High Efficiency of Neural Induction in Parkinson's Disease-Specific Induced Pluripotent Stem Cells

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) reprogrammed from patient-derived tissue pave the way for generating better *in vitro* models for biomedical research and innovative drug screening. Disease-specific cell lines from Parkinson's Disease (PD) mutation carriers seem to be more difficult to consistently differentiate into the neuronal lineage as compared to hiPSC control lines.

Our goal for this study is to use a combination of small molecules to promote neural induction of hiPSCs. We applied dorsomorphin (DM) and SB-431542 (SB), both of which inhibit the bone morphogenic protein and the activin/nodal signaling pathways.

Embryoid bodies (EBs) from five hiPSCs (3 controls and 3 PD cases) were treated with or without DM and SB for four days in hESC media without FGF2. EBs were induced in neural induction medium (DMEM/F12, N2 and FGF2) for additional 2 days. At Day 7, EBs were plated onto Geltrex™-coated plates. Neural rosettes appeared as early as day 2 after plating. The number of individual rosettes increased over time. The rosette formation in the group without DM/SB was between 0-31.9%; whereas, with addition of these small molecules the rosette formation increased to 48-97.5%. EBs expressed Pax6 and Sox1. Also, there was a remarkable difference of >150 fold neuroectodermal markers Sox1 and Nestin gene expression in Dor/SB treated EBs compared with EBs without small molecules.

This improvement of neural induction is a key for the production of homogenous neural progenitor cells (NPCs), which leads to a more efficient dopaminergic neuronal differentiation in patient-specific cell lines.

INTRODUCTION

- Patient-specific hiPSC-derived differentiated cells have become an attractive tool to study disease mechanisms in a human background and are a vanguard into a new era of science and certainly personalized medicine.
- The goal is that the human/patient-derived cell lines are "authentic" in their cellular response compared to any cell culture or animal model to date.
- But one of the challenges is to differentiate these patient-derived iPSCs into the desired specialized cell type of interest with high efficiency, reproducibility, and region specificity.
- Caveats of the EB/neurosphere mediated differentiation are clonal expansion of subgroup of cells and potential forebrain specification. However, the usefulness of NPCs is that they can be expanded and cryopreserved and be a starting pool for final maturation.
- The NPCs are also important for scientific questions of developmental phenotypes related to disease. Direct neuronal differentiation from embryonic stem cells or iPSCs gives the promise of shortening the timeline while reaching similar efficiency of dopaminergic neurons.
- We describe a 5-stage EB differentiation approach using small molecules to enhance neural induction consistently in patient and control hiPSC lines.

MATERIALS AND METHODS

iPSC Maintenance and Propagation

iPSCs were cultured and maintained on mitomycin C inactivated mouse embryonic fibroblasts (iMEF) in hESC media containing DMEM/F12, 20% KnockoutTM Serum Replacement, 1x NEAA, 1x P/S, 0.1% β-Mercaptoethanol, 0.5x L-Glu and 6 ng/ml of basic fibroblast growth factor (FGF2). Cells were split every week manually without enzymatic treatment.

Generation and Maintenance of Neural Progenitor Cells (NPCs)

To derive NPCs, iPSC colonies were harvested using 1 mg/ml of Collagenase IV.. Forming embryoid bodies (EB) were cultured in suspension with agitation on rocker for 4 days in EB media, which consisted of hESC media minus FGF2 with or without 5 µM Dorsomorphin (Dor) and 10 µM SB431542 (SB). Next, EBs were cultured for additional 2-3 days with agitation in neural induction media (NIM) consisting of DMEM/F12, 1×NEAA, 0.5x L-Glu, and freshly made and sterile filtered N2, which contained 1.55 g/L glucose, 2 g/L sodium bicarbonate, 100 µM putrescine, 30 nM sodium selenite, 20 nM progesterone, 0.1 mg/ml transferrin, 0.025 mg/ml insulin and FGF2 (20 ng/ml). Neural rosettes formed 2–5 days in adherent culture. To obtain a pure population of NPCs, rosettes were manually isolated using No. 15 scalpel cutting in squares with distance to edges of colonies, dissected pieces of rosettes were lifted using a pipette, re-plated onto Geltrex™ coated culture dishes and maintained with neural progenitor cell media (NPC media) containing Neurobasal media, 1× NEAA, 1× L-Glu, 1x P/S, 1× B27 supplement, and FGF2 (20 ng/ml). Manual isolation of rosettes as described above was repeated once to obtain more pure population of NPCs. Approximately 5-10 pieces of rosettes were dissociated into single cells using Accutase™. Cells were treated with Accutase™ for 2-5 minutes, then the cells were collected, centrifuged, resuspended in NPC media and plated onto one 96-well coated with Geltrex™ and cultured at 37 °C and 5% CO2. When confluent, NPCs were split in single wells with larger surface area such as 48 well, 24 well, 12 well etc. in NPC media. NPCs were enriched using anti PSA-NCAM microbeads (Miltenyi Biotech) according to the manufacturer's instruction.

Quantitative PCR (qPCR)

EBs were collected at day 7. Total RNA (500 ng) was extracted using a RNeasy Micro kit (Qiagen) and reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (BioRad). qPCR was performed using a PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA).

Immunocytochemistry

EBs were fixed with 4% paraformaldehyde at room temperature for 10 minutes. Fixed cells were permeablized with 0.3% Triton X-100 for 5 minutes and were blocked in blocking buffer (5% normal goat serum) in 1x Phosphate buffered saline (PBS) and followed by incubation with the primary antibody at 4 °C overnight in 5% normal goat serum and PBS. The following primary antibodies were used: Sox1 (Millipore, AB15766), 1:100; Pax6 (Developmental Studies Hybridoma Bank, Iowa City, IA), 1:20 and secondary antibodies were Alexa Fluor 488 Goat Anti-Mouse IgG and Alexa Fluor 555 Goat Anti-Rabbit IgG (Invitrogen), 1:300. Coverslips were mounted with Vectashield Mounting Medium with DAPI (Vector Labs). Fluorescent images were captured on an inverted fluorescence microscope (Nikon Instruments Inc, Melville, NY, Eclipse Ti). Phase contrast images were taken with an Axiovert 25 (Carl Zeiss Inc, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 4, GraphPad Software, San Diego, CA). Data were analyzed by one-way analysis of variance (ANOVA). Newman–Keuls post hoc analysis was employed when differences were observed in ANOVA testing ($P < 0.05$). Data were presented as the means + standard error of the mean (SEM). All results were derived from at least three independent experiments, except results of cell line 1679 which was n=2.

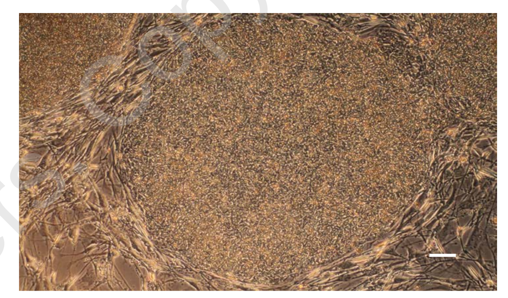
Neuronal differentiation into dopaminergic neurons

We have adapted and optimized embryoid body (EB) mediated neuronal differentiation in combination with dual SMAD inhibition. The goal is to fulfill criteria for an optimal culture of mDA neurons: a) purity of differentiated cultures, b) correct region identity of derived neurons, c) reproducibility of the method, and d) throughput of method.

Stage I	Stage II	Stage III	Stage IV	Stage V
Expansion of iPSCs →	Embryoid body formation →	Neural rosette formation →	Expansion of neural progenitor cells →	Dopaminergic maturation

Days in culture: 0 — 4 — 10

Media Name	EB 4 days	NIM 4 days	NPC 2-3 days	DA1 10 days	DA2 20-40 days
Basal Media	DMEM/F12	DMEM/F12	Neurobasal medium	Neurobasal medium	Neurobasal medium
Media supplement	β-ME	Bottenstein N2 FGF2	B27 FGF2	B27 FGF8	B27 BDNF GDNF
Test components	Dorsomorphine SB431524			Purmorphamine SAG Sonic Hedgehog	



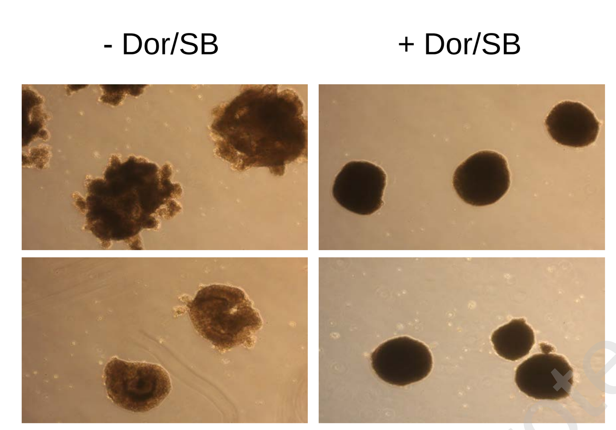
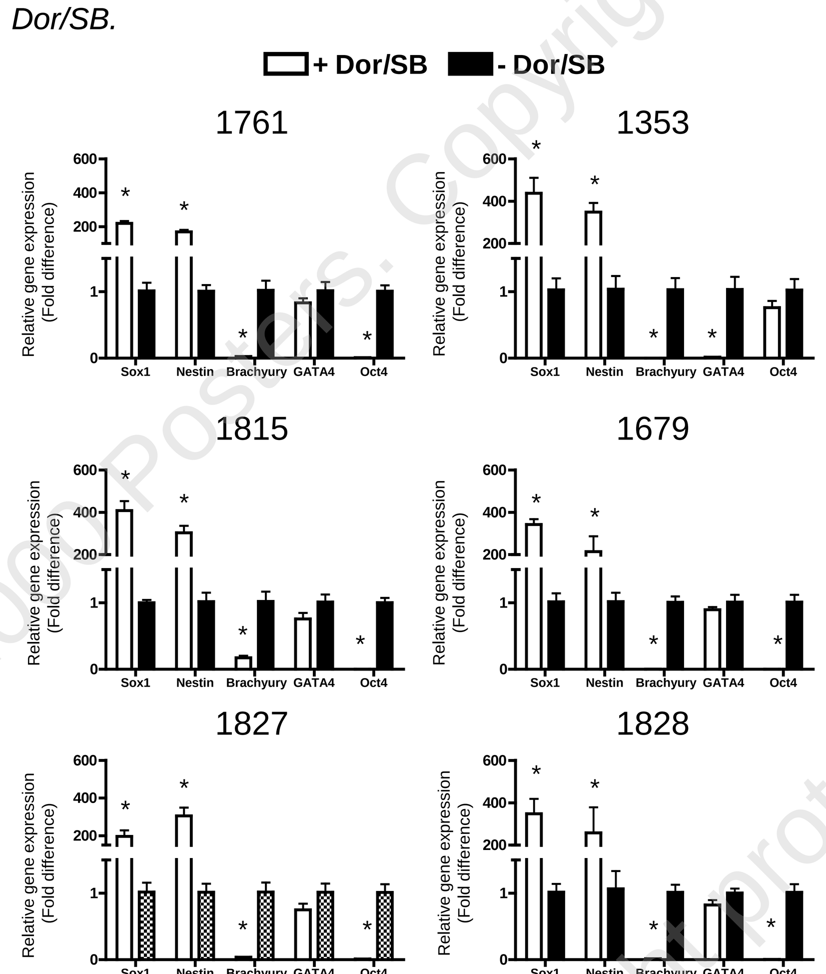
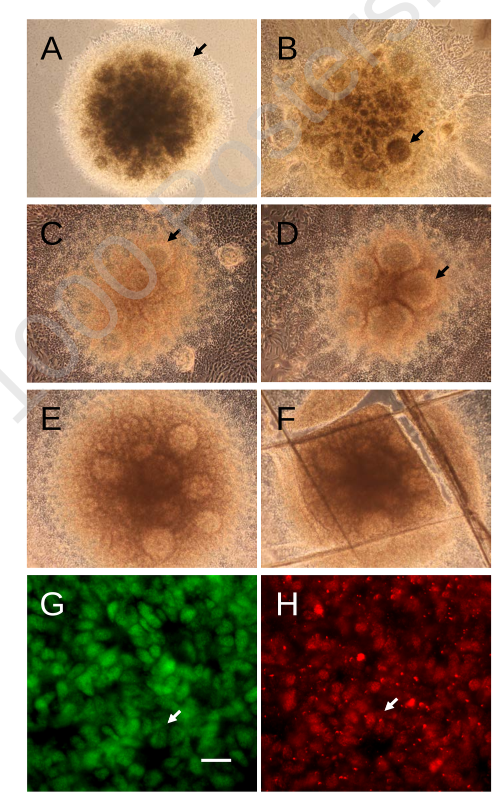


Figure 3. (A) Example of a iPSC colony with distinct border and no signs of differentiation ~2 mm in diameter. Scale bar 100 µm. (B) In control and PD lines, EBs were loose and had no clearly defined structures when cultured without Dor/SB; EBs were round, uniform and showed a distinct boundary line in EB media with addition of Dor/SB.





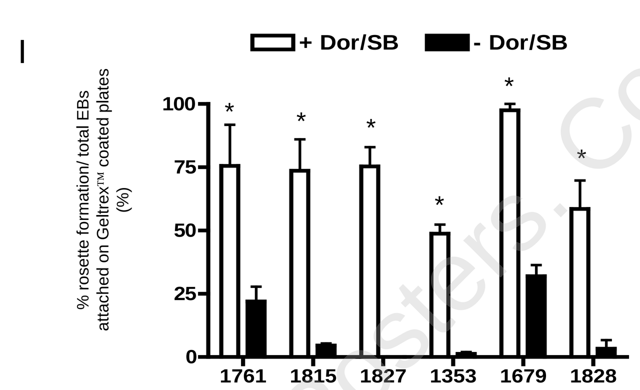
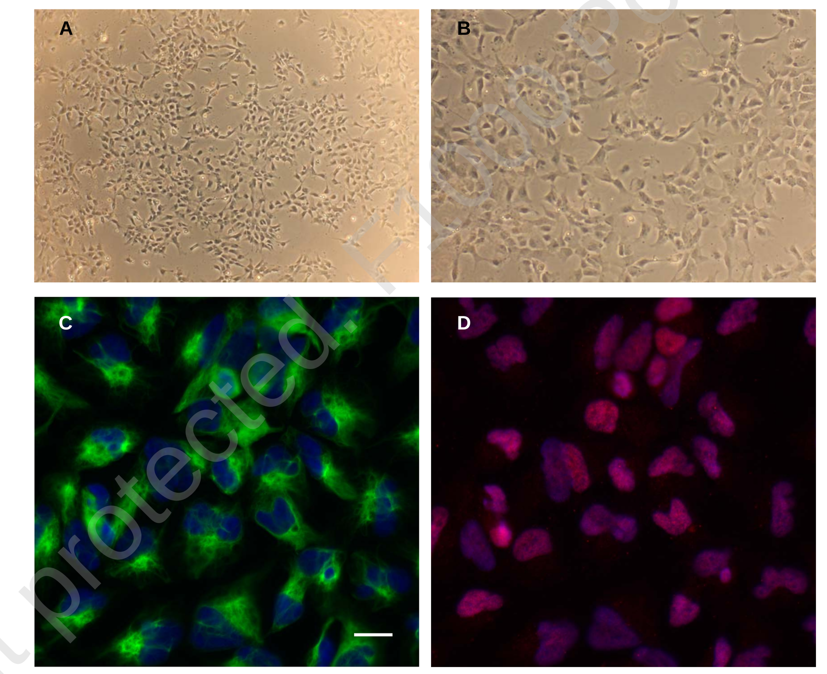


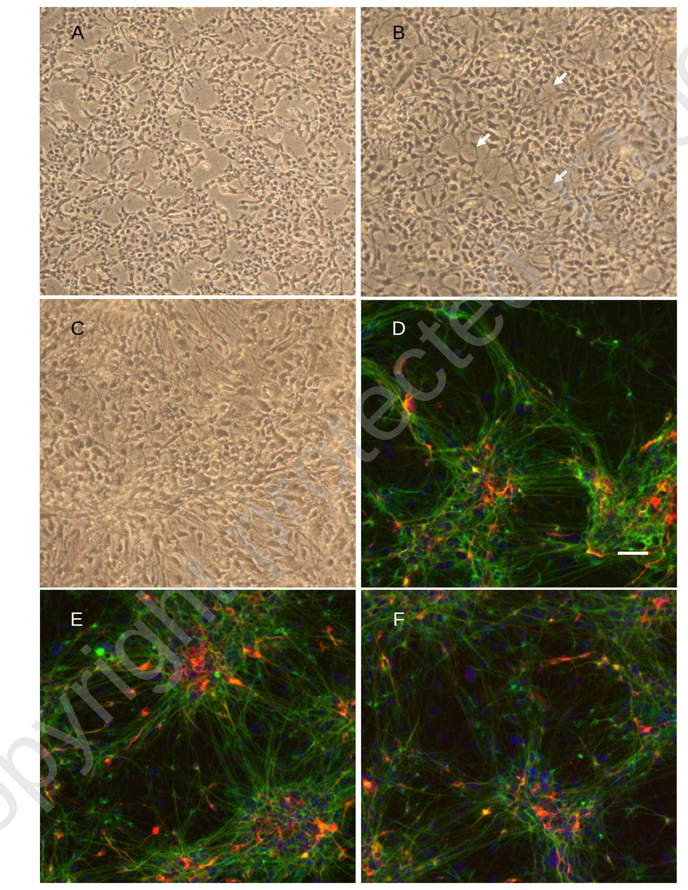
*Figure 3. (A-D) Examples of neural rosettes after Dor/SB treatment for days 7-12, stage III (5x mag.). Arrows indicate early and late stages of rosettes. (E and F) Manual dissection of rosettes using scalpel before (E) and after (F) dissection. (G and H) Arrows indicate positions of neural rosettes immunoreactive for Pax6 (green, G), Sox1 (red, H). Scale bar 20 µm. (I) Significant difference in neural rosette formation in all control and PD specific EBs treated with Dor/SM compared no Dor/SB treatment during stage II (*p<0.01).*

*Figure 4. Expression levels of neuroectoderm (Sox1 and Nestin), mesoderm (Brachyury), endoderm (GATA4) and pluripotency markers (Oct4) measured by Q-PCR. The y-axis represents means ± SEM of relative expression levels of each gene in the EBs with Dor/SB relative to no Dor/SB treatment (*p<0.01). Left panel=healthy donors; right panel= PD patient derived lines.*

NPC Stage and Final Neuronal Maturation



Stage 4: Expansion of neural progenitors NPC: morphology under phase-contrast microscopy with 5x (A) and 10x (B) magnification. (C and D) NPCs expressed Nestin (green) (C) and Sox1 (red) (D) Nuclei were counterstained with DAPI (blue). Scale bar represents 20 µm.



	Stereology			Flow cytometry		
	TUJ1/ Total cells	TH/TUJ1	TH/Total	TUJ1/ Total cells	TH/TUJ1	TH/Total
Pur	80.42 ± 2.21	8.58 ± 0.66	6.44 ± 1.12	71.30 ± 8.72	5.94 ± 1.55	4.47 ± 0.08
SAG	85.84 ± 3.10	7.00 ± 0.75	6.31 ± 0.63	80.52 ± 6.17	3.93 ± 0.88	3.28 ± 0.23
Shh	68.41 ± 12.49	7.58 ± 0.36	5.44 ± 0.55	68.89 ± 11.22	2.73 ± 1.32	1.81 ± 0.42

Figure 6. (A-C) Phase-contrast microscopy showed plating density of NPCs at day 1 in DA1 media (5x) (A) cell morphology at day 12 (arrows indicate processes) (B, 10x), and neurons at day 30 during DA maturation (C, 10x magnification). (D-F) Neurons treated with Pur (D), SAG (E) and Shh (F) were stained for TUJ1 (green), TH (red) and DAPI (blue). Scale bar 200 µm. G. Table: percentage of TUJ1 relative to total cells, TH-pos. neurons relative to TUJ1 pos. neurons and relative to total cells quantified using stereology and flow cytometry in control NPCs after dopaminergic maturation.

RESULTS and CONCLUSIONS

- This 5-stage protocol has several advantages to generate neural precursors that can be easily expanded without loosing its differentiation potential [1], to be suitable for studying disease-mechanism at neuroprogenitor stage, and to have potential for derivation of other CNS cell types [2].
- Small molecules have been reported to improve directing ESC/iPSCs into neural lineage [3, 4]. We tested a combination of small molecules, Dor and SB.
- For successful generation of NPCs, it is crucial to start with pristine undifferentiated iPSC cultures. IPSC colonies should be densely packed, show low nucleus/cytoplasma ratio, and have discrete borders and no differentiation on periphery and/or in the center of the colony.
- In the early stage of NIM induction (around at days 8-10), neural rosettes showed darker center of "flower-shaped" structures with indiscrete boundary line. In the latter stage, more distinct and clear edge of "flower-shaped" morphology showed.
- We evaluated neural differentiation of EB on day 10 by immunocytochemistry using neural markers Pax6 and Sox1 as well as the pluripotent cell marker Oct4. Pax6 and Sox1 showed positive staining in EBs but there was no Oct4 immunoreactivity.
- We quantified the percentage of neural rosettes formed with and without addition of Dor/SB. Without Dor/SB we observed low rosette formation between 0-31.9%, but the combination of Dor/SB increased the neural rosette formation to surprisingly 48-97.5% of EBs in both control and PD-specific cell lines.
- At day 6, we performed gene expression analysis of multiple markers in attached EBs. There was a striking difference of >150 fold neuroectodermal markers Sox1 and Nestin gene expression in Dor/SB treated EBs compared with EBs without small molecules. This suggests that the two small molecules very efficiently modulate the SMAD signaling pathway leading to this enormous increase in neuroectodermal markers.
- In conclusion, small molecules can enhance the efficiency of inducing iPSCs to differentiate into the neural lineage and also accelerate the efficacy to generate dopaminergic neurons for *in vivo* modeling study as well as drug screening.

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