A 3D model to characterize the maturation of an iPSC-derived mDA progenitor cell therapy

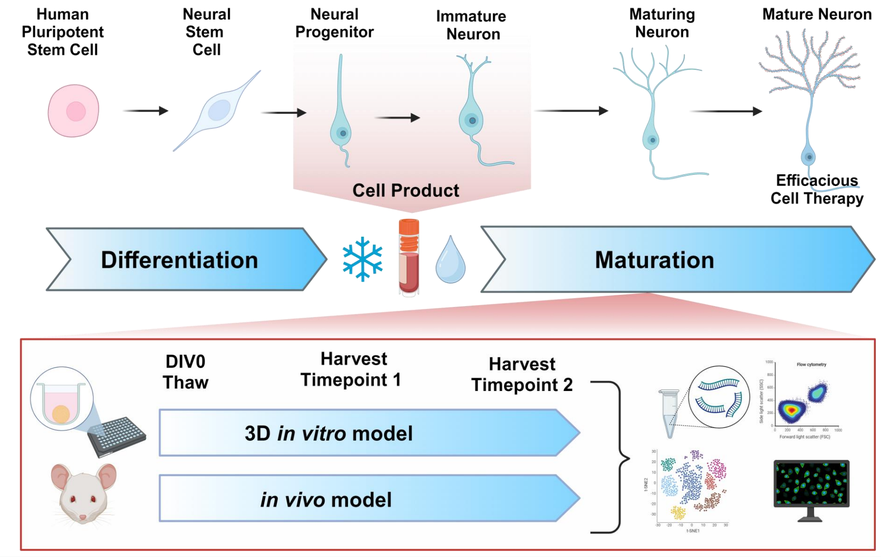
Sonia Bernal, Haley Clark, Melissa Jones, Dan Wilkinson, Mark Ebel, Kevin Michael, Nathanial Adams, Lilia Carpenter, Lucas Harvey, Luendreo Barboza, Deven LoSchiavo, Jim Xu, Maya Srinivas, Ryan Smith, Carlos Paladini

BlueRock THERAPEUTICS

ABSTRACT

Human induced pluripotent stem cells (hiPSCs) can be leveraged to generate ventral midbrain floorplate progenitor cell therapies to replace the dopaminergic (DA) neurons lost to Parkinson's Disease (PD). The efficacy of these therapies is dependent on the successful differentiation of the floorplate progenitor cells into adult DA neurons that acquire physiological maturity over several months in the *in vivo* environment. To study this protracted transition from progenitors to neurons, it is important to have *in vitro* culture systems that support long-term neuronal maturation and mimic the *in vivo* environment. Conventional *in vitro* models have relied on monolayer (2D) cell culture systems, which do not accurately recapitulate the architectural complexity of the *in vivo* environment and can be confounded by cell detachment. Here we describe a 3D cell culture model developed and leveraged to characterize the maturation of an hiPSC-derived midbrain dopaminergic progenitor cell therapy. Three batches of ventral midbrain DA (vmDA) progenitors were cultured long-term in a 3D *in vitro* environment and submitted for molecular (qPCR, flow cytometry, LCMS, single nuclei RNA sequencing) and immunohistochemical assays at various timepoints across maturation to understand cell fate and function. To assess the relevance of the 3D modeling environment, results were compared to the immunohistochemical characterization of hiPSC-vmDA cells grafted into the 6-OHDA rodent model of PD. These findings provide insights into the transition of midbrain floorplate progenitors to DA neurons in the context of a cell therapy for PD and demonstrate the potential of a 3D culture system for modeling the dynamics of iPSC-derived cell therapies upon transplantation.

GRAPHICAL ABSTRACT AND METHODS



After differentiation and cryopreservation, three batches (B1, B2, B3) of hiPSC-derived ventral midbrain floorplate progenitor cells were thawed, and cultured long-term in a 3D *in vitro* model. These cell batches were also transplanted into the striatum of the unilaterally lesioned 6-hyroxydopamine (6-OHDA)-lesioned rat model of Parkinson's disease. Both *in vitro* neurospheres and *in vivo* grafts were harvested at various timepoints for molecular analysis of cell fate and maturation.

FIGURE 1. Molecular characterization of vmDA progenitor maturation across time by RT-qPCR

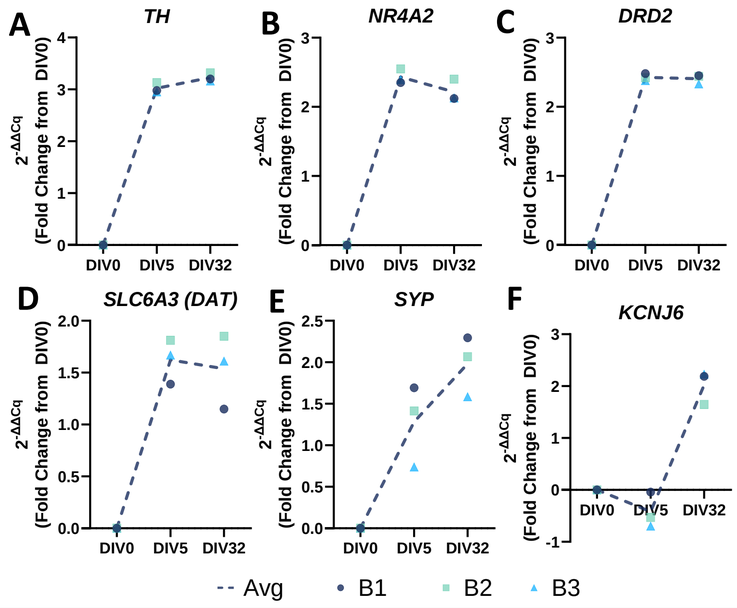


Figure 1. Ventral midbrain DA progenitors mature into functional DA neurons. (A-B) Dopaminergic identity is marked by the expression of *TH* and *NR4A2* (quantified by RT-qPCR). (C-F) Over 32 days *in vitro* (DIV), there is an increase in expression of markers associated with neurotransmitter signaling (*DRD2*, dopamine receptor D2; *SLC6A3*, dopamine transporter), synaptic function (*SYP*, synaptophysin), and signal propagation (*KCNJ6*, G protein-activated inward rectifier potassium channel 2). These transcriptional changes suggest acquisition of functional maturity as early as DIV5.

FIGURE 2. Molecular characterization of dopamine synthesis and release in maturing vmDA progenitors

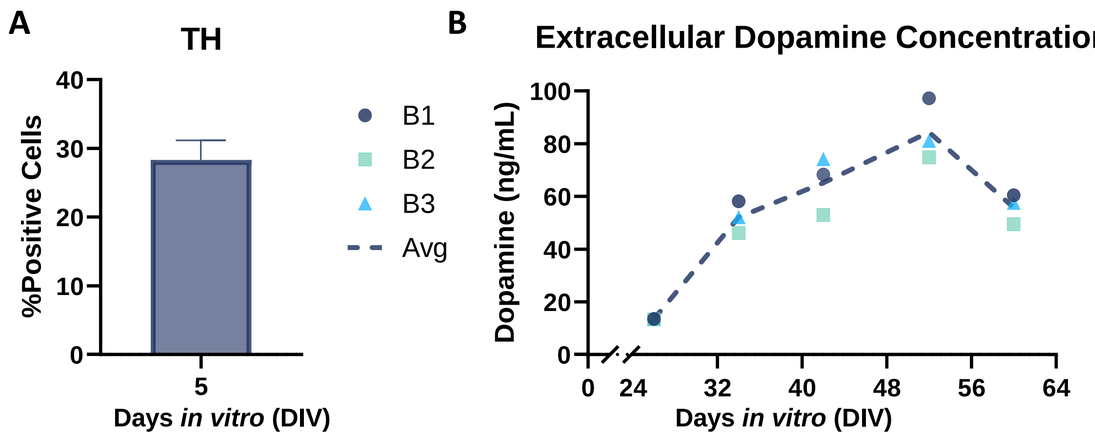


Figure 2. hiPSC-derived vmDA neurons cultured in a 3D model produce and release dopamine. (A) Protein expression of tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis, was captured early in maturation by flow cytometry. As the cells continued to mature *in vitro*, the spent media was sampled and collected for dopamine quantification by LCMS. Extracellular dopamine concentration increases over the course of maturation of all three batches, and peaks at DIV52.

FIGURE 3. Transcriptomic analysis of 3D *in vitro* matured vmDA progenitors across time

Figure 3. Transcriptomic analysis reveals cell fate transitions over time in 3D model of vmDA progenitor maturation. (A) Single nuclei were extracted from neurospheres cultured for five days (DIV5; blue), 32 days (DIV32; orange), or 60 days (DIV60; green) and underwent RNA sequencing (10x) for whole-transcriptome analysis. (B) Expression profiles of genes associated with midbrain floorplate progenitors (*FOXA2*, *SOX2*), differentiated (*TH, NR4A2*, *PITX3, DDC)* and matured dopaminergic neurons (*KCNJ6*, *DDC*, *SLC18A2, SNCA*), (C) with cells of a glial lineage (*SOX9*), and (D) vascular leptomeningeal like cells (VLMCs) described previously (*COL1A1*) reveal transitions from a progenitor state at DIV5 to differentiated cell states at later timepoints.

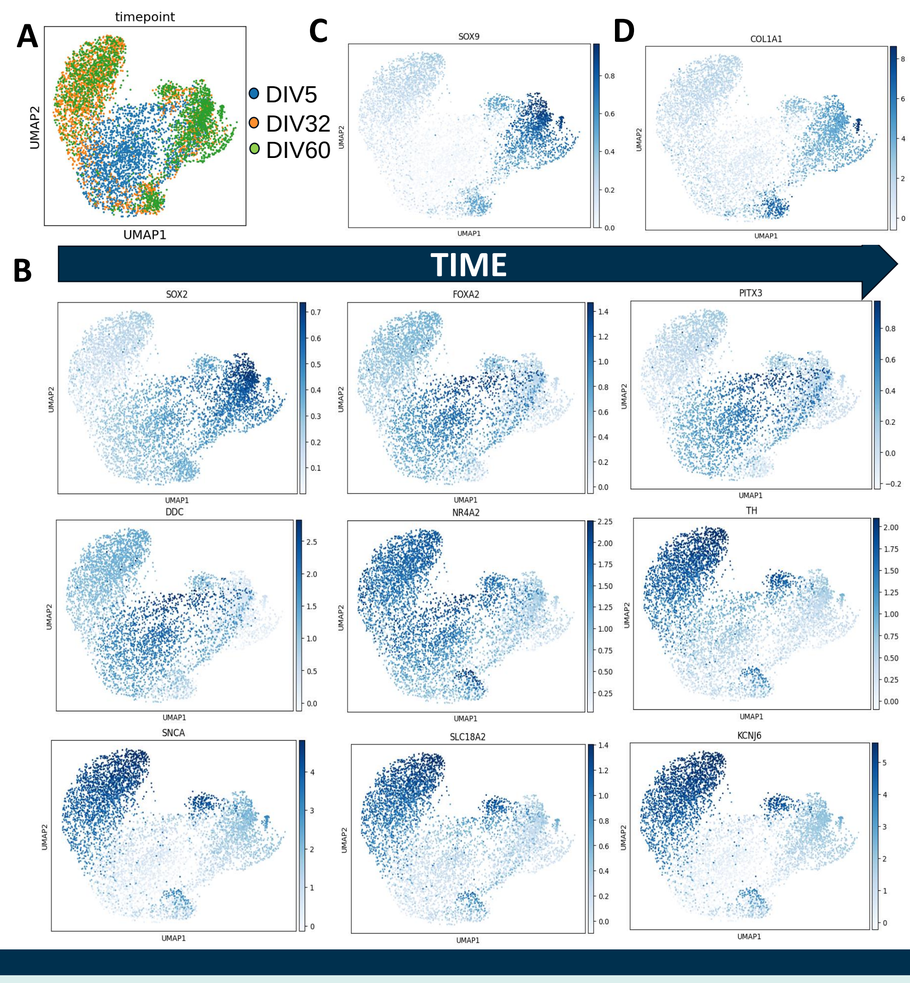


FIGURE 4. Immunohistochemical characterization of cell fate of vmDA progenitors *in vitro* and *in vivo*

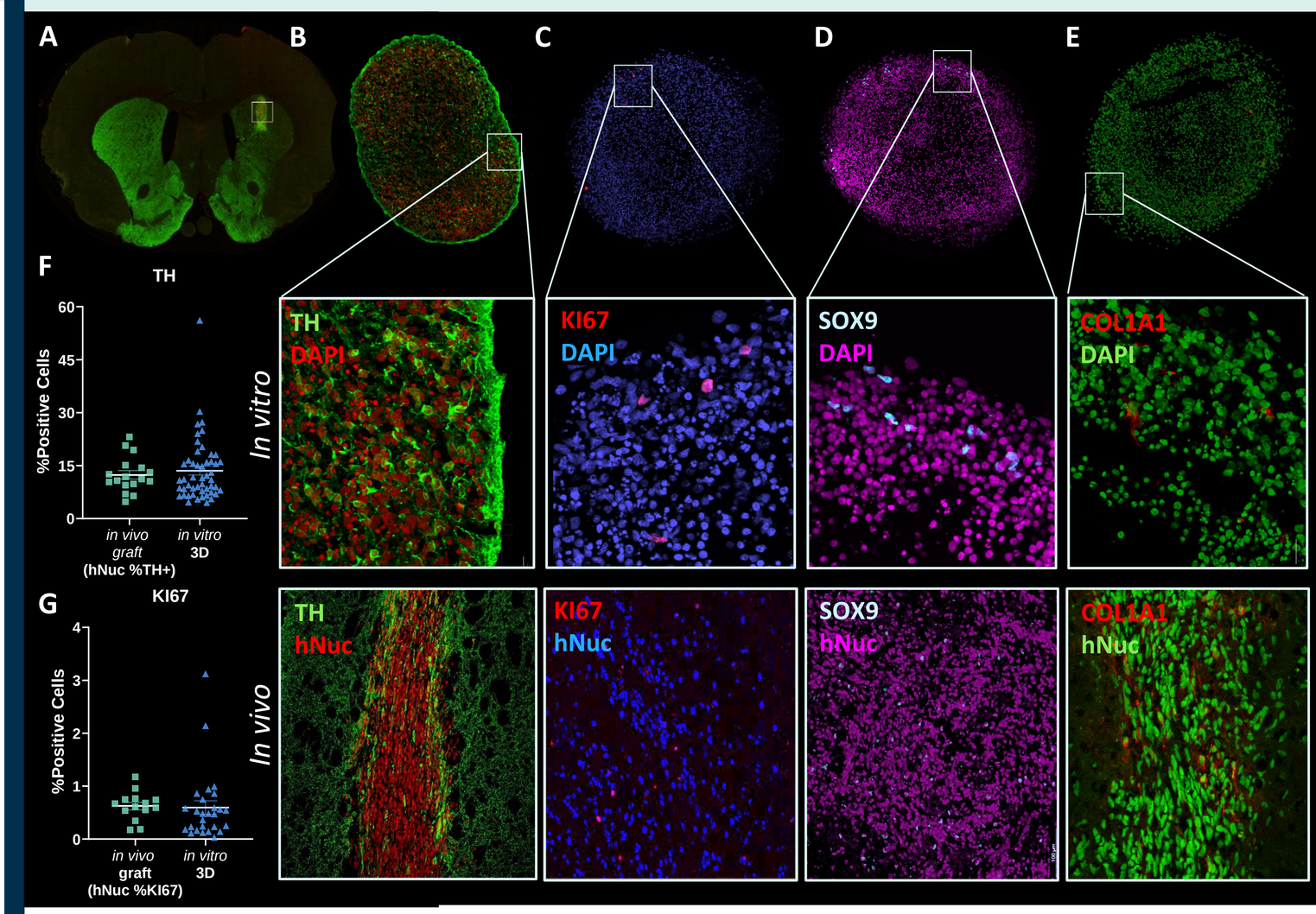


Figure 4. Cell fate characterization of hiPSC-vmDA progenitors in the 3D *in vitro* model reflects the cell fate of vmDA grafts *in vivo*.(A-B,F) Neurospheres at DIV32 show comparable proportion and spatial distribution of TH+ neurons to the *in vivo* grafts 8 months post-transplantation. (C,G) After maturation, there are fewer than 1% KI67+ cells in both the *in vivo* graft and the *in vitro* model. (D-E) In addition to dopaminergic neurons, these hiPSC-derived ventral midbrain floorplate progenitors also give rise to other cells types in the *in vivo* and *in vitro* context, consistent with what has been frequently reported in the literature to date. Here we show glial cells, marked by SOX9, as well as vascular leptomeningeal cells (VLMCs), marked by COL1a1, both of which can also be found in the 3D maturation model.

CONCLUSIONS

- Human induced pluripotent stem cells differentiated towards a ventral midbrain floorplate progenitor identity can form neurospheres in a 3D *in vitro* maturation model.
- Transcriptional analysis by RT-qPCR and snRNA-Seq reveal that the 3D environment supports terminal differentiation and subsequent functional maturation of mDA neurons, capable of synthesizing and releasing dopamine into the extracellular space. This maturation occurs as early as 5 days post-thaw (DIV5).
- Consistent with the literature, the hiPSC-vmDA progenitors characterized here give rise to glial cells and endothelial-like cells termed vascular-leptomeningeal cells (VLMCs) in addition to mDA neurons.
- Histological assessment demonstrates the 3D model reflects the cell fate and proportion of hiPSC-vmDA progenitor cells grafted into a rodent model of PD, thereby highlighting the 3D system's potential for modeling the dynamics of iPSC-derived cell therapies upon transplantation.