Hunting(ton) for the Correct Differentiation: The Validation of a Striatal GABAergic Differentiation Protocol

David S. Yi[1#]✉ | Lunden P. Friberg[1#]✉ | Rekha C. Balachandran[1]
Hyunjin Kim[1] | Morgan Thomas[1] | Aaron B. Bowman[1]

[1]School of Health Sciences, Purdue University, West Lafayette, IN
#These authors contributed equally to the current project. ✉ yi79@purdue.edu, lfriberg@purdue.edu

Introduction

- Huntington's disease (HD) is a neurodegenerative disorder caused by the polyglutamine (CAG) repeat in the Huntingtin gene; the disease onset is between 30 to 45 years of age, and marked by progressive decline in intellect, emotional control, and physical ability
- HD is "the disease of the striatum": it predominantly affects the neural striatum, resulting in the degradation of medium spiny neurons (MSN), which make up 95% of neurons in the striatum
- Using human induced pluripotent stem cell (hiPSC)-derived MSNs, it is possible to model HD *in vitro*. We seek to validate the efficacy of a protocol that differentiates hiPSCs into striatal GABAergic MSNs

Hypothesis & Aim

- ***Hypothesis:*** The Adil et al. differentiation protocol[1], with modification by R. C. Balachandran, will generate hiPSC-derived MSNs that express the canonical marker genes for MSNs
- ***Aim 1:*** Confirm successful differentiation into MSNs through differential gene expression analysis between hiPSC-derived MSNs, cortical (CTX) glutamatergic neurons, floor plate (FP) dopaminergic neurons, and pancreatic islet (ISL) cells—each derived from two controls & two HD patients
- ***Aim 2:*** Upon validation, replicate the differentiation protocol and confirm successful differentiation via reverse transcription quantitative polymerase chain reaction (RT-qPCR) and immunocytochemistry

Experimental Design

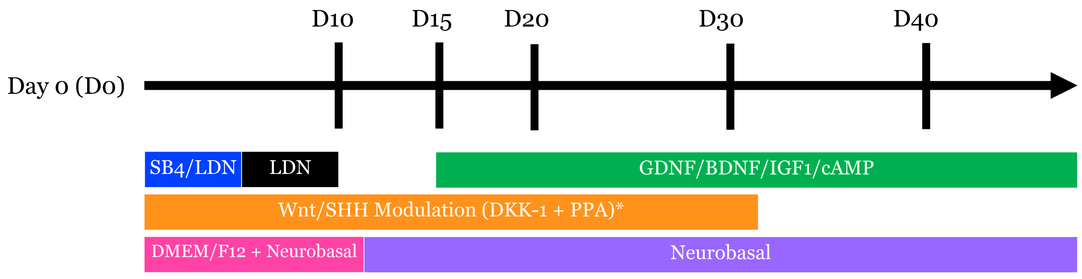

Fig. 1 Schematic and media conditions for hiPSC-derived MSN differentiation[1]. hiPSCs were differentiated to striatal progenitors on Matrigel-coated 2D surfaces. Both Wnt pathway inhibitor DKK-1 and the ventralizing morphogen SHH agonist purmorphamine were added from day 2 to day 31. The neurogenic factors BDNF, GDNF, cAMP, and IGF1 were used in media formulations from day 15 onwards.
* R. C. Balachandran's modification: DKK-1 + PPA were used in media until D31. Adil et al.'s original protocol used these until D26.

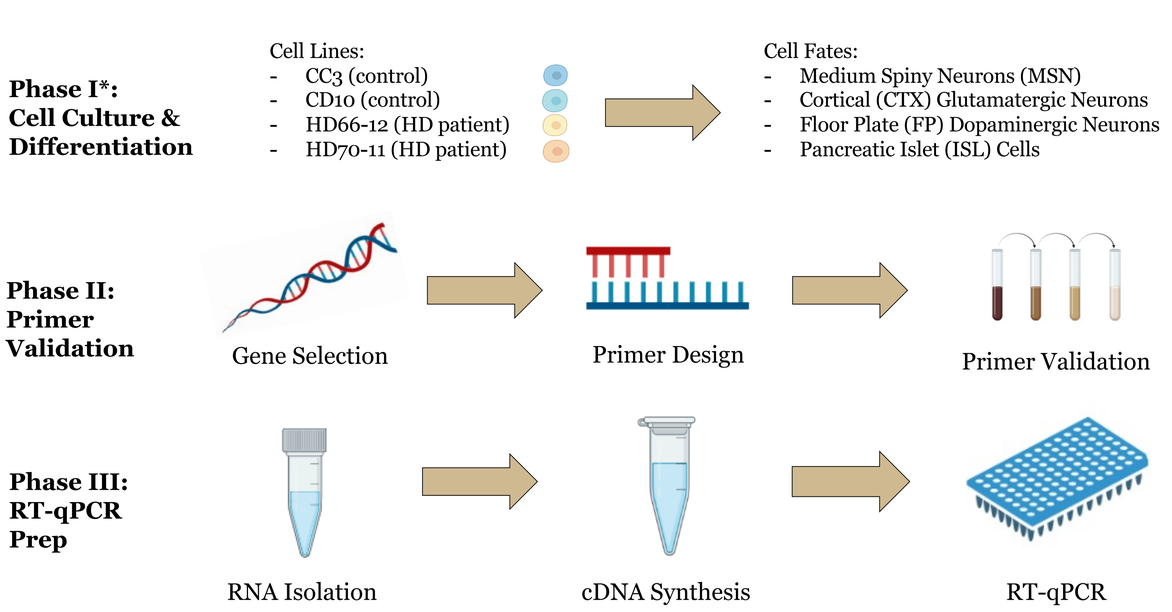

Fig. 2 Work flow for gene expression profiling via RT-qPCR. In Phase I, each cell line was differentiated into each of the four distinct cell fates. Cell lysates were harvested on day 11, 61, and 88. Lysates were stored in -80° C until prepped for RNA isolation. In Phase II, primers were designed using Primer3 and *in silico* PCR in the UCSC genome browser.
*Phase I was solely completed by R. C. Balachandran.

Results

Category	Gene
Housekeeping	GAPDH
Astrocytes	GFAP
Neural Progenitors	GLI3, PAX6, SOX1
Neuronal	MAP2
Striatal GABAergic Medium Spiny Neurons	DARPP-32, CTIP2, FOXP1, ISL1, vGAT,* GAD1,* GAD2*
Cortical Glutamatergic Neurons	vGLUT1, vGLUT2, SATB2
Floor Plate Dopaminergic Neurons	FOXA2, TH, LMX1A

Table 1 Gene Selection. Genes were selected according to the established canonical markers associated with the neuronal lineages and cell types studied in this project.
*These are used to generally characterize GABAergic neurons.

Gene	PCR Efficiency (%)	R^2	Gene	PCR Efficiency (%)	R^2
GAPDH	103.09	0.9994	vGAT	102.74	0.9997
GFAP	105.23	0.9980	GAD1	102.92	0.9994
GLI3	98.25	0.9946	GAD2	101.77	0.9986
PAX6	94.03	0.9989	vGLUT1	99.8	0.9994
SOX1	94.57	0.9973	vGLUT2	104.56	0.9981
MAP2	109.91	0.9991	SATB2	100.16	0.9975
DARPP-32	102.99	0.9922	FOXA2	100.49	0.9984
CTIP2	101.97	0.9819	TH	106.96	0.9961
FOXP1	99.97	0.9994	LMX1A	96.04	0.9958
ISL1	103.29	0.9996			

Table 2 Primer Validation. PCR efficiency and coefficient of determination of all primers used in this study are displayed.

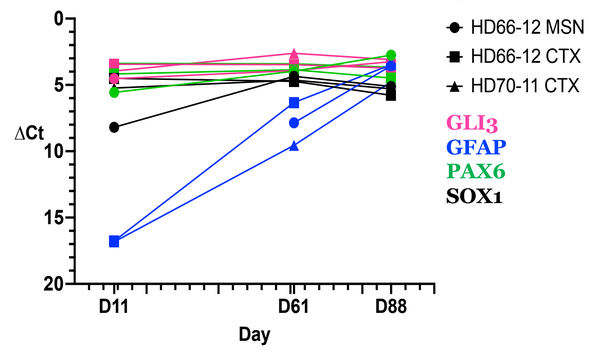

Fig. 3 Neural Progenitor Marker & Astrocyte Marker Gene Expression. mRNA levels were measured by RT-qPCR and normalized to GAPDH. GLI3 (pink), GFAP (blue), PAX6 (green), and SOX1 (black) are displayed. A higher ΔCt value represents a lower gene expression at the mRNA level.

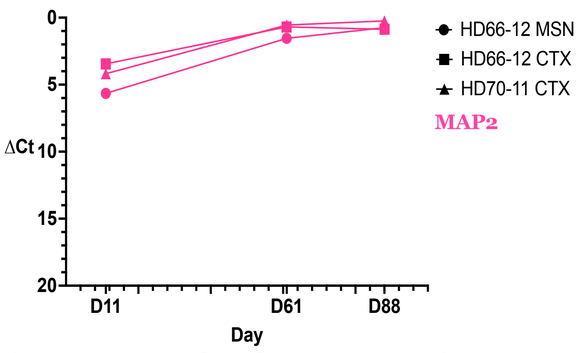

Fig. 4 Pan-Neuronal Marker Gene Expression. mRNA levels were measured by RT-qPCR and normalized to GAPDH. MAP2 (pink) is displayed. A higher ΔCt value represents a lower gene expression at the mRNA level.

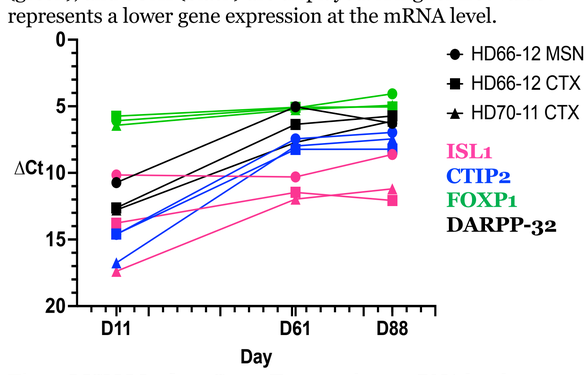

Fig. 5 MSN Marker Gene Expression. mRNA levels were measured by RT-qPCR and normalized to GAPDH. ISL1 (pink), CTIP2 (blue), FOXP1 (green), and DARPP-32 (black) are displayed. A higher ΔCt value represents a lower gene expression at the mRNA level.

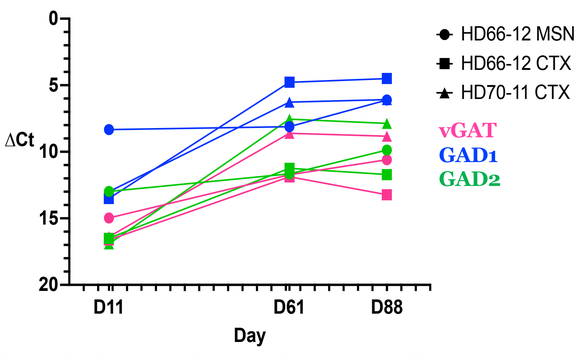

Fig. 6 GABAergic Neuron Marker Gene Expression. mRNA levels were measured by RT-qPCR and normalized to GAPDH. vGAT (pink), GAD1 (blue), and GAD2 (green) are displayed. A higher ΔCt value represents a lower gene expression at the mRNA level.

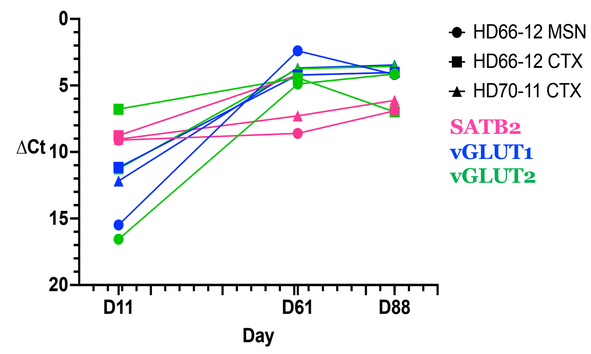

Fig. 7 CTX Marker Gene Expression. mRNA levels were measured by RT-qPCR and normalized to GAPDH. SATB2 (pink), vGLUT1 (blue), and vGLUT2 (green) are displayed. A higher ΔCt value represents a lower gene expression at the mRNA level.

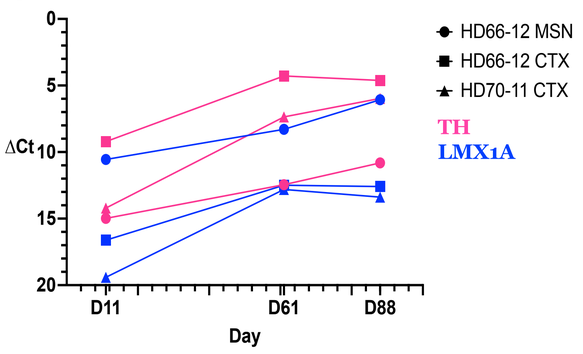

Fig. 8 FP Marker Gene Expression. mRNA levels were measured by RT-qPCR and normalized to GAPDH. TH (pink) and LMX1A (blue) are displayed. A higher ΔCt value represents a lower gene expression at the mRNA level.

Conclusions

- Neural progenitors and pan-neuronal markers indicate a neuronal differentiation
- From earlier differentiation to maturity, HD66-12 MSN exhibited the greatest change in DARPP-32 expression compared to other canonical MSN markers
- CTIP2 and FOXP1 may not adequately distinguish between MSN and CTX cultures based on D11 to D88 expression trajectories
- GAD1 and GAD2 expression in HD66-12 MSN stayed relatively the same over time. CTX lines showed substantial changes
- vGLUT1 and vGLUT2 expression in HD66-12 MSN greatly increased over time. CTX lines had modest increases
- There is no difference in FP marker gene expression trajectory between MSN and CTX lines

Future Directions

- Continue performing RT-qPCR assays to validate the protocol and quantify gene expression
- Replicate the protocol through additional differentiations and validate the differentiations using RT-qPCR and immunocytochemistry
- Use this protocol to generate hiPSC-derived MSN cultures for investigating both HD *in vitro* and gene x environment interactions in neurological disease

Thank you to Bingying Han for technical assistance and guidance.

[1]Adil, M. M., Gaj, T., Rao, A. T., Kulkarni, R. U., Fuentes, C. M., Ramadoss, G. N., Ekman, F. K., Miller, E. W., & Schaffer, D. V. (2018). HPSC-derived striatal cells generated using a scalable 3D hydrogel promote recovery in a huntington disease mouse model. *Stem Cell Reports*, *10*(5), 1481–1491. https://doi.org/10.1016/j.stemcr.2018.03.007