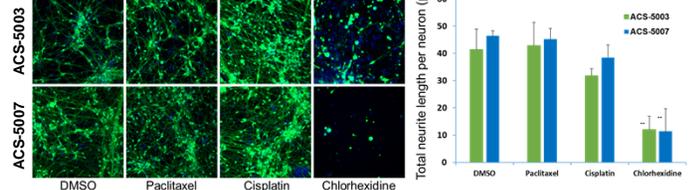
© 2015 American Type Culture Collection. The ATCC trademark and trade name, and any other trademarks listed in this publication are trademarks owned by the American Type Culture Collection unless indicated otherwise. NanoLuc<sup>®</sup>, HaloTag<sup>®</sup> are registered trademarks of Promega Corporation.



**Figure 7. Neurotoxicity testing of ACS-5003 and ACS-5001 NPCs treated with paclitaxel, cisplatin, piperine, vincristine, hydroxyurea, amiodarone, and chlorhexidine at 1, 10, or 100  $\mu\text{M}$  for two days.** Paclitaxel and vincristine at 1, 10, or 100  $\mu\text{M}$  significantly induced neurotoxicity ( $p < 0.001$ ) in both ACS-5003 and ACS-5001 NPCs. Furthermore, amiodarone and chlorhexidine at 10 or 100  $\mu\text{M}$  also significantly decreased cell viability ( $p < 0.001$ ) in both ACS-5003 and ACS-5001 NPCs. Cisplatin and hydroxyurea at 100  $\mu\text{M}$  significantly induced neurotoxicity in ACS-5003 NPCs, but not in ACS-5001 NPCs ( $n=3$ ,  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  vs. DMSO control, Student's T-test). Similar neurotoxicity was observed in ACS-5007 cells (data not shown).



**Figure 8. Dose-response curves for cell viability of ACS-5003 and ACS-5007 NPCs treated with paclitaxel for two days.** The neurotoxic effect of paclitaxel was similar in both types of NPCs.  $\text{IC}_{50}$  of paclitaxel on ACS-5003 and ACS-5007 NPCs was 0.9  $\mu\text{M}$  and 1.6  $\mu\text{M}$ , respectively ( $n=2$ ).



**Figure 9. High content imaging analysis of neurotoxicity of paclitaxel, cisplatin, and chlorhexidine in ACS-5003 and ACS-5007 NPCs-derived neurons.** ACS-5003 and ACS-5007 NPCs-derived neurons were treated with 10  $\mu\text{M}$  of paclitaxel, cisplatin, or chlorhexidine for two days and then stained with Calcein Green AM, and Hoechst 33342. Both paclitaxel and cisplatin didn't induce any significant neurotoxicity in both ACS-5003 and ACS-5007 NPCs-derived neurons. However, 10  $\mu\text{M}$  chlorhexidine significantly inhibited the neurite outgrowth ( $n=2$ ,  $^{**}p < 0.01$  vs. DMSO, Student's T-test). Original magnification, x20.

## Summary

- Human iPSC-derived NPCs and neurons are an appealing resource for *in vitro* disease modeling, toxicity screening and drug screening.
- ATCC has developed five types of normal non-reporter, normal lineage-specific GFP or Nanoluc-HaloTag reporter NPCs, and Parkinson's NPCs.
- ATCC normal and Parkinson's NPCs have the potential to be differentiated into dopaminergic, GABAergic, glutamatergic, motor, and cholinergic neurons after treatment with ATCC Dopaminergic Differentiation Media.
- ATCC NPCs and NPC-derived neurons have been validated for drug screening with several neurotoxicants by using a viability assay and high content imaging assessment.
- Paclitaxel is very toxic to NPCs with an  $\text{IC}_{50}$  of  $\sim 1 \mu\text{M}$ , but not in NPC-derived neurons.
- ATCC NPCs, NPC Expansion Media, and Dopaminergic Differentiation Media are suitable for studying neurological development and neurotoxicity screening.
- For a complete list of NPCs and other media types, please visit the ATCC website ([www.atcc.org/neuro](http://www.atcc.org/neuro)).