

# Deciphering the Role of VLMCs in Dopaminergic Graft Function Using Lineage-Restricted hPSCs

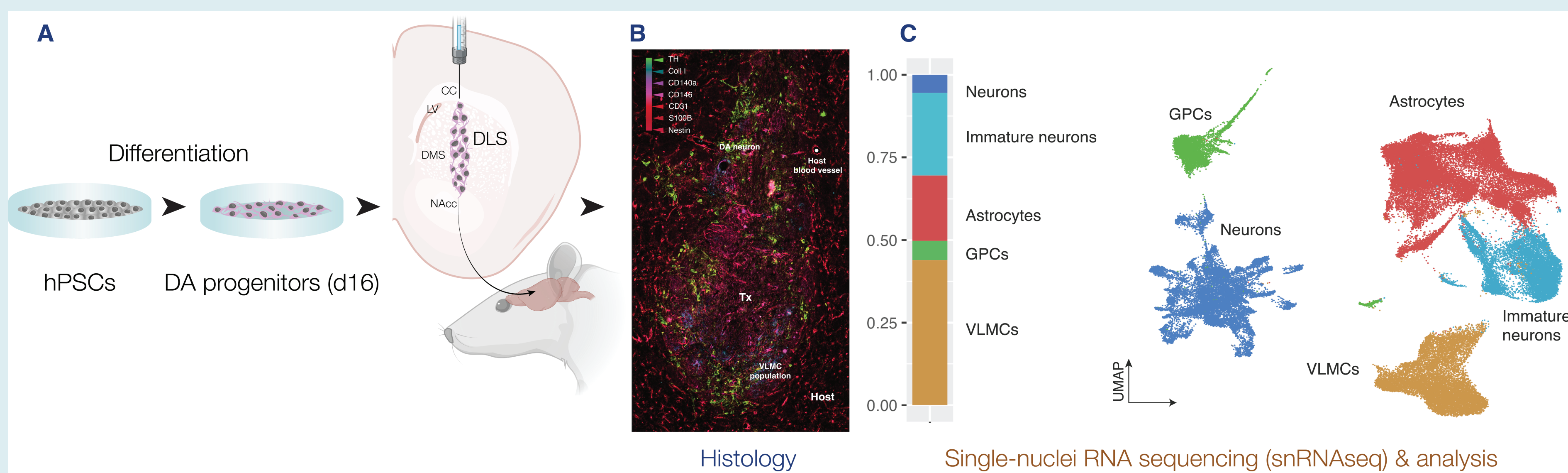
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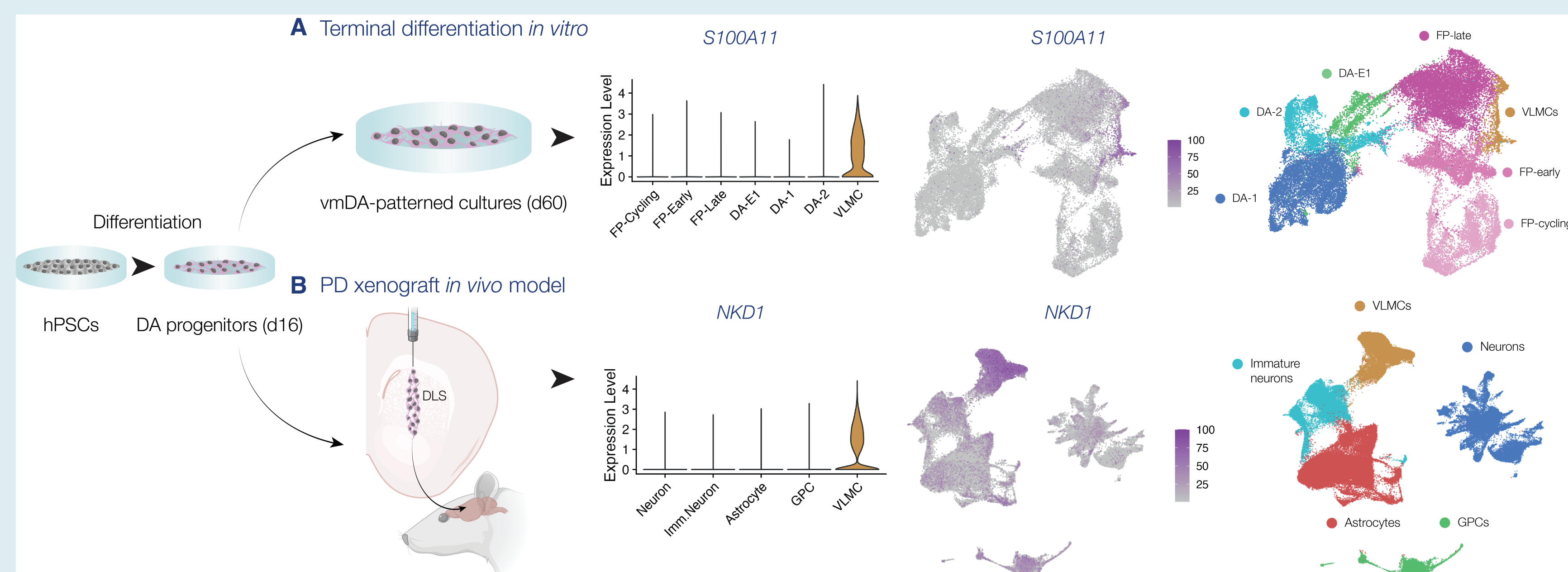
## INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder and it is caused by the degeneration of a well-defined population of ventral midbrain (VM) dopaminergic (DA) neurons. PD is an ideal candidate for cell replacement therapies as it predominantly affects a single DA neuronal subtype. DA progenitors derived from human pluripotent stem cells (hPSCs) represent a promising disease-modifying strategy, currently being tested in clinical trials aiming to restore striatal dopamine levels and motor function in PD patients.

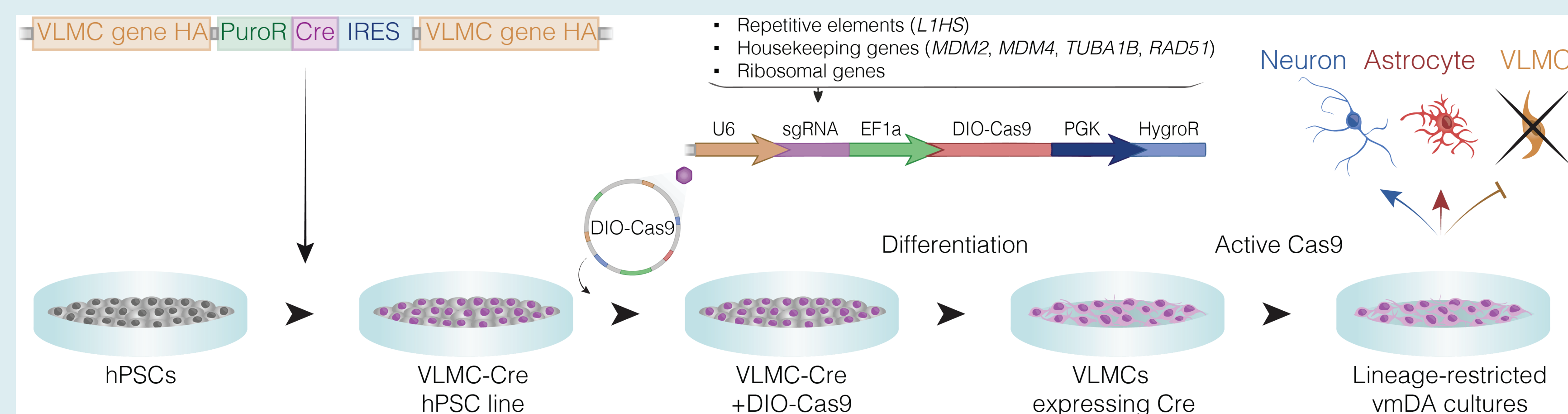
Interestingly, although the progenitor population is homogenous at the time of transplantation, they develop into DA grafts containing a heterogenous mix of mature cell types, including DA neurons, astrocytes and vascular leptomenigeal cells (VLMCs) (**Figure 1**). It is unknown to what extent graft composition affects the survival, maturation and subtype specification of the DA neurons, key parameters for graft functionality.



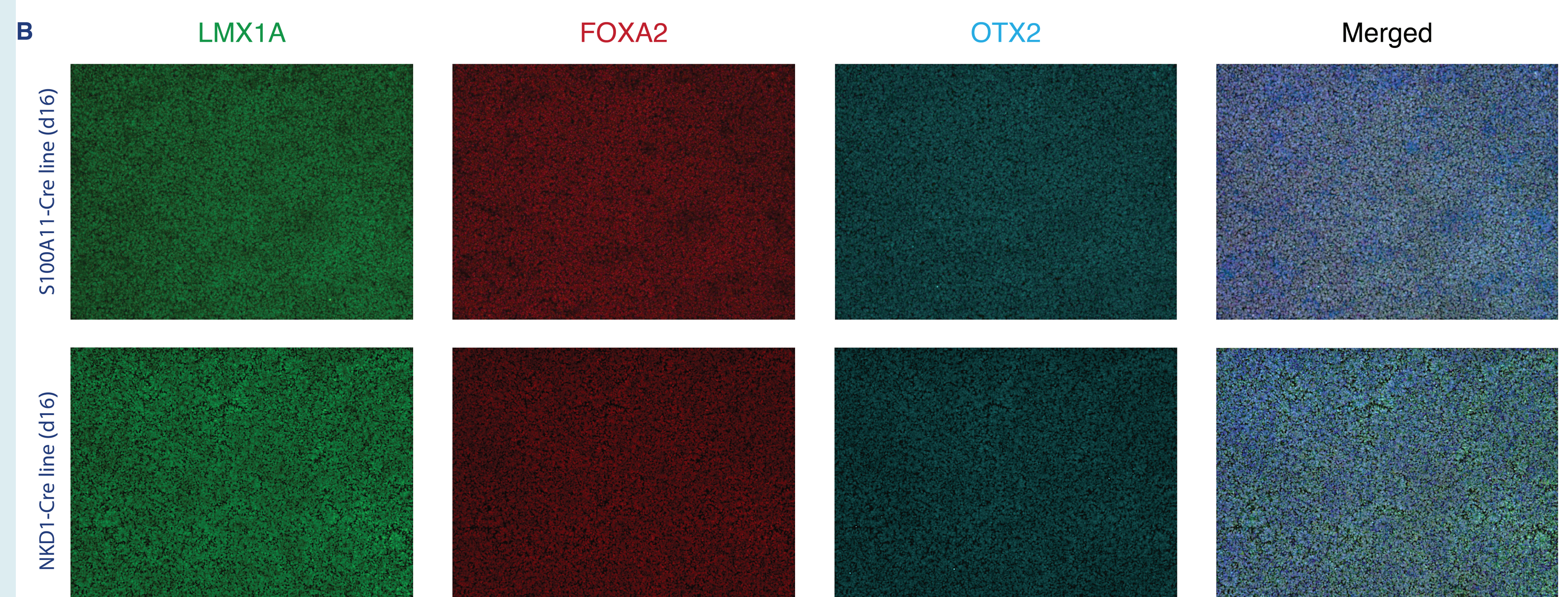
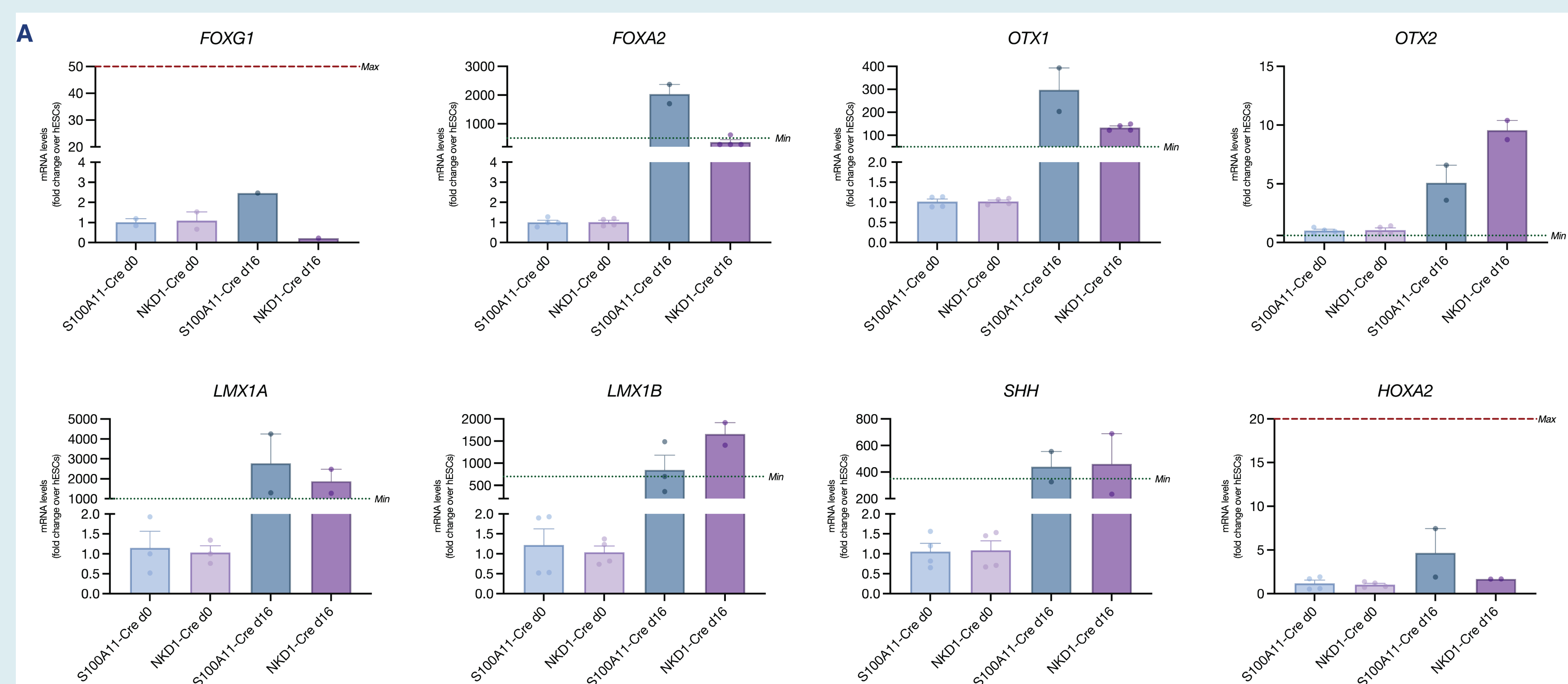
**Figure 1.** Vascular leptomenigeal cells (VLMCs) are a major component of highly heterogenous hPSC-derived grafts. (A) hPSC-derived DA progenitors grafted to the striatum of 6-OHDA nude rats and analyzed at 6 months post-transplantation. (B) Representative intra-striatal graft showing VLMC clusters - marked by COL1A1 (teal), CD146 (MCAM; pink) and CD140a (PDGFR; fuchsia) expression - around therapeutic DA neurons (green) and closely associated with CD31+ (PECAM; red) host blood vessels. (C) Bar plot and UMAP showing the different graft composites (neurons, immature neurons, astrocytes, GPCs and VLMCs) and their relative abundance within hPSC-derived grafts. CC = corpus callosum; DLS = dorsolateral striatum; GPCs = Glial Progenitor Cells; LV = lateral ventricle; NAcc = nucleus accumbens.



**Figure 2.** Identification of key regulators of VLMC development. (A) Violin plot and UMAP showing *S100A11* expression levels across clusters in vmDA-patterned hPSC cultures at day 60 of differentiation. (B) *NKD1* expression is restricted to the VLMC lineage in hPSC-derived grafts obtained from DA progenitors transplanted into a PD xenograft model and analyzed 6 months post-transplantation.



**Figure 3.** Engineering a lineage-restricted hPSC line for selective VLMC ablation. A Cre cassette was inserted into the genome of our clinical-grade hPSC line flanked by VLMC-specific gene homology arms (HA). Cre-expressing progenitors—fated to become VLMCs—activate a co-delivered DIO-Cas9 and sgRNA cassette. Cas9 is catalytically active only in VLMC-biased progenitors, where it targets essential genes for cell survival, leading to the selective ablation of VLMCs from vmDA-patterned cultures.



**Figure 4.** Validation of CRISPR/Cas9-mediated S100A11-Cre and NKD1-Cre knock-in hPSC lines. (A) qRT-PCR analysis of vmDA markers (*FOXA2*, *OTX1*, *OTX2*, *LMX1A*, *LMX1B*) and markers for contaminating rostral (*FOXG1*) and caudal (*HOXA2*) populations at day 16 of differentiation. S100A11-Cre or NKD1-Cre hPSC lines were patterned toward vmDA fate. Data are shown as fold change relative to undifferentiated hPSCs. Dotted and dashed lines indicate recommended minimal and maximal fold change thresholds for confirming successful vmDA identity. (B) Immunocytochemistry for LMX1A, FOXA2, and OTX2 at day 16 in S100A11-Cre (top) and NKD1-Cre (bottom) lines patterned toward vmDA fate. Nuclei were counterstained with DAPI.

## ENGINEERING LINEAGE-RESTRICTED hPSC LINES TO STUDY VLMC FUNCTION

VLMCs are a major component of hPSC-derived grafts (**Figure 1**) but their role in graft functionality remains unclear. To investigate this, we aim to generate mature DA grafts devoid of VLMC lineage to assess whether their absence impacts the identity, survival and maturation of DA neurons within the transplant. We sought to identify key regulators of VLMC fate that could be targeted to alter hPSC ability to generate VLMCs (**Figure 2**).

First, we searched for early drivers of VLMC fate using our previously generated scRNAseq datasets from vmDA-patterned hPSC cultures which recapitulate human VM development (**Figure 2A**). *S100A11* emerged as an early and specific marker of VLMC phenotype (**Figure 2A**). We then searched for later-stage regulators of VLMC development *in vivo*. Using snRNAseq data from our PD xenograft model, we identified *NKD1* as a robust marker of mature VLMCs (**Figure 2B**).

Recent barcode-based lineage tracing from our group revealed that DA neurons, astrocytes and VLMCs share a common progenitor, with lineage commitment occurring at the final cell division. Thus, FACS sorting of DA progenitors prior to transplantation cannot prevent the presence of VLMCs in mature grafts. To overcome this limitation, we aim to leverage the identified key regulators of VLMC fate as genetic switches to drive a cell-intrinsic ablation system. We engineered lineage-restricted hPSC lines by knocking-in a Cre cassette into the endogenous *S100A11* or *NKD1* loci using CRISPR/Cas9-mediated homology-directed repair (HDR), enabling VLMC-specific Cre expression without disrupting gene function (**Figure 3**). We then implemented an all-in-one lentiviral system delivering a double-floxed inverted Cas9 (DIO-Cas9) and sgRNA. Cas9 is active exclusively in VLMC-fated cells, where it targets either repetitive genomic elements (e.g. LINES), ribosomal DNA, or essential genes (e.g., *TUBA1B*, *RAD51*, *MDM2/4*) to selectively ablate VLMC lineage.

## VALIDATION OF CRISPR/CAS9-MEDIATED VLMC-CRE KNOCK-IN LINES FOR vmDA DIFFERENTIATION

To assess whether genome editing at the *S100A11* and *NKD1* loci affected the differentiation capacity of hPSCs, we induced both S100A11-Cre and NKD1-Cre lines to a vmDA fate using our GMP-compatible, high-yield protocol (**Figure 4**). RT-qPCR at day 16 confirmed accurate midbrain patterning, with expression of key ventral and caudal markers (*FOXA2*, *OTX1*, *OTX2*, *LMX1A*, *LMX1B*). To exclude progenitors patterned toward adjacent rostrocaudal identities relative to the VM, we included *FOXG1* marking rostral (forebrain) lineages and *HOXA2* marking caudal (hindbrain) lineages in our gene panel (**Figure 4A**).

Immunocytochemistry further validated VM identity, showing a high proportion of LMX1A<sup>+</sup>/FOXA2<sup>+</sup> and LMX1A<sup>+</sup>/OTX2<sup>+</sup> progenitors (**Figure 4B**), consistent with successful vmDA lineage specification.

Taken together, these findings indicate that Cre knock-in at the *S100A11* and *NKD1* loci does not impair the developmental potential of the targeted hPSC lines, validating these lines for further functional studies. Our strategy establishes a versatile CRISPR-based toolkit for lineage-specific ablation of a defined non-DA populations within DA mature grafts.

## HIGHLIGHTS

- Vascular leptomenigeal cells (VLMCs) are a major component of hPSC-derived DA grafts, whose contribution to graft functionality remains unclear.
- VLMCs and clinically relevant DA neurons arise from a shared progenitor, with lineage commitment occurring post-mitotically.
- S100A11* and *NKD1* are key regulators of VLMC lineage.
- Using CRISPR/Cas9-mediated HDR, we engineered S100A11-Cre and NKD1-Cre lines to selectively ablate VLMCs from vmDA-patterned populations.
- VLMC-Cre lines efficiently differentiate into transplantable vmDA progenitors.

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