Novel Neural Induction Method For Efficient Generation Of Neural Stem Cells Derived From Parkinson's Disease Patient-Derived Sample iPSC Lines

Marian S. Piekarczyk[a], Tori Sampsell-Barron[a], Kun Bi[a], Spencer Hermanson[a], Connie Lebakken[a], Laurie J. Reichling[a], Mohan Vemuri[b], Yiping Yan[b], Ramya Sundararajan[c], Malini Vangipuram[c], J. William Langston[c], Birgitt Schuele[c]

[a]Primary and Stem Cell Systems, Life Technologies, 501 Charmany Drive, Madison, WI 53719
[b]Primary and Stem Cell Systems, Life Technologies, 7335 Executive Way, Frederick, MD 21704
[c]The Parkinson's Institute and Clinical Center, 675 Almanor Avenue, Sunnyvale, CA 94085

ABSTRACT

Parkinson's disease (PD) is one of the most common neurodegenerative disorders affecting a million people in the United States alone, with 50,000 Americans being diagnosed with PD each year. The absence of physiologically relevant cellular models for PD represents a major bottleneck for PD research. Novel models are urgently needed to accelerate the discovery of disease mechanisms and drug targets and for screening purposes which could rapidly translate into a wide range of clinical and therapeutic applications. Patient-specific iPSC-derived cell types have become an attractive tool for disease modeling *in vitro*.

For neuronal differentiation, one commonly used approach is embryoid body (EB) formation followed by neural rosette isolation and expansion. This approach can generate neural stem cells (NSCs) which can differentiate into different neuronal cell types and glia and can be cryopreserved for further maturation. The current limitation is that the process is laborious, inefficient, and the cells usually need to be further purified. To overcome these limitations, we developed a novel neural induction method that allows for the generation of NSCs from iPSCs within 7 days without the need for EB formation. In this study, we differentiated 4 PD iPSC lines and 2 age-matched control lines into neural stem cells using a novel neural induction/expansion media to differentiate iPSCs from an adherent monolayer on different matrices or feeder cells. We demonstrate that the generated NSCs are karyotypically normal, and express known NSC markers: Nestin, Sox1 and Sox2. Furthermore, gene expression analysis distinguishes these NSCs from their parental iPSCs and fibroblasts, and clusters them together with control NSCs derived from H9 ESCs.

In summary, the novel neural induction medium allows for efficient and robust generation of NSCs from PD patient-derived sample iPSCs and has the potential for large scale NSC generation to be utilized for high throughput/high content screening and drug discovery.

INTRODUCTION

Human pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are excellent sources for studies of cell fate specification, disease modeling and drug screening. In order to produce various neural cells from PSCs, the induction to neural stem cells (NSCs) is the first important step. Conventional methods of NSC derivation from human PSCs involving embryoid body (EB) formation or co-cultures with stromal cell lines have several disadvantages including a time-consuming protocol and variability in the quality of resulting NSCs. We have developed a chemically defined neural induction medium which can convert human PSCs into NSCs in one week with 80-90% of efficiency but without the time consuming laborious processes of EB formation and mechanical NSC isolation.

MATERIALS AND METHODS

Six donor fibroblast samples were received from the Parkinson's Institute and reprogrammed using Life Technologies' **CytoTune® -iPS Sendai Reprogramming Kit.** All six iPSC lines were tested for known human pluripotency markers and had normal karyotypes prior to neural induction. Using **Gibco® PSC Neural Induction Medium**, a serum-free medium that provides high efficiency neural induction of human pluripotent stems cells (PSCs), we were able to generate neural stem cell in just seven days. Unlike existing methodologies, use of **Gibco® PSC Neural Induction Medium** does not require the intermediary step of embryoid body (EB) formation, which adds time, labor, and variability. Neural Stem Cells (NSCs) generated using Gibco® PSC Neural Induction Medium have high expression of NSC markers and can be further differentiated into other neural cell types.

Table 1. Starting Material :Patient –Derived Samples From the Parkinson's Institute

ID	Age	Gender	Ethnicity	Disease	Gene	Mutation
PD-1	19	female	Cau	YOPD	PARKIN	Ex2del, c.102delAG
Ctrl-1	25	female	Cau	control	n/a	n/a
PD-2	74	male	A-J	PD	LRRK2	G2019S, heterozygous
Ctrl-2	69	female	Cau	control	n/a	n/a
MSA	62	female	Cau	MSA	unknown	unknown
PD-3	61	male	A-J	PD	LRRK2/GBA	G2019S, p.N370S

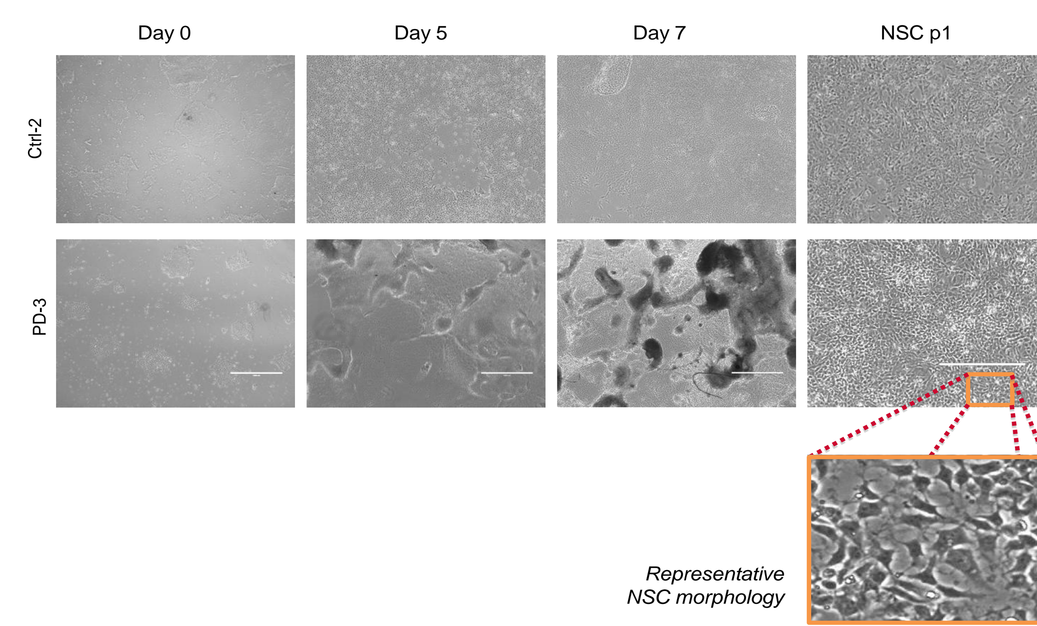
We used six donor -derived fibroblast cell lines for this study. Four were disease patient -derived samples and two were age matched healthy patient control samples.
All samples were processed in parallel for the duration of these studies.

Figure 1. Neural Induction and Expansion Timeline

Timeline illustrates the rapid and efficient strategy for the generation of patient derived NSCs in just seven days followed by banking and characterization.

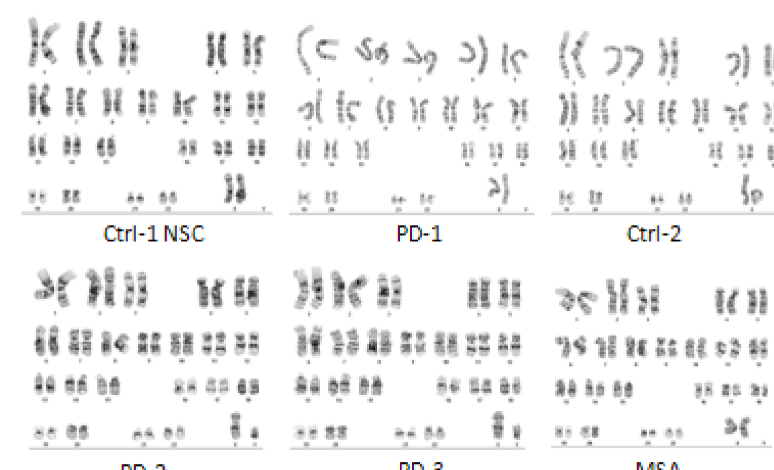
RESULTS

Figure 2. NSCs generated from PD patient-derived iPSCs in 7 days



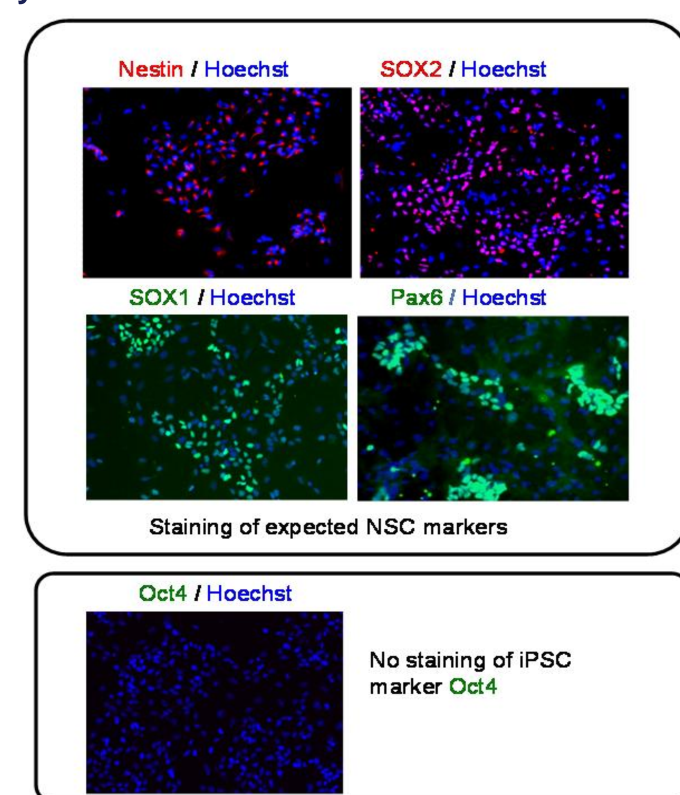
Progression of reprogrammed human iPSC towards neural induction which bypasses the need for EB formation and/or neural rosettes. Although some morphology of the neural cells varied between samples, all NSCs generated from patient-derived iPSCs demonstrated the ability to stain for known neural markers and several of the lines are currently being used in downstream glial and astrocyte differentiation studies.

Figure 3. All iPSC-Derived NSC Lines Displayed a Normal Karyotype



Karyotype analysis was performed on all six iPSC patient-derived NSCs. All lines displayed normal karyotypes via G-banded analysis. In addition, all six lines were banked and fully characterized through immunocytochemistry (ICC), and flow cytometry using known neural markers. Neural gene expression analysis was also carried out. (Karyotype analysis courtesy of WiCell Research Institute).

Figure 4. All iPSC-Derived NSC Lines Demonstrate Expected Neural Markers by ICC



All six iPSC-derived NSC lines stained positive for known neural markers Sox1, Sox2, and Nestin. All NSCs were negative for Oct4. In addition, NSCs stained positive for Pax6, a known regulator in neurogenesis and molecular regulation of the central nervous system. ICC samples were analyzed on the **FLoid® Cell Imaging Station.**

Figure 5. All iPSC-Derived NSC Lines Exhibit Expected Neural Markers by Flow Cytometry

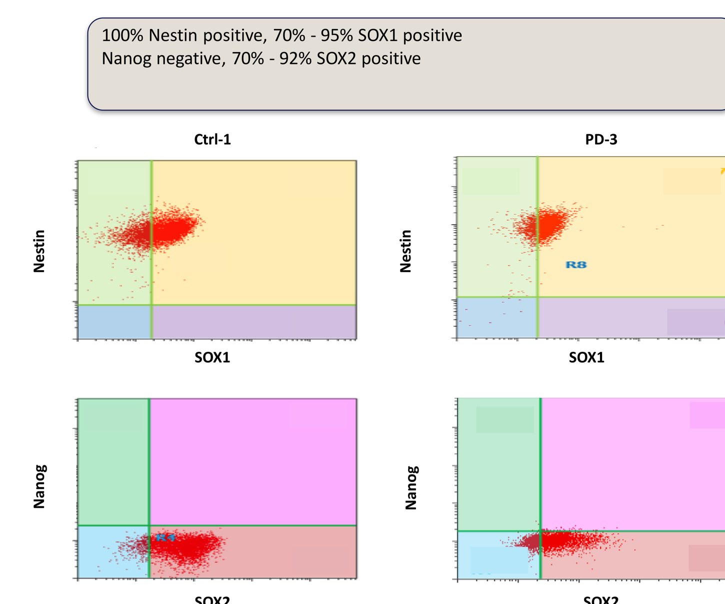


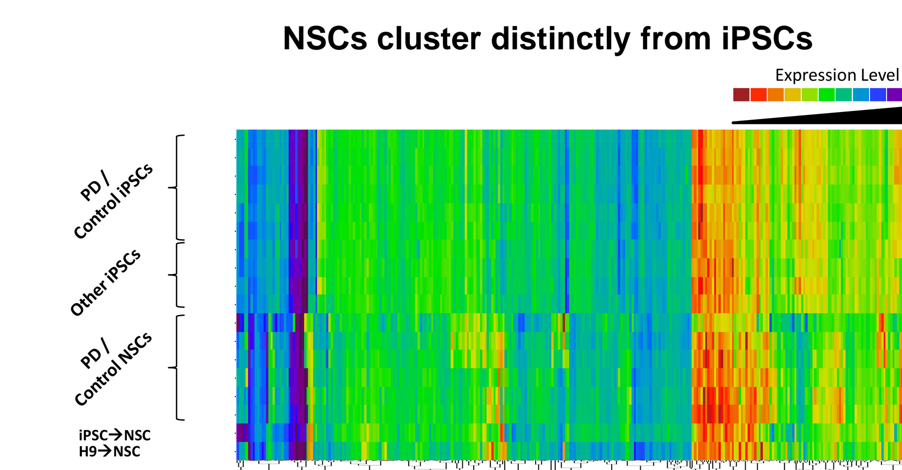
Table 2. Summary of Flow Cytometry Analysis

	Nestin	SOX1	SOX2	Nanog
PD-1 NSCs	99%*	88%*	98%*	0*
Ctrl-1 NSCs	98%*	64%*	89%*	1%*
Ctrl-2 NSCs	100%	70%	92%	0
PD-2 NSCs	100%	74%	95%	1%
PD-3 NSCs	100%	75%	71%	0
MSA NSCs	99%	72%	93%	0
MSA iPSCs	100%	5%	92%	93%

% positive cells compared to Isotype Controls.
*%positive cells compared to single stained and unstained control samples.

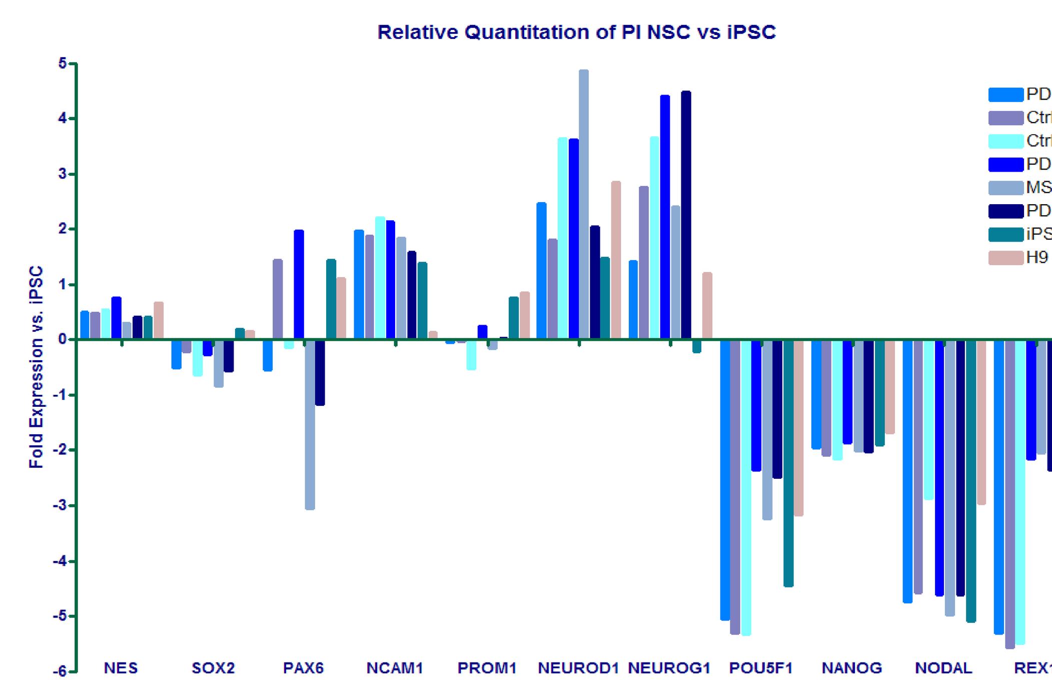
All iPSC-derived NSC lines exhibited positive expression for Nestin, Sox1, and Sox2. All NSCs were negative for Nanog expression. All flow cytometry samples were run and analyzed on the **Attune® Acoustic Focusing Cytometer.**

Figure 6. Heat Map Comparison Showing NSCs Cluster Distinctly from iPSCs



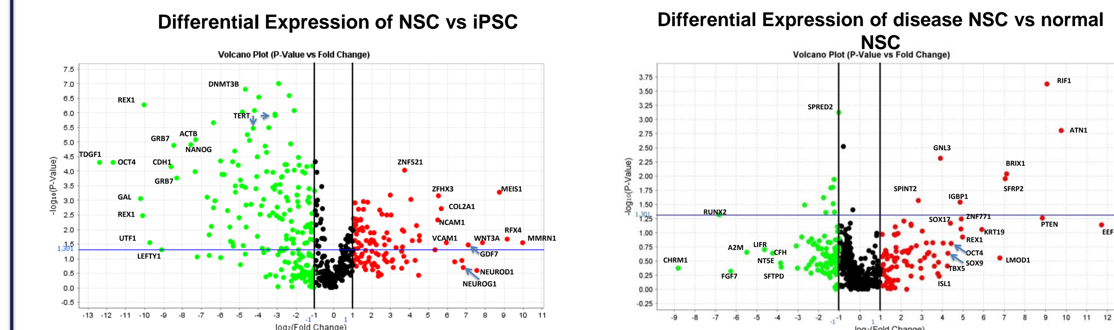
Cluster Analysis and heat map clearly distinguishes cell populations; NSCs are distinct from iPSCs in their clustering and cells of the same type (i.e. NSCs, iPSCs, and ESCs) cluster together.

Figure 7. Pluripotency Markers Are Down regulated in NSCs



Expression level of NSC and pluripotency markers in NSCs compared relative to source iPSCs. Some NSC markers show mixed results between samples, depending on the patient-derived source. Pluripotency markers are down regulated in NSCs. Data generated in Figures 5, 6, and 7 were generated using the **QuantStudio™.**

Figure 8. Differential Gene Expression of NSC vs.. iPSC and Disease vs. Normal NSC



For the purpose of identifying differentially expressed genes, patient-derived iPSCs were treated as biological replicates and all NSCs derived from patient-derived iPSCs were treated as biological replicates. Expression level of pluripotency markers reduced in NSCs compared to source iPSCs. Expression of genes related to neural differentiation were up regulated in NSCs vs.. iPSCs. Several interesting gene candidates were identified by differential expression between disease and normal NSC populations.

Table 3. Genes Showing Highest Differential Expression Between Disease NSC and Normal NSC

	Gene	Description
Up Regulated	RIF1	Telomere repair/DNA repair. Checkpoint arrest response to DNA damage.
	ATN1	Transcriptional corepressor. Tied to neurodegenerative diseases.
	EEF1A1	Part of elongation factor-1 complex - delivers tRNAs to ribosome.
	PTEN	Protein Phosphase. Tumor suppressor - mutated in many cancers. Neg regulates AKT/PKB signalling.
	BRIX	Required for biogenesis of the 60S ribosomal subunit.
	SFRP2	Soluble frizzled-related proteins (sFRPS) function as modulators of Wnt signaling through direct interaction with Wnts. They have a role in regulating cell growth and differentiation.
	GNL3	May be required to maintain the proliferative capacity of stem cells and may play an important role in tumorigenesis.
Down Regulated	CHRM1	GPCR. Expressed in nervous system. Affects phosphatidylinositol turnover. Target for Alzheimer's and Parkinson's.
	RUNX	Transcription factor essential for osteoblast differentiation and skeletal morphogenesis.
	FGF7	Mitogenic and cell survival activites. Regulation of embryonic development.
	A2M	Protease inhibitor and cytokine transporter. A2M is implicated in Alzheimer disease (AD) due to its ability to mediate the clearance and degradation of A-beta, the major component of beta-amyloid deposits.
	LIFR	Cytokine receptor. Involved in cellular differentiation, proliferation and survival.
	CFH	Member of the Regulator of Complement Activation (RCA) gene cluster.
	SPRED2	Tyrosine kinase substrate that inhibits growth-factor-mediated activation of MAP kinase.

Genes represented here illustrate those that were upregulated and down regulated, respectively, in a comparison between patient-derived disease and normal NSCs.

CONCLUSIONS

Here we demonstrate a rapid, robust and efficient method for the generation of neural stem cells from disease and non-disease donors in just seven days , displaying normal karyotypes. We illustrate that these NSCs express known neural markers and can be further utilized in downstream screening applications. In addition it will be possible to generate other neural cell types, such as DA neurons and glia/astrocytes (these studies are currently underway). With the generation of these fully characterized Parkinson's disease patient -derived NSCs we can look at downstream gene editing methodologies to better understand the nature of Parkinson's and other neurodegenerative diseases.

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TRADEMARKS/LICENSING

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