Single cell transcriptomics identifies stem cell-derived graft composition in a model of Parkinson’s disease

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Cell replacement is a long-standing and realistic goal for the treatment of Parkinson’s disease (PD). Cells for transplantation can be obtained from fetal brain tissue or from stem cells. However, after transplantation, dopamine (DA) neurons are seen to be a minor component of grafts, and it has remained difficult to determine the identity of other cell types. Here, we report analysis by single-cell RNA sequencing (scRNA-seq) combined with comprehensive histological analyses to characterize intracerebral grafts from human embryonic stem cells (hESCs) and fetal tissue after functional maturation in a pre-clinical rat PD model. We show that neurons and astrocytes are major components in both fetal and stem cell-derived grafts. Additionally, we identify a cell type closely resembling a class of recently identified perivascular-like cells in stem cell-derived grafts. Thus, this study uncovers previously unknown cellular diversity in a clinically relevant cell replacement PD model.
authentic and functional midbrain dopamine (DA) neurons and their progenitors can now be generated from human pluripotent stem cells (hPSCs) via a floor plate intermediate\(^1\). These cell preparations are both safe and functional when transplanted to animal models of Parkinson’s disease (PD)\(^2\). However, although resulting grafts from hPSCs contain large numbers of desired DA neurons, these therapeutic cells are a minor component of the grafts which is the case also for transplants from fetal brain tissue. A comprehensive understanding of the cellular composition of the graft has remained difficult to achieve due to limitations in histological methods that rely on pre-conceived notions concerning the cell types likely to be present in the graft.

Here, we use single-cell RNA sequencing (scRNA-seq) combined with histological analyses to characterize intracerebral grafts from ventral midbrain (VM)-patterned human embryonic stem cells (hESCs) and VM fetal tissue after long-term survival and functional maturation in a pre-clinical rat model of PD. The analyses shows that while both cell preparations gave rise to neurons and astrocytes, oligodendrocytes is only detected in grafts of fetal tissue. On the other hand, a cell type closely resembling a class of newly identified perivascular-like cells is identified as a previously unknown component of hESC-derived grafts. We also confirm the presence of these cells in transplants from three different hESC lines, as well as from iPSCs. Thus, these experiments address an outstanding question in the field of cell replacement in neurological disease by identifying graft composition of hESC- and fetal cell-derived grafts. These results have important implications when designing clinical trials.

**Results**

**scRNA-seq identifies composition of fetal VM and hESC-derived neural graft.** To compare the developmental potential of hESC-derived VM progenitors with fetal VM cells after transplantation in a rat model of PD, hESCs were subjected to VM-patterning by a well-established protocol intended for generation of clinical-grade cell preparations\(^4\) and human fetal tissue was dissociated from the VM of a 7.5-week-old human embryo by the same protocol used for the fetal cell clinical transplantation trial, TRANSEURO\(^5\). Both types of cells were transplanted into the striatum of adult rats that had been unilaterally lesioned with 6-hydroxydopamine (6-OHDA) (Fig. 1a). Both fetal and hESC-derived cell preparations gave rise to neuron-rich grafts (Supplementary Fig. 1a, d) with innervation extending from the graft core to dorsolateral striatum (Supplementary Fig. 1b, e) and prefrontal cortex (Supplementary Fig. 1c, f). The grafts from both sources also contained the expected component of DA neurons as detected by the expression of tyrosine hydroxylase (TH) 6 months following transplantation (Fig. 1b). In agreement with previous observations\(^6\), TH-expressing DA neurons with mature morphology were found concentrated in the periphery of the grafts (Fig. 1b, insets). In addition, paw use and rotational asymmetry induced by 6-OHDA lesioning was corrected in animals transplanted with hESC-derived VM progenitors, confirming functional maturation after transplantation (Fig. 1c, d).

Cells were sampled for scRNA-seq both before grafting and 6 months after transplantation as illustrated in Fig. 1a. To separate intact cells from cell fragments and debris, fetal and hESC-derived progenitors were isolated by fluorescence-activated cell sorting (FACS) based on cell size (forward and side scatter) before grafting (Supplementary Fig. 1g). t-Distributed neighbor embedding (t-SNE) and graph-based clustering of the scRNA-seq data from fetal and hESC-derived cells before grafting resulted in four major clusters (green, blue, orange, and yellow in Fig. 1e). The two top clusters (green, blue) consisted almost exclusively of VM-patterned hESCs while the two bottom clusters (orange, yellow) consisted mostly of fetal cells (Fig. 1f), suggesting that while both fetal- and hESC-derived cells generate DA neurons with similar subtype specificities and functional properties after grafting\(^7\),\(^8\), the two different sources of cells were transcriptionally distinct at the time of transplantation.

SAMseq was used to identify genes enriched in each cluster (Supplementary Data 1, Supplementary Fig. 2a, b). Neural progenitor/radial glial cell markers including NES, SOX2, SOX9, and RFX4 were prominently expressed in the yellow fetal cell-dominated cluster as well as in the blue and green hESC-dominated clusters consistent with the expression of these genes under DA neurogenesis in mouse and humans\(^9\),\(^10\) (Fig. 1g, Supplementary Fig. 2c). Regulators of neurogenesis (NEUROG2, ASCL1, HES1, and HES5) were prominently expressed in the yellow fetal cell-dominated cluster while the blue and green hESC-dominated clusters mainly expressed HES1 (refs.\(^9\),\(^10\)) (Fig. 1h, Supplementary Fig. 2d). Importantly, transcription factors such as LMX1A, FOXA2, and OTX2 that are critical in VM development and DA neurogenesis, as well as genes that are predictive of successful graft outcome (EN1 and DLK1)\(^11\),\(^12\) were expressed by both fetal- and hESC-derived cells (Fig. 1k, Supplementary Fig. 2e–g). Cells in the fetal cell-dominated orange cluster (Fig. 1e) expressed genes that are normally active in early developing neurons including DDX, NCAM1, SYT1, and STMN2, as well as genes that are normally expressed in maturing DA neurons including NRR4A2, TH, DDC, PITX3, and PBX1 (Fig. 1i–k, Supplementary Fig. 2e–g). Of note, cells expressing markers for more mature DA neurons could be distinguished in one part of the orange cluster, thus indicating neuronal diversity among more mature fetal cells (Supplementary Fig. 2g). Markers of pluripotency (POUSFI, NANO1), mesoderm (T) or endoderm (SOX17) development were not expressed in any of the cell clusters (Supplementary Fig. 2h, i).

Cell cycle scores were predicted based on a list of known cell cycle genes and cells assigned to different cell cycle phases, which indicated the presence of cycling cells within the blue (hESCs) and yellow (fetal) clusters (Fig. 1l, Supplementary Fig. 3a). From the list of enriched genes, it is also evident that the main difference between the green and blue clusters is expression of cell cycle genes in the blue cluster (Supplementary Data 1). To assess if clustering was critically influenced by cell cycle, the cell cycle scores were regressed out and included in an independent clustering experiment. This clearly shows that cell cycle genes are the distinguishing feature of the blue and green clusters (and also drives sub-clustering of the yellow (fetal) cluster) (Supplementary Fig. 2j, k). However, no additional divergence of other cell clusters became evident when cell cycle genes were omitted from the analysis.

Six months after transplantation, when grafts had reached functional maturity, the transplants were dissected from the striatum, dissociated into a single-cell suspension, and subjected to FACS (see Fig. 1a, Supplementary Fig. 1h). The transplanted hESCs contained a GFP-encoding transgene expressed in virtually all transplanted cells (Supplementary Fig. 1k–m). Fetal cells did not carry a GFP-encoding transgene and were therefore isolated by FACS based on cell size (forward and side scatter) to separate single cells from debris (Fig. 1a, Supplementary Fig. 1g). Notably, since fetal cells did not express GFP, the vast majority of single cells isolated from fetal grafts were contaminating rat cells and scRNA-seq data from fetal transplants was therefore limited to only 63 fetal cells. In contrast, several hundred cells in each condition could be analyzed from hESC-derived graft cells as well as from both fetal- and hESC-derived cells before grafting (Supplementary Fig. 1j).

Using graph-based clustering, fetal- and hESC-derived cells sampled from the rat striatum six months after grafting
segregated into four main clusters (Fig. 2a). We used SAMseq\textsuperscript{13} for the identification of genes enriched in each cluster (see Methods; Supplementary Data 2, Supplementary Fig. 4b). From inspection of the most highly enriched genes in each cluster it was evident that three of the clusters contained cells expressing genes characteristic of astrocytes (\textit{AQP4} and \textit{GFAP}), oligodendrocytes (\textit{OLIG1} and \textit{OLIG2}), and neurons (\textit{GAP43} and \textit{RBFOX3}), respectively (Fig. 2b–d, Supplementary Fig. 4c–e). Of note, \textit{PMP2}, one of the most significantly enriched genes in the oligodendrocyte cluster, is a marker for peripheral Schwann cells in mouse but was recently shown to be prominently expressed in human oligodendrocytes\textsuperscript{14}. In addition, dopaminergic markers including \textit{TH}, \textit{SLC18A2}, and \textit{DDC} were expressed in cells in the neuron cluster indicating, as expected, a high proportion of DA neurons (Fig. 2e, Supplementary Fig. 4f). Further analyses of publicly available datasets reporting both bulk and scRNA
Vascular leptomeningeal cells are a previously unknown component in hESC-derived grafts. The scRNA-seq data also identified a fourth cluster of cells which could not, based on the top enriched genes, be easily assigned to a known neural cell type (Supplementary Data 2, Supplementary Fig. 4b). Gene enrichment analysis using the top distinguishing genes of this cluster identified highly significant biological functions associated with vasculature and connective tissue, including genes encoding collagen and other extracellular components (Fig. 2f, Supplementary Fig. 6a, Supplementary Data 3). We then probed a large scRNA-seq dataset from the mouse brain interrogating more than 400,000 cells organized into 250 annotated clusters representing diverse cell types such as central and peripheral neurons, astrocytes, oligodendrocytes, Schwann cells, immune cells, and brain vascular cell types15 (Mouse Brain Atlas, http://mousebrain.org/). When this dataset was searched for cells co-expressing enriched genes of the fourth cluster, “enteric glia” and “vascular leptomeningeal cells” (VLMCs) were identified as cell type categories with highly related gene expression profiles (Supplementary Fig. 6b, c). Enteric mesothelial fibroblasts, the closest matching cell type among “enteric glia”, and VLMCs belong to a family of highly related organ barrier-forming fibroblast populations15. Recent scRNA-seq analyses have identified related cells associated with the mouse brain vasculature16,22, characterized by the co-expression of Pdgfra, Col1a1, Col1a2, Lum, and Dcn15,16,22. Additionally, the human homologs of these genes were all expressed in the unknown cluster (Fig. 2f, Supplementary Fig. 4g). Therefore, we refer to these cells as VLMCs as this cluster consists of cells with striking transcriptional resemblance to previously described populations of barrier-forming fibroblasts, including VLMCs of the mouse central nervous system15,16,22.

The analysis of graft composition uniquely allowed us to discern similarities and potential differences between the cellular composition of fetal- and hESC-derived grafts after transplantation. Strikingly, tracing the origin of grafted cells visualized by t-SNE suggested prominent differences in generated cell types (Fig. 2g). Accordingly, two of the clusters (astrocyte and neuron clusters) contained cells both from grafted VM-patterned hESCs and from fetal cells. In contrast, the oligodendrocyte cluster contained only fetal-derived cells while the VLMC cluster contained only hESC-derived cells (Fig. 2g). The scRNA-seq analysis of cells after grafting thus suggested that VM-patterned hESCs and fetal VM cells have distinct developmental potential after grafting. Based on the unexpected identification of VLMCs in the hESC grafts, we examined highly expressed genes found in the VLMC cluster (COL3A1, FBLN1, S100A11, and IFITM2), and additional genes encoding components of the extracellular space (e.g. EMP2 and MMP2), also in the cells prior to grafting (Supplementary Fig. 6d). We found that several of these VLMC-associated genes were expressed together with DA progenitor markers in the same hESC-derived cells suggesting that a common hESC-derived precursor has potential to generate neurons, astrocytes, and VLMCs. t-SNE and graph-based clustering including all sequenced cells before and after grafting resolved the same clusters as when cells were analyzed separately (Supplementary Fig. 6e, f).

To validate the key observations from the scRNA-seq analysis of transplanted cells, and to investigate in more detail the properties of graft-derived VLMCs, we first confirmed the presence of VLMCs by histological analysis of brain sections of animals grafted in parallel with the same batches of cells that were used for scRNA-seq. Using antibodies recognizing both rodent and human COL1A1 (uniquely expressed in the VLMC cluster among the transplanted cells), and an antibody that is specific for the human protein (hCOL1A1) we confirmed the presence of VLMCs of human origin in the hESC-derived grafts (Fig. 2h). These COL1A1+ cells in the hESC-derived grafts were commonly located in close association with blood vessels penetrating into grafts (arrows in Fig. 2h, and high magnification in Fig. 2i), which is consistent with previous studies describing VLMCs associated with the brain vasculature15,16,22. The histological analysis also indicated that the hESC-derived VLMCs were intermingled with rat cells of similar appearance and location within the transplants (Fig. 2h, i, j), suggesting that cells originating from both host and graft contributed to the vascularization as previously reported for rodent-to-rodent neural transplants23,24. Only weak and non-distinctive signal was seen in transplants originating from the fetal cells grafted at the same time as the cells used for sequencing.
**Fig. 2 scRNA-seq analysis and histological validation of grafted cells into the striatum.**

- **a**  t-SNE showing clustering of 746 cells grafted to striatum (683 cells of hESC origin, grafted rats \( n = 2 \); 63 cells of fetal origin, grafted rats \( n = 2 \)). Cell type assignments are indicated: OL oligodendrocyte, N Neuron, AC astrocyte, VLMC vascular leptomeningeal cells.
- **b–d**  Expression level per cluster for indicated genes. All indicated genes are significantly enriched and established markers for astrocytes, oligodendrocytes, and pan-neuronal cells, respectively.
- **e**  Expression level per cluster for selected dopaminergic markers.
- **f**  Expression level per cluster for genes that are significantly enriched in the VLMC cluster and established markers for barrier-forming fibroblasts including VLMCs.
- **g**  t-SNE of grafted cells as shown in Fig.2a. Cells are marked according to their origin from either hESC-derived (red circles) or fetal-derived (blue circles) transplants.
- **h**  Staining using antibodies recognizing both rat and human COL1A1 or only hCOL1A1 as indicated in the core of a hESC-derived graft from the same experiment as used for scRNA-seq. Human nuclei were counterstained with HUNU.
- **i**  Representative immunofluorescence micrograph of hCOL1A1-positive cells intermingled with host-derived COL1A1-positive cells in close association with blood vessels. Boxed area shows the localization of the close-ups.
- **j–k**  Representative micrographs of hCOL1A1/HUNU immunostaining from a hESC-derived graft (j) and from a fetal-derived grafts from the same experiment as used for scRNA-seq. Scale bars, 200 µM (h), 20 µM (i), and 100 µM (j, k).
(Fig. 2k). In addition, and in line with findings obtained from the scRNA-seq dataset, histological analysis also confirmed the presence of graft-derived oligodendrocyte progenitors expressing OLIG2 and PDGFRA at the edge of fetal VM grafts (Supplementary Fig. 7c–e) but not in hESC-derived grafts (Supplementary Fig. 7f). To further assess the observed absence of fetal VM-derived VLMCs, we established primary cultures from two more embryos (gestational age 7.5 and 9 weeks). Both embryos gave rise to cultures rich in TH neurons in vitro, but no COL1A1-expressing cells were detected in the cultures (Supplementary Fig. 7a, b). Thus, although it is impossible to exclude the presence of VLMCs in fetal grafts, the immunostaining of cells in vitro and after transplantation in vivo are consistent with the findings from scRNA-seq suggesting different potential to generate VLMCs and oligodendrocytes depending on the origin of the transplanted cells (Fig. 2g).

To assess if VLMCs are also found in DAergic grafts derived from other pluripotent stem cell lines and when using other differentiation protocols, we next assessed the presence of VLMCs in grafts from previous transplantation experiments using VM-patterned progenitors derived from three different hESC lines, RC17 (differentiated using a GMP protocol showing minimal variability), H9 (differentiated using a research grade protocol), and HS980 (refs. 4,7,8,11) (differentiated using a GMP protocol) (Fig. 3a–c), and an iPSC line (differentiated using a GMP protocol and including a MACS purification step prior to grafting (Fig. 3d)25. Importantly, graft-derived hCOL1A1+ cells were present in all analyzed VM-patterned hPSC-derived grafts independent of cell line or differentiation protocol (Fig. 3a–d). Clinical delivery will be done with cells that have been cryopreserved prior to transplantation, and VLMCs were shown to be present to a similar extent in grafts from fresh and cryopreserved cells (Fig. 3e, f).

To investigate whether the derivation of VLMCs was a characteristic uniquely associated with the specific differentiation protocols used in this study, we assessed hESCs after terminal differentiation in vitro using our standard protocol (Fig. 3g, Supplementary Fig. 7g) and two additional recently published protocols for generating VM progenitors for clinical use from hPSCs12,26; see also (https://patents.justia.com/patent/20180094242) (Fig. 3h, i, Supplementary Fig. 7h, i) as well as in three-dimensional self-organizing organoids (Fig. 3j, Supplementary Fig. 7j). These protocols all gave rise to cultures that are rich in TH+ neurons and cells co-expressing the VLMC markers COL1A1 and PDGFRA (Fig. 3g–j, Supplementary Fig. 7g–j).

**Graft placement does not affect cellular composition of VM-patterned hESC-derived grafts.** The scRNA-seq analysis of grafted cells show that hESC-derived transplanted cells give rise to neurons, astrocytes, and VLMCs, but not to oligodendrocytes. In these experiments, detection of GFP (from the SYN-GFP transgene) was used to isolate grafted hESC-derived cells by FACS. To validate and extend the findings, hESCs patterned by the same protocol as initially used (Figs. 1 and 2) were grafted to the midbrain of nude rats (homotopic graft placement) and analyzed by scRNA-seq after 9 months of in vivo maturation (Fig. 4a–d). Importantly, to ensure that the GFP reporter and transgene or bias the results, cells known to be part of neural grafts. These cells resemble normal vascular fibroblasts/VLMCs that were recently identified in the mouse brain using scRNA-seq16,22. The graft-derived VLMCs were primarily found localized around blood vessels and intermingled with endogenous vascular cells. scRNA-seq of the cells prior to transplantation showed that markers typical of VLMCs and DA progenitors are expressed by the same cells at the time of transplantation suggesting that the hESC-derived progenitors at this timepoint might have the developmental potential to generate both neural and perivascular cell types. In our analysis of fetal tissue (ranging from gestational week 7.5 to 9) we could not document the presence of VLMCs or their progenitors in the fetal VM itself or in transplants or primary cultures derived thereof, leading us to conclude that VLMCs are most likely a unique component of hPSC-derived VM-patterned DAergic grafts.
However, although the developmental origin of these cells remains unknown, previously published scRNA-seq data of human fetal cortical tissue have reported the presence of cells with a transcriptional profile of VLMCs, including COL1A1, COL3A1, and PCOLCE in the early human fetus at age 5–6 weeks (29), and it can therefore not be ruled out that VLMCs may be a component of early neural development. It will clearly be intriguing in future studies to establish the origin of VLMCs, both under normal development and in cultures of differentiating hESCs.

Efforts to use stem cell-derived DA neurons in clinical applications have progressed immensely and they are now on the verge of entering clinical trials (3). In addition to providing an unbiased and comprehensive characterization of the cells in the graft, the scRNA-seq analysis also resulted in several findings with significant implications for transplanting hPSC-derived VM progenitors in patients. First, scRNA-seq of cells after grafting demonstrated that only very few hESC-derived cells belonging to the astrocyte cluster remained proliferative. Second, serotonergic neurons that could potentially cause dyskinesias were not evident in hESC-derived grafts (30,31). Thus, the transcriptional analysis presented here provides support to the conclusion that these cells are safe to use in cell therapy. The discoveries presented in this study enables future focused studies to investigate the contribution of each individual cell type to graft integration and function. Further studies using grafts of controlled compositions are needed to better understand the role of astrocytes, oligodendrocytes, and VLMCs in graft vascularization, survival, maturation, and integration of DA neurons.

Methods
Animals. Athymic nude female rats (180 g) were purchased from Harlan laboratories (Hsd:RH-Foxn1nu) and housed in individual ventilated cages under a 12:12 h dark–light cycle with ad libitum access to sterilized food and water. All
**Fig. 4** scRNA-seq analysis and histological validation of grafted cells into the midbrain. 

**a** Schematic overview of experimental design. VM-patterned hESCs grafted to midbrain of 6-OHDA rats and analyzed at 9 months (n = 6). These rats were used as follows: scRNA-seq n = 3; histology n = 3, functional recovery n = 6. 

**b** Overview of hNCAM fiber outgrowth from hESC-derived intranigral graft showing a neuron-rich graft core and extensive re-innervation of the host striatum. 

**c** Immunohistochemistry showing TH staining in graft core of a hESC-derived intranigral graft at 9 months post-transplantation. 

**d** Drug-induced rotation test showing functional recovery in rats that have been transplanted to the midbrain with hESC-derived cells (n = 6 rats; mean ± SEM; **p < 0.01; compared to post-lesion; two-tailed paired t-test). 

**e** UMAP embedding showing clustering of 7875 analyzed cells after grafting to the midbrain (grafted rats n = 3). 

**f–i** Expression level per cluster for indicated genes. Indicated genes are established markers for astrocytes, VLMCs, neurons, and DA neurons, respectively. All indicated markers are the same as in Fig. 2. 

**j** UMAP of grafted cells as shown in Fig. 4e. Cells isolated by FACS (blue circles, n = 5958) or not by FACS (magenta circles, n = 1917) are indicated. Scale bars, 1 mm (b); 200 µM (c).
procedures on research animals were in accordance with the European Union directive (2010/63/EU) and approved by the local ethical committee at Lund University as well as the Swedish Department for Agriculture (Jordbruksverket). For all surgical procedures, rats (minimum 225 g/16–18 weeks) were anesthetized via i.p. injections using a 20:1 mixture of fentanyl–dormitor (Apopteksbolaget). The animals were rendered hemiparkinsonian via unilateral injection of 4 µL of the neurotoxin 6-hydroxypaminostyle at a concentration of 3.5 µg/µL (prepared from free-base HCL) aimed at the following stereotaxic coordinates (in mm): anterior −4.4, medial −1.2, and dorsal −7.8 with the incisor bar set at 2.4. Injections were performed as described at an injection speed of 1 µL/min with an additional 3 min allowed for diffusion before careful retraction of the needle. Drug-induced rotations were assessed 3 weeks post-lesion for 10 min using apomorphine, 2.5 mg/kg (Apopteksbolaget, Sweden) for 90 min in automated rotometer bowls (Omnitech Electronic Inc., USA). Rotational data were expressed as net turns per minute. The cylinder test was also used by placing animals in a glass cylinder (diameter 30 cm) for 5 min while video recording. We counted the total number of touches the animals performed over the 300 s interval with the paw ipsilateral and contralateral to the lesion specifically. Paw preference was expressed as 1. After matching rats into groups according to their behavioral performance each rat received a single-cell suspension of the respective hESC or human VM tissue. For intranigral grafts of hESCs-derived VM-patterned cells, we injected a total of 300,000 cells in 4 µL over four 1 µL deposits (75,000 cells per deposit) at the following coordinates relative to bregma (in mm): anterior +1.2, medial −2.6, dorsal −4.5 and −4.0 and anterior +0.5 medial −3.0. For intranigral grafts of hESC-derived VM progenitors, we injected a total of 150,000 cells over two deposits (75,000 cells per deposit) to the midbrain at the following coordinates AF −5.2, ML −2.3, DV −6.0/−6.5, TB flat head. At each deposit the injection was left in place for an additional 2 min before careful retraction to allow for settling of the tissue. The maturation of the graft was monitored using apomorphine-induced rotations as described above. On the day before perfusion a second cylinder test was performed. After behavioral data had been collected, the rats were transcardially perfused using 50 mL physiological saline (90 °C) followed by 1 min perfusion of 3% PFA (pH 7.4) solution over 5 min. For hESC-derived DA progenitors eight animals were transplanted in the intrastriatal grafting experiments. Out of eight, seven rats had surviving grafts and were used for analysis (two for sequencing and five for histology). For the intranigral transplants, nine rats were transplanted: one died after surgery but the intrastriatal grafting experiments. Out of eight, seven rats had surviving grafts and were used for analysis (two for sequencing and five for histology).

Human fetal tissue preparation. Human fetal tissue from legally terminated embryos was collected in accordance with existing guidelines with approval of the Swedish National Board of Health and Welfare and informed consent from women seeking elective abortions. To determine the gestational age of the embryos, the crown–rump length was measured and the embryo was staged according to week post-conception.

Fetal VM tissue (7,5 weeks) was prepared for transplantation according to the same standard operating procedures (SoPs) as were approved for the clinical TRANSEURO trial. Fetal VM was dissected, cut into smaller pieces, and incubated in TrypLE Select/Dornase alpha (DA, 20 U/mL) for 20 min at 37 °C for further dissociation. To achieve a crude cell suspension the tissue was triturated first with a large tip (1000 µL) and then with a small tip (100 µL) in HBSS/Tirilazad for 5 min while video recording. We counted the total number of touches the animals performed over the 300 s interval with the paw ipsilateral and contralateral to the lesion specifically. Paw preference was expressed as 1. After matching rats into groups according to their behavioral performance each rat received a single-cell suspension of the respective hESC or human VM tissue. For intranigral grafts of hESCs-derived VM-patterned cells, we injected a total of 300,000 cells in 4 µL over four 1 µL deposits (75,000 cells per deposit) at the following coordinates relative to bregma (in mm): anterior +1.2, medial −2.6, dorsal −4.5 and −4.0 and anterior +0.5 medial −3.0. For intranigral grafts of hESC-derived VM progenitors, we injected a total of 150,000 cells over two deposits (75,000 cells per deposit) to the midbrain at the following coordinates AF −5.2, ML −2.3, DV −6.0/−6.5, TB flat head. At each deposit the injection was left in place for an additional 2 min before careful retraction to allow for settling of the tissue. The maturation of the graft was monitored using apomorphine-induced rotations as described above. On the day before perfusion a second cylinder test was performed. After behavioral data had been collected, the rats were transcardially perfused using 50 mL physiological saline (90 °C) followed by 1 min perfusion of 3% PFA (pH 7.4) solution over 5 min. For hESC-derived DA progenitors eight animals were transplanted in the intrastriatal grafting experiments. Out of eight, seven rats had surviving grafts and were used for analysis (two for sequencing and five for histology). For the intranigral transplants, nine rats were transplanted: one died after surgery but the intrastriatal grafting experiments. Out of eight, seven rats had surviving grafts and were used for analysis (two for sequencing and five for histology).
t-SNE plot (Rtsne: T-Distributed Stochastic Neighbor Embedding using a Barnes-Hut implementation, https://github.com/kjrille/Rtsne). To extract the cluster-specific genes we used the SAMseq software package. Differentially expressed genes for each cluster are in Supplementary Data 1 and 2. Genes were considered significantly differentially expressed if \( q \)-value < 0.01. To analyze the effect of the cell cycle on the cluster separation we performed CellCycleScoring using a Rtsne implementation, https://github.com/jkrijthe/Rtsne). To extract the cluster-specific gene set consisting of top 100 up-regulated gene per cluster among the cells after grafting (for either Smartseq2 data alone or integrated with 10x data), comparison of the clusters merged into individual datasets as well as grafted cells originating from hESCs in differentiating dopamine and subthalamic nucleus neuronal lineages.

**Pathway analysis.** The gene clusters after transplantation (genes from Supplementary Data 1 and 2) were analyzed for biological functions using the QuPath Analysis program (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenenuity-pathway-analysis). Only functional categories related to System Development functions were selected (Supplementary Data 3) and ten most significant System Development functions of each cluster were plotted in Supplementary Fig. 6a.

**Comparison with other datasets.** Gene expression profiles per cluster from the Mouse brain atlas was downloaded from their website (http://mousebrain.org). A marker gene set consisting of top 100 up-regulated gene per cluster among the cells after grafting (for either Smartseq2 data alone or integrated with 10x data), combined with marker genes for all the 256 cell types in the atlas was used in the comparison (Supplementary Figs. 6b, c and 11). The 256 atlas cell types were grouped into master clusters at Taxonomy rank 4 (39 groups) and mean expression per group was calculated using the marker gene set. These were compared to the mean expression in our clusters using spearman correlation.

**Cell cycle scores.** G2M and S phase scores were calculated using the function CellCycleScoring in the Seurat package. As described (41) the cells were classified as cycling if either of the scores were larger than 1. If scores were less than 1 they were defined as non-cycling.

**Immunohistochemistry.** After perfusion, brains were post-fixed for 24 h in 4% PFA and then cryopreserved in a 30% sucrose solution before being sectioned coronally on a freezing sledge microtome at a thickness 35 µm in series of 1:8 sections. PFA and then cryopreserved in a 30% sucrose solution before being sectioned coronally on a freezing sledge microtome at a thickness 35 µm in series of 1:8 sections. After the sections were incubated with secondary biotinylated-horse antibodies (1:200, DABCO containing DAPI (1:1000). For di-amino-benzidine (DAB) stainings, the sections were incubated with secondary antibodies for 2 h and finally washed three times.

**Microscopy and images.** Images were captured using either a flatbed scanner Epson Perfection V850 PRO, a Leica DMM6000B widefield microscope or a Leica TCS SP8 laser scanning confocal microscope. The image acquisition software was Leica LAS X and images were processed using Velocity 6.5.1 (Quorum Technologies) and Adobe Photoshop. Any adjustments were applied equally across the entire image, and without the loss of any information. The following images were digitally stitched from multiple images: Figs. 1b, 2h, 3a–f, 4b, c, Supplementary Figs. 1k, 5b and 7d, e–g. Figures 1a and 4a were created by the authors. Each image represents a typical result obtained from analysis of minimum 2–3 graft sections per animal.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request.

Raw data have been deposited in the GEO database under accession codes: GSE118412 and GSE32758. An application for analyzing single cell data is available at http://perlmanlab.org.

The source data underlying Figs. 1c, 1d, 4d and Supplementary Fig. 5c are provided as a Source Data file.

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