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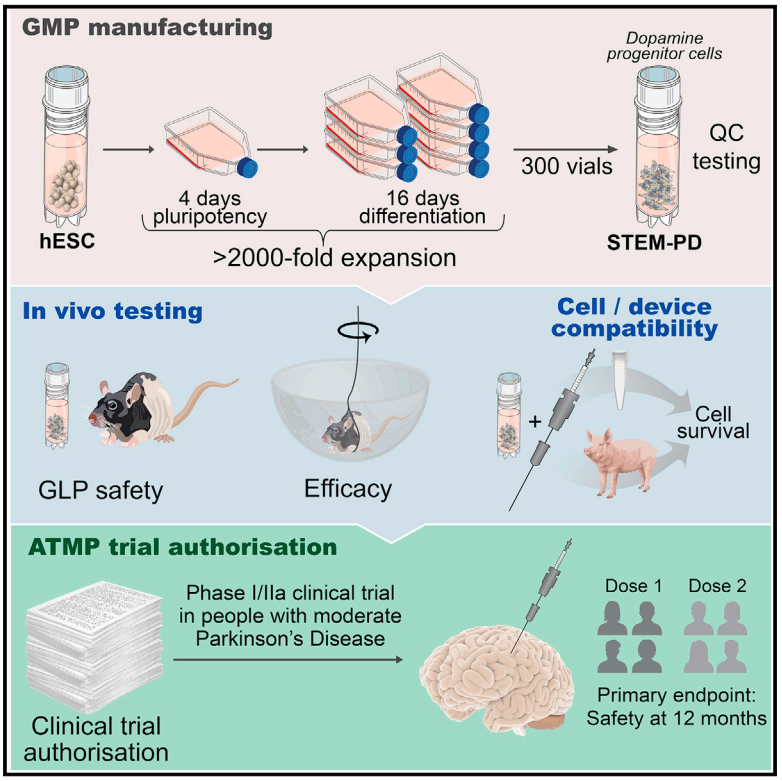
Clinical and Translational Report

Preclinical quality, safety, and efficacy of a human embryonic stem cell-derived product for the treatment of Parkinson's disease, STEM-PD

Graphical abstract



Highlights

- GMP manufacturing of cryopreserved dopamine progenitor cell product STEM-PD from hESCs
- STEM-PD shows no adverse effects or tumor formation in rats up to 9 months
- STEM-PD grafts innervate correct brain regions and reverse motor deficits in rat PD model
- A first-in-human ATMP trial with 8 patients is approved and initiated in Sweden

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In brief

Kirkeby et al. report in this issue on the manufacturing and preclinical testing of a pluripotent stem cell product for replacement of lost dopamine neurons in the brains of Parkinson's disease patients. A clinical trial using this stem cell transplantation product has been approved and initiated within the EU.

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Preclinical quality, safety, and efficacy of a human embryonic stem cell-derived product for the treatment of Parkinson's disease, STEM-PD

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SUMMARY

Cell replacement therapies for Parkinson's disease (PD) based on transplantation of pluripotent stem cell-derived dopaminergic neurons are now entering clinical trials. Here, we present quality, safety, and efficacy data supporting the first-in-human STEM-PD phase I/IIa clinical trial along with the trial design. The STEM-PD product was manufactured under GMP and quality tested *in vitro* and *in vivo* to meet regulatory requirements. Importantly, no adverse effects were observed upon testing of the product in a 39-week rat GLP safety study for toxicity, tumorigenicity, and biodistribution, and a non-GLP efficacy study confirmed that the transplanted cells mediated full functional recovery in a pre-clinical rat model of PD. We further observed highly comparable efficacy results between two different GMP batches, verifying that the product can be serially manufactured. A fully in vivo-tested batch of STEM-PD is now being used in a clinical trial of 8 patients with moderate PD, initiated in 2022.

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the loss of midbrain dopamine (DA) neurons, predominantly of the A9 subtype located in the substantia nigra pars compacta (SNc). These neurons send projections to the forebrain, where DA is released and functions to modulate key aspects of movement control and some aspects of cognition.[1] In PD, the loss of dopaminergic (DAergic) innervation to the striatum, especially the putamen, leads to motor impairments such as bradykinesia and rigidity.[2] There are currently no disease-modifying treatments, and medical management is mainly focused on controlling the motor symptoms using drugs that act on the DAergic system, such as levodopa (L-DOPA) or

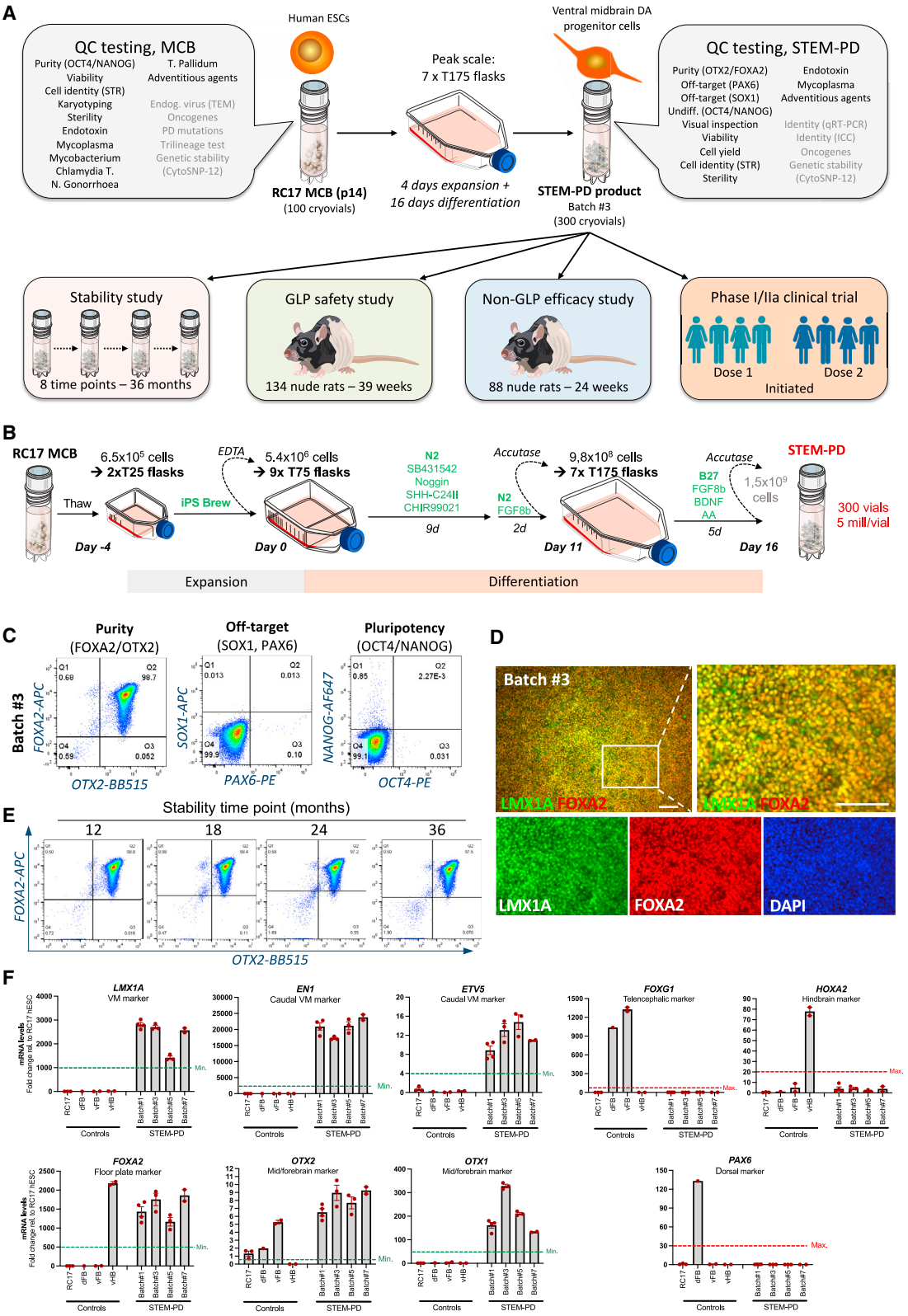

Figure 1. Manufacturing and quality control of STEM-PD

(A) Overview of manufacturing and testing of RC17 MCB and STEM-PD product. Quality control (QC) tests shown in black font were defined as release criteria and conducted under GMP according to European Pharmacopoeia (E.P.) standards, where applicable. Tests listed in gray font were conducted for information only.

(legend continued on next page)

DA agonists. These drugs provide significant clinical benefit in early disease stages, but with time, they lose efficacy and give rise to adverse effects including neuropsychiatric complications, L-DOPA-induced dyskinesias (LIDs), and exacerbation of non-motor aspects of PD such as postural hypotension.[3]

Based on a large number of preclinical studies in animal models of PD, DA cell replacement therapies are being pursued as a potential attractive alternative to oral DA medications.[4] The concept behind this is that physiological and targeted release and reuptake of DA provided by implanted DA neurons would lead to restoration of motor function, while avoiding the loss of efficacy and prominent side effects associated with long-term oral DA medications. Previous studies using DA neurons sourced from the ventral midbrain (VM) of aborted fetuses have demonstrated that intracerebrally grafted human fetal-derived DA neurons can survive long-term in the Parkinsonian brain with physiological release of DA and symptomatic benefit for decades.[5–11] However, the results for patients in receipt of fetal VM transplants have been highly variable, and two double blind placebo-controlled trials failed to reach their primary endpoints, while side effects in the form of graft-induced dyskinesias (GIDs) were observed in some patients.[7,12]

The reasons for these variable trial outcomes have been extensively discussed elsewhere,[13] and led us to undertake another transplant trial using human fetal VM tissue—TRANSEURO (NCT01898390). The TRANSEURO trial initiated in 2012 was designed with the aim to reduce the variation in outcome seen in the previous trials and included more stringent patient selection, cell preparation, handling and dosing, surgical technique, and immune suppression.[14] However, major logistical problems relating to tissue availability meant that recruitment to the TRANSEURO trial had to be terminated before the originally planned number of patients could be grafted.[14] Transplantation of human fetal tissue is further complicated by the fact that each patient receives a different composition and quantity of cells determined by the number and gestational age of the donated tissue, and there is limited possibility to perform quality testing of the cells prior to grafting.

Developing a DA cell replacement product that can be standardized, tested, and manufactured at a large scale will not only allow for adequate control of cell quality and dose but will also enable the therapy to be available to a globally impactful number of patients if successful. We, and others, have therefore focused our efforts toward the derivation of human midbrain DA neurons from pluripotent stem cells through directed differentiation.[15–18] In preclinical studies, we have shown that such differentiated human embryonic stem cell (hESC)-derived DA neurons can fully reverse motor impairments in experimental models of PD and that the neurons function en par with human fetal DA neurons.[19] Efforts to adapt such protocols to clinical standards[20–22] have already led to initiation of clinical trials in Japan and the US[23,24] as well as a single-case medical intervention.[25] Our team has now initiated a first-in-human clinical trial in people with moderate PD in Europe using the recently developed hESC-derived DAergic cell product STEM-PD.[26]

The STEM-PD product was generated using a good manufacturing practice (GMP)-compliant protocol for DA progenitor cell differentiation with scalable manufacturing and includes a method for cryopreservation of the progenitors to facilitate reproducibility, increase safety, and enable shipment of cells between centers.[27] This has enabled us to perform extensive quality control and *in vivo* assessment of the safety and efficacy of the exact same batch of cells that is now being delivered to patients in the STEM-PD clinical trial (EudraCT 2021-001366-38, NCT05635409). This trial is a single arm, first-in-human, phase I/IIa multicenter dose escalation trial to assess the safety and tolerability of hESC-derived DA progenitor cells grafted into the putamen of patients with moderate PD, including secondary efficacy and imaging endpoints. We present here the preclinical data for STEM-PD, some supportive data from research-grade cells, and the clinical trial design forming the basis for the regulatory approval of STEM-PD—a stem-cell-based trial for intracerebral grafting to receive regulatory approval in Europe under the EU advanced therapeutic medicinal product (ATMP) regulation.

RESULTS

Manufacturing and quality control of STEM-PD

A master cell bank (MCB) of 100 hESC cryovials was manufactured from the GMP-grade cell line RC17 obtained under license from Roslin Cells (Edinburgh, UK). This MCB was subjected to a battery of quality tests according to regulatory requirements including confirmation of pluripotency marker expression by flow cytometry (75.3% OCT3/4^{+}NANOG^{+}) and assessment of genetic integrity and stability by karyotyping, CytoSNP array and sequencing of oncogenes and PD-associated genes (Figure 1A; Tables S1 and S2). The MCB was subsequently used for manufacturing of the STEM-PD product (see STAR Methods).[27]

The differentiation protocol involves an initial patterning phase of 9 days using dual SMAD inhibition together with regionalizing growth factors. This includes Noggin and SB431542 for efficient neuralisation[28] combined with the Glycogen Synthase Kinase 3 (GSK3) inhibitor CHIR99021 for posteriorizing toward midbrain fates and sonic hedgehog (SHH) for ventralization to obtain VM progenitor cells (Figure 1B; STAR Methods).[27] At day 9, fibroblast growth factor 8b (FGF8b) expressed at the midbrain-hindbrain boundary was added to fine-tune patterning toward a caudal VM fate, which we have shown to be associated with successful graft outcome in a preclinical rat model of PD.[17]

(B) Detailed overview of the STEM-PD GMP manufacturing process.
(C) Results from flow cytometric release analysis of batch #3 for purity markers FOXA2 and OTX2 as well as off-target markers SOX1 and PAX6 and the pluripotency markers OCT4 and NANOG.
(D) Confirmation of VM identity in STEM-PD batch #3 by ICC. Scale bars, 100 μm.
(E) Stability assessment of STEM-PD batch #3 by flow cytometry for up to 36 months after cryopreservation.
(F) Quantitative real-time PCR analysis for VM and non-VM markers in 4 GMP STEM-PD batches compared with positive and negative non-VM neural progenitor control populations generated from RC17 hESCs. Minimum and maximum fold change cutoff levels for each gene were applied.[27] Data are represented as mean ± SEM. dFB, dorsal forebrain; vFB, ventral forebrain; vHB, ventral hindbrain.

Brain-derived neurotrophic factor (BDNF) and ascorbic acid were added at day 11 to enable progression of the cells toward post-mitotic neuronal progenitor cells, and the cells were harvested and cryopreserved at day 16 (Figure 1B). Each STEM-PD manufacturing run of 20 days resulted in a >2,300-fold expansion in cell number from the initially seeded MCB cells and was conducted at a peak scale of only 7× T175 flasks, generating a total of 300 vials (5×10^6 cells/vial) of the STEM-PD product (Figure 1B).

The cryopreserved STEM-PD product was quality tested for cell line identity, viability, yield, sterility, endotoxins, viruses, and unknown adventitious agents according to EU ATMP guidelines (Figure 1A; Table 1). We present here the results of the full *in vitro* batch release testing from 4 STEM-PD GMP batches, including the batch that is being used in our ongoing STEM-PD clinical trial (batch #3). This batch complied with release specifications and was subsequently used in all our *in vivo* safety and efficacy testing (see full list of specifications and test results in Table 1, and *in vivo* testing results in Figures 2, 3, 4, and 5). Correct differentiation toward VM progenitor fate was assessed by flow cytometry for the purity of FOXA2 and OTX2 double-positive cells (release specification of $\geq$70% FOXA2$^+$/OTX2$^+$) and by the relative absence of off-target non-VM neural cells (release specification of $\leq$2% SOX1$^+$ and $\leq$2% PAX6$^+$ cells, Figure 1C; Table 1). Batch #3 showed a purity of 98.7% FOXA2$^+$/OTX2$^+$ cells, and through stability studies, we demonstrated that the purity was not affected by long-term storage in vapor-phase liquid nitrogen (Figure 1E). The absence of any residual undifferentiated pluripotent cells in the product was confirmed through flow cytometry for OCT3/4 and NANOG (release specification of $\leq$ 0.1% OCT3/4$^+$/NANOG$^+$) (Figure 1C; Table 1). Correct VM identity was further confirmed by immunocytochemistry (ICC) for the VM markers LMX1A and FOXA2 and by quantitative real-time PCR of VM (*EN1*, *ETV5*, *FOXA2*, *LMX1A*, *OTX1*, and *OTX2*) and non-VM (*PAX6*, *FOXG1*, and *HOXA2*) neural markers (Figures 1D and 1F). Genetic stability and the potential presence of any oncogenic mutations was assessed with the same assays as for the MCB (Figure 1A; Table S3).

GLP safety testing in nude rats shows no adverse effects and no biodistribution outside the transplant region

To assess the safety profile of STEM-PD cells prior to use in the clinical trial, we performed a 39-week toxicity, tumorigenicity, and biodistribution study of STEM-PD batch #3 in immunodeficient athymic nude rats under full GLP conditions. This study involved a total of 52 animals administered with vehicle and 70 animals administered with STEM-PD cells at the maximum feasible dose (MFD) through stereotactic implantation unilaterally into the striatum. The MFD was defined as the maximum dose which could feasibly be administered to the rat striatum without graft core necrosis or displacement of the striatal parenchymal boundaries and was established as 700,000 cells distributed over 4 deposits (1.5 μL per deposit) in two tracts. Animals were sacrificed at 4, 26, or 39 weeks post-grafting for assessment of histopathology, organ weight, hematology, blood chemistry, and biodistribution. The 39-week group was further assessed for any ophthalmological adverse events as well as for central nervous system (CNS)-associated signs of abnormalities in motor function or behavior through modified Irwin testing (Figure 2A). A satellite group of 12 animals implanted with 700,000 undifferentiated pluripotent RC17 cells served as a positive control for teratoma formation. At necropsy, the brain was collected from all animals, and 2/3 of the most lateral non-transplanted hemisphere was collected for biodistribution analysis, whereas the remaining part of the brain was used for general histopathological assessment at 7 different rostro-caudal levels according to Bolon sectioning practice.[29] The tissue block containing the graft was additionally used for collection of 20 sections covering the graft area (Figure 2B).

The safety study found no treatment-related effects on mortality, body weight, body temperature, organ weight, food consumption, ophthalmological findings, or on the Irwin assessment of behavior. Some non-adverse inter-group variations in hematology and blood chemistry were observed (not shown), as well as the appearance of peripheral tumors in both treated and untreated animals that were unrelated to the graft, but likely attributable to the immunodeficient nature of the nude rat model (Table S4). Premature euthanasia for welfare reasons occurred in 5.8% of animals in the vehicle group (3 out of 52 animals) and 11.4% of animals in the STEM-PD treated group (8 out of 70 animals). None of the premature terminations were attributed to the STEM-PD treatment (Table S4).

Histopathological assessment of the transplanted hemisphere described findings that would normally be expected from a cell implantation procedure, including needle/trephination tracks and the presence of graft tissue (Figures 2C and 2D). Xenografts with a human neural identity were detected by immunohistochemistry (IHC) for human neural cell adhesion molecule (hNCAM) in 19 of 20 and 39 of 40 STEM-PD-treated animals at 26 and 39 weeks post-transplantation, respectively (Figures 2E and 2F). Only very few scattered Ki67-positive proliferative cells were observed in the graft at 26 and 39 weeks in 47% and 46% of the animals, respectively (Figure 2G). Together with the observation of similar graft volume at 26 and 39 weeks, this indicated that the size of the grafts had stabilized by 26 weeks post-transplantation. In contrast, transplantation of undifferentiated RC17 hESCs resulted in massive teratoma formation (Figure 2H), and 2 out of 12 animals in this group had to be euthanized for welfare reasons before the 12-week endpoint (Table S4). Although not required for regulatory approval, we have subsequently assessed graft proliferation over time in more detail through collection of single nucleus sequencing data from 22 individual animals grafted with 4 different research-grade batches of cells produced with same protocol as STEM-PD. Bioinformatic scoring of cell-cycle phase showed that approximately 0.5% of the cells in the grafts were detected as cycling at 3 months post-transplantation, and this decreased over time. By 12 months, the % cycling cells and % Ki67$^+$ cells were both <0.1%, (Figure S1).

Biodistribution analysis was performed on the transplanted rats by using a high-sensitivity quantitative PCR for human Alu Y elements. Analysis of 20 different organ tissues from a total of 20 vehicle treated and 27 STEM-PD-treated rats at 4 and 26 weeks confirmed that no human cells could be detected outside of the transplanted brain hemisphere (Table S5). Therefore, the remaining animals sacrificed at 39 weeks were assessed only for biodistribution within the CNS (i.e., in the contralateral hemisphere, the cerebrospinal fluid [CSF], and three segments of the spinal cord). Out of 113 samples analyzed,

Table 1. STEM-PD release specification and results

Test parameter	Method of analysis	Specification	Result from 4 GMP batches	Result, batch #3	PASS/FAIL
Purity/identity	flow cytometry: FOXA2 and OTX2	≥70% of the population is positive for both markers	87%–99%	98.7%	PASS
Off-target cells	flow cytometry: SOX1	≤2% of the population is positive	<0.5%	<0.5%	PASS
Off-target cells	flow cytometry: PAX6	≤2% of the population is positive	<0.5%	<0.5%	PASS
Undifferentiated cells	flow cytometry: OCT3/4 and NANOG	≤0.1% of the population is positive for both markers	<0.1%	<0.1%	PASS
Viability	NucleoCounter NC-200	≥70% viability	78%–83%	83%	PASS
Yield	NucleoCounter NC-200	$\geq 3 \times 10^6$ viable cells per vial	$3–4 \times 10^6$	4×10^6	PASS
Appearance	visual assessment	clear to cloudy, colorless to pink dispersion, and free of foreign particles	complies	complies	PASS
Cell line ID	STR DNA amplification	the cell line identity must be identified as the MCB	complies	complies	PASS
Sterility	BacTEC FX40 and BacT/ALERT 3D rapid microbial detection system in accordance to Ph. Eur. 2.6.27	no growth	no growth	no growth	PASS
Endotoxin	LAL test using turbidimetric method, according to Ph. Eur. 2.6.14.	<5 EU/mL	<0.1 EU/mL	<0.1 EU/mL	PASS
Mycoplasma	real-time PCR in accordance with Ph. Eur. 2.6.7	mycoplasma not detected	negative	negative	PASS
Adventitious agent testing	*in vitro* assay (28 days) on MRC-5, Vero and HeLa cells, in accordance with ICH Q5A. Porcine virus testing in PPK and Vero cells; bovine virus testing in Vero and BT cell lines, according to CHMP guidelines and Ph. Eur. requirements; evaluation of reverse transcriptase activity by ultracentrifugation and QFPERT assay	virus not detected	no virus detected	no virus detected	PASS

STEM-PD cells were tested for release according to the specifications in the table. Results from the GMP batches are shown in the last two columns. BT, bovine turbinate; CHMP, Committee for Medicinal Products for Human Use, European Medicines Agency; ICH, International Council for Harmonization; LAL, limulus amebocyte lysate; MCB, master cell bank; PPT, primary porcine kidney; QFPERT, quantitative fluorescent product enhanced reverse transcriptase.

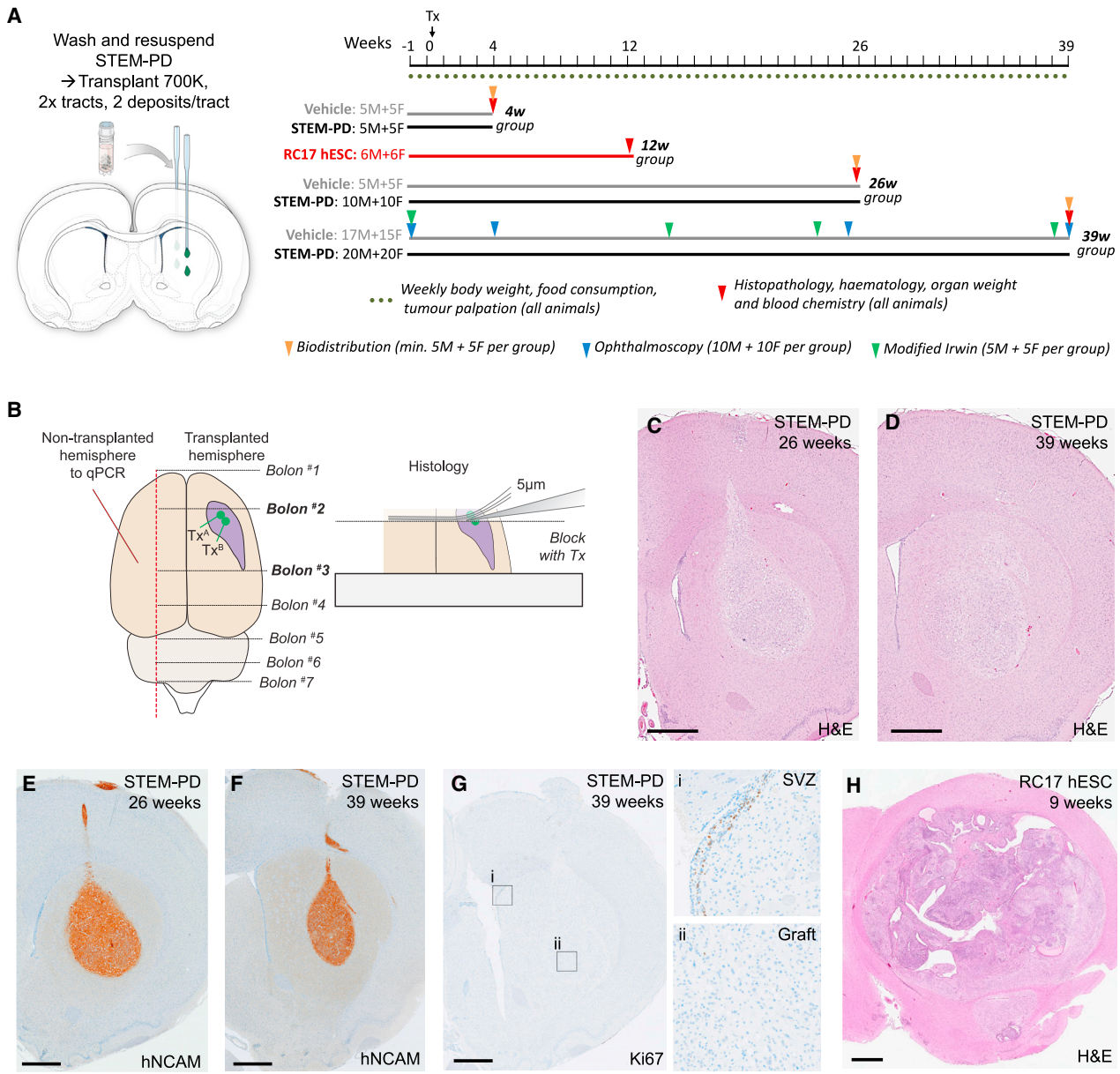

Figure 2. GLP safety study for toxicity, tumorigenicity, and biodistribution

(A) Overview of the GLP safety study design. The rats were divided into 3 groups, receiving either vehicle, an MFD of 700,000 STEM-PD cells, or 700,000 RC17 hESC. The cells were distributed over 4 deposits into one striatum (2 deposits per needle tract), and the animals were assessed at several time points.

(B) The rat brains were dissected to allocate 2/3 of the non-transplanted hemisphere for qPCR and the remaining part for paraffin-embedding and histopathology with Bolon sectioning.

(C–F) Staining by hematoxylin and eosin (H&E) was used to assess general tissue histopathology (C and D), and staining for hNCAM was used to confirm neural identity of the transplanted cells (E and F).

(G) Staining for Ki67 revealed few or no Ki67+ cells in the graft tissue (Gi), as opposed to the neighboring subventricular zone (SVZ, Gii), which showed clear immunoreactivity for Ki67.

(H) H&E brain section from an early sacrificed animal from group 3 (transplanted with 700,000 RC17 hESCs), showing a large teratoma structure at 9 weeks post-transplantation. See Tables S4 and S5 for further results from the study. M, male; F, female; Tx, transplant. All scale bars, 1 mm.

only 1 CSF sample from a STEM-PD-treated animal at 39 weeks showed a human DNA signal above the limit of detection (LoD), although below the limit of quantification (LoQ) (Table S5). Together, the GLP safety study of STEM-PD batch #3 showed that STEM-PD administered at the MFD was well tolerated with no adverse in-life or histopathological findings and with no detectable biodistribution of STEM-PD cells outside of the transplanted brain hemisphere.

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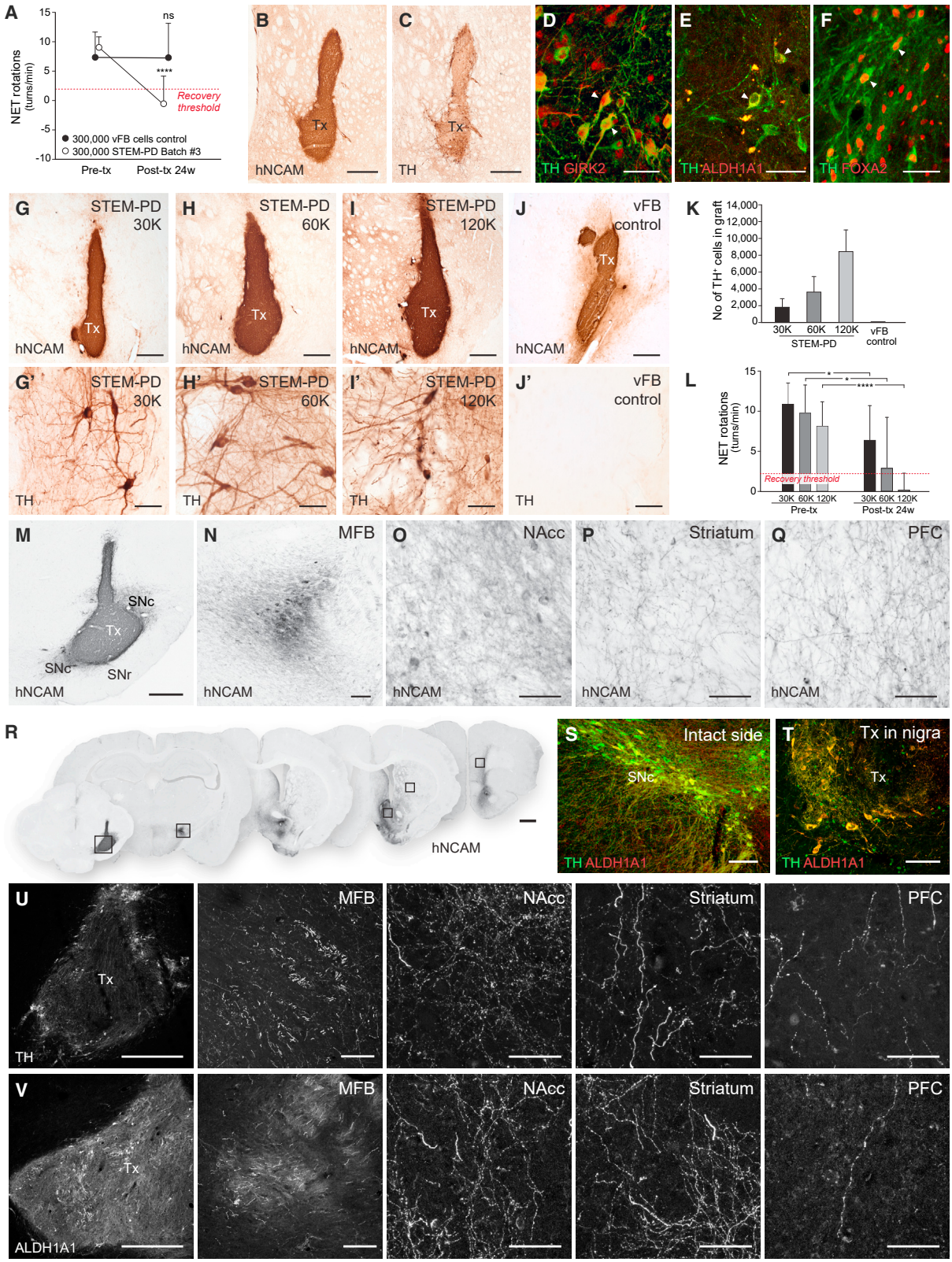

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STEM-PD grafts are rich in DA neurons, innervate the host brain, and reverse amphetamine-induced rotations in rats

Efficacy, potency, and outgrowth capacity of STEM-PD batch #3 was evaluated in the unilateral 6-hydroxydopamine (6-OHDA)-lesioned model of PD (Table S6) with immunodeficient nude rats as recipients. To confirm functional efficacy, 300,000 STEM-PD cells were transplanted to the denervated striatum (group 1 in Table S6), and motor function was evaluated using the amphetamine-induced rotation test prior to transplant surgery (baseline) and at 24 weeks post-transplantation. The mean value for the pre-transplantation rotational scores in this group of animals was 9.0 ± 1.9 turns/min and −0.54 ± 4.58 turns/min at 24 weeks post-transplantation ($p < 0.0001$ for pre-Tx vs. 24 weeks post-Tx in paired t test, Figure 3A). Furthermore, all surviving animals with correct graft placement in group 1 (n = 16) reduced from their pre-transplantation score, and 14 out of 16 displayed rotation scores below 2 turns/min at 24 weeks. No recovery was observed in the lesioned animals transplanted with non-DA ventral forebrain (vFB) control cells (Figure 3A).

At 24 weeks post-transplantation, the rats were perfused, and the brain tissue processed for histology. The transplants were detected by hNCAM and Tyrosine Hydroxylase (TH) (Figures 3B and 3C). The TH^+ neurons co-expressed mature mesencephalic DAergic (mesDA) markers typical of A9 SN neurons such as GIRK2 (KCNJ6), ALDH1A1, and FOXA2 (Figures 3D–3F). Quantification of the total number of TH^+ neurons in the grafts showed that the mean (±SD) content of TH^+ cells was 8,504 ± 4,397 cells/graft (Table S7). Hence, the yield of TH^+ neurons from STEM-PD batch #3 was determined to be 2,835 TH^+ neurons per 1×10^5 transplanted cells, which is in the range of what we have previously observed.[17] Finally, since it has been hypothesized that GIDs observed in some patients receiving fetal human VM transplants may relate to the presence of contaminating serotonergic neurons in the grafts,[7,12,30,31] we performed IHC for 5-hydroxytryptamine (5-HT). No 5-HT^+ cells were detected in any of the 16 analyzed grafts in group 1 (Figure S2).

To investigate dose dependency, we included 3 additional groups (groups 2–4, Table S6), transplanted with lower numbers of STEM-PD cells (i.e., 30,000, 60,000, and 120,000 cells, respectively). To avoid confounding effects of graft size and density of cell suspension on cell maturation, the STEM-PD cells in these experiments were diluted with non-DA carrier cells of vFB identity to retain the same density of cell preparation used for transplantation and same total cell number as in group 1. hNCAM immunostaining confirmed graft viability in all three groups (Figures 3G–3J). Immunostaining for TH (Figures 3G′–3J′), confirmed a dose-response correlation between the number of TH^+ cells and the increasing dose of STEM-PD (Figures 3G′–3J′ and 3K). As a control, we analyzed transplants of vFB carrier cells alone (group 7, Table S6), and confirmed that these cells did not give rise to any TH^+ neurons (Figures 3J and 3J′). We found that 120,000 STEM-PD cells diluted with vFB cells produced almost the same amount of DA neurons as 300,000 STEM-PD cells transplanted alone. This indicates that co-transplantation with non-DA progenitors may potentially be supportive for DA neuron survival or maturation—something that was not expected and would be relevant to investigate further experimentally for future product improvement.

Prior to grafting, the animals in groups 2–4 (dosed with 30K, 60K, and 120K STEM-PD, respectively) showed a mean rotational score of 11.05 ± 2.53, 9.82 ± 3.46, and 8.21 ± 3.01 turns/min, respectively (Figure 3L; Table S7). At 24 weeks post-transplantation, the respective rotation scores for these groups were 6.49 ± 4.27, 2.97 ± 6.32, and 0.15 ± 2.12, thus showing significant reductions in mean rotational scores for all three dosing groups ($p = 0.011$, $p = 0.026$, and $p < 0.0001$, respectively; Figure 3L) but only full recovery in animals dosed with 120K STEM-PD cells. From the low-dose groups (30K and 60K STÈM-PD), we identified two rats that had grafts with <2,000 TH^+ cells (rat #21 with 912 TH^+ cells and rat #36 with 1,720 TH^+ cells, Table S7) while still showing complete behavioral recovery with rotation scores <2 at 24 weeks after dosing. This suggests that even a small number of STEM-PD DA cells can support recovery of motor asymmetry in rats as has been reported previously for fetal cells[32] and research-grade ESCs.[19]

Another important criterion for clinical efficacy is the extent of DAergic innervation into the host brain parenchyma. To assess the growth capacity of graft-derived axons, we transplanted 20 6-OHDA-lesioned nude rats with STEM-PD cells into the midbrain (group 5, Table S7). At 24 weeks post-transplantation, hNCAM staining confirmed the presence of a graft in the substantia nigra (SN) in all 19 surviving animals (Figures 3M

Figure 3. Efficacy testing of STEM-PD batch #3

Efficacy of STEM-PD batch #3 was tested in a long-term *in vivo* study in nude rats.

(A) Recovery of amphetamine-induced rotational assessment pre-transplantation and 24 weeks post-transplantation. One way ANOVA: $F(2,22) = 60.97$, ****$p < 0.0001$ for pre-Tx vs. 24 weeks post-Tx in paired t test, n = 16 for STEM-PD and n = 4 for vFB, ns: non-significant.

(B and C) (B) The presence of human neural grafts was confirmed by hNCAM-positive immunohistochemical staining and (C) DAergic content was confirmed with TH staining.

(D–F) High magnification images of cells confirming their mature DA phenotype and midbrain identity by co-labeling of TH with GIRK2, ALDH1A1, and FOXA2.

(G–J) Transplants of increasing numbers of STEM-PD cells, diluted with non-DA vFB cells (J) to reach a total of 300,000 grafted cells per animal in all groups. (G′–I′) Staining for TH confirmed DAergic content of the grafts in animals dosed with STEM-PD but not with vFB carrier cells alone (J′).

(K and L) (K) TH counts and (L) amphetamine-induced rotational assessment for animals dosed with 30,000, 60,000, and 120,000 STEM-PD cells at 24 weeks post-transplantation, *$p < 0.05$, ****$p < 0.0001$ by paired t test.

(M–Q) (M) hNCAM staining of intranigral grafts and their innervation from the graft core into the (N) MFB, (O) NAcc, (P) striatum, and (Q) PFC.

(R) Whole-brain visualization of hNCAM staining from an intranigral graft. Boxes show locations for magnified images in (M)–(Q).

(S and T) Staining for A9 DA markers TH and ALDH1A1 in endogenous neurons in the intact SNc (S), and in an intranigral STEM-PD transplant (T).

(U and V) TH and ALDH1A1 staining in animals with intranigral STEM-PD grafts, showing A9-like innervation to known DA target structures. All graphs are showing mean ± SD. MFB, medial forebrain bundle; NAcc, nucleus accumbens; PFC, prefrontal cortex; SNc, substantia nigra pars compacta; Tx, transplant; vFB, ventral forebrain. Scale bars: 50 μm in (D)–(F), (G′)–(J′), (O)–(Q), (U), and (V) (NAcc, Striatum, PFC); 100 μm in (N) and (S)–(V) (MFB); 500 μm in (B), (C), (G)–(J), (M), (U), and (V) (Tx); and 1 mm in (R). See Table S6 for details on study outline.

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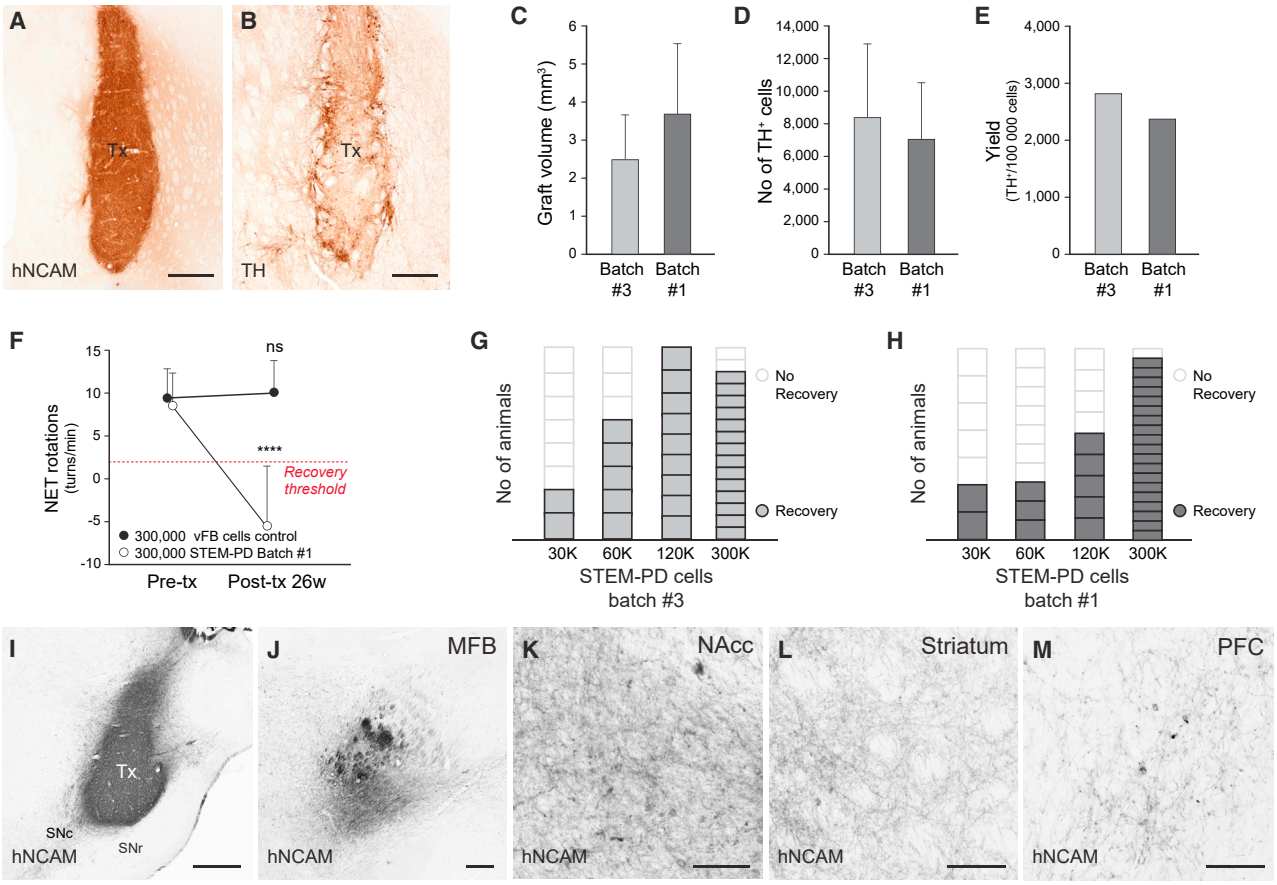

Figure 4. Batch-to-batch comparability on *in vivo* efficacy

Long-term efficacy testing in nude rats was performed with one additional batch of STEM-PD (batch #1), and the results were compared with batch #3.

(A and B) (A) hNCAM and (B) TH staining of batch #1. Scale bars, 500 μm.

(C) Quantification of graft volume in batch #1 and #3 based on HuNu staining.

(D and E) (D) Quantification of TH^+ neurons in batch #1 and #3, and (E) yield of TH^+ neurons per 100,000 grafted cells.

(F) Amphetamine-induced rotational assessment pre-transplantation and 26 weeks post-transplantation for animals dosed with batch #1 ($t = 9.083$, $^{****}p < 0.0001$ by paired t test, $n = 20$).

(G and H) Panels showing the number of recovered animals (i.e., with post-transplantation rotational scores <2) out of total assessed animals in each group transplanted with 30,000, 60,000, and 120,000 cells, respectively, from batch #3 (G) and batch #1 (H).

(I–L) (I) Grafts placed in the midbrain (scale bars, 500 μm; SNc, substantia nigra pars compacta; SNr, substantia nigra reticulata) were confirmed to innervate through the medial forebrain bundle (MFB) (J, scale bars, 100 μm); nucleus accumbens (NAcc) (J), striatum (K), and prefrontal cortex (PFC) (L). Scale bars: 100 μm in (K)–(M). All graphs are showing mean ± SD.

and 3R). hNCAM-positive fibers could be traced in large numbers along the nigrostriatal pathway (NSP) and the medial forebrain bundle (MFB; 19/19 animals) toward the A9 target area in the striatum (15/19 animals), as well as the A10 target areas in nucleus accumbens (NAcc, 19/19 animals) and prefrontal cortex (PFC; 19/19 animals), extending approximately 7–8 mm from the graft core, which is at a scale relevant for re-innervation of the human putamen in a clinical setting (Figures 3M–3R). Co-staining of TH and ALDH1A1 confirmed midbrain DAergic identity of the grafted cells, which were of a similar appearance to the endogenous nigral neurons and further confirmed prominent A9-like DAergic innervation of the DA-depleted dorsolateral striatum (Figures 3S–3V).

Taken together, these data show that STEM-PD cells survive transplantation long-term and mature into DA neurons with the capacity for long-distance fiber outgrowth and the ability to mediate full functional recovery in the DA-lesioned recipient animal.

STEM-PD shows robust and reproducible *in vivo* efficacy in batch-to-batch comparison

To assess batch-to-batch comparability with respect to graft outcome and *in vivo* efficacy, we performed a second full efficacy study with 80 animals using a different batch of GMP-manufactured STEM-PD cells (batch #1), which had a $FOXA2^+$/$OTX2^+$ purity of 86.5% and complied with all release criteria, thereby showing characteristics similar to batch #3 (Figure S3; Table 1). Likewise, batch #1 gave rise to DA-rich grafts with a similar appearance to batch #3 as determined by hNCAM and TH immunostaining (Figures 4A and 4B vs. Figures 3B and 3C), and yielding a similar graft volume and number of TH^+ neurons (Figures 4C–4E). Moreover, batch #1 also mediated complete

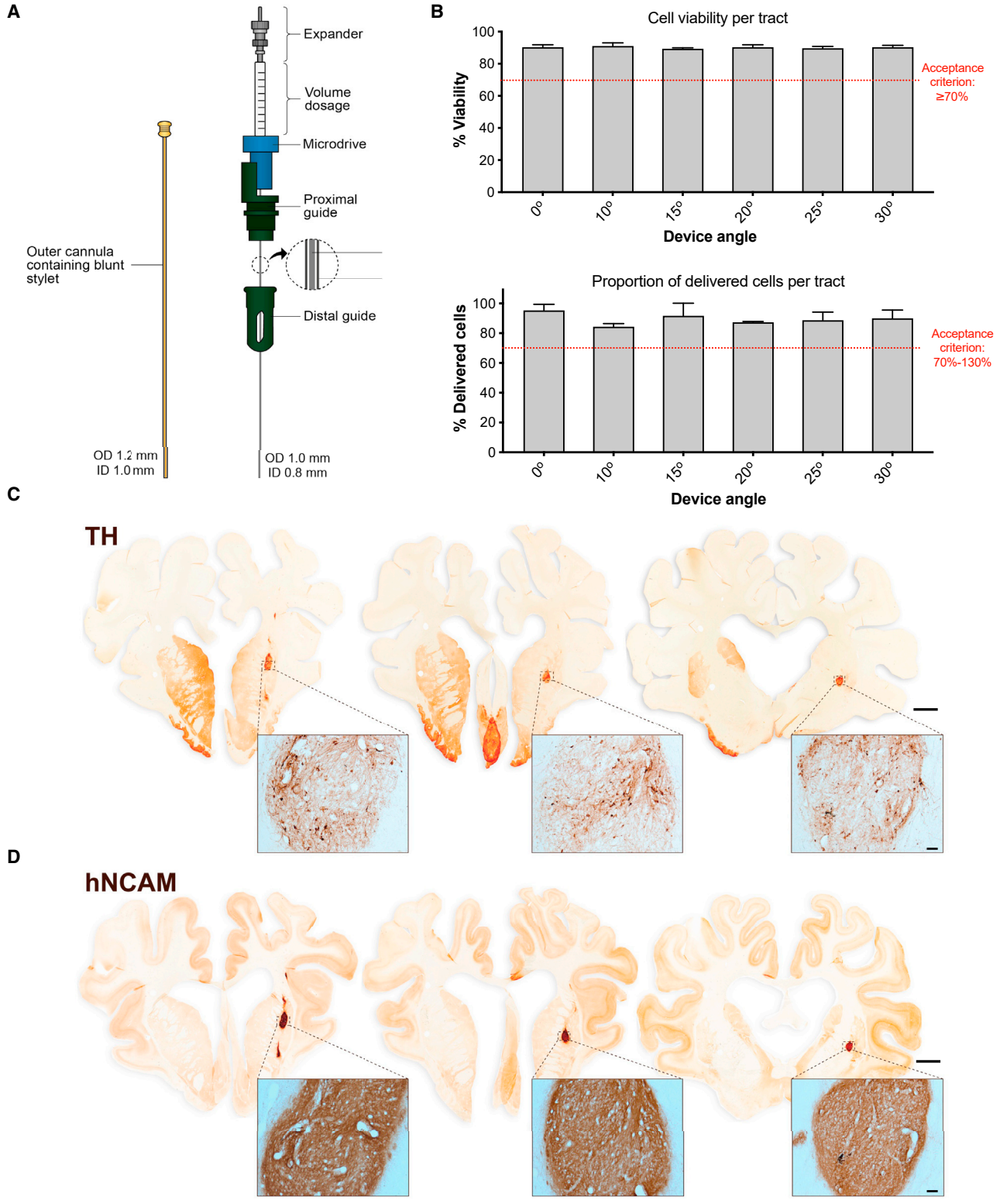

Figure 5. Testing of R-L device compatibility with STEM-PD cells *in vitro* and in minipig

The in-house manufactured Rehncrona-Legradi (R-L) device was tested with STEM-PD cells *in vitro* and in a pig transplantation study for compatibility and feasibility.

(A) Schematic drawing of the R-L device design with an inner and outer cannula and stepwise microdrive at the top to allow accurate serial delivery of up to 8 × 2.5 μL deposits in each tract.

(legend continued on next page)

functional recovery in the 6-OHDA rat model of PD at 26 weeks post-transplantation (Figure 4F) with a dose dependency similar to batch #3 (Figures 4G and 4H). When transplanted to the SN, this batch was similarly able to regrow fibers along the NSP and MFB (in 8/8 animals with correctly placed grafts) to the NAcc (8/8 animals), striatum (6/8 animals), and PFC (in 6/8 animals, Figures 4I–4M).

STEM-PD cells survive long term in the pig brain upon transplantation using the clinical delivery device

For the clinical trial, all surgeries will be performed in one site (Skåne University Hospital, Sweden), and the cells delivered using the Rehncrona-Legradi (R-L) device (Figure 5A) that has successfully been used in the clinic for human fetal VM tissue implants.[5,33–35] We tested the compatibility of this device to deliver viable STEM-PD cells *in vitro* and confirmed that the viability after passing through the device ranged from 89% to 92% (Figure 5B). We further showed that the number of delivered viable cells per simulated tract ranged from 82% to 99% of the expected pre-loaded number of cells at all tested angles of the device position (0°–30°).

The R-L device is too large for use in the rat brain, so to assess the feasibility and compatibility of this device for delivery of STEM-PD cells in a large brain, we conducted a small transplantation study in a single 6-OHDA lesioned Göttingen minipig immunosuppressed with daily PO administration of tacrolimus. Using the R-L device, a total of 1.25 × 10^6 STEM-PD cells (batch #3) were implanted into the right putamen, distributed over 10 deposits of 2.5 μL each. Histological assessment of the pig brain 11 months post-transplantation showed the presence of a surviving human graft in the putamen, rich in TH^+ DA neurons (Figures 5C and 5D). This confirmed that the R-L device is compatible with delivery of STEM-PD cells to the correct target structures in a large brain and that the implanted cells survive long term and mature to DA neurons.

Clinical dose calculation and implantation approach

To set the clinical dose for the STEM-PD trial, we were guided by experiences gained from previous human fetal tissue transplantation trials in PD patients where several post-mortem studies have reported the number of surviving DA neurons in patients with clinically effective grafts (see Table S8). Based on these data, a minimal dose for clinical recovery was estimated to be approximately 1 × 10^5 human TH^+ neurons per grafted putamen, which is the dose we have selected as the starting dose in the STEM-PD trial (dose 1). To determine how many STEM-PD cells should be transplanted to achieve 1 × 10^5 mature TH^+ neurons in the grafts after transplantation, we made use of the DA yield obtained from the STEM-PD batch #3 efficacy study (i.e., 2,835 TH^+ neurons derived per 1 × 10^5 transplanted cells). From this, the clinical dose 1 was set at 3.5 × 10^6 STEM-PD cells/putamen, distributed over 5 needle tracts in each putamen—two tracts targeting the anterior putamen and three tracts targeting the posterior putamen—with 4 × 2.5 μL deposits per tract and 7.1 × 10^4 cells/μL to achieve optimal coverage of the host tissue. Magnetic resonance imaging (MRI) is performed on each patient prior to surgery for individual calculation of target region, burr hole locations, and needle angles to achieve optimal targeting of the putamen while avoiding major blood vessels during the implantation surgery. To explore the optimal dose range, a second dose (dose 2) with twice the number of transplanted cells, targeting 2 × 10^5 TH^+ neurons/putamen = 7.1 × 10^6 STEM-PD cells administered per putamen at the same cell density but in the double number of deposits (i.e., 5 tracts with 8 deposits/tract in each putamen) has been included in the trial design. The human SN contains approximately 4 × 10^5 TH^+ neurons,[36] and based on the assumption that approximately half of these innervate the putamen, this corresponds to approximately 2 × 10^5 DA neurons. Thus, the selected doses (1 × 10^5 and 2 × 10^5 TH^+ neurons/hemisphere) are within the range of the number of DA neurons that normally innervate the putamen in healthy humans. At present, we plan for 4 patients to receive dose 1, and 4 patients to receive dose 2; however, a decision on dose escalation will be dependent on an interim safety assessment and positron emission tomography (PET) imaging of the first 4 patients transplanted with dose 1.

STEM-PD clinical trial design

The STEM-PD trial design is similar to that of the TRANSEURO trial (NCT01898390) and is based on lessons learned from previous fetal tissue transplantation trials, with rationales discussed in part previously.[14,37] Clinical trial participants will be transplanted in a pre-determined staggered schedule in which each patient will receive bilateral grafts of the STEM-PD product in a single neurosurgical session (Figure 6A). The primary objective of the trial is to assess the safety, tolerability, and feasibility of intraputamenal transplantation of the STEM-PD product in patients with moderate PD at 12 months post-transplantation. Secondary objectives related to efficacy are assessed at 36 months (Figure 6B). The secondary objectives are 3-fold, (1) to evaluate the course and efficacy on clinical features post-grafting using validated clinical measures such as the unified PD rating scale (UPDRS) in the defined OFF state (i.e., the patient is off DAergic medication during testing), (2) to assess the survival of DAergic cells following transplantation using F-DOPA and PE2I PET imaging to label functional DA terminals, (3) to determine the safety and clinical efficacy between doses (if dose escalation is undertaken) of the STEM-PD product, including assessment of whether there

(B) Viability and cell yield results from device delivery of STEM-PD cells *in vitro* at various angles relevant for the surgical setup. The top graph shows cell viability per simulated tract and the bottom graph shows the cell number delivered per simulated tract as a percentage of the number of cells loaded into the device. Predefined acceptance criteria for each test are noted in red. Data are represented as mean ± SD.

(C and D) A minipig transplanted with STEM-PD batch #3 using the R-L device was sacrificed at 11 months post-transplantation, and brain sections were immunostained for TH and hNCAM.

(C) TH^+ staining at the coronal level of the grafted neurons in the putamen of the 6-OHDA-lesioned minipig. Insert: high magnification images of the grafted neurons and fibers.

(D) hNCAM-positive graft staining of coronal minipig sections. Insert: images of positive fiber staining within the graft. The coronal sections are presented in anterior to posterior direction (corresponding to the minipig atlas S9, S11, and S13 https://auinstallation34.cs.au.dk/fileadmin/cense.au.dk/Atlas/atlas/index.html). Scale bars, 1,000 μm (coronal section); Scale bars, 100 μm (insets). Cd, caudate nucleus; ic, internal capsule; Pu, putamen; Acb, accumbens nucleus.

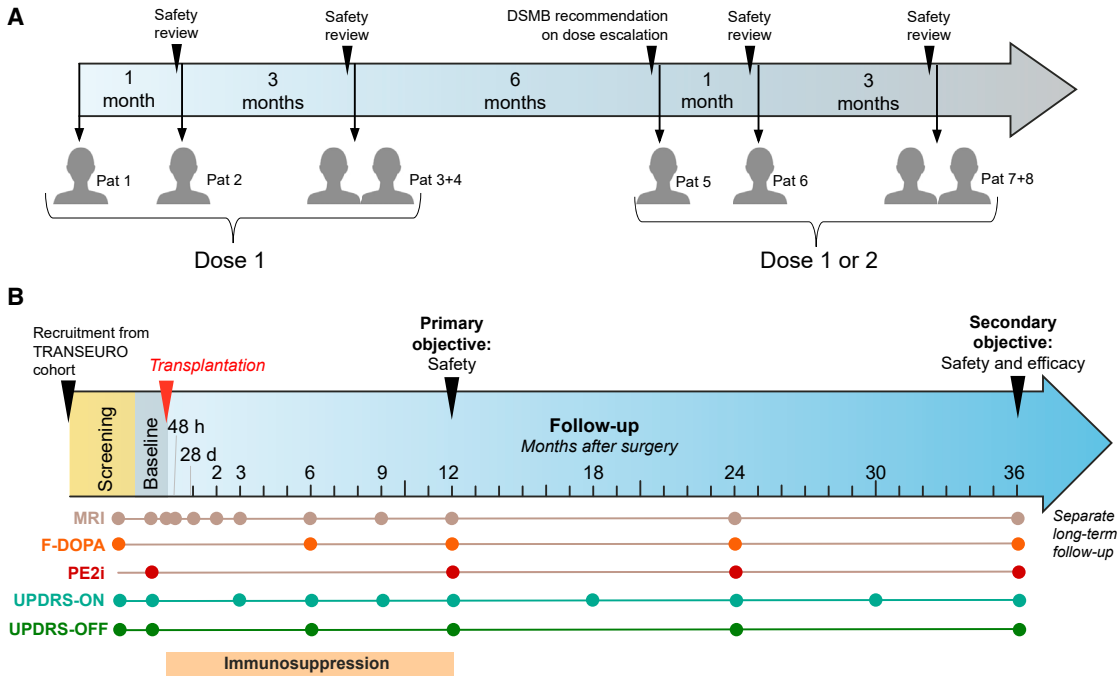

Figure 6. Overview of the STEM-PD trial

The STEM-PD trial is a 36-month trial including 8 patients.

(A) Schematic summarizing the staggered dosing scheme for the 8 patients. Patients 1–4 will be staggered with interim safety assessments, and all 4 patients will receive dose 1. At 6 months after patient 4 is dosed, once data are available for safety, imaging, and clinical measurements, the Data and Safety Monitoring Board (DSMB) for the trial will make a recommendation for the dosing of the remaining 4 patients. The DSMB can recommend to either (1) remain at dose 1, (2) proceed to dose 2, or, (3) wait longer to collect more data. The final decision will be made by the Trial Management Group, after receiving the DSMB's recommendation.

(B) Overview of trial design. Patients will be recruited from the ongoing TRANSEURO observational study[14] and at the end of the STEM-PD trial, the patients will be asked to take part in a long-term follow-up study. Patients will be assessed over the first 12 months for the primary objectives concerning safety, tolerability, and feasibility. Secondary objectives at 36 months include safety as well efficacy of the graft, based on clinical measures and DAergic PET imaging (F-DOPA and PE2i). Structural imaging with MRI is included to check for space-occupying lesions at the graft site as well as transplant placement. Clinical assessments include validated tests such as the unified Parkinson's disease rating scale (UPDRS) in ON and OFF states (i.e., the patient is on or off DAergic medication at the time of testing). Additional clinical assessments not shown here include a range of timed motor tasks as well as cognitive, affective, and quality-of-life assessments.

is a dose-response effect. All participants will be followed up for 3 years within the trial and also offered participation in a separate long-term follow-on study where they would be followed up indefinitely.

The trial will be open label involving 8 participants with moderately advanced PD who are entering the phase of the condition when an advanced interventional therapy such as deep brain stimulation (DBS), DuoDopa, or apomorphine infusions are being considered. Furthermore, and in line with TRANSEURO, patients with significant LIDs are excluded from the trial, as pre-existing LIDs may predispose participants to develop GIDs and may further require additional medical interventions, which could interfere with the trial. In addition, a relatively preserved DAergic innervation of the ventral striatum has been linked to better clinical outcome in patients grafted with human fetal VM tissue[10,38]; therefore, patients are required to show a good response to L-DOPA clinically and to be devoid of any major cognitive, neuropsychiatric, or autonomic problems. The patients selected have all been part of the TRANSEURO observational study for a minimum of 12 months, providing good long-term pre-intervention data on their disease progression.

All participants will receive immunotherapy similar to that used in renal transplant programs: basiliximab, tacrolimus (or cyclosporine if tacrolimus is not tolerated), azathioprine, and steroids for 12 months post-grafting.

DISCUSSION

There are currently at least two other ongoing clinical trials using induced pluripotent stem cell (iPSC)—and hESC—derived DAergic progenitor cells in patients with PD.[23,24] These trials, like the TRANSEURO trial and our STEM-PD trial, are allogeneic investigational therapies with immune suppression given to the recipients for 12 months post-grafting. The iPSC-based trial in Japan is using a fresh cell product that is manufactured separately for each patient starting from the same iPSC line, while the BlueRock trial in the US and Canada makes use of a cryopreserved cell product from the H9 hESC line, which was re-derived from a research-grade cell line. When designing STEM-PD, we opted for a cryopreserved cell product from the hESC line RC17, which was derived fully under GMP conditions.[39] The STEM-PD product has been manufactured in batches large enough to allow for quality assessment and *in vivo* safety and efficacy, as well as use in the entire clinical trial. While full characterization of all cells in the grafts after maturation *in vivo* was not requested and has not been performed, thorough testing of the

clinically administered batch for safety and efficacy in long-term animal studies gives high confidence in the functionality and safety of the exact product batch used in patients. This is particularly important for first-in-human studies using neural progenitor cells derived from pluripotent stem cells, since such cells undergo terminal differentiation, diversify, and become functional only after transplantation *in vivo*. In addition, the use of a single clinical batch ensures minimal product-related variation between participants, and the availability of a banked, cryopreserved product makes surgical and clinical planning logistically easier. However, in view of developing a global therapy at a scale that demands serial batch manufacturing, it is unlikely to be practically feasible or financially viable to perform extensive long-term *in vivo* testing of all batches intended for clinical use. It is therefore important to verify that the product is efficacious also across several GMP batches, as we have done here.

Whereas the other ongoing stem cell trials for PD involve patients with more advanced PD,[40,41] the STEM-PD trial targets patients with moderate disease, many of which would under normal circumstances be eligible for some form of invasive interventional therapy in the next few years, such as DBS, MRI-focused ultrasound lesioning, DuoDopa, and/or apomorphine infusions. The "moderate" inclusion criterion developed for the STEM-PD trial aims to strike a balance by avoiding the treatment of early-stage patients who may be managed well on their current standard of care for years, and late-stage patients who may be more vulnerable to lengthy surgery and less likely to respond to DA cell replacement therapy. If cell replacement therapies such as STEM-PD are shown to be safe and effective, we anticipate that earlier-stage patients, who would potentially benefit the most from cell replacement therapy, could be recruited for future trials. The STEM-PD trial has the major advantage that it is designed in a nested fashion, with patients recruited to the STEM-PD trial directly from the TRANSEURO observational study which began in 2011. This means that some of the patients in the trial have been followed for up to 10 years prior to the intervention with regular motor testing, ensuring that the clinical team has considerable data on the patients' pre-intervention disease trajectories. Although the approach of recruiting patients from a pre-existing cohort may potentially introduce a risk of selection bias toward patients with less aggressive disease progression, we will nevertheless be able to crucially monitor disease progression trajectories of each patient pre- and post-transplantation given that they will have been followed and assessed over many years. The TRANSEURO patients are further accustomed to the trial assessment tests over several years, thereby minimizing any initial training effects, which could confound the interpretation of clinical assessment scores.

Preclinical studies in rats have shown that transplanted hESC-derived VM progenitor cells can survive and mature into DA-producing neurons, innervate the host striatum, integrate into the host circuitry, mediate functional recovery in DA depletion models through DA secretion, and alleviate LIDs in those same models through DA reuptake.[16,17,19,42–45] Based on these data, the aim for the ongoing stem cell trials is that the grafted cells will survive, produce DA, and mediate clinical benefits in patients. As a first step, it will be crucial to demonstrate through PET imaging, that the stem-cell-derived neurons survive and give rise to local DAergic innervation of the putamen in humans, as has been shown in animal models. If safety and feasibility as well as cell survival and functionality can be shown, the ability to generate virtually unlimited numbers of standardized and quality-controlled DA cells from stem cells could transform cell replacement into a clinically useful therapy for PD. A standardized cell product further allows one to conduct properly controlled studies to determine the effect of the transplantation procedure, cell placement, and dose. It is estimated that approximately 100,000 surviving DA neurons per grafted putamen are needed to mediate clinical benefit, based on human fetal VM transplant data. A controlled dose-finding study with fetal tissue has previously not been possible, given severe issues with tissue availability and the inherent variation in tissue quality and preparation. Using manufactured stem cell-derived DA neurons, dose escalation studies can now be performed, such that the optimal number of cells can be deduced. Detailed analysis of the quantitative cellular composition of grafts from the clinical batches of STEM-PD was not requested by the authorities. However, as complementary data, experimental studies from our group have shown that stem-cell-derived VM grafts as well as in vitro organoids generated with same protocol as STEM-PD also contain other cell types including non-DA neurons, astrocytes, and vascular leptomeningeal cells (VLMCs).[46] The potential effect or function of these other cell types in the grafts is not known, although one study suggests that grafted astrocytes may be protective against alpha-synuclein pathology,[47] and data presented in this study suggest that mixing of non-DA neurons to the graft might be beneficial for maturation of the DA neurons. Future studies are required to elucidate this, and to determine if a controlled cellular composition may be harnessed for further improving graft function. Lastly, to enable upscaling and development of globally available therapies, refinements in manufacturing procedures, identification of validated potency markers, and exploration of safety assessments that rely less on long-term *in vivo* experiments are required. However, global regulatory consensus is needed, such that comparable data packages and standards can be applied for approval in the US, UK, EU, and Asia Pacific. With stem cell-based products now in clinical trials across three different continents, the prospects of such products going toward market authorization are getting closer.

Limitations of the study

The data presented in this study reflect the data package provided to the regulatory authorities in Sweden for the purpose of demonstrating preclinical safety and efficacy of the STEM-PD product to gain clinical trial authorization. Although additional experiments aimed at investigating biological or mechanistic details of the product may be of interest from a scientific point of view, further studies looking at this have not been requested by the authorities and have therefore not been performed. In general, preclinical assessment of clinical products does not involve exploratory studies, since each analysis performed on the product must have clear and predefined acceptance criteria that the product must adhere to. Furthermore, most of these analyses must be performed under highly controlled GMP/GLP conditions at outsourced facilities, which severely limits further scientific explorations. To investigate comparability of GMP manufacturing runs, ideally more than 2 production batches

should be tested *in vivo*; however, additional *in vivo* batch testing has not been possible due to the high costs incurred with manufacturing and testing. When setting the dose in STEM-PD, we made the assumption that the stem-cell-derived DA neurons have similar potency to fetal DA neurons based on previous experimental data[19] and the few individual animals in the efficacy study which contained a low number of TH^+ cells and yet demonstrated full recovery. The limited experimental data on potency, combined with potential host species differences in graft survival and differences in instrument used in rats and humans, bring uncertainty in the clinical dose calculation but are the best data available from the experimental models at hand. The clinical trial design presented here reflects the trial design at the initiation of the clinical study. Changes to the trial design may potentially occur during the study in agreement with the relevant authorities, if prompted by safety concerns, patient considerations, clinical data, or regulatory requirements.

CONSORTIA

The members of Novo Nordisk Cell Therapy R&D Consortium are Josefine Rågård Christiansen, Nicolaj Strøyer Christophersen, Simona Graziano, Kevin A. Keane, Ida Stenfeldt Mathiasen, Jonathan Christos Niclis, Lisbeth Palm, Sonja Pikkupeura, Lina A. Thorén, and Dorthe Bach Toft.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stem.2023.08.014.

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AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: trial conception and design, A.K., J.N., D.B.H., N.R., H.B., N.N.C.T., A.M.L., J.C.S., O.L., T.v.V., A.B., B.M., B.H., E.C., H.W., G.P., R.A.B., and M.P.; performance of experiments, data collection, analysis, and interpretation of results, A.K., J.N., D.B.H., N.R., H.B., N.N.C.T., A.F., A.F.A., T.C., S.V., J.M., P.S., Y.Z., A.M.L., T.P.L., M.L., C.C., O.B., B.M., and M.P.; draft manuscript preparation, A.K., M.P., and R.A.B. All authors reviewed the results, gave input to the manuscript, and approved the final version of the manuscript.

DECLARATION OF INTERESTS

M.P. is the owner of Parmar Cells AB. A.K. is the owner of Kirkeby Cell Therapy APS. M.P. and A.K. are co-inventors on patents WO2016162747A2/A3 and WO2019016113A1. M.P., A.K., R.A.B., H.W., H.B., A.B., E.C., D.B.H., G.P., and B.H. have performed paid consultancy for Novo Nordisk A/S, and members of NNCT R&D are current or previous employees of Novo Nordisk A/S. T.C., A.F.A., Y.Z., S.V., and D.B.H. performed the work as employees of Lund University but are currently employed by Novo Nordisk A/S (T.C., A.F.A., and S.V.), Takara Bio (Y.Z.), and D.B.H. at Eli Lilly and Company, where

she is also a minor share holder. Novo Nordisk A/S is developing the STEM-PD product for commercial use.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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REFERENCES

1. Kehagia, A.A., Barker, R.A., and Robbins, T.W. (2013). Cognitive impairment in Parkinson's disease: the dual syndrome hypothesis. Neurodegener. Dis. *11*, 79–92. https://doi.org/10.1159/000341998.
2. Bloem, B.R., Okun, M.S., and Klein, C. (2021). Parkinson's disease. Lancet *397*, 2284–2303. https://doi.org/10.1016/S0140-6736(21)00218-X.
3. Obeso, J.A., Rodriguez-Oroz, M.C., Chana, P., Lera, G., Rodriguez, M., and Olanow, C.W. (2000). The evolution and origin of motor complications in Parkinson's disease. discussion S21–S13. Neurology *55*, S13–S20.
4. Barker, R.A., and Björklund, A. (2023). Restorative cell and gene therapies for Parkinson's disease. Handb. Clin. Neurol. *193*, 211–226. https://doi.org/10.1016/B978-0-323-85555-6.00012-6.
5. Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K.L., Sawle, G., Rothwell, J.C., and Marsden, C.D. (1990). Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science *247*, 574–577.
6. Lindvall, O., Sawle, G., Widner, H., Rothwell, J.C., Björklund, A., Brooks, D., Brundin, P., Frackowiak, R., Marsden, C.D., and Odin, P. (1994). Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. Ann. Neurol. *35*, 172–180. https://doi.org/10.1002/ana.410350208.
7. Freed, C.R., Greene, P.E., Breeze, R.E., Tsai, W.Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, H., Culver, S., Trojanowski, J.Q., et al. (2001). Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N. Engl. J. Med. *344*, 710–719. https://doi.org/10.1056/NEJM200103083441002.
8. Kordower, J.H., Goetz, C.G., Freeman, T.B., and Olanow, C.W. (1997). Dopaminergic transplants in patients with Parkinson's disease: neuroanatomical correlates of clinical recovery. Exp. Neurol. *144*, 41–46. https://doi.org/10.1006/exnr.1996.6386.
9. Mendez, I., Sanchez-Pernaute, R., Cooper, O., Viñuela, A., Ferrari, D., Björklund, L., Dagher, A., and Isacson, O. (2005). Cell type analysis of functional fetal dopamine cell suspension transplants in the striatum and substantia nigra of patients with Parkinson's disease. Brain *128*, 1498–1510. https://doi.org/10.1093/brain/awh510.
10. Piccini, P., Pavese, N., Hagell, P., Reimer, J., Björklund, A., Oertel, W.H., Quinn, N.P., Brooks, D.J., and Lindvall, O. (2005). Factors affecting the clinical outcome after neural transplantation in Parkinson's disease. Brain *128*, 2977–2986. https://doi.org/10.1093/brain/awh649.
11. Kefalopoulou, Z., Politis, M., Piccini, P., Mencacci, N., Bhatia, K., Jahanshahi, M., Widner, H., Rehncrona, S., Brundin, P., Björklund, A., et al. (2014). Long-term clinical outcome of fetal cell transplantation for Parkinson disease: two case reports. JAMA Neurol. *71*, 83–87. https://doi.org/10.1001/jamaneurol.2013.4749.
12. Olanow, C.W., Goetz, C.G., Kordower, J.H., Stoessl, A.J., Sossi, V., Brin, M.F., Shannon, K.M., Nauert, G.M., Perl, D.P., Godbold, J., and Freeman, T.B. (2003). A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann. Neurol. *54*, 403–414. https://doi.org/10.1002/ana.10720.
13. Barker, R.A., Barrett, J., Mason, S.L., and Björklund, A. (2013). Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. Lancet Neurol. *12*, 84–91. https://doi.org/10.1016/S1474-4422(12)70295-8.
14. Barker, R.A.; TRANSEURO consortium (2019). Designing stem-cell-based dopamine cell replacement trials for Parkinson's disease. Nat. Med. *25*, 1045–1053. https://doi.org/10.1038/s41591-019-0507-2.
15. Kikuchi, T., Morizane, A., Doi, D., Onoe, H., Hayashi, T., Kawasaki, T., Saiki, H., Miyamoto, S., and Takahashi, J. (2011). Survival of human induced pluripotent stem cell-derived midbrain dopaminergic neurons in the brain of a primate model of Parkinson's disease. J. Parkinsons Dis. *1*, 395–412. https://doi.org/10.3233/JPD-2011-11070.
16. Kirkeby, A., Grealish, S., Wolf, D.A., Nelander, J., Wood, J., Lundblad, M., Lindvall, O., and Parmar, M. (2012). Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. Cell Rep. *1*, 703–714. https://doi.org/10.1016/j.celrep.2012.04.009.
17. Kirkeby, A., Nolbrant, S., Tiklova, K., Heuer, A., Kee, N., Cardoso, T., Ottosson, D.R., Lelos, M.J., Rifes, P., Dunnett, S.B., et al. (2017). Predictive markers guide differentiation to improve graft outcome in clinical translation of hESC-based therapy for Parkinson's disease. Cell Stem Cell *20*, 135–148. https://doi.org/10.1016/j.stem.2016.09.004.
18. Kriks, S., Shim, J.W., Piao, J., Ganat, Y.M., Wakeman, D.R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature *480*, 547–551. https://doi.org/10.1038/nature10648.
19. Grealish, S., Diguet, E., Kirkeby, A., Mattsson, B., Heuer, A., Bramoulle, Y., Van Camp, N., Perrier, A.L., Hantraye, P., Björklund, A., and Parmar, M. (2014). Human ESC-derived dopamine neurons show similar preclinical efficacy and potency to fetal neurons when grafted in a rat model of Parkinson's disease. Cell Stem Cell *15*, 653–665. https://doi.org/10.1016/j.stem.2014.09.017.
20. Doi, D., Magotani, H., Kikuchi, T., Ikeda, M., Hiramatsu, S., Yoshida, K., Amano, N., Nomura, M., Umekage, M., Morizane, A., and Takahashi, J. (2020). Pre-clinical study of induced pluripotent stem cell-derived dopaminergic progenitor cells for Parkinson's disease. Nat. Commun. *11*, 3369. https://doi.org/10.1038/s41467-020-17165-w.
21. Kim, T.W., Piao, J., Koo, S.Y., Kriks, S., Chung, S.Y., Betel, D., Socci, N.D., Choi, S.J., Zabierowski, S., Dubose, B.N., et al. (2021). Biphasic activation of WNT signaling facilitates the derivation of midbrain dopamine neurons from hESCs for translational use. Cell Stem Cell *28*, 343–355.e5. https://doi.org/10.1016/j.stem.2021.01.005.
22. Piao, J., Zabierowski, S., Dubose, B.N., Hill, E.J., Navare, M., Claros, N., Rosen, S., Ramnarine, K., Horn, C., Fredrickson, C., et al. (2021). Preclinical efficacy and safety of a human embryonic stem cell-derived midbrain dopamine progenitor product, MSK-DA01. Cell Stem Cell *28*, 217–229.e7. https://doi.org/10.1016/j.stem.2021.01.004.
23. BlueRock_Therapeutics (2021). BlueRock therapeutics in collaboration with memorial sloan kettering cancer center receives IND clearance for DA01 in Parkinson's disease. https://bluerocktx.com/bluerock-therapeutics-in-collaboration-with-memorial-sloan-kettering-cancer-center-receives-ind-clearance-for-da01-in-parkinsons-disease/.
24. CiRA (2017). News and events. https://www.cira.kyoto-u.ac.jp/e/pressrelease/news/180730-170000.html.
25. Schweitzer, J.S., Song, B., Herrington, T.M., Park, T.Y., Lee, N., Ko, S., Jeon, J., Cha, Y., Kim, K., Li, Q., et al. (2020). Personalized iPSC-derived dopamine progenitor cells for Parkinson's disease. N. Engl. J. Med. *382*, 1926–1932. https://doi.org/10.1056/NEJMoa1915872.
26. Lund_University (2022). First patient receives milestone stem cell-based transplant for Parkinson's disease. https://www.lunduniversity.lu.se/article/swedish-medical-products-agency-grants-approval-clinical-study-new-stem-cell-based-parkinsons. https://www.lunduniversity.lu.se/article/first-patient-receives-milestone-stem-cell-based-transplant-parkinsons-disease.
27. Nolbrant, S., Heuer, A., Parmar, M., and Kirkeby, A. (2017). Generation of high-purity human ventral midbrain dopaminergic progenitors for in vitro maturation and intracerebral transplantation. Nat. Protoc. *12*, 1962–1979. https://doi.org/10.1038/nprot.2017.078.

28. Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. *27*, 275–280. https://doi.org/10.1038/nbt.1529.

29. Bolon, B., Garman, R.H., Pardo, I.D., Jensen, K., Sills, R.C., Roulois, A., Radovsky, A., Bradley, A., Andrews-Jones, L., Butt, M., and Gumprecht, L. (2013). STP position paper: recommended practices for sampling and processing the nervous system (brain, spinal cord, nerve, and eye) during nonclinical general toxicity studies. Toxicol. Pathol. *41*, 1028–1048. https://doi.org/10.1177/0192623312474865.

30. Politis, M., Oertel, W.H., Wu, K., Quinn, N.P., Pogarell, O., Brooks, D.J., Bjorklund, A., Lindvall, O., and Piccini, P. (2011). Graft-induced dyskinesias in Parkinson's disease: high striatal serotonin/dopamine transporter ratio. Mov. Disord. *26*, 1997–2003. https://doi.org/10.1002/mds.23743.

31. Politis, M., Wu, K., Loane, C., Quinn, N.P., Brooks, D.J., Rehncrona, S., Bjorklund, A., Lindvall, O., and Piccini, P. (2010). Serotonergic neurons mediate dyskinesia side effects in Parkinson's patients with neural transplants. Sci. Transl. Med. *2*, 38ra46. https://doi.org/10.1126/scitranslmed.3000976.

32. Rath, A., Klein, A., Papazoglou, A., Pruszak, J., Garcia, J., Krause, M., Maciaczyk, J., Dunnett, S.B., and Nikkhah, G. (2013). Survival and functional restoration of human fetal ventral mesencephalon following transplantation in a rat model of Parkinson's disease. Cell Transplant. *22*, 1281–1293. https://doi.org/10.3727/096368912X654984.

33. Li, W., Englund, E., Widner, H., Mattsson, B., van Westen, D., Lätt, J., Rehncrona, S., Brundin, P., Björklund, A., Lindvall, O., and Li, J.Y. (2016). Extensive graft-derived dopaminergic innervation is maintained 24 years after transplantation in the degenerating parkinsonian brain. Proc. Natl. Acad. Sci. USA *113*, 6544–6549. https://doi.org/10.1073/pnas.1605245113.

34. Widner, H., Tetrud, J., Rehncrona, S., Snow, B., Brundin, P., Gustavii, B., Björklund, A., Lindvall, O., and Langston, J.W. (1992). Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). N. Engl. J. Med. *327*, 1556–1563. https://doi.org/10.1056/NEJM199211263272203.

35. Lindvall, O., Rehncrona, S., Brundin, P., Gustavii, B., Astedt, B., Widner, H., Lindholm, T., Björklund, A., Leenders, K.L., Rothwell, J.C., et al. (1989). Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. A detailed account of methodology and a 6-month follow-up. Arch. Neurol. *46*, 615–631.

36. Hardman, C.D., Henderson, J.M., Finkelstein, D.I., Horne, M.K., Paxinos, G., and Halliday, G.M. (2002). Comparison of the basal ganglia in rats, marmosets, macaques, baboons, and humans: volume and neuronal number for the output, internal relay, and striatal modulating nuclei. J. Comp. Neurol. *445*, 238–255. https://doi.org/10.1002/cne.10165.

37. Kirkeby, A., Parmar, M., and Barker, R.A. (2017). Strategies for bringing stem cell-derived dopamine neurons to the clinic: A European approach (STEM-PD). Prog. Brain Res. *230*, 165–190. https://doi.org/10.1016/bs.pbr.2016.11.011.

38. Ma, Y., Tang, C., Chaly, T., Greene, P., Breeze, R., Fahn, S., Freed, C., Dhawan, V., and Eidelberg, D. (2010). Dopamine cell implantation in Parkinson's disease: long-term clinical and (18)F-FDOPA PET outcomes. J. Nucl. Med. *51*, 7–15. https://doi.org/10.2967/jnumed.109.066811.

39. hPSCReg (2011). RCe021-A. https://hpscreg.eu/cell-line/RCe021-A.

40. Takahashi, J. (2020). iPS cell-based therapy for Parkinson's disease: A Kyoto trial. Regen. Ther. *13*, 18–22. https://doi.org/10.1016/j.reth.2020.06.002.

41. NIH (2021). Phase 1 Safety and Tolerability Study of MSK-DA01 Cell Therapy for Advanced Parkinson's Disease. https://classic.clinicaltrials.gov/ct2/show/NCT04802733.

42. Cardoso, T., Adler, A.F., Mattsson, B., Hoban, D.B., Nolbrant, S., Wahlestedt, J.N., Kirkeby, A., Grealish, S., Björklund, A., and Parmar, M. (2018). Target-specific forebrain projections and appropriate synaptic inputs of hESC-derived dopamine neurons grafted to the midbrain of parkinsonian rats. J. Comp. Neurol. *526*, 2133–2146. https://doi.org/10.1002/cne.24500.

43. Elabi, O.F., Pass, R., Sormonta, I., Nolbrant, S., Drummond, N., Kirkeby, A., Kunath, T., Parmar, M., and Lane, E.L. (2022). Human embryonic stem cell-derived dopaminergic grafts alleviate L-dopa induced dyskinesia. J. Parkinsons Dis. *12*, 1881–1896. https://doi.org/10.3233/JPD-212920.

44. Grealish, S., Heuer, A., Cardoso, T., Kirkeby, A., Jönsson, M., Johansson, J., Björklund, A., Jakobsson, J., and Parmar, M. (2015). Monosynaptic tracing using modified rabies virus reveals early and extensive circuit integration of human embryonic stem cell-derived neurons. Stem Cell Rep. *4*, 975–983. https://doi.org/10.1016/j.stemcr.2015.04.011.

45. Steinbeck, J.A., Choi, S.J., Mrejeru, A., Ganat, Y., Deisseroth, K., Sulzer, D., Mosharov, E.V., and Studer, L. (2015). Optogenetics enables functional analysis of human embryonic stem cell-derived grafts in a Parkinson's disease model. Nat. Biotechnol. *33*, 204–209. https://doi.org/10.1038/nbt.3124.

46. Tiklová, K., Nolbrant, S., Fiorenzano, A., Björklund, Å.K., Sharma, Y., Heuer, A., Gillberg, L., Hoban, D.B., Cardoso, T., Adler, A.F., et al. (2020). Single cell transcriptomics identifies stem cell-derived graft composition in a model of Parkinson's disease. Nat. Commun. *11*, 2434. https://doi.org/10.1038/s41467-020-16225-5.

47. Yang, Y., Song, J.J., Choi, Y.R., Kim, S.H., Seok, M.J., Wulansari, N., Darsono, W.H.W., Kwon, O.C., Chang, M.Y., Park, S.M., and Lee, S.H. (2022). Therapeutic functions of astrocytes to treat alpha-synuclein pathology in Parkinson's disease. Proc. Natl. Acad. Sci. USA *119*, e2110746119. https://doi.org/10.1073/pnas.2110746119.

48. Christensen, A.B., Sørensen, J.C.H., Ettrup, K.S., Orlowski, D., and Bjarkam, C.R. (2018). Pirouetting pigs: A large non-primate animal model based on unilateral 6-hydroxydopamine lesioning of the nigrostriatal pathway. Brain Res. Bull. *139*, 167–173. https://doi.org/10.1016/j.brainresbull.2018.02.010.

49. Glud, A.N., Bech, J., Tvilling, L., Zaer, H., Orlowski, D., Fitting, L.M., Ziedler, D., Geneser, M., Sangill, R., Alstrup, A.K.O., et al. (2017). A fiducial skull marker for precise MRI-based stereotaxic surgery in large animal models. J. Neurosci. Methods *285*, 45–48. https://doi.org/10.1016/j.jneumeth.2017.04.017.

50. Lillethorup, T.P., Glud, A.N., Alstrup, A.K.O., Mikkelsen, T.W., Nielsen, E.H., Zaer, H., Doudet, D.J., Brooks, D.J., Sørensen, J.C.H., Orlowski, D., and Landau, A.M. (2018). Nigrostriatal proteasome inhibition impairs dopamine neurotransmission and motor function in minipigs. Exp. Neurol. *303*, 142–152. https://doi.org/10.1016/j.expneurol.2018.02.005.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ALDH1A1: Rabbit polyclonal anti-ALDH1A1	Abcam	Cat#ab24343, RRID:AB_2224007
FOXA2: Recombinant human monoclonal anti-FOXA2 (clone REA506), APC	Miltenyi Biotec	Cat#130-123-850, RRID:AB_2819525
FOXA2: Polyclonal goat anti-FOXA2	R&D Systems	Cat#AF2400, RRID:AB_2294104
GIRK2: Rabbit polyclonal anti-GIRK2	Alomone Labs	Cat#APC-006, RRID:AB_2040115
Ki67: Rabbit polyclonal anti-Ki67	Novus	Cat#NB110-89717, RRID:AB_1217074
KU-80: Rabbit monoclonal anti-KU80 (clone C48E7)	Cell Signaling Technology	Cat#2180, RRID:AB_2218736
LMX1A: Rabbit polyclonal anti-LMX1A	Millipore	Cat#AB10533, RRID:AB_10805970
NANOG: Mouse monoclonal anti-Nanog (clone 23D2-3C6), Alexa Fluor® 647	BioLegend	Cat#674010, RRID:AB_2632605
NCAM: Mouse monoclonal anti-NCAM (clone ERIC 1)	Santa Cruz Biotechnology	Cat#sc-106, RRID:AB_627128
NCAM: Rabbit monoclonal anti-NCAM1 (clone EP2567Y)	Abcam	Cat#ab75813, RRID:AB_2632384
OTX2: Recombinant human monoclonal anti-OTX2 (clone REA1178), VioB515	Miltenyi Biotec	Cat#130-121-193, RRID:AB_2801807
PAX6: Recombinant human monoclonal anti-PAX6 (clone REA507), PE	Miltenyi Biotec	Cat#130-123-250, RRID:AB_2819456
PDGFRB: Recombinant human anti-CD140b (clone REA363), PE	Miltenyi Biotec	Cat#130-123-772, RRID:AB_2819521
SOX1: Recombinant human monoclonal anti-SOX1 (clone REA698), APC	Miltenyi Biotec	Cat#130-111-044, RRID:AB_2653491
SOX2: Mouse monoclonal anti-SOX2 (clone O30-678), V450	BD Biosciences	Cat#561610, RRID:AB_10712763
SOX17: Mouse monoclonal anti-SOX17 (clone P7-969), Alexa Fluor® 488	BD Biosciences	Cat#562205, RRID:AB_10893402
OCT3/4: Mouse monoclonal anti-OCT3/4 (clone 40/Oct-3), PE	BD Biosciences	Cat#560186, RRID:AB_1645331
TH: Rabbit polyclonal anti-Tyrosine Hydroxylase	Millipore	Cat#AB152, RRID:AB_390204
TH: Sheep polyclonal anti-Tyrosine Hydroxylase	Millipore	Cat#AB1542, RRID:AB_90755
TH: Rabbit polyclonal anti-Tyrosine Hydroxylase	Abcam	Cat#ab112, RRID:AB_297840
VE-Cadherin: Recombinant human anti-CD144 (clone REA199), FITC	Miltenyi Biotec	Cat# 130-123-688, RRID:AB_2819510
5-HT: Rabbit polycloncal anti-5-HT antibody	Immunostar	Cat#20080 RRID: AB_572263
Chemicals, peptides, and recombinant proteins		
iPS Brew GMP Basal Medium and Supplement	Miltenyi Biotec	Cat#170-076-317, Cat#170-076-318
MACS® GMP Recombinant Human TGF-β1	Miltenyi Biotec	Cat#170-076-166
Recombinant Human Laminin 521 CTG	Biolamina	Cat#CT521

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant Human Laminin 111	Biolamina	Cat#LN111
CryoStor® CS10	Stem Cell Technologies	Cat#07930
StemPro™ Accutase™	Thermo Fisher Scientific	Cat#A1110501
UltraPure™ 0.5M EDTA	Thermo Fisher Scientific	Cat#15575020
DMEM/F-12 medium	Thermo Fisher Scientific	Cat#21331-020
CTS™ Neurobasal™ Medium	Thermo Fisher Scientific	Cat#A13712-01
L-Glutamine	Thermo Fisher Scientific	Cat#25030-081
CTS™ N-2 Supplement	Thermo Fisher Scientific	Cat#A13707-01
CTS™ B-27 Supplement without vitamin A	Thermo Fisher Scientific	Cat#A3353501
StemMACS™ Y27632	Miltenyi Biotec	Cat#130-106-538, CAS:129830-38-2
SB431542	Miltenyi Biotec	Cat#130-106-543, CAS:301836-41-9
CHIR99021	Miltenyi Biotec	Cat#130-106-539, CAS:252917-06-9
Recombinant Human Noggin, GMP	R&D Systems	Cat#6057-GMP
Recombinant Human Sonic Hedgehog (C24II), GMP	R&D Systems	Cat#1845-GMP
Recombinant Human BDNF, GMP	R&D Systems	Cat#248-GMP
Recombinant Human FGF8b, Premium Grade	R&D Systems	Cat#130-095-740
L-Ascorbic Acid	Sigma	Cat#A4403-100MG, CAS:50-81-7
Zenalb® 4.5 (Human Serum Albumin 4.5%)	Bio Products Laboratory Ltd	N/A
Zenalb® 20 (Human Serum Albumin 20%)	Bio Products Laboratory Ltd	N/A
Dimethyl Sulfoxide (DMSO)	WAK-Chemie Medical GmbH	Cat#WAK-DMSO, CAS:67-68-5
LIVE/DEAD™ Fixable Violet Dead Cell Stain	Thermo Fisher Scientific	Cat#L34955
UltraComp eBeads™ Compensation Beads	Thermo Fisher Scientific	Cat#01-2222-41
MACS® Comp Bead Kit, anti-REA	Miltenyi Biotec	Cat#130-104-693
Transcription Factor Buffer Set	BD Biosciences	Cat#562574
LightCycler® 480 SYBR Green I Master	Roche Life Sciences	Cat#04707516001
Pulmozyme® (Dornase alpha, 2500U/2.5ml) ampoules	Roche	N/A
6-OHDA	Sigma	Cat# H4381
Amphetamine	Skåne University Hospital Pharmacy	Item no. 699258
Critical commercial assays		
HumanCytoSNP-12 v2.1 BeadChip Kit	Illumina	Cat#WG-320-2101
QIAamp DNA Mini Kit	QIAGEN	Cat#51306
AmpliSeq™ Cancer HotSpot Panel v2	Illumina	Cat#20019161
RNeasy Micro Kit	QIAGEN	Cat#74004
Maxima First Strand cDNA Synthesis Kit	Thermo Fisher Scientific	Cat#K1641
StemMACS™ Trilineage Differentiation Kit	Miltenyi Biotec	Cat#130-115-660
Deposited data		
CytoSNP data on MCB and STEM-PD	This paper, https://www.ncbi.nlm.nih.gov/geo	GSE229769
Illumina cancer Hot-spot panel data on MCB	This paper, https://www.ncbi.nlm.nih.gov/geo	GSE229769
Experimental models: Cell lines		
hPSCReg RCe021-A (RC17), passage 12, 46XX	Roslin Cells Ltd (now available through University of Edinburgh)	RRID:CVCL_L206
Experimental models: Organisms/strains		
Hsd:RH-*Foxn1*^{rnu} nude rats (female)	Envigo, France	Item no. 505F
Göttingen minipig (female)	Ellegaard Minipigs ApS	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primers for qRT-PCR, see Table S9	This paper	N/A
Software and algorithms		
Beeline, version 2.0.3.3	Illumina	https://support.illumina.com/downloads/beeline-software-2-0.html
BlueFuse Multi, version 4.5	Illumina	https://support.illumina.com/downloads/bluefuse-multi-software-download.html
Illumina Variant Studio, version 3.0	Illumina	https://support.illumina.com/sequencing/sequencing_software/variantstudio/downloads.html
FACSDiva™, version 9.0	BD Biosciences	https://www.bdbiosciences.com/en-us/products/software/instrument-software/bd-facsdiva-software
FlowJo™, version 10.6.2	BD Biosciences	https://www.flowjo.com/solutions/flowjo/downloads/previous-versions
GraphPad Prism 9	GraphPad Software, Inc.	https://www.graphpad.com/updates/prism-900-release-notes
Other		
STEM-PD Clinical trial design, NCT05635409	STEM-PD clinical protocol, deposited at NIH clinical trial registry	https://clinicaltrials.gov/ct2/show/NCT05635409

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Malin Parmar (malin.parmar@med.lu.se).

Materials availability

The RC17 cell line is available under an MTA agreement with the University of Edinburgh and the UK Stem Cell Bank. The GMP-grade STEM-PD product is a clinical product which is currently not available for distribution, however, research-grade versions of the product may be supplied under a collaboration agreement. This study did not generate any other new unique reagents.

Data and code availability

- Raw data for the Illumina HumanCytoSNP-12 v2.1 array and the Illumina Cancer Hot-Spot Panel v2 sequencing are deposited and publicly available at https://www.ncbi.nlm.nih.gov/geo. Accession numbers are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines

GMP-grade RC17 hESCs (hPSCReg RCe021-A, 46XX, passage 12) were obtained from Roslin Cells Ltd., and a master cell bank (MCB) was generated at passage 14 at the Royal Free Hospital (London, United Kingdom). A vial from a parallel research-grade stock of the same cell line was also obtained from Roslin Cells at passage 20 and applied for research-grade studies (i.e. single nuclear sequencing of grafts in this study).

Animals and housing conditions, GLP safety study

The GLP safety study was performed at Covance Laboratories Ltd in Huntingdon, UK, study no. RC53QY for the main toxicity, tumourigenicity and biodistribution study, and study no. 8433861 for RC17 hESC positive tumour control group. A total of 134 athymic nude rats (Hsd:RH-Foxn1^{rnu} strain from Envigo RMS UK Ltd., 68 males and 66 females) were used for these studies, and the animals were housed in individually ventilated cages (IVC), with up to 4 animals of the same sex per cage, at 20-24^{o}C with a 12h:12h light/dark cycle and unrestricted access to sterilized water and food pellets. Interventions were performed after a minimum of 16 weeks of age. All experimental groups contained male and female animals to allow for detection of potential sex-specific responses, and all study animals were subject to weekly assessments of body weight, food consumption and palpation for peripheral tumour masses.

Animals and housing conditions, non-GLP efficacy study

The non-GLP efficacy study was performed in the lab of Malin Parmar, Lund University, Sweden under ethical permit no. M-8579/2017. A total of 88 female athymic nude rats (Hsd:RH-Foxn1^{rnu} strain from Envigo RMS UK Ltd.) were used for this study, and the animals were housed in individually ventilated cages (IVC) with up to 2-3 animals per cage at room temperature with a 12h:12h light/dark cycle and unrestricted access to sterilized water and food pellets. Interventions were performed after 12 weeks of age.

Minipig model for device testing

The Danish Animal Experiments Inspectorate (2016-15-0201-00878) approved the minipig study and all procedures were conducted in compliance with the 2010/63/EU directive for animal experiments and reported according to the ARRIVE guidelines. One female Göttingen minipig (6.2 months, 16.4 kg) from Ellegaard Minipigs ApS (Dalmose, Denmark) was housed with a cage-mate (4.6 m^2, humidity 50-55% and temperature 20°C) at the Aarhus University farm with free access to tap water and fed with hay and a restricted pellet diet.

METHOD DETAILS

STEM-PD manufacturing

The RC17 MCB and the STEM-PD product were both manufactured under GMP by the Centre for Cell, Gene and Tissue Therapeutics, Royal Free Hospital (London, United Kingdom). The cells were cultured in humidified temperature- and gas-controlled incubators at 37°C and 5% CO2. For MCB manufacturing, a vial of GMP-grade RC17 hESCs (hPSCReg RCe021-A, passage 12) from Roslin Cells Ltd. (Edinburgh, United Kingdom) was thawed and expanded in iPS Brew medium (Miltenyi Biotec, GMP-grade) on laminin-521 (1 μg/cm^2, Biolamina, cell therapy grade). The cells were expanded for two passages with daily media changes and cryopreserved in CryoStor® CS10 cryopreservation medium (Stem Cell Technologies). For STEM-PD manufacturing, RC17 MCB cells were thawed on day -4 of differentiation and seeded onto laminin-521-coated T25 flasks at 13,000 cells/cm^2 in iPS Brew medium with 10 μM Y27632 (Miltenyi Biotec). The differentiation protocol applied for STEM-PD manufacturing is a scaled-up version of a previously published protocol.[27] On day 0 of differentiation, the hESCs were dissociated with 0.5 mM EDTA (Thermo Fisher Scientific) and seeded onto T75 flasks coated with laminin-111 (2 μg/cm^2, Biolamina) at 8,000 cells/cm^2 in N2 medium consisting of a 1:1 mix of DMEM/F12 medium (Thermo Fisher Scientific) and CTS Neurobasal medium (Thermo Fisher Scientific) supplemented with 1% CTS N2 (Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific), with 10 μM SB431542 (Miltenyi Biotec), 100 ng/mL Noggin (R&D Systems, GMP-grade), 0.7 μM CHIR99021 (Miltenyi Biotec), 200 ng/mL SHH-C24II (R&D Systems, GMP-grade), and 10 μM Y27632. The medium was changed on days 2, 4, and 7, without Y27632. On day 9, the medium was changed to N2 medium supplemented with 100 ng/mL FGF8b (Miltenyi Biotec, premium grade). On day 11, the cells were dissociated with Accutase (Thermo Fisher Scientific) and replated onto T175 flasks coated with laminin-111 at 800,000 cells/cm^2 in B27 medium consisting of CTS Neurobasal medium, 2% CTS B27 without vitamin A (Thermo Fisher Scientific), and 2 mM L-glutamine with 100 ng/mL FGF8b, 20 ng/mL BDNF (R&D Systems, GMP-grade), 200 μM L-ascorbic acid (Sigma), and 10 μM Y27632. The medium was changed on day 14, without Y27632. After 16 days of differentiation, the cells were dissociated with Accutase and cryopreserved in vials of 5×10^6 cells in a cryopreservation medium consisting of N2 medium supplemented with 2% CTS B27 without vitamin A and 10% DMSO (WAK-Chemie Medical). MCB and STEM-PD cryovials were stored in a temperature-monitored vapour-phase nitrogen tank at the Royal Free Hospital.

Genetic testing on MCB and STEM-PD

Karyotyping analysis of expanded MCB cells was carried out under GMP by BioReliance Ltd (UK) by fixing live cells in metaphase and a total of 50 cells in metaphase were analysed by standard G-banding by microscopical inspection of the chromosomes for aberrations. STR analysis of the MCB and STEM-PD was performed by determining the alleles of 20 autosomal short tandem repeat (STR) markers (D3S1358, D1S1656, D6S1043, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433 and FGA) and a sex chromosome marker (AMEL). The STR profile of STEM-PD was compared to the STR profile of the MCB and the initial RC17 cell line from Roslin Cells for confirmation of identity. For assessment of potential novel copy number variations (CNVs) or copy neutral loss of heterozygosity (CN-LOH) events, both the MCB as well as one batch of STEM-PD was analysed by CytoSNP analysis. To assess the genetic stability of the MCB and STEM-PD cells over time, the MCB was analysed directly after thawing as well as after expansion for an additional 2 and 4 passages. Likewise, STEM-PD cells were analysed after thawing and additionally after 14 days of extended culturing. The CytoSNP analysis was done using the Illumina HumanCytoSNP-12 v2.1. gDNA was extracted using a QIAamp DNA Mini Kit (QIAGEN) and arrays were scanned with an Illumina iScan System. The raw data files were analysed using Beeline 2.0.3.3 and BlueFuse Multi v4.5 software. Copy number variations (CNVs) were identified using the log R ratio and B allele frequency. CNV regions >75Kb and copy-neutral loss of heterozygosity (CN-LOH) >5Mb were reported. The list of genes encompassing each CNV/CN-LOH region was cross-referenced to public databases to look for any association with human malignancies and/or diseases. CNVs were further cross-references to the Database of Genomic Variants for known constitutive genomic variants. The presence of somatic mutations in potential oncogenes was investigated in the RC17 MCB, in two different STEM-PD GMP batches, by targeted next generation sequencing (NGS) with the Illumina Cancer Hot-Spot Panel v2. This Panel covers approximately 2,800 COSMIC mutations in 50 cancer and tumour suppressor genes: *ABL1, JAK3, AKT1, KDR, ALK, KIT, APC, KRAS, ATM, MET, BRAF,MLH1, CDH1,MPL, CDKN2A, NOTCH1, CSF1R, NPM1,*

CTNNB1, NRAS, EGFR, PDGFRA, ERBB2, PIK3CA, ERBB4, PTEN, EZH2, PTPN11, FBXW7, RB1, FGFR1, RET, FGFR2, SMAD4, FGFR3, SMARCB1, FLT3, SMO, GNA11, SRC, GNAQ, STK11, GNAS, TP53, HNF1A, VHL, HRAS, IDH1, IDH2, JAK2, with a sensitivity of 5% mosaicism. This analysis identified only benign genomic variants across all analysed samples, allowing us to conclude that there were no detectable oncogenic variants in the cell line or the manufactured product. To ensure that the RC17 cell line was free from PD-associated mutations which might compromise DA neuron function, DNA from the RC17 MCB cells was sequenced for pathogenic variants in a panel of genes known to be associated with PD and complex parkinsonism. The panel involved whole-exome sequencing of 31 PD-associated genes at a depth of minimum 20X. The genes in the panel were: *ATP13A2, ATP1A3, CSF1R, DCTN1, DNAJC6, FBXO7, FTL, GCH1, GRN, LRRK2, LYST, MAPT, OPA3, PANK2, PARK7, PINK1, PLA2G6, PRKN, PRKRA, RAB39B, SLC30A10, SLC6A3, SNCA, SPG11, SPR, SYNJ1, TH, TUBB4A, VPS13A, VPS35, WDR45*. From this analysis, no clear pathogenic variants were detected.

Flow cytometry release testing

The STEM-PD product was characterised by flow cytometry analysis using three panels of fluorophore-conjugated antibodies against on-target ventral midbrain markers FOXA2 (clone REA506, Miltenyi Biotec) together with OTX2 (clone REA1178, Miltenyi Biotec), off-target neural markers PAX6 (clone REA507, Miltenyi Biotec) together with SOX1 (clone REA698, Miltenyi Biotec), and pluripotency markers OCT3/4 (clone 40/Oct-3, BD Biosciences) together with NANOG (clone 23D2-3C6, BioLegend). Cryovials of STEM-PD were thawed with the automated ThawSTAR Thawing System (Biocision), and after washing and counting with an automated cell counter (NC-200, Chemometec), the cells were stained with a violet LIVE/DEAD™ Fixable Dead Cell Stain (Thermo Fisher Scientific) for 15 minutes at room temperature and then fixed and permeabilised using the Transcription Factor Buffer Set (BD Biosciences) according to the manufacturer's instructions. For the pluripotency panel and on- and off-target panels, 1.0×10^6 and 0.5×10^6 cells, respectively, were stained by incubation with 100 μL of an antibody cocktail per 0.5×10^6 cells in 1X Perm/Wash buffer (BD Biosciences) for 30 minutes at 4°C, washed once with 1X Perm/Wash buffer, then washed once with FACS buffer (1% bovine serum albumin in PBS without Ca^{2+} and Mg^{2+}), and finally resuspended in 200 μL of FACS buffer per 0.5×10^6 cells. The stained cells were acquired on a BD Celesta flow cytometer (BD Biosciences) with BD FACSDiva™ software (BD Biosciences) and 50,000–100,000 single live cell events were recorded per sample. Compensation was performed using UltraComp eBeads (Thermo Fisher Scientific) and Anti-REA Compensation Beads (Miltenyi Biotec). FCS files were exported and analyzed using FACS Diva™ software. Debris, doublets, and dead cells were filtered out, and the fluorescent channels were gated based on fluorescence-minus-one (FMO) control samples. Cells from the MCB were analysed using the same method but applying only the pluripotency panel (OCT3/4 and NANOG).

Quantitative real-time PCR

RNA was isolated from STEM-PD cells using RNeasy Micro kit (both from Qiagen), running on a QIAcube instrument, according to the manufacturer's procedures. Reverse transcription was performed with random hexamer primers and a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) using up to 1 μg of RNA from each sample. The complementary DNA was pipetted onto a 384-well plate, together with SYBR green Mastermix (Roche Life Sciences) and primers using an automated liquid handler (I.DOT One, Dispendix). Samples were analyzed by real-time quantitative PCR on a LightCycler 480 instrument (Roche Life Sciences) using a two-step protocol with a 60°C annealing/elongation step, for 40 cycles (Ct calculations capped at 35). All qRT-PCR samples were run in duplicate wells, and the averaged Ct values were used for calculations. For each gene, the fold change was calculated as the average fold change relative to undifferentiated RC17 hESCs using the ΔΔCt method against two different housekeeping genes (ACTB and GAPDH). Each sample was analysed at least twice in separate qRT-PCR runs, representing separate dots in the graphs. Primer sequences are provided in Table S9.

Immunocytochemistry for FOXA2 and LMX1A

STEM-PD cells were thawed and cultured for 2 days in B27 medium supplemented with 20 ng/mL BDNF, 200 μM L-ascorbic acid and 10 μM Y27632 prior to fixation and immunofluorescent labelling for key vmDA markers LMX1A and FOXA2. The cells were fixed in 4% paraformaldehyde (PFA) for 15 min and washed three times in PBS (Gibco). The fixed cells were incubated with primary antibodies, goat anti-FOXA2 (1.1000, R&D AF2400) and rabbit anti-LMX1A (1:1000, Millipore 10533) diluted in blocking solution ($PBS^{-Ca2+/-Mg2++}$ + 0.1% Triton X-100 + 5% donkey serum) at 4°C overnight followed by 3x wash, 30 minutes pre-incubation in blocking solution and 2 h incubation with a fluorophore-conjugated secondary antibody (Molecular Probes or Jackson Laboratories, 1:200 in blocking solution) at room temperature.

STEM-PD stability testing

STEM-PD batches were placed in a stability programme prior to trial initiation. The batches will be monitored at long-term storage condition in vapour phase liquid nitrogen at < -135°C, for up to 60 months. The stability programme contains tests for appearance, purity, impurity, yield, viability, sterility and endotoxin, the first five being stability indicating parameters as they are most likely to change systematically. Sterility and endotoxin are tested at a minimum at the beginning and the end of the stability study.

Trilineage test

Trilineage differentiation of MCB cells was performed using the StemMACS Trilineage kit from Miltenyi following the manufacturer's instructions, with optimised coating and seeding conditions. After differentiation, the cells were harvested using Accutase (StemPro)

and fixed using 4% formaldehyde (Cell Signaling) prior to flow cytometry analysis for detection of ectoderm (PAX6, 1:50, Miltenyi and SOX2, 1:50, BD Biosciences), endoderm (SOX17, 1:100, BD Biosciences and FOXA2, 1:80, Miltenyi) and mesoderm (CD140b, 1:50, Miltenyi and CD144, 1:50, Miltenyi). The cells were stained by incubation for 30 minutes at room temperature and protected from light. The stained cells were acquired on a BD A5 Symphony flow cytometer (BD Biosciences) with BD FACSDiva™ software (BD Biosciences) and 20,000-single live cell events were recorded per sample. Compensation was performed using UltraComp eBeads (Thermo Fisher Scientific) and Anti-REA Compensation Beads (Miltenyi Biotec). FCS files were exported and analysed using FlowJo™ software. Debris and doublets cells were gated out, and the quadrant gate on fluorescent channels was based on a control sample fixed before the start of the trilineage differentiation, i.e., the undifferentiated MCB sample.

Procedure for preparation of STEM-PD cells for transplantation and device testing

To prepare STEM-PD cells for transplantation in both the GLP safety study and the non-GLP efficacy study for device testing, we applied the same washing and resuspension procedure as is applied in the clinical trial for patient cell preparation. STEM-PD cells were thawed using a ThawStar automated cell thawing system (BioCision), and then washed twice in wash buffer consisting of Hank's Balanced Salt Solution (HBSS, Thermo Fisher or Lonza) without Ca^{2+} and Mg^{2+}, supplemented with 0.5% human serum albumin (ZENALB, Bio Products Laboratory). During a second wash, prior to centrifugation, cells were counted using an automated NC-200 Nucleocounter (ChemoMetec), and the resulting cell pellet was resuspended in delivery vehicle (HBSS without Ca^{2+} and Mg^{2+}, supplemented with 20 U/ml Pulmozyme from Roche) at a concentration of 75,000-116,000 cells/μl. During the transplantation procedure, cells were kept at a cooled environment (2-8°C) for up to 10 hours.

Surgical procedures, GLP safety study

Surgery was conducted under isofluorane gaseous anesthesia. A bupivacaine/lidocaine solution was injected subcutaneous (s.c.) as local anesthesia. The animals were placed in a stereotactic frame and adjusted to a "flat head" position (dorsoventral vertical difference between bregma and lambda is ±0.2 mm or less). Cell implantations were performed with a glass capillary attached to a blunt needle of a Hamilton syringe. Intrastriatal cell transplantations of 700,000 cells were made via two tracts with two deposits per tract (1.5 μl/deposit, 117,000 cells/μL, 1 μL/min, 2 min diffusion time per deposit) using the following coordinates: A/P: +0.9, M/L: -3.0, D/V: -4.0/-5.0 and A/P: +1.4, M/L: -2.6, D/V: -4.0/-5.0

GLP biodistribution testing

Tissues were collected from the animals from the GLP safety study at necropsy (see Table S5 for full list of collected tissues) for assessment of the presence of human DNA by qPCR analysis. Each organ was cut in half upon collection, and one half was used for histopathology whereas the other half was used for qPCR. In case of bilateral organs, one organ on each side was allocated to either qPCR or histopathology. To avoid contamination of brain samples with fibers from the intrastriatal graft cells, the most lateral 2/3 of the untransplanted hemisphere was collected for qPCR analysis to assess the potential biodistribution of human cells to the contralateral side of the brain. DNA was extracted from each tissue and 1 μg of DNA (or 5 μl of eluate in case of liquid samples) was used for each qPCR reaction. Full validation of the DNA extraction and qPCR procedure was performed prior to analysis, and the method was determined to reliably detect human DNA in a rat DNA background with a sensitivity of LoD = 0.1 human cell per 1 μg of rat DNA and LoQ = 1 human cell per 1 μg of rat DNA. Technical triplicates were performed on all qPCR samples, and data was reported as the mean value of the triplicate values. A Taqman probe was applied for AluY detection, and the PCR reaction was performed with the following primers/probe: Alu Y, forward: 5'-CCGAGGCGGGCTGATC-3', Alu Y, reverse: 5'-TCACTGTGT TAGCCAGGATGGT-3', Alu Y, probe: 5'6FAM-CTA GGT CAG GAG ATC GA-MGB 3'

Surgical procedures, efficacy study

All surgical procedures on the nude rats for the efficacy study took place under general anesthesia by intraperitoneal (i.p.) injection of a mixture of ketaminol® (ketamine hydrochloride, 45 mg/kg) and domitor® (medetomidine, 0.3 mg/kg) according to weight. Marcain® (bupivacaine, 0.1 ml) was injected s.c. as local anesthesia. A post-operative s.c. injection of Antisedan® (atipamezole, 0.28 mg/kg) and Temgesic® (buprenorphine, 0.04 mg/kg) was administered for anesthesia-reversal and analgesia, respectively. The animals were placed in a stereotactic frame and adjusted to a "flat head" position (dorsoventral vertical difference between bregma and lambda is ±0.2 mm or less). Surgical procedures were performed with a glass capillary attached to a blunt needle of a Hamilton syringe. 6-OHDA (3.5 μg/μl free base, 3 μL, 0.3 μL/min, no diffusion time) lesions were carried out in the MFB with the following coordinates A/P: -3.9, M/L: -1.2, D/V: -7.3 Intrastriatal cell transplantations were made via two tracts with two deposits per tract (1 μl/deposit, 75.000 cells/μL, 1 μL/min, 2 min diffusion time per deposit) using the following coordinates: A/P: +0.9, M/L: -3.0, D/V: -4.0/-5.0 and A/P: +1.4, M/L: -2.6, D/V: -4.0/-5.0 and intranigral in one tract A/P: -4.9, M/L: -2.3, D/V: -6.4

Amphetamine-induced rotations

Amphetamine-induced rotation tests were used to evaluate the extent of the 6-OHDA lesions at a minimum of 3 weeks after lesioning, and to assess potential recovery after transplantation. The rats were injected with amphetamine (3.5 mg/kg) i.p., placed in a harness and put in a bowl which is connected to an automated counter that records their rotations over 90 minutes. Clockwise turns were recorded with a positive value and counter-clockwise turns were recorded with a negative value. The criterion for a complete lesion was set to $\geq$5 net clockwise turns per minute over a 90-minute period. Only animals with complete lesions were included in Group 1

for assessment of transplant efficacy. The lesion was confirmed with histology post-mortem. For full motor recovery there should be a statistically significant decrease in net turns per minute on a group level combined with the requirement that more than half the group should have a net turn value of 2 or less. Animals with surviving grafts (e.g. evidence of graft detection based on hNCAM staining) and appropriate graft placement (defined as majority of cells placed in the dorso-lateral striatum) were retained in study and used to determine graft efficacy. Out of a total group size of n=20 for Group 1 with STEM-PD batch #3, 2 animals were prematurely euthanized and were not included in the rotational analysis. An additional two animals were found at post-mortem to have graft placement outside of the target site in the striatum, and their data was excluded from the rotational analyses. For STEM-PD batch #1, 5 out 26 animals from Group 1 were prematurely euthanized and 1 animal was excluded due to poor graft survival.

Immunohistochemistry, efficacy study

At the end of the experiment, the animals were terminally anaesthetised with 1.2 mL pentobarbital injected i.p. and perfused transcardially with room temperature 0.9% saline solution for 3–5 min followed by ice-cold 4% PFA solution (pH 7.4 ± 0.2) for 5 minutes. Thereafter, the brains were taken out and put in 4% PFA at 4°C for 24 hours. Subsequently, the PFA was poured off and the brains were put in 25% sucrose solution at 4°C for 2-3 days until the brains had sunk to the bottom of the vial. The brains were sectioned coronally on a freezing microtome at 35 μm thickness in a 1:8 series. Sections that were analyzed immediately were put in 0.1M KPBS and sections analyzed later were put in antifreeze and stored at -20°C until analysis.

Sections were incubated with primary antibodies overnight in 0.1 M KPBS solution containing 0.25% Triton-X and 5% serum for the species specific to the secondary antibody. Sections were then incubated with fluorophore-conjugated (fluorescent detection) or biotin-coupled (DAB detection) secondary antibodies for 1 hr in the same solution. All stained sections were mounted on gelatin-coated microscope slides. Fluorescent sections were coverslipped using polyvinyl alcohol mounting medium with DABCO (Sigma-Aldrich). DAB-developed sections were dehydrated in an ascending series of alcohols, cleared with xylene, coverslipped using DPX mountant and left to dry overnight.

The primary antibodies for DAB staining were mouse anti-hNCAM (1:1000, Santa Cruz Biotechnology sc106), rabbit anti-TH (1:2000, Merck Millipore ab152) rabbit anti-5-HT (1:10,000, Immunostar 20080). The sections were incubated with secondary biotinylated antibodies (1:200, Vector Laboratories). The primary antibodies for fluorescent immunolabeling were: rabbit anti-TH (1:2000, Merck Millipore ab152), sheep anti-TH (1:1000, Merck Millipore ab1542), rabbit anti-GIRK2 (1:2000, Alamone APC 006), goat anti-FOXA2 (1:500 R%D systems AF2400) and rabbit anti-ALDH1A1 (1:1000 Abcam AB24343). The sections were incubated with fluorophore-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories)

Immunohistochemical quantification, efficacy study

Images of hNCAM stained coronal sections were taken at the level of the striatum by an Epson Perfection V850 Pro scanner. To determine the DA neuron yield, the number of DAB-stained TH+ neurons in each section was counted manually using the Olympus AX70 microscope at 20x magnification in brightfield. Final counts were adjusted for the number of series (1:8) to get an estimate of the total number of TH+ cells within the graft. The serotonergic content was assessed by the analysis of one section from the graft core per animal in the group transplanted with 300,000 cells to striatum (group 1). All quantifications were performed by a single investigator who was blinded to the treatment. For graft volume quantification, images of HuNu stained coronal sections were taken at the level of the striatum by Epson Perfection V850 Pro scanner. To determine graft volume, the area of the cellular graft core in every eighth section through the graft was measured using ImageJ (version: 2.9.0) and calibrated by associating the number of pixels with a known measurement. The graft volume was calculated according to Cavalieri's principle, given the known distance between each section and the known section thickness.

Single nucleus RNA-sequencing of research-grade grafts, and bioinformatic analysis

Individual nuclei were isolated from tissue transplanted to the striatum (3 and 6 months) or the nigra (at 6, 9, and 12 months after transplantation) using a dounce homogenizer and nuclei lysis buffer. This suspension was sorted based on size using the BD FACSAria III Cell Sorter to remove cell debris and fractured nuclei. 7-10,000 nuclei per sample were loaded onto a 10x Chromium Next GEM Chip G following the manufacturer's instructions and processed in a Chromium controller (both 10x Genomics, Pleasanton, USA). Briefly, after encapsulating single nuclei with barcoded beads, cell lysis and reverse transcription were carried out in droplets containing polyT primers with cell-specific barcodes, Unique Molecular Identifiers (UMI), and sequencing adaptor sequences. Successful library preparation was confirmed using the Bioanalyzer (DNA HS kit, Agilent), and the libraries were sequenced on the Illumina NovaSeq 6000 100 cycles flow cell (Illumina) with run settings 28-10-10-90 cycles. Cell Ranger (version 3.0, 10x Genomics) was used to demultiplex base-call files to FastQ files and align the reads. Default alignment parameters were used, and a combined human/rat reference (Both version 93 from Ensembl) was utilized. Seurat (version 4) was applied for preprocessing and downstream analysis of the snRNA-seq data. Cell cycle analysis was performed using Seurat's CellCycleScoring with default parameters, with a score > 0.4 being taken as indicative of cycling cells. The percentage of Ki67+ cells was defined as cells with $\geq$1 transcript detected for *MKI67*. Individual cell values for cell cycle scores and MKI67 transcript numbers are shown in Table S10.

Necropsy, histopathology and immunohistochemistry of animals from safety study

The animals were killed by carbon dioxide asphyxiation with subsequent exsanguination and a detailed necropsy was performed. Tissues were routinely preserved in 10% Neutral Buffered Formalin (NBF) with the exception of testes, eye and CNS fluid that

was preserved initally in Davidsons fluid and then in NBF, in Davidsons's fluid or as smears, respectively. Tissue samples were dehydrated, embedded in paraffin wax, sectioned at a nominal four to five micron thickness and stained with hematoxylin and eosin.

The non-transplanted brain hemisphere was divided into two parts – 1/3 (sagittal) for qPCR and the remaining non-transplanted hemisphere remained intact along with the transplanted hemisphere for histology. The transplanted brain hemisphere was assessed by histology in all transplanted animals. Seven coronal samples were sectioned as per "Sampling and preparation according to Bolon et al.,[29] see also Figure 2B. For the section including the graft (level 2) the brain was embedded on the rostral surface and ~2.8 mm (2800 μm or 560 x 5 μm) was discarded. For animals transplanted with 700,000 STEM-PD or RC17 hESC cells (group 2 and 3) the first and then every 5th 5 μm section up to a total of 20 sections (representing 0.1 mm or 100 μm of tissue) were collected and mounted onto slides for histopathological assessment of the graft tissue. Five interspersed sections were studied with a routine hematoxylin and eosin (H&E) stain under GLP by a study pathologist with peer review from an external pathologist. The remaining unstained sections were used for IHC investigations (non-GLP).

The staining was performed in blocking buffer consisting of 0.1M Tris-HCl with 0.15M NaCl and 0.5 % TSA® Blocking reagent (Perkin Elmer). The hNCAM antibody was a rabbit monoclonal obtained from Abcam (Ab75813) and was diluted to a final concentration of 1:800. The KU80 stain was a rabbit monoclonal antibody obtained from Cell Signalling (2180) and was diluted to a final concentration of 1:200. Positive and negative control slides were included in each run. Detection was performed with an anti-rabbit antibody and horseradish peroxidase – diaminobenzidine (DAB) detection system in an automated immunostainer (Ventana Medical Systems) which produces a brown reaction product in a nucleus staining pattern. Tissues were counterstained with haematoxylin.

The Ki67 antibody was a rabbit polyclonal antibody obtained from Novus (NB110-89717) and was diluted to a final concentration of 1:2000. Positive and negative control slides were included in each run. Detection was performed using the anti-rabbit Bright Vision detection system in an automated immunostainer (Ventana Medical Systems) which produces a purple reaction product in a cell nucleus staining pattern. Tissues were counterstained with haematoxylin. Histopathological evaluation of the IHC slides was performed by the contributing scientist and the results were compared to the routine H&E sections from the study.

In vitro device testing

A preclinical batch of VM DA progenitor cells was prepared according to the procedure outlined for STEM-PD preparation for transplantation. The R-L device was filled with 20 μl cell suspension and the STEM-PD solution was delivered as 2.5 μl deposits into 8 separate vials containing HBSS buffer with 0.5% HSA. The 8 × 2.5 μl deposits correspond to the number of deposits delivered per tract during clinical transplantation of the high dose (Dose 2). Each deposit was delivered at the same speed and inter-deposit waiting times as is applied during clinical transplantation. The number of viable cells delivered per aliquot *in vitro* was quantified using the NC200 automated cell counting system (ChemoMetec). The cell delivery process was performed at 6 different angles of the R-L device within the stereotactic frame to simulate all possible injection angles during surgery (i.e. at 0°, 10°, 15°, 20°, 25° and 30° from horizontal plane). Delivery at each angle was performed in triplicate experiments.

Transplantation to minipig

The minipig was injected unilaterally with 6-hydroxydopamine (6-OHDA) and two months later with stem cells using MR stereotaxic surgery. Eleven months post-grafting, the brain was removed and studied using immunohistochemistry. Unilateral lesion with 6-OHDA was done in the anesthetised minipig according to,[48] targeting the medial forebrain bundle with 2 injections of 25 μL of 8 μg/μL 6-OHDA hydrobromide mixed in 0.9% NaCl with ascorbic acid (Sigma Aldrich, Denmark).[48] Infusion rates were 5 μL/min and retraction of needle 1 mm/min for the first 2 mm and then full retraction. Anaesthesia and stereotaxic procedures were done according to Glud et al.[49] and Lillethorup et al.[50] The lesion was verified on post-MRI and confirmed with amphetamine-induced rotations 3 weeks later (3.9 mg/kg amphetamine).

For transplantation of STEM-PD, the minipig was anesthetised, intubated and ventilated, similar to previous reports[49,50] and kept under anaesthesia using 2% sevoflurane during the whole procedure. Coordinates were determined through 3D MRI-based guidance, and the clinical R-L device was used for the implantation procedure. The minipig received a total dose of 1,250,000 STEM-PD Batch #3 cells into 5 tracts in the right putamen (2 mm apart in anterior-posterior direction) with 2 deposits/tract (2-3 mm apart ventral-dorsal) and 2.5 μL/deposit with a cell suspension of 50,000 cells/μL (total volume of 25 μL). The infusion rate was 2.5 μL/min and diffusion time was 1 min between injections and needle retractions. The minipig received immunosuppression with 10 mg Basiliximab (Simulect) IV 2 hours pre-grafting and 4 days post-grafting. Furthermore, Tacrolimus (Envarsus) was administered twice daily (approximately 0.7 mg/kg PO, Chiesi Farmaceutici). Post-operative tacrolimus was monitored for adjustment of dose every 2-3 weeks by measuring trough blood levels (target blood range 10-15 μg/L). The minipig was perfused at 11 months post-grafting and the brain processed for histological analysis. Immunohistochemistry was performed as previously described[50] on free-floating sections incubated with rabbit anti-TH (1:1000, ab112, abcam) and mouse anti-hNCAM (1:1000, SC-106, Santa Cruz Biotechnology).

QUANTIFICATION AND STATISTICAL ANALYSIS

Procedures for graft quantification are described in sections above. Procedures for statistical analysis of rotational data in group 1 of the efficacy study were as follows: Only animals with pre-transplantation scores ≥5 were included in Group 1 for assessment of transplant efficacy. For information on excluded animals, see section on *Amphetamine-induced rotations*. Criteria for successful recovery was defined as > 50% of the animals in the tested group having a reduction in rotational scores to <2 rotations/minute

post-transplantation with a group analysis showing a significant reduction from pre-transplantation rotation scores ($p < 0.05$ by student's paired t-test, parametric) The statistical analyses were performed by using the software GraphPad Prism 8/9 and Excel.

ADDITIONAL RESOURCES

More information on the STEM-PD trial can be found here: https://stem-pd.org/. Press release from the first patient: https://www.lunduniversity.lu.se/article/first-patient-receives-milestone-stem-cell-based-transplant-parkinsons-disease. Details on the clinical trial design are available at https://clinicaltrials.gov/ct2/show/NCT05635409.