High-efficiency differentiation into functional dopaminergic neurons from Parkinson's patients-derived induced pluripotent stem cells

Faria Zafar[1], Soojung Shin[2], Michael Derr[2], Andrew Nguyen[1], Adrian Flierl[1], Birgitt Schüle[1]

[1]Parkinson's Institute and Clinical Center, Sunnyvale, CA and [2]Thermo Fisher Scientific Inc., Carlsbad, CA

Abstract

The objective of the study was to compare two neuronal differentiation protocols: 1) Pluripotent Stem Cell (PSC) Dopaminergic (DA) Neuron Differentiation Kit (Prototype, Thermo Fisher, catalog Number A30416SA) and 2) our dopaminergic differentiation protocol (Mak et al. 2012) for length of protocol, efficiency of DA neuron generation, and electrophysiological properties.

Parkinson disease (PD) patient specific induced PSCs (iPSC) differentiated into DA neurons lay the foundation for exploring disease mechanism and drug screening. However, there remain critical challenges that must be addressed to reduce variability. It is important to develop standardized and validated iPSC laboratory protocols to generate and characterize cultures. Critical factors for successful neuronal differentiation are yield of desired, functional target tissue type, reproducibility, duration, and cost of the protocol.

We tested two different protocols to achieve high yields of DA neurons using iPSCs from PD patients. Two approaches were 1. embryoid body (EB)/rosette derived neural stem cells induced with dual SMAD inhibition and NCAM sorting that are further differentiated into mature neurons (via GDNF/BDNF) in ~60 days (Mak et al. 2012) and 2. direct differentiation of iPSCs through floorplate progenitor into mature DA neurons via PSC Dopaminergic Neuron Differentiation Kit in 35 days. We detected higher amounts of the DA neuron specific enzyme, tyrosine hydroxylase after 35 days of differentiation with the PSC Dopaminergic Neuron Differentiation Kit compared to cells derived with EB/dual SMAD inhibition protocol after 60 days. The PSC Dopaminergic Neuron Differentiation Kit showed a sharp increase in floorplate/mesencephalic markers such as FOXA2, Corin, LMX1A, and EN-1 at Day 10 (expression range of 10-10,000 fold increase in comparison to day 0). Neuronal cultures showed spontaneous activity on multielectrode arrays (MEA, Axion Biosystems) of about 3000 spikes and an average amplitude of 22µV(range of 17µV to 40µV).

In summary, dopaminergic differentiation with the ThermoFisher PSC Dopaminergic Neuron Differentiation Kit provided reproducible culture conditions, shorter differentiation time, and a high-yield of functional dopaminergic neurons.

Materials and methods

iPSC culture and maintenance: iPSCs were cultured either on Geltrex with manual passaging every 6-7 days or on hLaminin-521 (Thermo Fisher Cat. No. A29249) passaging with Accutase at 1:6 split every 4-5 days. Essential 8 media was changed daily.

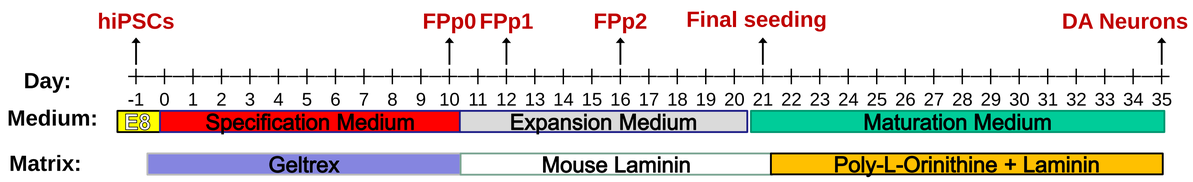
PSC midbrain DA neuron differentiation: iPSCs were cultured for 10 days with Specification media to generate floor plate progenitors (FPps). FPps were expanded for 10 more days with Expansion media to be either cryopreserved or further maturation. The last 15 days of the differentiation process, FPps were developed into functional DA neurons with Maturation media.

Immunocytochemistry: Cells were fixed at day10 and at day35 in 4% PFA for 10 minutes at room temperature (RT). Cells were permeabilized with 0.3% Triton X-100 in PBS for 5 minutes (except cells stained with Tyrosine Hydroxylase (TH, Millipore, 2234) and β-III-Tubulin (TUJ1) antibodies), washed with PBS, blocked with 5% goat serum for 1 hour at RT, and incubated with primary antibodies in 5% goat serum for overnight at 4C. Indirect immunofluorescence staining was performed with Alexa fluor 488 and 555 conjugated H+L antibodies. Fluorescent images were captured on an Nikon Eclipse Ti inverted fluorescence microscope and analyzed with ANDOR Zyla software. TH count was performed by HSC Studio 2.0 software's Neuronal Profiling BioApplication at Thermo Fisher Scientific.

mRNA expression analysis: Total RNA was collected by treating cells with Trizol at day0, day10, and day35 and Taqman probes were used for relative expression analysis.

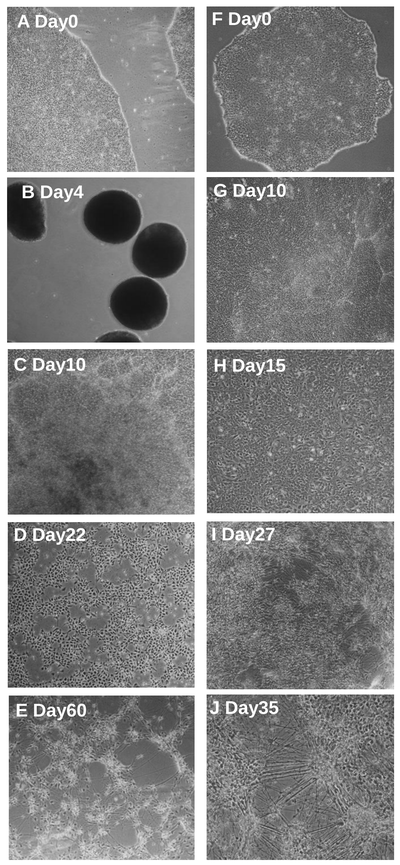
Electrophysiological activity measurement: Day 27 neurons were seeded on 0.1% Polyethylenimine (PEI) coated single-well microelectrode array (MEA) plates. ~150,000 neurons were plated in MEA array and measured daily for 10 min per session for spontaneous activity in Muse (Axion Biosystems). Spike activity of was analyzed via AXIS 2.1 software.

Timeline for *in vitro* dopaminergic differentiation



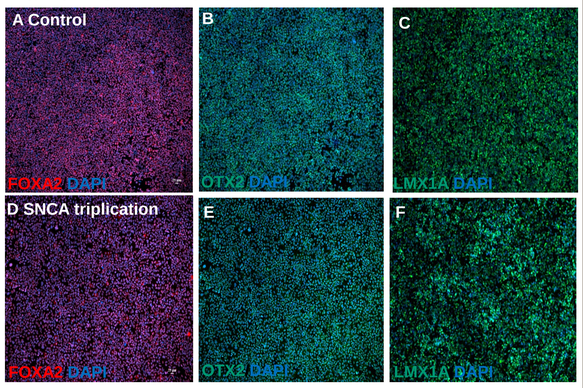
Results

Figure 1: Comparison of two protocols, Thermo Fisher kit and Mak protocol, to derive DA neurons from iPSCs in terms of length of differentiation and efficiency



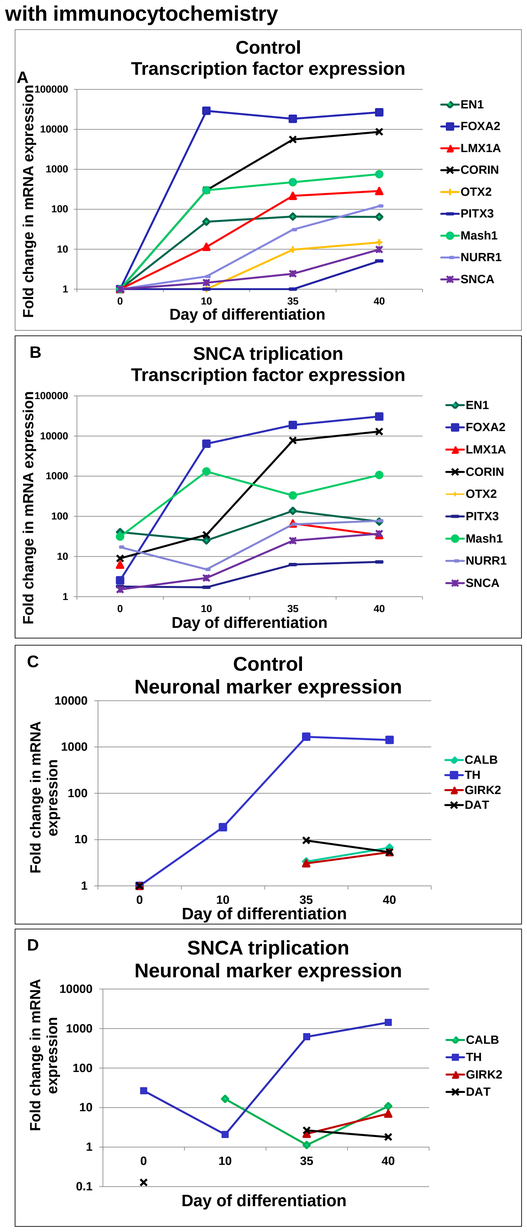
Left panel (A-E) describes derivation of DA neurons from iPSCs following Mak protocol. A) 5 day old iPSC colonies; B) EBs at day4 on rocker; C) rosette formation at day 10; D) NCAM-sorted NSCs (10 days after dissociating rosettes); E) mature DA neurons at day35 starting from NSCs.
Right panel (F-J) describes Thermo Fisher DA neuron derivation. F) 3 day old iPSCs; G) 10 days in specification media; H) FPps at day15 in expansion media; I) immature neurons; J) mature DA neurons at day35.

Figure 2: FPps at day10 expressing both floor plate marker FOXA2 and other midbrain markers



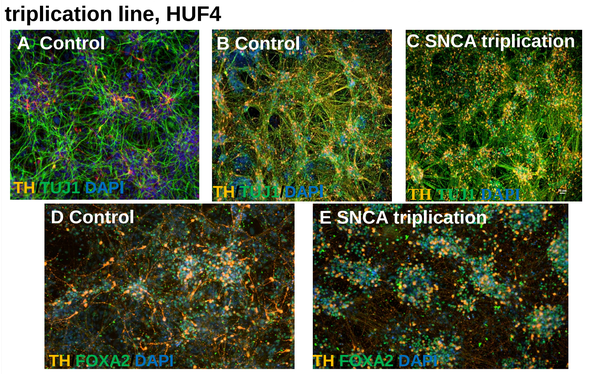
iPSCs treated with specification media for 10days modified into floor plate progenitors. FPps from both control line (upper panel) and SNCA triplication line (lower panel) expressed A&D) floor plate marker FOXA2 (Thermo Fisher, 100024245) B&E) midbrain marker OTX2 (Abcam, ab114138) C&F) LMX1A (Millipore, AB10533). Counterstained with DAPI. 10X magnification with scale bar 25µM.

Figure 3: mRNA expression holds invariable outcome as with immunocytochemistry



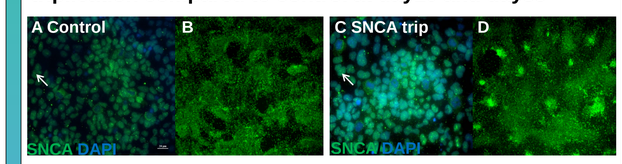
mRNA expressions were measured at day10 (after specification period), at day35 (in mature DA neurons) and at day40 (mature DA neurons derived from cryopreserved FPps), normalized with day0 control, HUF5, iPSCs. A-B) in both HUF5 and HUF4, floor plate marker, FOXA2 expressed 10,000 fold increase at day10 compared to day0 indicating most cells turning toward midbrain DA neuron progenitors after 10 days of specification treatment. All measured transcription factors expressed same level of intensity for both directly derived neurons (day35) and neurons derived from cryopreserved FPps (day40). Other mid brain markers, EN1, CORIN, LMX1A, PITX3 also showed higher level of changes at day10 and day35 compared to day0. C-D) dopaminergic neuron marker, TH and GIRK2, for both cell lines showed fold increase at day35 compared to day10. Two more trials of differentiation illustrated similar results.

Figure 4: Day35 mature neurons expressing high % of DA neuron marker, TH, in both control, HUF5, and SNCA triplication line, HUF4



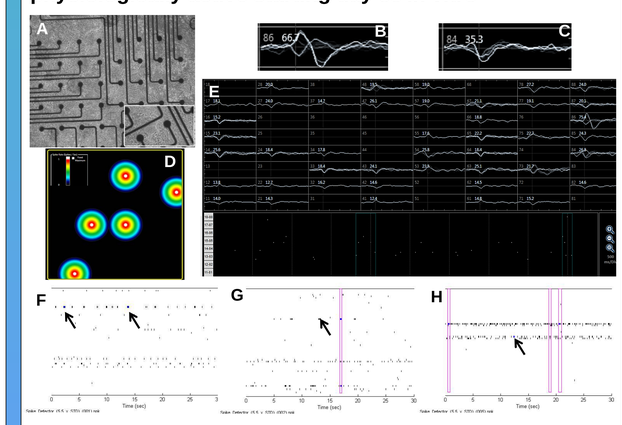
Efficiency of achieving TH+ cells was 10 fold higher in Thermo Fisher kit protocol compared to Mak protocol. A)~4-6% TH+ cells were detected in Mak protocol in controls. B-E) neurons obtained using Thermo Fisher kit. B) in HUF5, ~25.3% TH positive cells were measured over total cell count by DAPI staining (2 trials). C) in HUF4, ~60.9% TH positive cells were measured over total cell count by DAPI staining (in a 2nd trial, TH+ count was ~24.0%). D, E) Majority of TH+ neurons also stained for FOXA2. 10X magnification with scale bar 50µM. Count was performed by HSC Studio 2.0 software's Neuronal Profiling BioApplication.

Figure 5: α-Synuclein expression is increased in SNCA triplication compared to control at day10 and day35



α-Synuclein polyclonal antibody (Stressgen, ADI9055651) was used for ICC in control A) day10, 60X magnification and B) day35, 20X magnification. SNCA trip C) day10, 60X magnification and D) day35, 20X magnification.

Figure 6 : iPSC-derived mature neurons are electro-physiologically active starting day 35 *in vitro*



Functional activity of iPSC derived differentiated neurons was evaluated using MEA MUSE system to measure spontaneous action potentials. A) neurons at day35 B-C) amplitude of spike ranging 15µV to 75µV on an individual electrode. D) Activity map shows neuronal activity on different electrodes. E) Snapshot of spike patterns on all 64 electrodes at a single time-point. Raster plot shows functional activity for all electrodes for 30s time period at F)day 35 G) day 38 and H) day 42. Arrow on a blue box at F indicates network bursts. Pink column at G-H shows bursts synchrony.

Conclusion

- FPps can be cryopreserved and banked to achieve steady final differentiation for drug screening
- Consistent dopaminergic differentiation >20.0%, and up to 60% observed
- Seeding density for each passage, coating consistency of culture vessel and attachment are crucial factors
- Spontaneous neuronal activity can be measured to compare neurons from PD patients to control

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1. Sally K. Mak, and *et al.*. "Small Molecules Greatly Improve Conversion of Human-Induced Pluripotent Stem Cells to the Neuronal Lineage." *Stem Cells International.* Vol. 2012, Article ID 140427. 2012.
2. Thermo Fisher PSC Dopaminergic Neuron Differentiation Kit, catalog # A30416SA https://tools.thermofisher.com/content/sfs/manuals/psc_da_neuron_diff_kit_man.pdf

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