Generation of large number of floor plate derived, midbrain-specified DA neurons from human PSCs for scaled application

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ABSTRACT

We developed a simplified and standardized system to make midbrain floor plate cells and further matured to DA neurons. For scaled application we developed an alternative workflow enabling stable growth of mFP cells up to passage 10 (p10). The cryopreserved mFPp10 were able to be directly recovered on assay format and after 7 days in maturation, phenotype marker of TH was examined using high throughput imaging system. The standardized work flow resulted in consistent differentiation of our reference PSC line over time. However we have noticed significant variation in maturation efficiency can occur across PSC lines and looked for ways to reduce this variability. To improve the differentiation efficiency, we tested a number of conditions and ultimately incorporated an extended differentiation procedure for low performing lines. As a result, we could get comparable differentiation efficiency from this line. Thus, we could use our system successfully across multiple cell lines.

INTRODUCTION

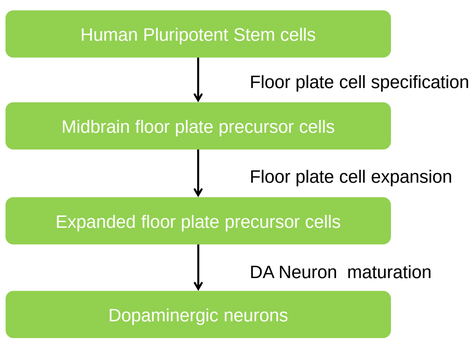
Stem cell derived midbrain dopaminergic (DA) neurons provide an excellent cell source for disease modeling and drug screening for Parkinson's disease. To expedite this effort, we have developed a culture system which can make authentic midbrain DA neurons starting from human pluripotent stem cells. Our system was designed to help simplify and standardize the whole differentiation process while compressing timelines and adding increased flexibility in to this complex differentiation workflow (Diagram below). The process has 3 distinct steps: (1) specification of hPSC to midbrain floor plate (mFP) cells, (2) expansion and cryopreservation of derived mFP cells, and (3) maturation to DA neurons. We examined further to narrow down the intrinsic variation coming from the PSC lines and that extended way of use of the kit such as large scale expansion and application on neural stem cells.

MATERIALS AND METHODS

PSC DA neuron differentiation kit (P/N. A3147701)
- FP specification supplement (P/N. A3146801)
- FP cell expansion supplement and base (P/N. A3165801)
- DA neuron maturation supplement (P/N. A3147401)

Gibco™ Essential 8™ Medium (P/N. A1517001)
Gibco™ Neurobasal™ medium (P/N. 21103049)
Gibco™ DMEM/F-12 (P/N.10565018)
Natural Mouse Laminin (P/N.23017015)
Vitronectin (P/N. A14700)
PDL coated plate (BD P/N. 354413, 354414, 354640)
Stempro™ Accutase™ cell Dissociation Reagent (P/N. A11105)
Human DA neuron ICC kit (P/N. A29515)

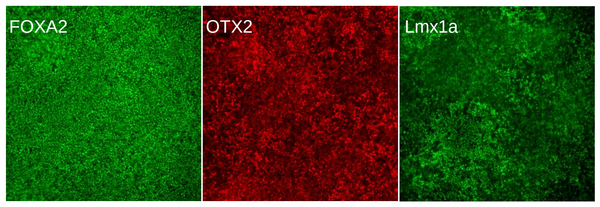
PSC DA neuron differentiation kit workflow:



First step is to specifiy hPSC to midbrain floor plate cells which can be characterized by the expression of Otx2,Engrailed1, Lmx1a and FoxA2. Specified floor plate cells can be expanded to be either cryopreserved or matured further to DA neurons.

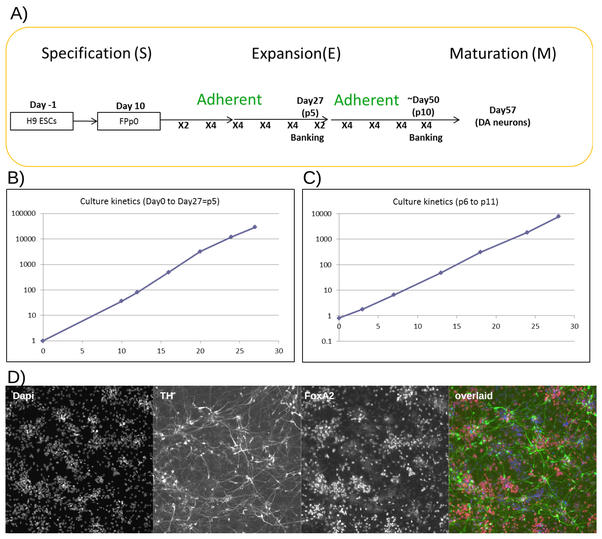
RESULTS

Figure 1. Induced midbrain floor plate cells express floor plate and midbrain marker



Marker expression of induced FP cells. H9 ESC cultured in Essential 8™ Flex medium was specified to midbrain floor plate with specification medium for 7 days. Population express floor plate marker FOXA2 and rostral marker OTX2 and dopaminergic neuron progenitor marker of LMX1a. 95.1% cells were double stained with FoxA2 and Otx2.

Figure 2. Expansion ability of hPSC-derived midbrain floor plate cells

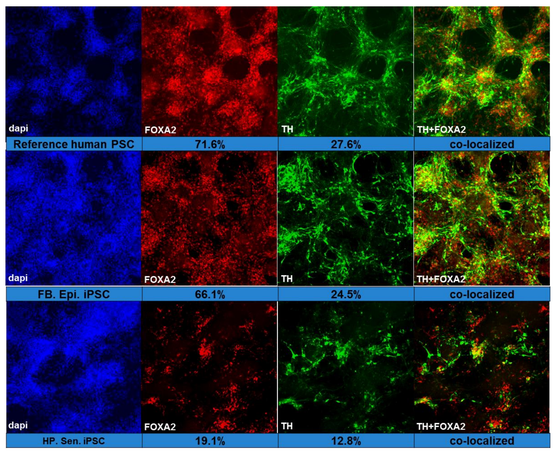


After 10 days of specification, Floor plate progenitors were expanded further to test their proliferation potential. A) Diagram of protocol used. B-C) Stable growth of cells >10 passages. Cells could be frozen down at p5 (day 27) and recovered as adherent culture and expanded another 6 passages before maturation. D) FPp10 cells were thawed and plated to be matured in DA kit maturation medium. Upon 7 days maturation, TH expression was captured using high content imaging system and HCS studio analysis resulted 42.9% TH expression. FoxA2 co-localization was observed around 20% of TH positive neurons.

Figure 3. Long term maintenance of matured TH neurons

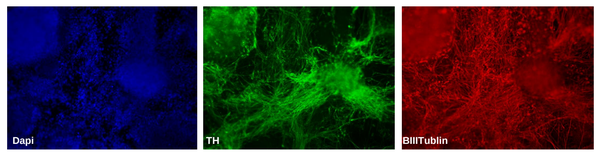
Maintain matured TH neuron. Upon maturation, population was maintained in either maturation medium or maintenance medium (Neurobasal + Maturation supplement) for 14 days. There was no significant difference in % TH neurons (A) or total Neuron (B) but cell healthiness (C) was improved by base medium changed from DMEM/F12 (DF12) to Neurobasal (N).

Figure 4. Intrinsic variation coming from cell line used



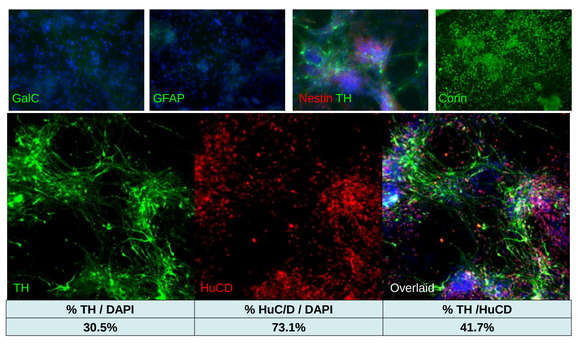
Kit performance on multiple lines. Reference human PSC (H9), iPSC derived from CD34+ cells reprogrammed with Episomal vector (FB. Epi.) and iPSC derived from fibroblast reprogrammed with Sendai virus (HF. Sen) were differentiated using DA kit. Low efficiency toward TH neurons (overall neuronal differentiation) was observed with one line.

Figure 5. Improved differentiation efficiency with customized protocol



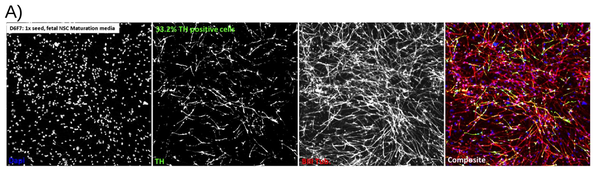
Improved efficiency toward neurons through customized protocol. FP progenitors were recovered from frozen vial and suspended for 10 days instead of 5 days. Upon maturation, improved efficiency toward TH neurons was obtained (Figure 4 vs. 5)

Figure 6. Characterization of matured populations



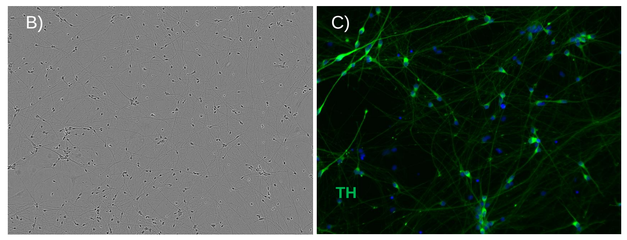
Phenotype examination. Population doesn't have glial population (GalC and GFAP, <1%) and major population was neuron (HuCD, >70%) or progenitor population (Nestin, Corin or FoxA2). Among neurons, TH neurons are around 40%.

Figure 7. Extended application of maturation medium on neural stem cells



Maturation medium can be used for Neural stem cells isolated from tissue (A) or derived from hPSC (B-C). Resulting population has enriched neurons devoid of progenitor population and more TH neurons compared to conventional condition (B27 + neurobasal, up to 4 fold increase). Matured neurons can further be maintained in neurobasal medium supplemented with maturation supplement more than 14 days.

Figure 7. continued



CONCLUSIONS

1.Formulation and protocol optimization were designed to simplify overall workflow and enable consistent, robust differentiation.
2.Floor plate precursor cells induced from hPSCs express the midbrain markers LMX1A & OTX2 and the floor plate marker FOXA2 with high efficiency.
3.Significant expansion of ifloor plate precursor cells can be obtained, although long term expansion accompanied down regulation of FOXA2 expression of differentiated TH neurons.
4.Differentiation efficiency can be influenced by intrinsic variation coming from starting PSC, amongst other factors, and modification steps in the protocol were identified to help overcome variation and aid in troubleshooting and in process QC.
5.Application of the kit can be extended to different progenitor population such as neural stem cells isolated from Tissue or neural stem cells derived from pluripotent stem cells.

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ACKNOWLEDGEMENTS

Authors thank project team members for their vision and support, Brent Samson, application scientist for CellInsight™ CX5 for excellent support.

TRADEMARKS/LICENSING