● PERSPECTIVE

Novel pluripotent stem cell lines for enriched grafting in Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting 1% of the population over 55 years of age and up to 4% of the population over 80 years of age (Blesa et al., 2012). This progressive and neurodegenerative condition results from an excessive loss of dopaminergic neurons (50–70%) of the substantia nigra pars compacta, leading to a significant decrease in dopamine (DA) levels in the striatum and consequently a functional deterioration of motor circuity (Blesa et al., 2012; Nielsen et al., 2016). The direct relationship between the loss of motor function and the degeneration of a single cell type makes PD an attractive prospect for cellular replacement therapy (Lindvall et al., 1990) and we are now on the verge of seeing the first clinical trials using human induced pluripotent stem cells (hiPSCs) as a source of transplantable dopamine neurons in clinical trials (Barker et al., 2017). The application of hiPSCs as a donor source for neural transplantation has progressed rapidly and may offer advantages over embryonic stem cells by avoiding ethical issues and potentially also complications related to immune response.

A parallel area of recent progress has been the refinement of genome-editing through CRISPR/Cas-9 technology. This presents opportunities for targetted editing of human iPSC lines including the introduction of fluorescent proteins driven by specific gene loci as reporters of gene expression. These tools are particularly attractive for neural transplantation studies and allow for applications such as pre-selection of specific pools of progenitor cells as a means to refine and standardize donor preparations or for unambiguous identification of grafted cells to characterize electrophysiological properties and subtype-specific patterns of connectivity in the host brain. Both approaches have been useful in transplantation experiments to isolate dopamine progenitors obtained *in vitro* and grafted successfully into animal models of Parkinson's disease. Overall, new reporter lines represent promising tools for refinement of iPSC based neural transplantation for PD and potentially other neurodegenerative diseases.

How can hPSC reporter lines enhance graft outcomes? Many groups have established protocols for obtaining midbrain DA (mDA) neurons from human pluripotent stem cells, clarifying the molecular markers and transcriptional programs that govern their specification and differentiation. During the differentiation protocol several mDA progenitor, immature mDA neuron and mature mDA neuron transcriptional programs are sequentially activated. Key transcription checkpoints for validation of appropriate mDA specification include co-expression of marker combinations including: $Lmx1^+$/$Foxa2^+$/$Otx2^+$ (progenitor stage day 11); followed by TH^+/$Lmx1^+$/$Foxa2^+$/$Pitx3^+$ (day 19) and then other fully differentiated markers such as $MAP2^+$/$VMAT2^+$/DAT^+ by days 40–50 (Nolbrant et al., 2017). The integration of fluorescent reporters, such as eGFP or mCherry, in the gene loci selective for early mDA progenitors may allow for selection via fluorescence-activated cell sorting prior to transplantation in order to enrich the contribution of therapeutic mDA neurons in the resulting grafts. This would represent a significant refinement over currently available procedures given the low final fraction of mDA neurons (~5%). Using human ESCs, Niclis et al. (2017) reported the first fully defined Xeno-free differentiation protocol with two novel reporter cell-lines: LMX1A-eGFP and PITX3-eGFP for *in vitro* and *in vivo* characterization purposes. Furthermore, in a following study they characterise *in vivo* connectivity of implanted mDA neurons using the PITX3-eGFP line (Niclis et al., 2017). Recent work from Tiklová et al. (2019) is a major advancement toward the dechipering of subtype-specific molecular mDA identity via single-cell RNA sequencing, in doing so identifying new patterning markers. Moreover this approach may enhance subtype specific differentiation protocols towards a more directed, subtype-specific cell replacement therapy for PD (Tiklova et al., 2019).

CRISPR/Cas9 approaches for introduction of flourescent proteins in hPSCs: CRISPR technologies enable the modification of endogenous loci with unprecedented precision. Recent works demonstrate robust platforms for the endogenous tagging in silent hiPSCs genes that avoid the drawbacks of incorporation of recombinase associated Cre/Lox-sites. Roberts et al. (2019), developed a multistep method for the endogenous tagging of transcriptionally silent genes via homology direct-repair mechanism, through a monomeric EGFP fusion tag and a constitutively expressed mCherry fluorescence selection cassette. This, allowed the group to tag five genes that are not expressed in hiPSCs but are important in later stages of cardiac development *TTN*, *MYL7*, *MYL2*, *TNNI1*, and *ACTN2* (Roberts et al., 2019). Therefore, this approach is a robust tool to comprehend the dynamics of the differentiation and morphogenic changes and might be incorporated in neural development.

Currently in our group, we are using similar CRISPR-tag hiPSCs that are reporter lines for key developmental genes related to different DA maturation stages. In addition to the aforementioned applications related to cell-sorting and tracking connectivity, this has also permitted the understanding of the dynamic behaviour of some of the transcriptional programs in the immature and mature stages. Deciphering master development regulators in early progenitor stages could help refine differentiation protocols in addition to enrichment of appropriate progenitors for grafting (**Figure 1**).

Overall, CRISPR-modified hiPSCs derived DA neurons may be highly valuable tools for improving cell replacement strategies as well as improving understanding of transcriptional programs underlying mDA differentiation *in vitro*. Further studies that continue to look for early-transcriptional markers that determine mDA regionalization *in vitro* will help identify new targets for new CRISPS-modified iPSC lines that can then be exploited to improve graft outcomes in transplantation studies.

This work was supported by funds from Consejo Nacional de Ciencia y Tecnología (CONACyT; 271307, 272968, to NEDM).

This work was performed by ACC in partial fulfillment of the requirements for the Doctorado en Ciencias en Innovación Biotecnológica, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco.

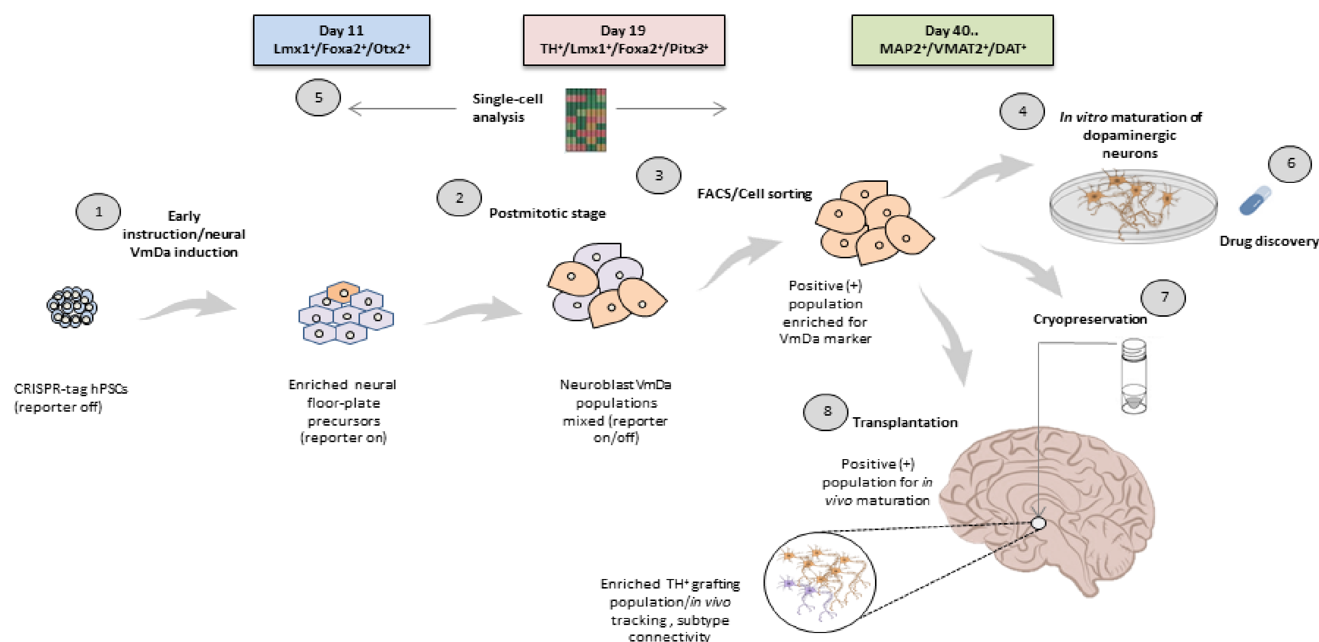

Figure 1 Diagram for the enrichment of VmDa populations prior to transplantation.
1) Regional identity and parallel neural induction via small molecules differentiation of hPSCs, can enrich the floor-plate VmDa precursors yields. 2, 3) The CRISPR-tag reporter switches-on in VmDa neuroblasts and allows the selection of dopaminergic precursors via cell-sorting approaches. 4, 7, 8) The selected population goes directly into the injured hemisphere for repopulate and *in vivo* maturation, or can be expanded *in vitro* for 6) drug discovery purposes and cryopreservation. 5) The single-cell analysis of the pre-grafting neuroblasts enables the identification of newly mDA neuron subgroups and give evidence of their role in the VmDa specification in the mixed populations generated by the *in vitro* protocols. FACS: Fluorescence-activated cell sorting; hPSCs: human induced pluripotent stem cells; mDA: midbrain dopamine; VmDa: ventral midbrain dopamine.

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***Received:** June 26, 2019*
***Accepted:** July 18, 2019*

***doi:** 10.4103/1673-5374.265548*
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***Plagiarism check:** Checked twice by iThenticate.*
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***Open peer reviewers:** Abraam M. Yakoub, Stanford University, USA; Leonardo Cocito, Universita degli Studi di Genova, Italy.*
***Additional file:** Open peer review reports 1 and 2.*

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P-Reviewers: Yakoub AM, Cocito L; C-Editors: Zhao M, Li JY; T-Editor: Jia Y