Reproducible Differentiation of Dopaminergic Progenitors from Patient-Derived iPSCs via 3D Suspension Culture

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder, characterized by the progressive loss of midbrain dopaminergic (mDA) neurons in the substantia nigra [1]. These neurons are essential for dopamine production, having a critical role in motor control, regulating mood and other cognitive functions. While current therapies alleviate symptoms, they do not halt disease progression. Restoring the dopaminergic neurons is vital in developing an effective treatment for PD, and the use of induced pluripotent stem cells (iPSCs) provides a promising autologous source of mDA progenitors (mDAPs) for transplantation [2]. As a personalized treatment without complications of immune response, using mDAPs with high purity and differentiation potential from a patient's own reprogrammed iPSCs is an avenue to replenish the lost mDA neurons. When inducing mDAPs in conventional two-dimensional (2D) adherent culture systems, we observed high variability and limited scalability in the process. We compared 2D adherent differentiation with three-dimensional (3D) suspension-based induction protocols across multiple iPSC lines to create a more robust and reliable method of generating mDAPs for transplantation.

Methods

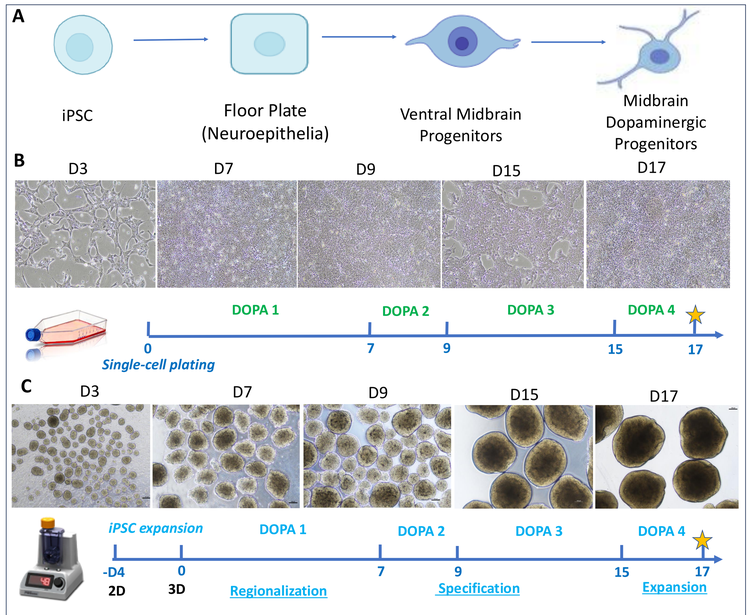


Figure 1. Overview of our induction protocol from iPSC to mDAP: A. Simplified schematic of the induction pathway. B. Format and timeline of 2D induction in tissue culture flask. Images taken before each change in media composition, which we call DOPA 1-4. C. Format and timeline of 3D induction in vertical wheel bioreactor. Images taken before each change in media composition. Scale bar: 100um

To induce mDAPs in our stem cells, we utilize a pair of small molecules at the start of the induction to achieve dual inhibition of SMAD signaling and drive neurogenesis. We use additional small molecules to control the signaling of Wnt and Sonic Hedgehog (SHH) to properly regionalize the neural progenitors and create a population of floor plate derived ventral midbrain progenitors by use of FGF8 [3]. In the 2D culture method, single cell iPSCs are plated into a T25 flask and passaged on day 7. On day 17, the cells are dissociated and cryopreserved. For the new 3D culture system, we began by plating single cell iPSCs in a flask and expanding them for 4 days in TeSR AOF media. After reaching about 70% confluency, we harvest the iPSCs as single cells and seed them into the PBS-0.1L mini or PBS-0.5L vertical wheel bioreactor. The 3D system grows spheroids of a uniform size resulting in a consistent response to differentiation cues. These methods are summarized in **Figure 1**.

References:
1. Surmeier DJ, Obeso JA, Halliday GM. (2017). Nat. Rev. Neurosci., 18(2), 101–113.
2. Song B, et al, (2018). J.Clin. Invest., 128(4), 1028–1042.
3. Xi J, Liu Y, Liu H, Chen H, Emborg ME, Zhang S-C. (2012). Stem Cells, 30(8), 1655–1663.

Results

To assess the induction success, we used flow cytometry analysis to classify the identity of our cell product, particularly looking at the co-expression of FOXA2 and OTX2 which is a defining marker combination for mDAPs. We tested for the presence of residual iPSC in our mDAPs from both culture methods by flow cytometry for OCT4/NANOG double-positive cells, which were consistently at or below the negative control level. The flow data revealed that 3D conditions increase the proportion and consistency of FOXA2+/OTX2+ double-positive progenitors compared to 2D **(Fig. 2A).** We calculated the mDAP percent yield of each induction across conditions and saw improvement in the 3D culture method, reaching up to 2800% yield **(Fig. 2B).** Nanostring gene expression profiling of the mDAP product further demonstrated reduced batch-to-batch variability in 3D conditions, with improved expression of mDAP lineage markers (FOXA2, OTX2, LMX1A) **(Fig. 2C)** and decreased off-target markers (NKX2-1, Pax6, SOX1), indicating improved lineage fidelity **(Fig. 2D).**

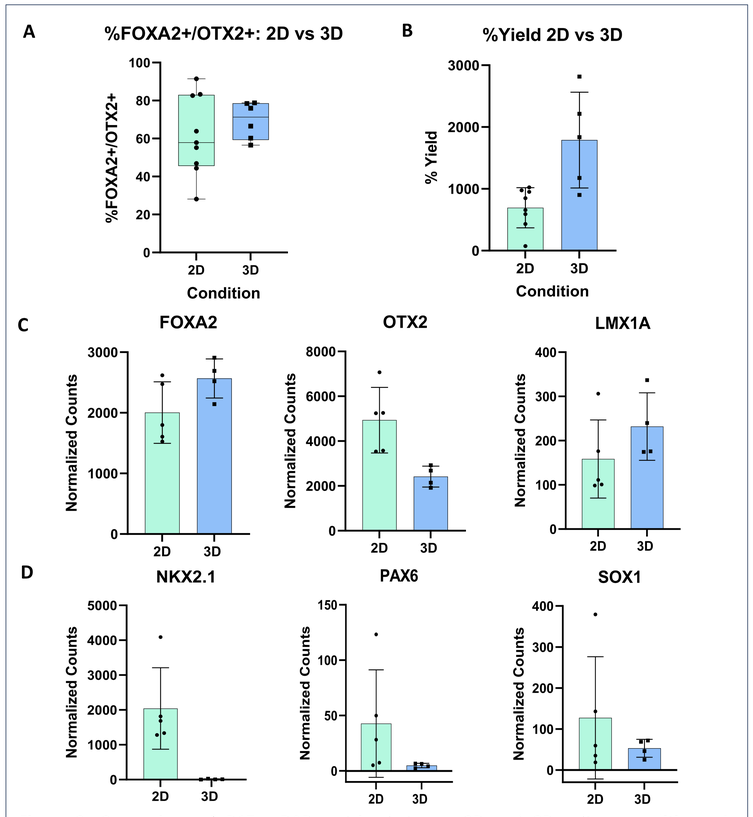
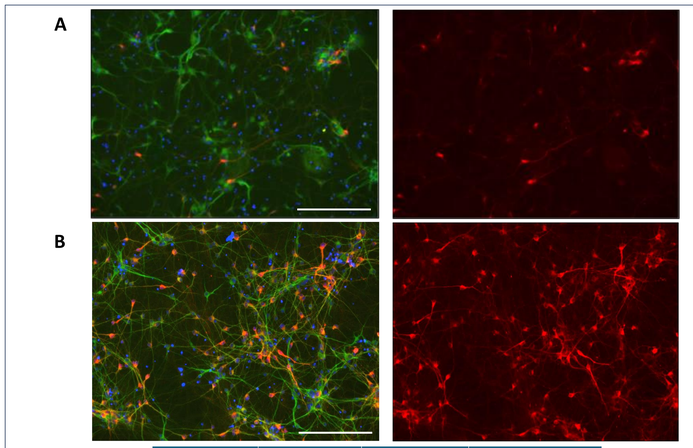


Figure 2. Comparison of D17 mDAP metrics between 2D and 3D culture conditions: A. Percentage of FOXA2+/OTX2+ cells under 2D and 3D conditions. B. % Yield of mDAP between 2D flasks and 3D bioreactors. C. Normalized nanostring data of on-target genes for dopaminergic progenitors. D. Normalized nanosting data of off-target genes which indicate forebrain (NKX2.1 & PAX6) and hindbrain (SOX1) contamination.

For therapeutic application, mDAPs must have the ability to differentiate into functional dopaminergic neurons. To evaluate the differentiation potential of our product we performed a 7–14-day differentiation protocol and used immunocytochemistry (ICC) to assess neuronal differentiation across several iPSC lines. Co-staining with MAP2 and TH enabled quantification of the total neuronal population and identification of dopaminergic neurons within it. The differentiated neurons from the 3D culture conditions showed much more expansive branching of neurites **(Fig. 3A, B).** The proportion of $TH^+/MAP2^+$ neurons was increased and exhibited high reproducibility between different batches and cell lines in the 3D culture system **(Fig. 3C).**

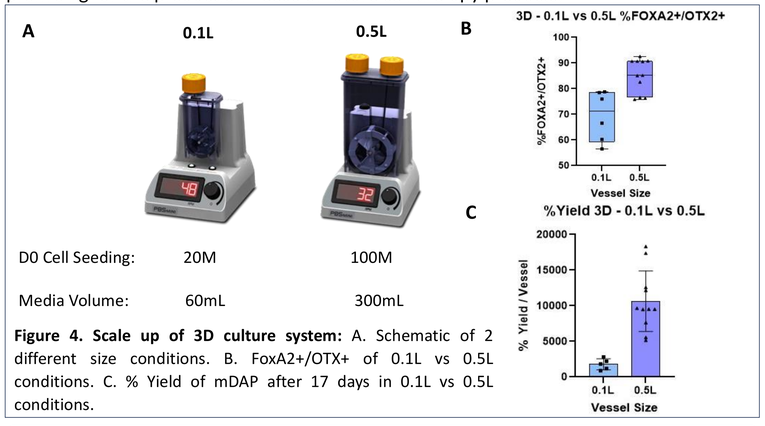


C

Condition	Cell Line	TH/MAP2	Neuron/Total
2D	PD5	27.29%	92.91%
2D	PD2.2	25.24%	91.85%
2D	PD2.2	26.26%	90.36%
2D	ABx2	30.34%	84.97%
2D	ABx2	29.79%	80.15%
3D	PD116.7	65.21%	99.82%
3D	PD116.7	51.11%	99.54%
3D	PD116.7	63.06%	98.02%
3D	PD238.1	55.69%	98.30%
3D	PD032.28	47.9%	97.19%

Figure 3. Fluorescent microscopy images of in vitro differentiated neurons with ICC staining from 2D vs 3D mDAP (Hoechst in blue, MAP2 in green, and TH in red): A. Neurons from 2D mDAP, overlay on the left and TH+ on the right. B. Neurons from 3D mDAP, overlay on the left and TH+ on the right. C. Quantification of ICC across 6 cell lines for 2D and 3D conditions. Scale bar 200um.

After developing the 3D suspension culture on the small scale 0.1L vessels, we investigated the scalability to a larger system for future manufacturing needs. For this scale-up, we used the PBS-0.5L mini vertical wheel bioreactor with a 300-500mL working volume **(Fig. 4A).** We tested the 0.5L vessels on 3 cell lines and observed an improvement in on-target identity **(Fig. 4B)** as well as a striking increase in yield **(Fig. 4C)**. This is highly promising for the production needs of future cell therapy products.



	0.1L	0.5L
D0 Cell Seeding:	20M	100M
Media Volume:	60mL	300mL

Figure 4. Scale up of 3D culture system: A. Schematic of 2 different size conditions. B. FoxA2+/OTX+ of 0.1L vs 0.5L conditions. C. % Yield of mDAP after 17 days in 0.1L vs 0.5L conditions.

Conclusion

Together, the data demonstrate that the 3D suspension method enhances lineage specificity, reproducibility, yield, and scalability in mDAPs production, providing a more reliable platform for translational cell therapy development in Parkinson's disease.

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