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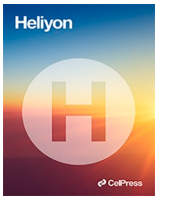

Research article

A cell therapy approach based on iPSC-derived midbrain organoids for the restoration of motor function in a Parkinson's disease mouse model

Chong-Lei Fu [a,b], Bo-Cheng Dong [a], Xi Jiang [a], Dan Li [c], Jun Yao [a,*]

[a] State Key Laboratory of Membrane Biology, Tsinghua-Peking Center for Life Sciences, IDG/McGovern Institute for Brain Research, School of Life Sciences, Tsinghua University, Beijing, 100084, China
[b] Shandong Institute of Brain Science and Brain-inspired Research, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, 250117, China
[c] Key Lab of Mental Health, Institute of Psychology, Chinese Academy of Sciences, Beijing, 100101, China

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease characterized by the degeneration of dopaminergic (DA) neurons in the substantia nigra and loss of DA transmission in the striatum, thus making cell transplantation an effective treatment strategy. Here, we develop a cellular therapy based on induced pluripotent stem cell (iPSC)-derived midbrain organoids. By transplanting midbrain organoid cells into the striatum region of a 6-OHDA-lesioned PD mouse model, we found that the transplanted cells survived and highly efficiently differentiated into DA neurons. Further, using a dopamine sensor, we observed that the differentiated human DA neurons could efficiently release dopamine and were integrated into the neural network of the PD mice. Moreover, starting from four weeks after transplantation, the motor function of the transplanted mice could be significantly improved. Therefore, cell therapy based on iPSC-derived midbrain organoids can be a potential strategy for the clinical treatment of PD.

1. Introduction

Parkinson's disease (PD) is one of the major health threats in an aging society, affecting more than 10 million people worldwide [1]. The main pathological change was the degeneration and death of dopaminergic (DA) neurons in the substantia nigra, resulting in a significant reduction of dopamine release in the striatum [2]. So far, there is no effective drug or treatment to cure the disease, and current drug and surgical therapies can only delay the deterioration of the condition and improve the life quality of the patients. The use of induced pluripotent stem cell (iPSC)-based cell transplantation therapy to introduce newborn neurons and restore the physiological functions in patients may be an effective strategy for the treatment of neurodegenerative diseases [3,4]. Recently, a number of studies have shown that DA progenitor cells or stem cell derived-neurons can be transplanted into the lesioned area of the midbrain of the PD mouse model to replace the dead cells. These new cells can restore dopamine levels and repair the motor disorder usually in 12–20 weeks [5–10].

Recently, the single-nucleus RNA-sequencing (snRNA-seq) analysis of postmortem brain tissues revealed that the dopamine-

* Corresponding author.
E-mail address: jyao@mail.tsinghua.edu.cn (J. Yao).

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releasing neurons in the substantia nigra region could be divided into 10 cell clusters and 68 molecular-specific subtypes, reflecting the diversity of cell types in the substantia nigra. Moreover, in the brains of PD patients, the proportion of DA neurons specific for 11 molecular subtypes was significantly reduced compared with the healthy control group [11]. These findings suggested that transplantation with more abundant types of cells may have better therapeutic effects for PD treatment. With the development of stem cell technology, the iPSC-derived organoid model has advantages in the complexity of simulating human organs and the diversity of cell types [12–16]. However, the therapeutic benefits of organoid-based cell transplantation for PD treatment have not yet been demonstrated.

In the present study, we developed a cellular therapy based on iPSC-derived midbrain organoids enriched with DA progenitor cells and DA neurons. We transplanted isolated midbrain organoid cells into the striatum of the 6-OHDA-lesioned PD model mice and found that the motor dysfunction of the PD mice was significantly restored; moreover, the therapeutic effect could be obviously detected at as early as 4 weeks after transplantation. Using the immunohistochemistry and dopamine sensor techniques, we found that approximately 84 % of transplanted cells differentiated into DA neurons that can highly efficiently release dopamine and integrate into the

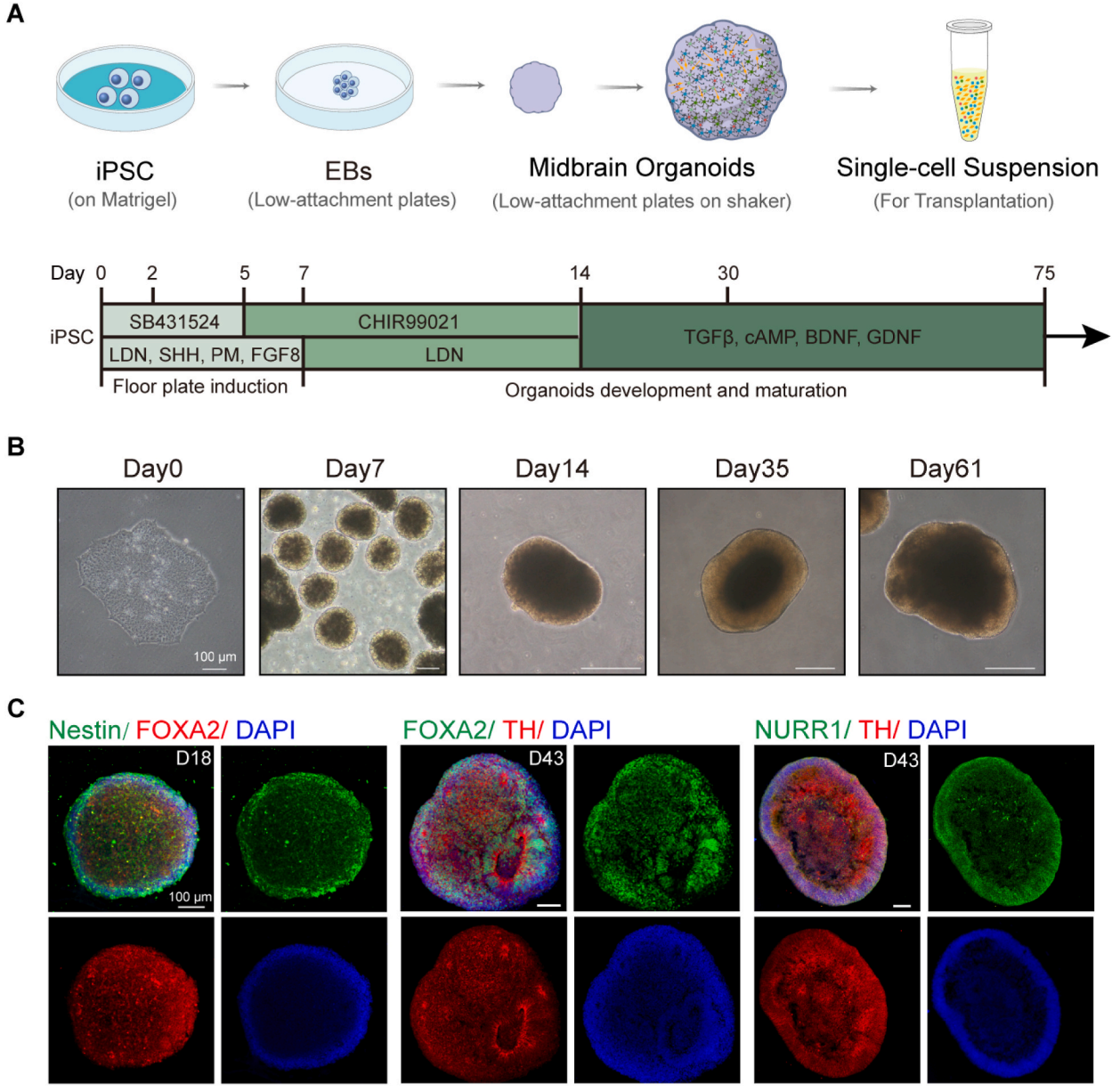

Fig. 1. Generation of Midbrain organoids derived from iPSCs. (**A**) Schematic diagram of midbrain organoids protocols. (**B**) Sample bright field images of midbrain organoids cultured from day 0 to day 61. (**C**) Sample immunostaining images of the midbrain organoids at day 18 and day 43. Scale bar, 100 μm.

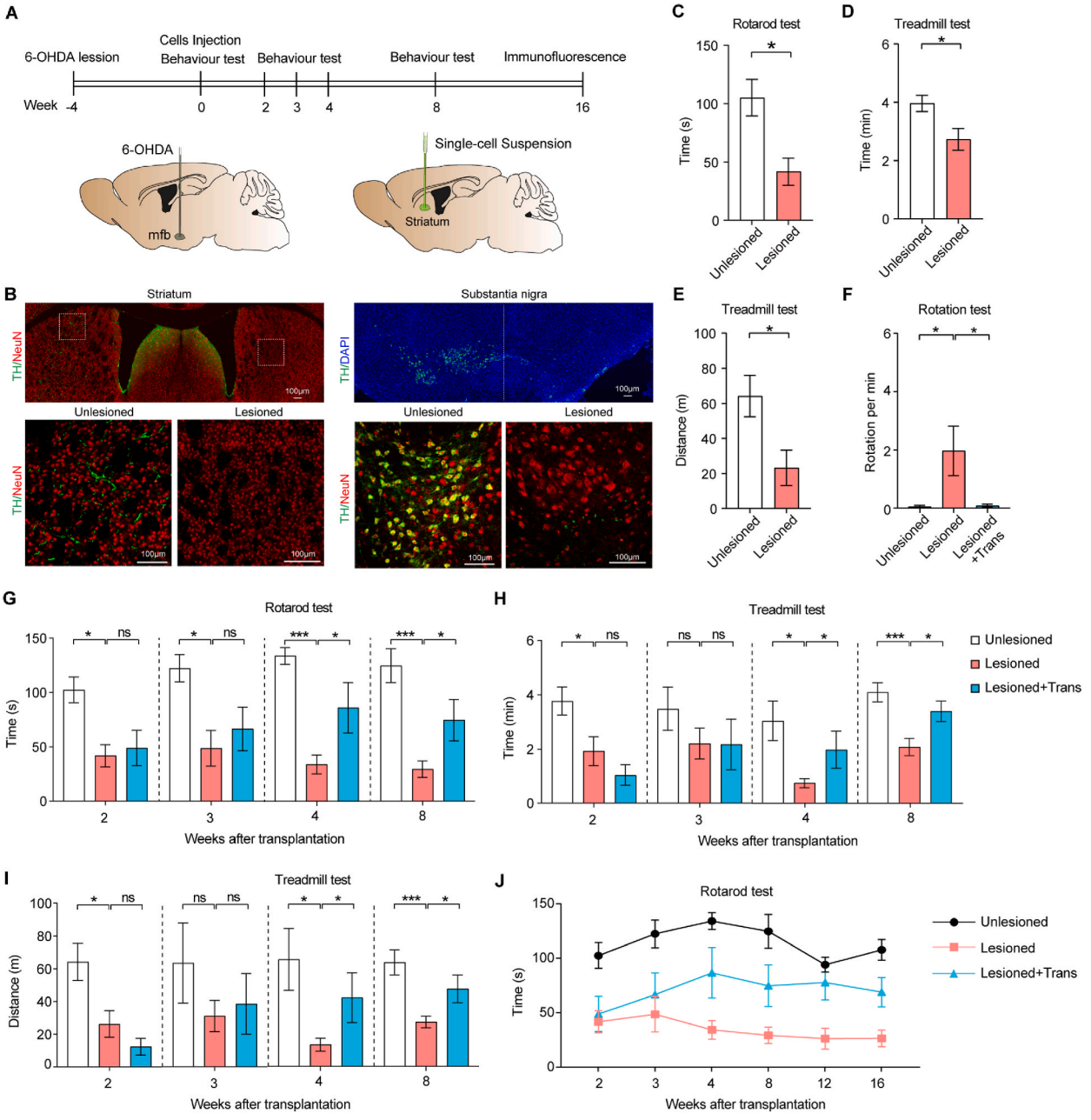

Fig. 2. Establishment of Parkinson's disease animal model and motor function correction after transplantation. (**A**) Schematic of the experimental schedule for 6-OHDA-induced lesion, transplantation, and behavioral tests. (**B**) Sample immunostaining images of the 6-OHDA-induced unilateral loss of TH^+ cells in striatum and substanita nigra. Scale bar, 100 μm. (**C**) Rotarod test of Unlesioned and Lesioned mice. Unlesioned, n = 10; Lesioned, n = 18. (**D-E**) Treadmill test of Unlesioned and Lesioned mice. Unlesioned, n = 10; Lesioned, n = 18. (**F**) Rotation test of mice after transplantation. (**G**) Rotarod test of mice after transplantation. (**H–I**) Treadmill test of mice after transplantation. (**J**) Line chart of rotarod test after transplantation. Unlesioned, n = 10; Lesioned, n = 10; Lesioned + Trans, n = 8. Student's t-test, *P < 0.05, ***P < 0.001. Bars, mean ± s.e.m.

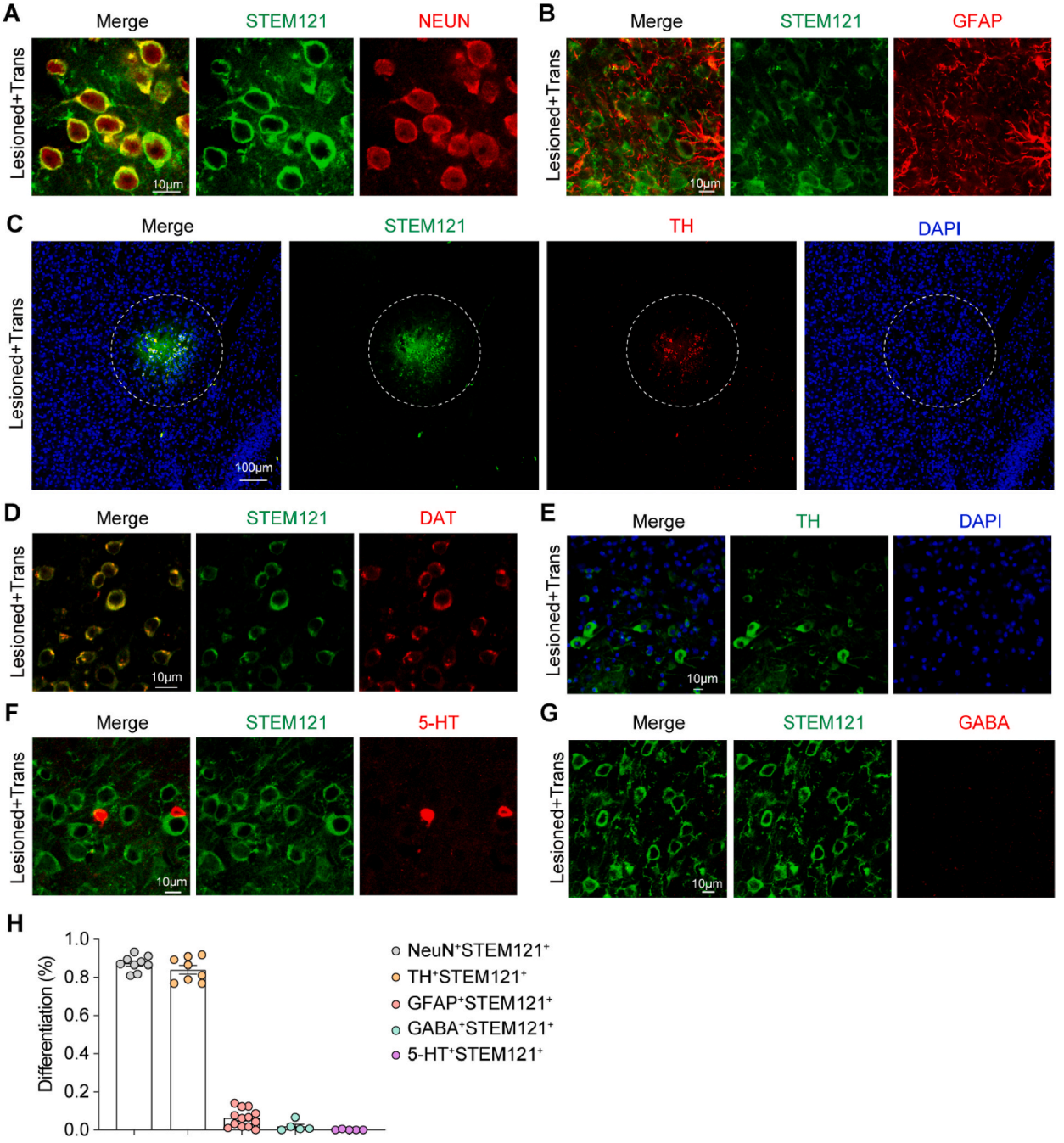

Fig. 3. In vivo survival and proportion of hiPSC-derived DA cells. (**A**) Immunofluorescence staining of the human-specific cell marker STEM121, and neuron maker NeuN in grafted region. Scale bar, 10 μm. (**B**) Immunofluorescence staining of STEM121, and astrocyte maker GFAP in the grafted region. Scale bar, 10 μm. (**C**) Immunofluorescence staining of STEM121 and the DA neuron marker TH in the grafted region. Scale bar, 100 μm. (**D**) Immunofluorescence staining of STEM121 and the DA neuron marker DAT in the grafted region. Scale bar, 10 μm. (**E**) Immunofluorescence staining of the DA neuron marker TH and DAPI in the grafted region. Scale bar, 10 μm. (**F**) Immunofluorescence staining of STEM121 and GABA in the grafted region. Scale bar, 10 μm. (**G**) Immunofluorescence staining of STEM121 and 5-HT in the grafted region. Scale bar, 10 μm. (**H**) Statistical results of cell differentiation ratio. $NeuN^+/STEM121^+$, n = 9 slices; $TH^+/STEM121^+$, n = 8; $GFAP^+/STEM121^+$, n = 13; $GABA^+/STEM121^+$, n = 5; $5\text{-}HT^+/STEM121^+$, n = 5. Bars, mean ± s.e.m. All graft analysis data were obtained 16 weeks after transplantation.

local neural network of the PD mouse brain. We therefore concluded that cell therapy based on iPSC-derived midbrain organoids could be a potential strategy for the clinical treatment of PD.

2. Results

2.1. Generation of midbrain organoids derived from human iPSCs

We used previously qualified iPSCs of a healthy human subject for functional examinations. Quality control examinations were carried out to ensure that all selected iPSC lines had pluripotency and normal karyotypes and could differentiate into the three germ layers [17]. Then we generated midbrain organoids from iPSCs referring to the experimental procedures that previously described [12] (Fig. 1A). At day 0–61 of differentiation, bright field imaging analysis revealed that the midbrain organoids developed into normal morphological structures (Fig. 1B). At day 18 of differentiation, immunofluorescence analysis revealed that the midbrain organoids developed into compact structures, including Nestin$^+$ neuron progenitor cells (NPCs) and FOXA2$^+$ cells that implicated the formation of the floor plate (FP)-like layer which is important in human midbrain development (Fig. 1C). At day 43 of midbrain organoid culture, we detected the expression of FOXA2, a DA progenitor cell marker NURR1, and a DA neuron marker TH (Fig. 1C). Hence, we successfully established the human iPSC-derived midbrain organoid model. We observed that at the day 18–43 stage, the midbrain organoids enriched DA progenitor cells and DA neurons, consistent with previous single-cell sequencing studies [18]. Analysis of midbrain organoid composition over time revealed that the cell types at day30 included 79 % FP cells, 20 % DA neurons, and 1 % vascular leptomeningeal cells, astrocytes, and oligodendrocyte progenitors [18]. This result means that the midbrain organoids at this stage could be suitable for transplantation and repair injured dopamine secretion in the PD mouse model.

2.2. Transplantation of cells from midbrain organoids improved the motor function of the Parkinson's disease mouse model

In the next step, we performed cell transplantation to investigate the potential of midbrain organoids to restore the injured midbrain dopamine pathway. For the establishment of the PD mouse model, we injected 6-hydroxydopamine (6-OHDA), a widely used dopamine analog, into one side of the medial forebrain bundle (mfb) to ablate the DA neurons in the nigrostriatal pathway [19] (Fig. 2A).

Four weeks after the 6-OHDA injection, we observed unilateral loss of TH$^+$ cells in the striatum and substantial nigra (Fig. 2B). Then, we performed three standard behavioral tests to assess the motor dysfunction of the mice, two based on spontaneous motor activities and one based on drug-induced rotation. The results of the rotarod test indicated that compared to the unlesioned group, the lesioned mice showed a significantly shorter duration on the rotarod equipment, thus exhibiting a state of balance lack (Fig. 2C). Analysis of the treadmill test revealed that compared to the unlesioned mice, the lesioned mice showed a significantly shorter duration and travel distance (Fig. 2D and E). Analysis of the rotation test revealed that compared to the unlesioned mice, the lesioned mice showed a significantly increased rotation induced by apomorphine (Fig. 2F). Those results indicate that the 6-OHDA lesioned mice showed a significant motor dysfunction, which is one of the classic symptoms of PD.

According to previous research [18], at about day 30 of midbrain organoids development, DA progenitor cells and DA neurons accounted for the highest proportion of cells within the midbrain organoids, while oligodendrocytes and astrocytes accounted for a very low ratio. Hence, 30-day-old midbrain organoids were likely suitable for the test of transplantation therapy. We prepared single-cell suspension from the day 30 midbrain organoids, with a $>$90 % cell survival rate and a $<$10 % agglomeration rate. Then, we performed transplantation into the striatum of 6-OHDA lesioned mice.

At 2, 3, 4, and 8 weeks after transplantation, we performed behavioral tests to assess the correction of motor dysfunction in the mice. The results of the APO-induced rotation test indicated that compared to the untreated lesion group, the lesioned mice receiving transplantation showed significantly reduced rotations, thus showing restoration of asymmetry (Fig. 2F). The results of the rotarod test indicated that compared to the untreated lesion group, the lesioned mice receiving transplantation showed a significantly longer duration on the rotarod equipment at 4 and 8 weeks, thus exhibiting a state of balance restoration (Fig. 2G). Analysis of the treadmill test revealed that compared to the untreated lesion group, the transplanted lesion group showed a significantly improved duration and travel distance at 4 and 8 weeks (Fig. 2H and I). The continuous behavioral testing of the mice showed that at 16 weeks after cell transplantation, the motor balance ability of the transplanted mice was still higher than that in the untreated PD group, indicating that the transplanted cells could stably survive and function in the midbrain of PD mice for an extended period (Fig. 2J).

Together, our results indicated that transplanting cells from midbrain organoids could efficiently improve the motor dysfunction of the PD mice from 4 weeks after the surgery.

2.3. The transplanted cells survived and differentiated into DA neurons in vivo

To investigate the survival of transplanted cells in the mouse brain, we performed immunofluorescence staining analysis in striatum slices at 16 weeks. By detecting the human cell-specific marker STEM121, we located the transplanted cells and observed that these STEM121$^+$ cells had normal morphology (Fig. 3A–G), indicating that the transplanted cells could survive in vivo.

To investigate the differentiation efficiency of the transplanted cells in the mouse brain, we carried out immunostaining analysis with biomarkers for different cell types (Fig. 3A–G). We found that 87 % of STEM121$^+$ cells were NeuN$^+$ neurons, of which 84 % of STEM121$^+$ cells were TH$^+$ DA neurons, 2 % were GABAergic interneurons, and 0.1 % were 5-HT$^+$ serotonergic neurons (Fig. 3H). In addition, we observed that 6 % of STEM121$^+$ cells were GFAP$^+$ glial cells. These results indicated that the transplanted cells could be

highly efficiently differentiated into DA neurons.

2.4. Restoration of striatal dopamine release level

To investigate the dopamine-releasing function and neural network integration of transplanted cells in vivo, we assayed the DA transmission by measuring the release of endogenous dopamine using a reported $GRAB_{DA2h}$ sensor in the acutely dissected striatal slices to detect presynaptic dopamine release from the postsynaptically expressed DA receptor-like fluorescence sensor [20].

In slices expressing $GRAB_{DA2h}$, we applied a train of electrical stimulation pulses and recorded the stimulation-evoked fluorescence changes (Fig. 4A–C). The unlesioned group showed a sharp elevation of fluorescence intensity (Fig. 4A and B), indicating functional dopamine release in response to the stimulation. In contrast, the peak of fluorescence intensity change was abolished in the lesioned slices, indicating that dopamine release was severely impaired in the PD mice. Moreover, compared to the untreated lesion group, the slices of lesion mice receiving transplantation showed significantly restored peak amplitude of fluorescence change in response to the electrical stimulation (Fig. 4A and B). We performed electrophysiological recordings of eGFP-labeled transplanted cells and observed significant Na^+/K^+ currents and spontaneous action potentials (sAPs), indicating that the transplanted cells had differentiated into functional neurons (Fig. 4D). These observations indicated that not only the dopamine release in the PD mice was repaired by cell transplantation, but also the differentiated DA neurons could be efficiently integrated into the neural network of the mouse brain.

Together, our results indicated that the cell therapy based on iPSC-derived midbrain organoid transplantation could effectively compensate for the loss of functional DA neurons and rescue the destroyed dopamine release in the PD mouse model.

3. Discussion

Neurodegenerative diseases are often difficult to cure completely [21]. PD is usually pharmacologically treated, using one single small molecule drug or a combination of drugs with different acting mechanisms to compensate for the reduction of DA transmission caused by neuronal degeneration [22]. The patients often require long-term use of these drugs to delay the disease progression and thus improve the quality of life [23]. However, prolonged medication not only leads to a shorter duration of action but may also induce side effects such as postural hypotension, cardiac arrhythmias, urinary problems, and sleep disturbances [24]. In recent years, stem cell-based cell transplantation has been introduced into PD treatment research. Cell transplantation can restore the reduced dopamine

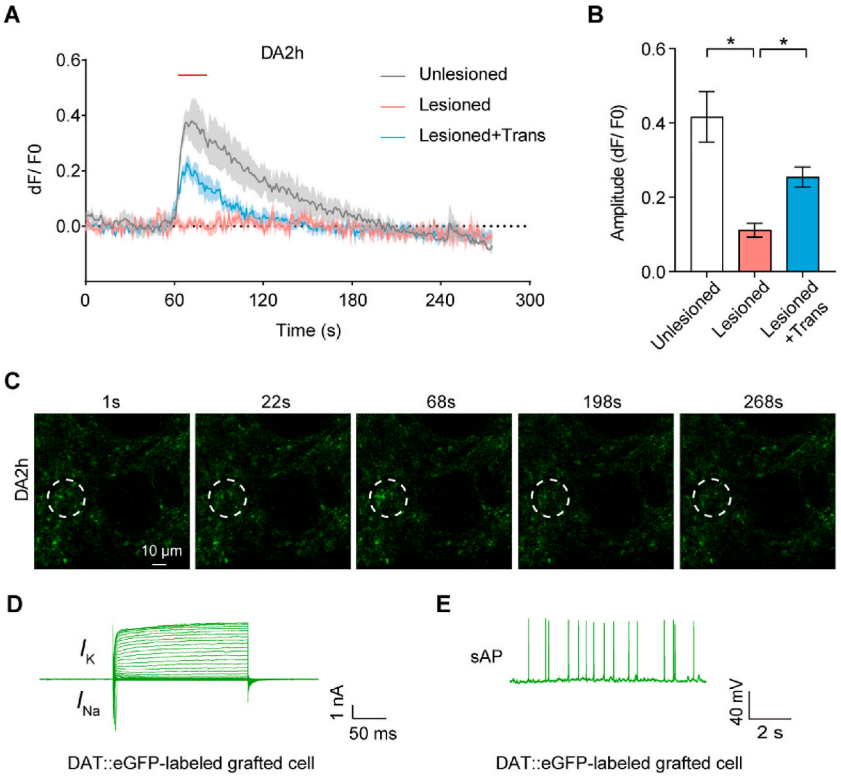

Fig. 4. Restoration of striatal dopamine release level. (**A**) $GRAB_{DA2h}$-indicated dopamine release in striatal slices. (**B**) The amplitude of striatal dopamine release in response to the electrical stimulation. Unlesioned, n = 5 mice; Lesioned, n = 5; Lesioned + Trans, n = 5. (**C**) Representative images of fluorescence responses in DA2h-expressing neurons following electrical stimulation. (**D**) Patch-clamp recording in DATeGFP expressing grafted cells showed Na^+/K^+ currents. (**E**) Sample trace of spontaneous APs. Student's t-test, *P < 0.05, ***P < 0.001. Bars, mean ± s.e.m.

level by introducing new neurons to replenish the degenerated DA neurons and thus provides an effective alternative treatment option.

Current cellular therapies for PD are mainly transplantation of monolayer cultured DA progenitor cells and DA neurons, and *trans*-differentiation of endogenous glial cells. However, compared to the process of 3D brain development, the 2D system lacks the complex temporal and spatial regulation required for DA neuron development and thus fewer subtypes of neurons are formed. In the present study, our results indicated that organoid-based cell transplantation therapy might have several advantages compared to 2D system. Firstly, the therapy showed a faster onset of action in treating motor dysfunction. It has been suggested that the 6-OHDA lesioned rats with homotopic transplants of iPSC-derived DA neurons could not show motor function correction until 24 weeks [6], and transplantation of hiPSC-derived DA progenitors into PD model rodents showed motor function improvement at 16 weeks [8,9]. Moreover, converting midbrain astrocytes to DA neurons could rescue the motor deficits 12 weeks after AAV treatment [5]. In our study, we observed that the organoid-based transplantation showed an improved therapeutic efficacy, in which the motor function in PD mice could be significantly restored in 4 weeks after transplantation. Secondly, our midbrain organoid culture system is more sophisticated and mature in inducing dopamine neurogenesis. In this study, using unsorted cells from midbrain organoids, we were able to compensate for the loss of functional DA neurons with high efficiency. Approximately 84 % of the transplanted and surviving cells eventually form DA neurons and could be efficiently integrated into the local neural network. Thus, the three-dimensional organoid system more closely mimics the process of human brain development than traditional monolayer neuronal culture, thus making the derived cells more suitable for transplantation therapy for neurological diseases. Lastly, we note that the chimeric transplantation of whole midbrain organoids is still not accessible for treating PD mice due to the difficulty of surgery in deeper brain regions and the potential tumorigenic risk within the "necrotic core" of the organoids [25]. For the development of clinical treatments, isolating single cells of the organoids for transplantation is more accessible and less risky.

In summary, we have developed a cell transplantation therapy based on midbrain organoids for Parkinson's disease. Through transplantation in a mouse model of Parkinson's disease, we have found that the treatment can replace damaged dopamine neurons to effectively rescue the motor disorder of Parkinson's disease mice and restore the dopamine release levels in the striatum. Therefore, the cell therapy approach based on midbrain organoids is a potential therapy for Parkinson's disease.

3.1. Limitation of the study

In the present study, as we have discussed, there are still several remaining questions. First, do DA progenitor cells from midbrain organoids have a higher differentiation potential than cells from monolayer cultures? Second, can DA progenitor cells from midbrain organoids differentiate into multiple subtypes of DA neurons? Third, it is unclear whether the therapeutic benefits of midbrain-based organoid transplantation are due to the differentiated viability of DA progenitor cells or to the diversity of DA neuron subtypes. Lastly, can flow sorting of DA progenitor cells in midbrain organoids before transplantation lead to better therapeutic outcomes than unsorted cells? These issues need to be addressed in future work.

4. Materials and methods

Table 1
Key resources table

Reagent or Resource	Source	Identifier
Antibodies		
Goat anti-FOXA2	R&D	Cat#AF2400; RRID:AB_2294104
Rabbit anti-TH	Abcam	Cat#ab112; RRID:AB_297840
Mouse anti-TH	Santa Cruz	Cat#sc-25269; RRID:AB_628422
Rabbit anti-DAT	Merck	Cat#MAB369
Mouse anti-Nestin	Millipore	Cat#MAB5326; RRID:AB_2251134
Rabbit anti-NURR1	Santa Cruz	Cat#SC-990; RRID:AB_2298676
Mouse anti-STEM121	Cellartis	Cat#Y40410; RRID:AB_2801314
Rat anti-GFAP	Abcam	Cat#ab279303
Rabbit anti-GABA	Calbiochem	Cat#PC213L
Rabbit anti-NeuN	Abcam	Cat#ab177487; RRID:AB_2532109
Rabbit anti-5-HT	novin	Cat#50-67-9
Goat anti-mouse Alexa Fluor 488	Jackson	Cat#715-545-151; RRID: AB_2341099
Goat anti-rat Alexa Fluor 568	Abcam	Cat#ab175476; RRID: AB_2813739
Goat anti-rabbit Alexa Fluor 647	Invitrogen	Cat#A-31573
Viruses		
AAV9-hSyn-DA1h(DA4.2)	WZ Biosciences	Cat#YL002002
Lenti-hDAT-eGFP	–	–
Chemicals		
6-OHDA	Topscience	Cat#28094-15-7
Albumin Bovine V	Biodee	Cat#0332
Agar	Biodee	Cat#DE0010
Agarose	lablead	Cat#AG0100
DMSO	Sigma	Cat#D2650
KCL	VETEC	Cat#V900068

(*continued on next page*)

Table 1 (*continued*)

Reagent or Resource	Source	Identifier
KH_2PO_4	Sigma	Cat#P5655
PFA	Sangon	Cat#A500684
NaH_2PO_4	Sigma	Cat#S0751
NaCL	General-Reagent	Cat#G81793J
SDS	BBI	Cat#A600485-0500
TRYPTONE	OXOID	Cat#LP0042B
Tris	amresco	Cat#77-86-1
Triton X-100	Biodee	Cat#0694
TRIzol Reagent	ambion	Cat#15596018
YEAST EXTRACT	OXOID	Cat#LP0021
Mediums and Cytokines		
DMEM/F12	Thermo Fisher	Cat#11330-032
DMEM/F-12 basic	Thermo Fisher	Cat#C11330500BT
Neurobasal Medium	Thermo Fisher	Cat#21103-049
PSCeasy human pluripotent stem cell medium	Cellapybio	Cat#CA1014500
PSCeasy-supplement	Cellapybio	Cat#CA1014500-2
Matrigel	CORNING	Cat#354234
ROCK inhibitor	Enzo Life Sciences	Cat#ALX-270-333
SB-431542	Cellagen Tech	Cat#C7243-5
β-ME	Thermo Fisher	Cat#21985-023
GlutaMax	Thermo Fisher	Cat#35050-061
MEM-NEAA	Thermo Fisher	Cat#11140-050
Pen/Strep	Thermo Fisher	Cat#15140-122
N2 supplement	Thermo Fisher	Cat#17502-048
B27 supplement	Thermo Fisher	Cat#17504-044
EDTA	Thermo Fisher	Cat#15575-038
Accutase	Invitrogen	Cat#00-4555-56
CHIR 99021	Cellagen Tech	Cat#C2447-2s
BDNF	Pepro Tech	Cat#450-02
GDNF	Pepro Tech	Cat#100-25
LAA	Sigma	Cat#A92902
TGF-β	Pepro Tech	Cat#100-21
cAMP	Sigma	Cat#A6885
SHH	Peprotech	Cat#100-45
LDN-193189	Stem Cell	Cat#72146
Purmorphamine	Stem Cell	Cat#72202
FGF-8	Peprotech	Cat#100-25A
Software		
Fiji/ImageJ	https://imagej.nih.gov/ij	N/A
GraphPad Prism9	www.graphpad.com	N/A
NIS-Elements AR	Nikon	N/A
Illustrator 2022	Adobe	N/A
EndNote 20	Thomson ResearchSoft	N/A

4.1. Materials availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Yao (jyao@mail.tsinghua.edu.cn). Materials used in this study will be available on reasonable request, but we may require a completed Materials Transfer Agreement if there is potential for commercial application (Table 1).

4.2. Subjects and iPSC

All human donor skin samples described in this study were obtained from healthy volunteers who had given informed consent, and all the experimental procedures were approved by the Ethics Committee of Tsinghua University. The induced pluripotent stem cells (iPSCs) were derived from fibroblasts using a CytoTune-iPS2.0 Sendai reprogramming kit (Thermo Fisher) according to the manufacturer's instructions.

4.3. Animals and Parkinson's disease model

All the mice were cared for and handled according to the Guidelines for Animal Experiments of Tsinghua University. All the mice were group-housed four per cage at a constant temperature room under a 12 h:12 h light/dark cycle (7:00 to 19:00). Water and food are freely available. Adult male mice at 7 weeks were used for the 6-OHDA-lesioned model. A total of 3.6 μg 6-OHDA per mouse in ice-cold saline solution was injected into the medial forebrain bundle (mfb) region in the left hemisphere. The coordinates were calculated in reference to the bregma: anterior–posterior (A/P), −1.2 mm; medio-lateral (M/L), 1.3 mm and dorso-ventral (D/V), 4.75 mm.

4.4. Generation of midbrain organoids

Midbrain organoids were derived from iPSCs referring to the experimental procedures previously described [12]. For midbrain organoid differentiation from feeder-free maintained hiPSCs, cells were maintained on matrigel-coated plates in a PSC medium. Embryoid bodies (EBs) were formed by dissociation of iPSC colonies using collagenase IV and plating onto low attachment plates in PSC supplemented with the ROCK inhibitor Y27632 (10 μM), SB-431542 (10 μM), SHH (100 ng/ml), LDN-193189 (100 nM), Purmorphamine (2 μM), and FGF-8 (100 ng/ml) for 4 days. Then, transfer the EBs into media consisting of DMEM/F12, 1 × N2, 1 × GlutaMax, LDN-193189 (100 nM), CHIR99021 (3 μM), SHH (100 ng/ml), Purmorphamine (2 μM), and FGF-8 (100 ng/ml) for 2 days. At day 7–14, change media to DMEM/F12, 1 × N2, 1 × GlutaMax, LDN-193189 (100 nM), and CHIR99021 (3 μM). After 14 days, to obtain mature organoids, transfer the organoids into media consisting of neurobasal, 1 × B27, 1 × GlutaMax, β-ME (0.1 mM), BDNF (20 ng/ml), GDNF (20 ng/ml), L-Ascorbic Acid (0.2 mM), cAMP (0.5 mM), and TGF-β (1 ng/ml).

4.5. Preparation of single cell suspension

The single-cell suspension was prepared using Neural Tissue Dissociation kits (Miltenyi Biotec) and a magnetic sorting instrument according to the instructions. The organoids were gently dissociated with a MACS™ Octo Dissociator with program 37C-NTDK-1. The cell suspension was filtered using a 70 μ m cell filter and centrifuged at 300g for 10 min, and the pellet was resuspended using the PBS+0.04 % BSA solution. Cell viability and clumping rate were measured with CountStar.

4.6. Cell transplantation

Cell transplantation was performed with stereotactic injection of 5×10^4 cells in 1 μl through a 5 μl Hamilton Neuros syringes into the left side of the striatum. The coordinates were calculated in reference to the bregma: anterior–posterior (A/P), 0.5 mm; mediolateral (M/L), 2 mm and dorso-ventral (D/V), 3 mm.

4.7. Behavioral assays

All behavioral data analysis was performed double-blinded to experimental conditions. The rotarod test is a widely used behavioral paradigm to detect motion coordination, which requires the animal to keep balance and move continuously on the rotarod. The mice were placed in the experimental chamber for 1 h in advance to acclimatize. The diameter of the rotarod is 6 cm, and the rotation speed is 20 r/min. After five acclimations, the duration of the mice on the rotarod was recorded. The interval of each test was 1min, and the average value was taken for five consecutive tests. For the treadmill test, three days before the formal examination, the mice were placed on the treadmill with a fixed 10 % slope for 5 min at a speed of 10 m/min. On the testing day, the mice ran on the treadmill at 10 m/min for 5 min and the speed was increased by 4 m/min every minute until they were exhausted or a maximal speed of 40 m/min was achieved. Running time and distance were recorded. For the rotation test, apomorphine (APO, 0.5 mg/kg) were injected to mice. APO-induced rotations were recorded for 5 min after 10min of injection.

4.8. Immunofluorescence

Midbrain organoids were fixed in 4 % paraformaldehyde for 12 h at 4 °C and then dehydrated in 20–30 % sucrose solution for 3 days. Then, the organoids were embedded in a Tissue-Tek cryomold with OCT embedding medium and stored at −80 °C overnight. Organoids were then sectioned into 40-μm-thick slices using the Leica CM1950 cryostat and permeabilized for 1 h. The organoid slices were then blocked in 3 % Albumin Bovine in PBS for 2 h, and incubated with primary antibody overnight at 4 °C. After washing with PBST three times, slices were incubated with secondary antibodies for 2 h at room temperature. Fluorescent signals of midbrain organoid slices were detected using a Nikon A1 confocal microscope, and images were processed with Adobe Photoshop CC 2019 and Adobe Illustrator 2022 software. The primary antibodies used were goat anti-FOXA2 antibody (1:500, R&D, AF2400), rabbit anti-TH antibody (1:300, Abcam, ab112), mouse anti-Nestin antibody (1:500, Millipore, MAB5326), rabbit anti-NURR1 antibody (1:500, Santa Cruz, SC-990), mouse anti-STEM121 antibody (1:300, Cellartis, Y40410), rat anti-GFAP antibody (Abcam, ab279303), rabbit anti-GABA antibody (1:300, Calbiochem, PC213L), rabbit anti-NeuN monoclonal antibody (1:300, Abcam, ab177487), rabbit anti-5-HT antibody (1:300, Novin, 50-67-9). The secondary antibodies used were donkey anti-mouse Alexa Fluor 488 (1:500, Jackson, 715-545-151), goat anti-rat Alexa Fluor 568 (1:500, Abcam, ab175476), and donkey anti-rabbit Alexa Fluor 647 (1:500, Invitrogen, A-31573).

4.9. Fluorescence imaging of dopamine release

AAVs expressing the $GRAB_{DA2h}$ sensor were injected into the mouse striatum. Two weeks later, horizontal 200 μm brain slices were prepared in an ice-cold solution and incubated in an artificial cerebrospinal solution (ACSF) containing 124 mM NaCl, 26 mM NaHCO3, 10 mM glucose, 3 mM KCl, 2 mM CaCl2, 1.25 mM KH2PO4, and 1 mM MgCl2, saturated with 95 % O2/5 % CO2. In slices expressing $GRAB_{DA2h}$, electrical stimulation consisting of a train of pulses was performed for 20 s, and the stimulation-evoked fluorescence changes were recorded using a Nikon A1 confocal microscope.

4.10. Electrophysiology

Cell suspensions were infected with a DAT promoter-driven lentivirus expressing eGFP (DATeGFP) prior to the transplantation. Brain slices were incubated at 33 °C in an artificial cerebrospinal solution (ACSF) for 60 min. The ACSF contains 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 10 mM Glucose and 1 mM $MgCl_2$, saturated with 95 % O_2/5 % CO_2. Whole-cell recordings were performed using a MultiClamp 700B amplifier (Molecular Devices). To record the Na^+/K^+ currents, the membrane potential was held at −70 mV, and cells were depolarized in 5 mV increments. Spontaneous action potentials (sAP) were recorded in the current clamp mode, with currents being injected to keep the neurons at the desired membrane potential.

4.11. Statistical analysis

The statistical analyses were performed using GraphPad Prism 9 software, and data were shown as mean values ± SEM. All data met the criteria of normal distribution. The P values were calculated using Student's t-test, and the differences were considered to be statistically significant for values of $p < 0.05$.

Data availability statement

Data included in article/supplementary material/referenced in article.

Ethics approval and consent to participate

All the experimental procedures were approved by the Institutional Animal Care & Use Committee of Tsinghua University and the Animal Welfare and Ethics Committee of Tsinghua University (Approval ID: 22-YJ1). Every effort was expended to minimize suffering.

CRediT authorship contribution statement

Chong-Lei Fu: Investigation. **Bo-Cheng Dong:** Investigation. **Xi Jiang:** Investigation. **Dan Li:** Investigation. **Jun Yao:** Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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