BIOLOGY

Long-Term Efficacy and Safety of Human Umbilical Cord Mesenchymal Stromal Cells in Rotenone-Induced Hemiparkinsonian Rats

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Several studies have shown functional improvements, neuroprotective, and neuroregenerative effects after mesenchymal stem cells transplantation to parkinsonian animal models. However, questions remain about the safety, feasibility, and long-term efficacy of this approach. In this study, we investigated migration, therapeutic, tumorigenesis, and epileptogenic effects of human umbilical cord mesenchymal stromal cells (HUMSCs) 1 year after transplantation into rotenone-induced hemiparkinsonian rats. Our data indicated that DiI-labeled HUMSCs migrated in the lesioned hemisphere, from corpus striatum (CPu) to substantia nigra. By integrating with host cells and differentiating into NSE, GFAP, Nestin, and tyrosine hydroxylase-positive cells, HUMSCs prevented 48.4% dopamine neurons from degeneration and 56.9% dopamine terminals from loss, both correlating with improvement of apomorphine-induced rotations. The CD50 and CD97 value of pentylenetetrazol and semiquantitative immunohistochemical analysis of proliferating cell nuclear antigen (PCNA), β-catenin, C-myc, and NF-κB expression showed no significant difference between HUMSCs transplanted and untransplanted groups, whereas the expressions of Bcl-2 and P53 in the grafted CPu were upregulated by 281% and 200% compared to ungrafted CPu. The results of this long-term study suggest that HUMSCs transplantation, 1 of the most potential treatments for Parkinson's disease, is an effective and safe approach.

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INTRODUCTION

Parkinson's disease (PD) is an age-related, progressive neurodegenerative disorder characterized by loss of dopaminergic (DA) neurons in substantia nigra pars compacta (SNc) and consequent loss of DA terminals in the corpus striatum (CPu). The etiology and pathogenesis of PD are not completely understood, and the treatments of PD are limited. Although several laboratory treatments have been shown to modify the course of PD, there is still no treatment that could halt or reverse the neurodegeneration in PD [1]. The pathologic characteristics and treatment status make PD a good target for cell therapy.

Several studies have shown the therapeutical benefit of neural stem cells [2,3], embryonic stem cells [4,5], bone marrow mesenchymal stem cells (BMSCs) [6,7], induced pluripotent stem cells [8], and HUMSCs [9-11] in parkinsonian animal models. However, embryonic stem cells can form teratomas [12] and are likely to elicit immune rejection if used for transplantation [13], and the source of human neural stem cells is limited. Moreover, the number of BMSCs, which can be used for autologous transplantation for the aging-associated PD [14], decreases steadily with age [15]. Furthermore, induced pluripotent stem cells,

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which could be derived from patients' own cells, might be associated with increased susceptibility to the degenerative process of PD [16], as increased a-synuclein expression was recently reported in Parkinson patient fibroblasts [17]. Although early studies have indicated the functional benefit from HUMSCs transplantation [9-11], there is no data about the long-term therapeutic effects, survivorship of HUMSCs, migration, fate of differentiation, and long-term safety of HUMSCs treatment.

Previous data demonstrated the potential antitumorigenic effects of human MSCs [18,19]. However, embryonic stem cells and multipotent tissue-specific stem cells still have the capacity to self-renew and to differentiate into many different mature cell types, and may share many signaling pathways with cancer stem cells. Therefore, the long-term tumorigenesis of HUMSCs must be carefully assessed before any clinical application.

It is reported that neurologic complications such as seizures attack might be among the consequences of allogeneic BM transplantation [20]. Moreover, we found a refractory epileptic patient who received umbilical cord blood transplantation 2 years ago and the relationship between engraftments and seizures was indistinct. It is rational for us to assess the epileptogenic effect in long-term stem cell therapy.

In this study, we transplanted 1,1′-dioctadecyl-3, 3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled HUMSCs to rotenone-induced hemiparkinsonian rats. After a period of 1 year, cellular migration in the rat brain was detected by monitoring the red flourescence of DiI from the continuous frozen section of brain tissue via a confocal microscopy. The efficacy was assessed by the apomorphine-induced rotational behaviors and tyrosine hydroxylase (TH) immunoreactivity in the CPu and SNc. The differentiation fate of HUMSCs was examined by the immunohistochemical staining of Nestin (neural stem cell marker), Neuron-Specific Enolase (NSE, neuronal marker), glial fibrillary acidic protein (GFAP, astrocyte marker) and TH (dopaminergic marker). The safety was evaluated by the p53, C-myc, H-RAS, β-catenin, Bcl-2, PCNA, and NF-κB immunofluorescent staining, and the CD50 and CD97 value of pentylenetetrazol (PTZ).

METHODS

Preparation of HUMSCs

The experiments described here were approved by the Ethical Committee of Tongji Medical College, Huazhong University of Science and Technology, China (HUST). With the permission of the parturient (Gynecology and Obstetrics Department, Union Hospital, HUST), umbilical cords were aseptically collected during Cesarean sections, stored at 4°C in 0.01 M sterilized phosphate-buffered solution (PBS) with heparin (20-30 U/mL) until further processing [11]. To avoid endothelial cell contamination, umbilical arteries and vein were removed and then the cords were cut into small pieces (0.5–1 cm). Subsequently, the cords were further cut into 1–2 mm^3 fragments and the explants were transferred into 6-well plates containing the DMEM/F12 (Invitrogen, Carlsbad, CA) along with 20% fetal bovine serum (Invitrogen) and 10 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The fragments were left undisturbed for 10-12 days to allow cells to migrate from the explants. They were refed every 48-72 hours and passaged (1:3) by trypsin (0.25%, Invitrogen) digestion until cells reached 80%-90% confluency.

HUMSCs Labeling

Cell labeling by DiI (Invitrogen) has been previously described [21,22]. Briefly, HUMSCs were harvested by treatment with 0.25% trypsin, washed with PBS, then resuspended and incubated for 30 minutes at 37°C in darkness with growth media containing 10 μM DiI. After washing with PBS 3 times (1000 rpm, 5 minutes), the DiI-labeled HUMSCs were used for transplantation, or the detection of labeling efficiency by flow cytometer or by confocal microscopy.

Rotenone-Induced Hemiparkinsonian Rat Model

The rotenone-induced slowly progressive hemiparkinsonian rodent model was chosen for this study (23). Briefly, inbred adult female Sprague-Dawley rats (220–260 g; from the Center of Experimental Animals, Tongji Medical College, HUST; 12-hour light/dark cycles, 22 ± 2°C, 60% ± 5% humidity; standard laboratory chow and water ad libitum) were anesthetized with chloral hydrate (400 mg/kg in 0.9% NaCl, intrasperitoneally [i.p.]) and fastened on a cotton bed over a stereotaxic frame (RWD Life Science, Shenzhen, China). Rotenone dissolved in DMSO was infused into the right ventral tegmental area (VTA, AP: 5.0 mm; L: 1.0 mm; DV: 7.8 mm) at a flow rate of 0.2 μL/minute. The needle was left in place for an additional 5 minutes for complete diffusion of the drug. Rotenone was infused into the right SNc (AP: 5.0 mm; L: 2.0 mm; DV: 8.0 mm) at a flow rate of 0.2 μl/minute, with a 5-minute needle retention. After needle withdrawal, proper postoperative care was given until the animals recovered completely. The animals were given ibuprofen and penicillin in their drinking water for 24 hours to alleviate potential postsurgical discomfort and to prevent infection.

HUMSCs Transplantation

Rats were divided randomly into 2 groups 4 weeks after surgery: Saline group, 10 μL saline alone (without donor cell administration) (n = 12); and the HUMSC group, 10 μL saline containing 1.0×10^6 DiI-labeled HUMSCs (n = 18). HUMSCs were transplanted into the CPu of each rat (anterior 1.0 mm, lateral 3.0 mm, ventral 5.0 mm), based on positioning from the bregma and skull surface. There was a 10-minute waiting period before the needle was removed to allow the cells to settle. No immunosuppressive medication was given to the animals.

Apomorphine-Induced Rotations

Apomorphine (APO)-induced rotational behavior was blindly tested every month for a total of 12 months with coded animals. Rats were placed on a table (1 m × 1 m) with railing and allowed to acclimate to the environment for 10 minutes, and then administered an intraperitoneal injection of APO (1.5 mg/kg) [23]. Subsequent rotational behavior was then recorded for 30 minutes. Following rotational testing, animals were put back into their cages.

PTZ-Induced Seizures

The threshold for clonic convulsions was measured in all rats by intraperitoneal injection of PTZ at doses ranging from 40 to 100 mg/kg. Following the injection of PTZ, rats were placed separately into transparent Plexiglas cages (80 × 80 × 40 cm) and monitored for 30 minutes for the occurrence of clonic seizures. Clonic seizure activity was defined as clonus of the whole body lasting for more than 3 seconds, with an accompanying loss of righting reflex. The number of animals convulsing out of the total number of rats tested was noted for each group. The convulsive action of PTZ was evaluated as the CD50 (median convulsive dose, that is, the dose of PTZ that produced clonic seizures in 50% of the mice tested) and CD97 (the dose necessary to induce clonic seizures in 97% of animals tested). To determine the CD50 and CD97 values for control animals, 20 mg/kg to 80 mg/kg PTZ was used for testing (10 rats per group). Subsequently, a dose-response curve was determined from the percentage of rats convulsing according to a log-probit method [24,25].

Histologic Examination of Grafted HUMSCs

One year after transplantation, rats were profoundly anesthetized with chloral hydrate and the brains were removed and postfixed for 12 hours in 4% paraformaldehyde in PBS at 4°C. Next, the specimens were equilibrated in PBS containing 15% sucrose for 24 hours at 4°C, then in 20% sucrose/PBS for 24 hours at 4°C, and finally, in 25% sucrose/PBS for another 24 hours at 4°C. Embedded in OCT compound, brains were cut into serial 10-μm-thick slices by using a cryostat. Hematoxylin and eosin (HE) stain was performed to detect tumor-like formations.

For differentiation and efficacy study, immunofluorescent staining method was employed to assess Nestin (mouse monoclonal antibody [mAb], Santa Cruz, Santa Cruz, CA), NSE (mouse mAb, Abcam, Cambridge, MA), GFAP (mouse mAb and rabbit polyclonal antibody, Santa Cruz), and TH (mouse mAb, Millipore, Billerica, MA) expression in the CPu (at 1.2 mm caudal to bregma), and TH (rabbit polyclonal antibody, Santa Cruz) expression in the SNc (from −4.5 mm to −6.2 mm caudal to bregma), and visualized by the fluorescein isothiocyanate (FITC)-conjugated goat-antimouse IgG or FITC-conjugated goat-antirabbit IgG (Proteintech, Chicago, IL). For the safety study, the primary antibody NF-κB, p53, C-myc, H-RAS, β-catenin, Bcl-2 (rabbit polyclonal antibody, Santa Cruz), PCNA (mouse mAb, Cell Signaling, Danvers, MA) and the corresponding secondary antibody FITC-conjugated goat-antimouse IgG or FITC-conjugated goat-antirabbit IgG were used. The slides from the anterior to posterior regions of the left and right striata were densitometrically observed and analyzed by employing the Image-Pro plus 6.0 software package (Media Cybernetics, Inc., Bethesda, MD) [23]. A design-based, unbiased, stereologic method and a morphometry/image analysis system has been previously described for counting TH-positive cells [23]. In each section, the region of interest was outlined (SNc and VTA), and the TH-positive cells/mm^2 in that region were selected and semiautomatically counted.

Statistical Analysis

In tissue immunofluorescence staining, all tissue manipulations were conducted in large batches to avoid batch to batch variability. Following an experimenter-blind fashion of data collection, analysis of variance (ANOVA) was used to evaluate group differences. In all statistical analyses, a P-value of $<.05$ was considered statistically significant. All data were presented as mean ± standard error.

RESULTS

HUMSCs Were Highly Efficiently Labeled by DiI

After coculture with DiI for 30 minutes, 99.8% ± 0.1% of HUMSCs were labeled (Figure 1B-D). The long spindle-shaped DiI-labeled cells were observed under confocal microscopy (Figure 1A).

HUMSCs Migrated from the CPu to the SNc

The migration of grafted HUMSCs was visualized by tracking the distribution of DiI fluorescence in

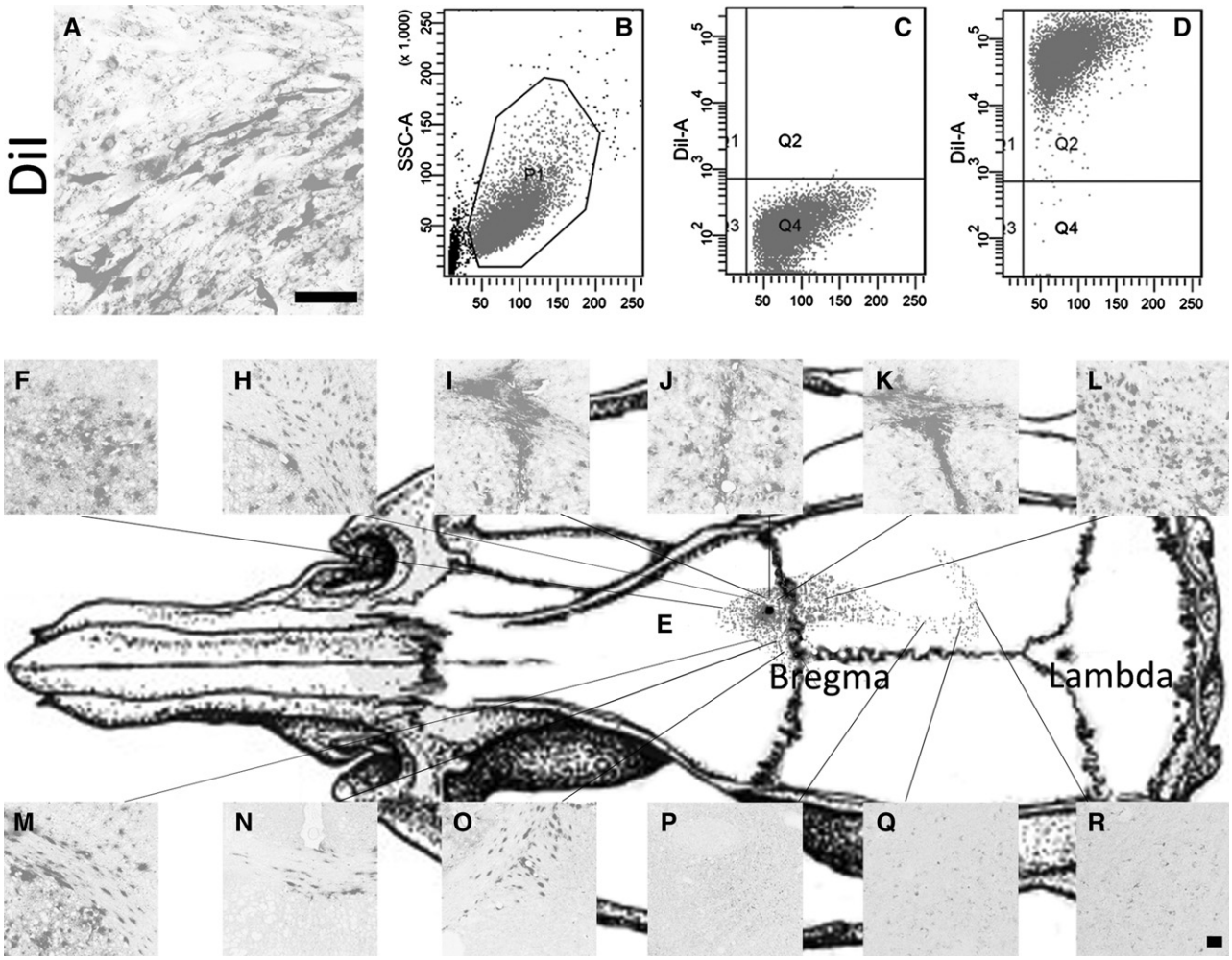

Figure 1. Labeling and migration of HUMSCs. (A) DiI-labeled HUMSCs were detected by confocal microscopy. (B) Scatter diagram of FSC/SSC gating. (C) Unlabeled HUMSCs as a negative control. (D) 99.8% of HUMSCs were labeled by DiI. (E) Dorsal view of skull of transplanted rats. (I-K) The grafted HUMSCs distribution in the cortex and CPu around the needle track. (F and L) HUMSCs distribution in the CPu 1 mm both rostrally and caudally to the needle track. (H and M) HUMSCs distribution in the cortex and corpus callosum. (N and O) HUMSCs migration to the contralateral cerebral hemisphere through the corpus callosum. (P) HUMSCs migration to the lesioned substantia nigra. (Q and R) HUMSCs migration to the lesioned VTA and SNc. (Scale bars = 50 μm.)

brain tissue. DiI-labeled HUMSCs were mainly distributed in the lesioned CPu (Figure 1F and I-L), and a portion of the HUMSCs migrated to the lesioned SNc and VTA (Figure 1P-R). HUMSCs even migrated to the contralateral cerebral hemisphere through the corpus callosum (Figure 1N-O).

HUMSCs Significantly Reduced APO-Induced Rotations in Parkinsonian Rats

After APO treatment, all the animals that received unilateral rotenone infusion showed rotational behavior. As expected, the rotational behavior of untreated animals advanced gradually until the 12th month (Figure 2A). Compared to the Saline group, the PD model rats transplanted by the HUMSCs showed a significant decrease in the number of rotations (Figure 2A). Our data indicated, 12 months after transplantation, the HUMSC group decreased rotations by 67.48% (Figure 2A).

HUMSCs Preserved TH Immunoreactivity in the CPu and SNc

The quantification study of TH-positive neurons in the SNc demonstrated that the animals that accepted the transplantation had significantly more TH-stained neurons in the SNc (Figure 2I: Saline group; Figure 2J: HUMSC group; Figure 2C), which was correlated with the TH immunoreactivity in the CPu and behavioral recovery (Figure 2E: Saline group; Figure 2F: HUMSC group; Figure 2B). Compared with the contralateral CPu, the TH immunoreactivity in the CPu of the Saline group was diminished by 92.08%, whereas it decreased by only 35.13% in the CPu of the HUMSC group (Figure 2B and D-F). Compared with the contralateral SNc, the number of

TH-positive neurons of the lesioned SNc of the Saline group was decreased by 79.68%, whereas it was reduced by only 31.28% in the lesioned SNc of HUMSC group (Figure 2C, and H-J).

HUMSCs Differentiated into Neuron-Like Cells after Transplantation

Twelve months after implantation, human-originated Nestin (Figure 3A-D), NSE (Figure 3E-H), GFAP (Figure 3I-L), and TH (Figure 3M-P, mouse mAb, Millipore) were detectable in the HUMSC group (Figure 3A-P). Furthermore, these positive stained cells were integrating with host cells (physical connection with host cells; Figure 3). The immunofluorescence staining of sections from the SNc showed that DiI-labeled HUMSCs migrated to the SNc and differentiated into TH-positive cells in the substantia nigra (Figure 3Q-T, rabbit polyclonal antibody; Santa Cruz).

Tumorigenesis Was Absent in Rats after Long-Term HUMSCs Treatment

Immunohistostaining of Bcl-2, p53, PCNA, β-catenin, and C-myc or HE staining (HE stain) was used to examine any tumorigenesis of HUMSCs. Our data showed that Bcl-2, an anti-apoptosis factor, and the P53 tumor-suppressor protein were highly expressed in the DiI-labeled HUMSCs 12 months after transplantation. Bcl-2 and P53 expressions in the CPu of the HUMSC group (Bcl-2: Figure 4A-D; P53: Figure 4I-L) were significantly different from that in the lesioned CPu of the Saline group (Bcl-2: Figure 4E-H; P53: Figure 4M-P): the expressions of Bcl-2 and P53 in the grafted CPu were upregulated by 281% and 200% compared to ungrafted CPu. PCNA, β-catenin, C-myc, and NF-κB expressions showed no difference between HUMSCs implanted and unimplanted CPu (Figure 4Q-AN). Based on the HE stain of the CPu (Figure 5B and C), especially the regions besides the needle track, and the cortex (Figure 5D and E), no tumor-like formations (duct-like structures, irregular nests, malignant cell infiltration, or specific ultrastructural organization) were observed, not even in proximity to the pin track.

Epileptogenic Test Showed No Difference between the Saline Group and the HUMSC Group

The epileptogenic effect after long-term HUMSCs transplantation was assessed by intraperitoneal injection of PTZ. CD50 and CD97 showed no significant difference between the HUMSCs treated and untreated groups (Figure 5F-H). Unfortunately, 3 rats from the Saline group and 2 rats from the HUMSC group died 24 hours after PTZ treatment, and the animal death was PTZ dose-related.

DISCUSSION

In the present study, we showed that intra-CPu transplantation of DiI-labeled HUMSCs 4 weeks after rotenone-administration ameliorated APO-induced rotations gradually in a period of 12 months, which indicates long-term therapeutic effect of this approach. HUMSCs migrated in the lesioned cerebral hemisphere, from the CPu to the SNc, or even to the opposite hemisphere through the corpus callosum. HUMSCs survived for up to 12 months after transplantation, and differentiated into Nestin-, NSE-, GFAP-, and TH-positive cells in the CPu and TH-positive cells in the SNc. No tumor-like structure was observed in the implanted CPu, as confirmed by immunostaining of Bcl-2, P53, PCNA, β-catenin, and C-myc. No immunologic rejection or seizure attack was found without immunosuppressant administration. CD50 and CD97 value of PTZ showed no significant difference between HUMSCs implanted and unimplanted animals. Our results indicate that HUMSC is an effective and safe therapeutic alternative for PD.

Compared to the "gold standard" BMSCs, HUMSCs showed a higher proliferative potential than BMSCs and were capable of osteogenic, chondrogenic, and adipogenic differentiation. However, there are abundant similar characteristics between HUMSCs and BMSCs that support the applicability of HUMSCs for cell-based therapies because they are all mesenchymal stem cells (MSCs) [26]. The underlying mechanisms of the therapeutic efficacy of MSCs could be summarized as follows: (1) neuroprotective effect: MSCs are capable of homing to the impaired area and secreting neurotrophic factors that support the survival of remanent neurons, induce endogenous cell proliferation, and promote nerve fiber regeneration [11,27-29]; (2) neuroregenerative effect: in the cerebral nervous tissue niche, grafted MSCs are able to differentiate into neurons and astrocytes [11,30-32]; (3) immunomodulatory effect [33-35]: MSCs suppress autoimmunity regardless of the conditioning regimen, as a previous study showed that MSC infusions lead to T cell anergy in experimental autoimmune encephalomyelitis, with a decrease in inflammatory infiltrates and demyelinization in treated mice. Although evidence showed MSCs transdifferentiated into neural cells, much remains to be done to increase the rate of HUMSC differentiation into a specified neuronal phenotype, such as DA neurons.

It was reported that 4 months after the transplantation of 1×10^5 in vitro HUMSCs-conversed TH-positive cells (12.7%) into the CPu of parkinsonism rats, these conversed cells migrated by 1.4 mm both rostrally and caudally [10]. However, our data showed that HUMSCs moved from the whole lesioned CPu to the lesioned SNc, which was the result of the homing capability of HUMSCs to the lesioned tissue. The

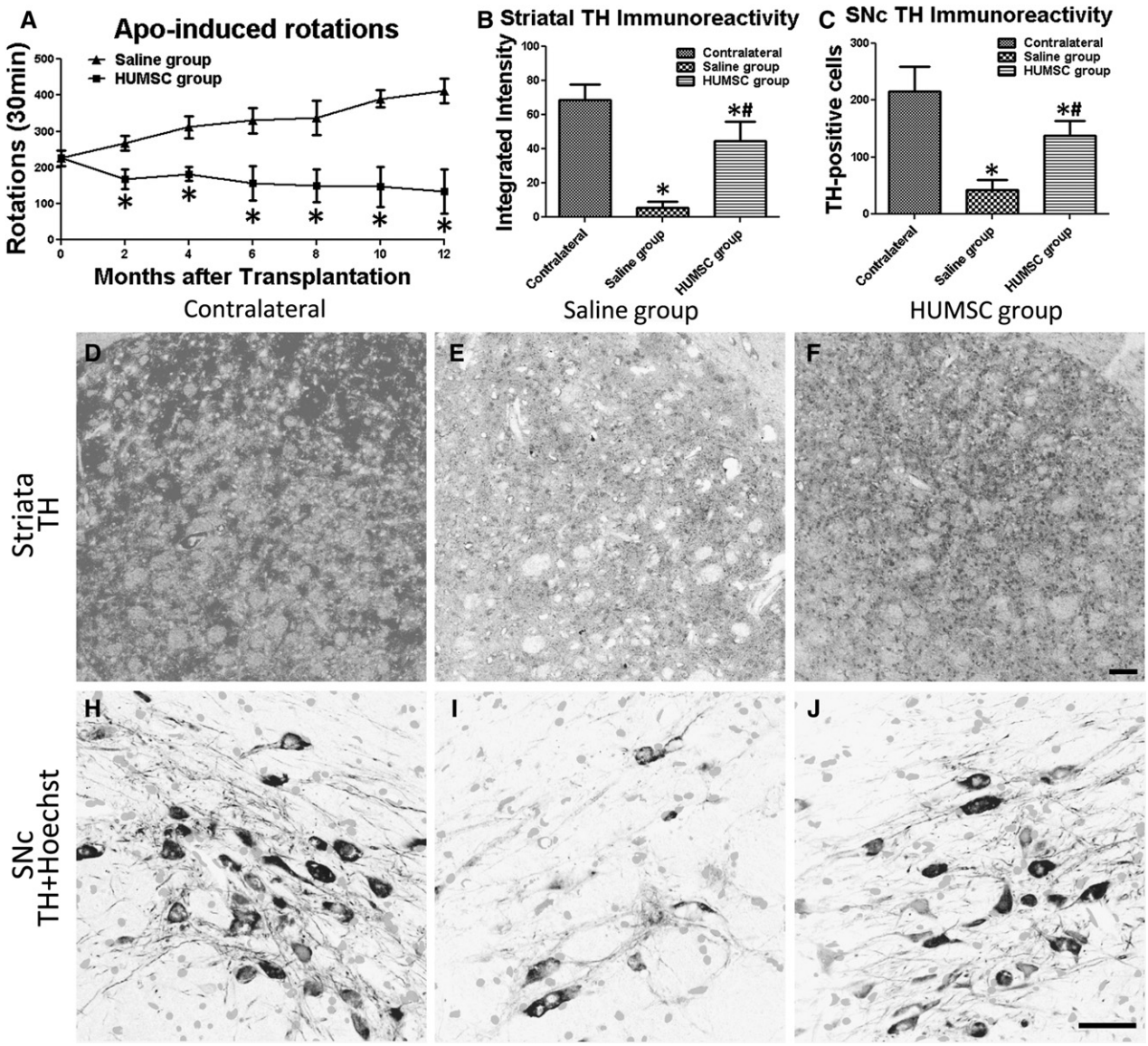

Figure 2. Effect of HUMSCs transplantation. (A) Rotational behavior of rats of Saline group versus HUMSC group. (B) TH immunoreactivity in the CPu of Saline group versus HUMSC group. (C) Number of TH-positive neurons in the SNc of Saline group versus HUMSC group. (D-F) Immunofluorescence staining of TH in the lesioned CPu of saline group (E), HUMSC group (F), and the contralateral CPu of both groups (D). (H-J) TH-positive cells in the lesioned SNc of Saline group (I), HUMSC group (J), and the contralateral CPu of both groups (H), with nuclear staining by hoechst33258 (statistics consisted of one-way ANOVA; $*P < .05$ compared to the contralateral side; $\#P < .05$ compared to the Saline group; scale bars = 50 μm).

difference may be attributed to the different ending time points of the 2 studies and quantity of HUMSCs implanted into the lesioned CPu, because ours were 10 times as much as that in the previous study. We found that HUMSCs migrated to the opposite hemisphere through the corpus callosum, consistent with previous findings [6,10]. Furthermore, we found that DiI-positive cells expressed TH in the lesioned SNc, suggesting that HUMSCs migrated to the lesioned SNc and differentiated into TH-positive cells. Unfortunately, it is still unknown whether these HUMSCs-derived TH-positive cells have the same functional characteristics, electrophysiologic characteristics, and synaptic connections as the DA neurons.

In vitro studies have shown that MSCs can differentiate into ecto- and endodermal derivates under specific induced culture conditions [11,36-41]. In vivo studies suggested that MSCs have the ability of differentiating into multilineage cells of various tissues [42-46]. However, other researchers have proved that most transplanted MSCs still retain their hematopoietic fate [47], and MSCs underwent fusion rather than differentiation in vitro and in vivo [48-50]. Our data showed that under a long-term induction by the CPu niche, HUMSCs differentiated into Nestin-, NSE-, GFAP-, and TH-positive cells, suggesting that HUMSC is also a good candidate for the treatment of other neurologic disorders.

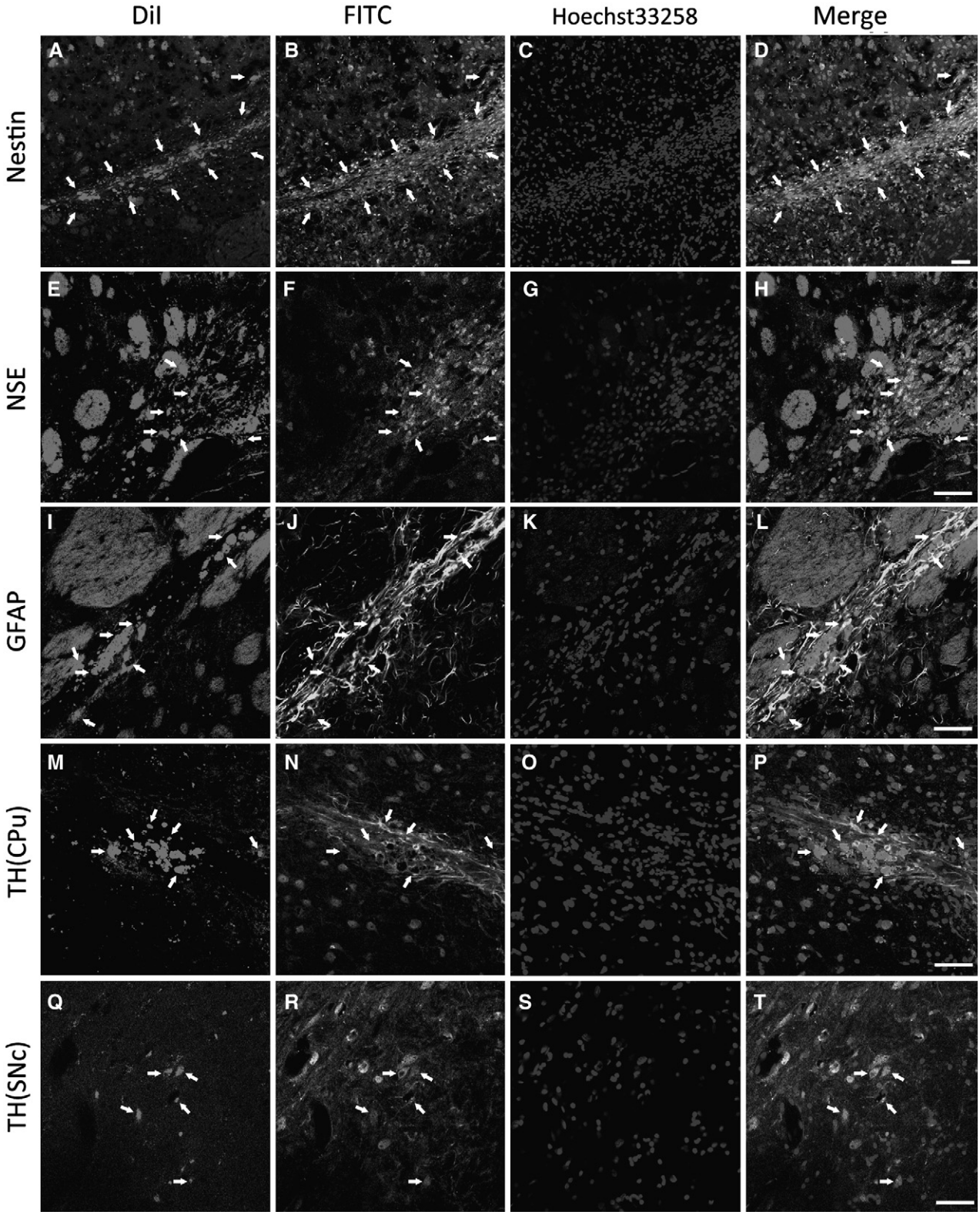

Figure 3. Differentiation fate of HUMSCs. DiI, HUMSCs tracker; hoechst33258, nuclear staining; Nestin, NSE, GFAP, and TH, all visualized by FITC-conjunct goat-antimouse (or rabbit) IgG. (A-D) Coexpression of DiI and Nestin around the needle track. (E-H) Coexpression of DiI and NSE around the needle track. (I-L) DiI-labeled HUMSCs and differentiation into GFAP-positive cells. (M-P) DiI-labeled HUMSCs and differentiation into TH-positive cells in the microenvironment of CPu. (Q-T) DiI-labeled HUMSCs and migration to the lesioned SNc and differentiated into TH-positive cells (scale bars = 50 μm).

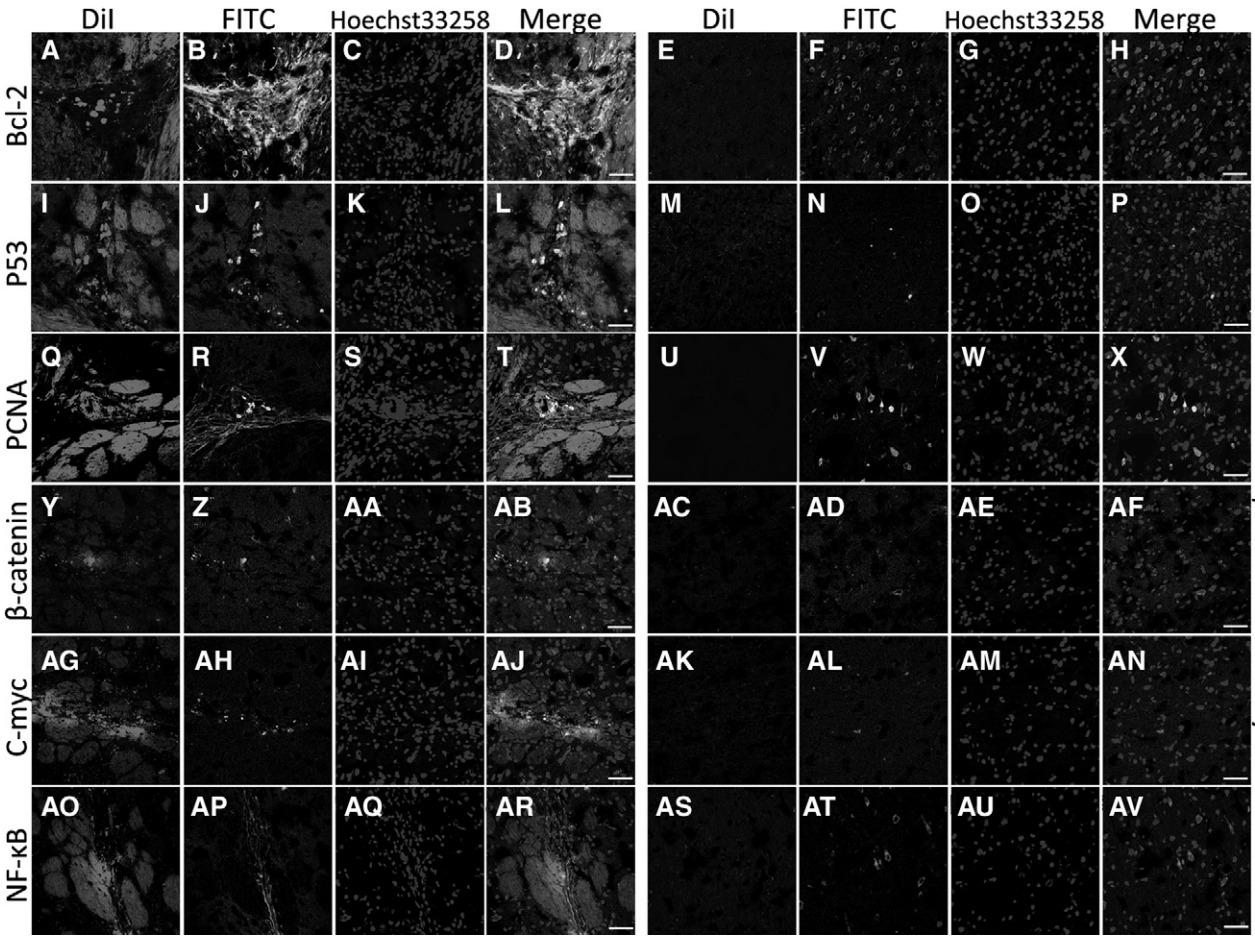

Figure 4. Safety of long-term HUMSCs treatment. DiI for HUMSCs; p53, C-myc, β-catenin, Bcl-2, PCNA, and NF-κB all visualized by FITC-conjunct goat-antirabbit or goat-antimouse IgG; Hoechst33258, nuclear staining. (A-D, I-L, Q-T, Y-AB, AG-AJ, and AO-AR) Bcl-2, P53, PCNA, β-catenin, C-myc, and NF-κB expression in the HUMSCs-grafted CPu, respectively. (E-H, M-P, U-X, AC-AF, AK-AN, and AS-AV) Bcl-2, P53, PCNA, β-catenin, C-myc, and NF-κB expression in the lesioned CPu of Saline group, respectively (scale bars = 50 μm).

The potential safety hazards related to stem cell transplantation is a predominant scruple in stem cell therapy, although with distinct origins, stem cells and tumor cells share many characteristics. Tumors may often originate from the transformation of normal stem cells, and similar signaling pathways may regulate self-renewal in stem cells and cancer cells [51,52]. Therefore, HE staining and oncoprotein immunohistostaining were employed in this study to assess tumorigenesis of long-term HUMSCs transplantation. Among the oncoproteins, Bcl-2, which was upregulated by 281%, was shown to promote cellular survival rather than proliferation [53]. Bcl-2 provided a distinct survival signal to the cells and may contribute to neoplasia by allowing a clone to persist until other oncogenes, such as c-myc, become activated [53]. C-myc was reported to be a key factor that involves tumorigenesis of stem cells [54]. P53, upregulated by 200% in this study, was known as the most important tumor suppressor, and its function is lost in many types of cancers by mutation or by excessive negative regulation, which initiates DNA repair, cell-cycle arrest, senescence, and apoptosis [55]. The p53 and nuclear factor NF-κB pathways play crucial roles in human cancer. Inactivation of p53 and hyperactivation of NF-κB are common occurrences, and activation of p53 and inhibition of NF-κB both promote apoptosis [56]. PCNA, a cell cycle marker protein, is well known as a DNA sliding clamp for DNA polymerase delta and as an essential component for eukaryotic chromosomal DNA replication and repair [57]. Aberrant activation of the canonical WNT/β-catenin pathway occurs in many cancers and contributes to their growth, invasion, and survival, and dysregulated β-catenin activity drives colon tumorigenesis [58]. Our data showed that Bcl-2 and P53 expressions were significantly different, whereas PCNA, β-catenin, C-myc, and NF-κB expressions were no different between HUMSCs implanted and unimplanted CPu. We speculated that overexpression of Bcl-2 is responsible for the long-term survival of grafted HUMSCs, that p53 upregulation may play an important role in tumor supression, and that the canonical expression of PCNA, β-catenin, C-myc, and NF-κB in the HUMSC-

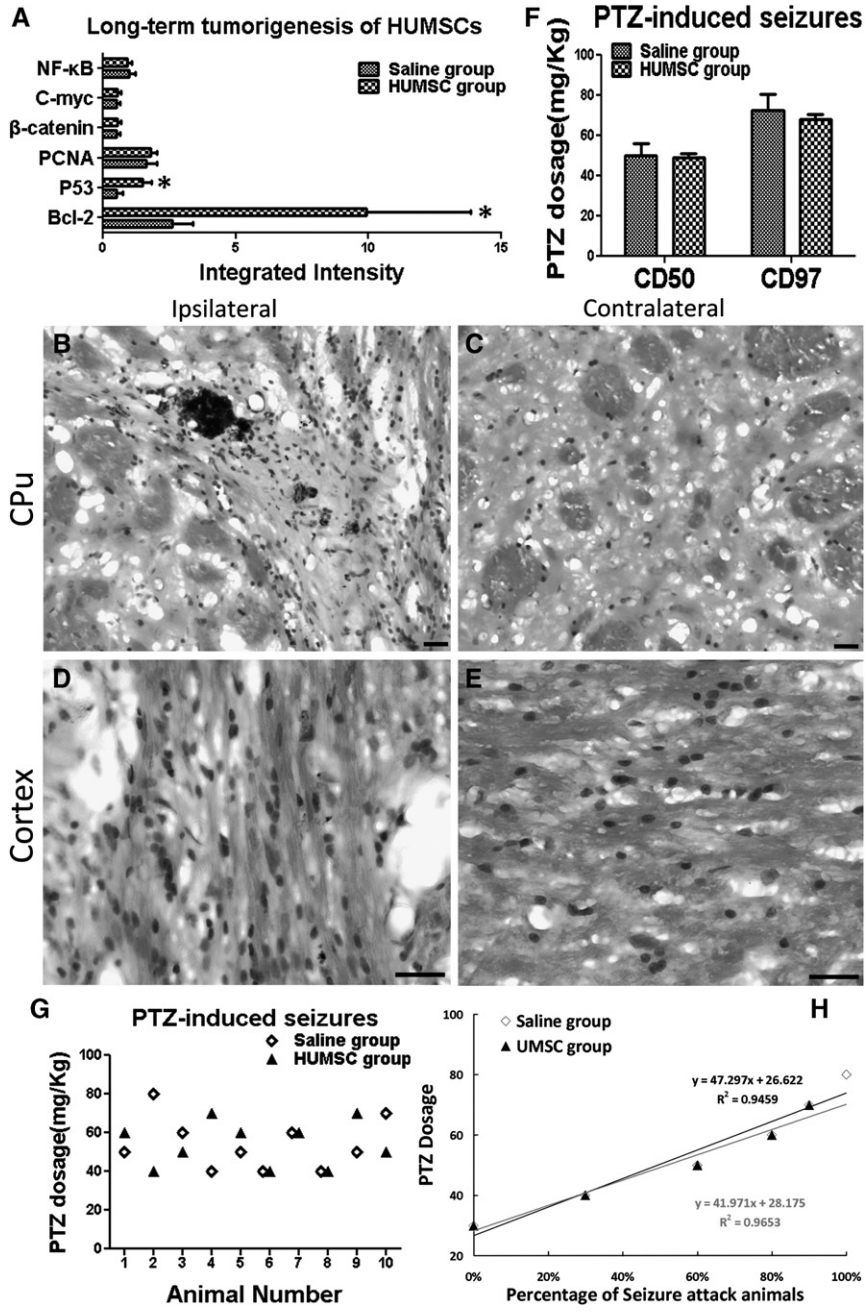

Figure 5. HE staining of brain tissue and PTZ-induced seizures. (A) Quantification of p53, C-myc, β-catenin, Bcl-2, PCNA, and NF-κB expression in the HUMSCs implanted and unimplanted CPu. (B) HE stain of needle track in the lesioned CPu of HUMSC group. (C) HE stain of the contralateral CPu of HUMSC group. (D) HE stain of the lesioned cortex of HUMSC group. (E) HE stain of the contralateral cortex of HUMSC group. (F) CD50 and CD97 of PTZ-induced seizures in HUMSCs treated versus untreated groups. (G) Quantitative analysis of PTZ dosage in experimental animals. (H) Regression analysis of PTZ-induced seizures in experimental animals ($*P < .05$ compared to Saline group; scale bars = 50 μm).

transplanted CPu might minimize the possibility of HUMSCs tumorigenesis. HE staining further confirmed this finding, as no tumor-like structure was observed in the grafted CPu.

Another concern about stem cell treatment is the epileptogenic effect of stem cells, which may result from the wrong connection of the grafted cells to the host cells, and induce abnormal cerebral neuronal discharge and evoke seizures. None of the HUMSC group rats showed any kind of seizure in the total period of 12 months. To better assess the epileptogenic effect of HUMSCs, PTZ, a chemical convulsant agent, was used in this study. We found that the CD50 and CD97 value of PTZ showed no significant difference between HUMSC transplanted and untransplanted rats, suggesting HUMSCs are unlikely induce seizures in rodent models when used for transplantation.

We acknowledge the drawback of this study. The long-term cell tracker DiI, with a highly lipophilic nature of the carbocyanine dyes, may stain host cells

when the grafted cells disintegrate and release the dye. Furthermore, the flourescence of DiI may quench during this long period of time. Although the human-specific primary antibodies were used as an independent method to confirm that the differentiated cells were originally from human tissue, it was still unable to calculate recovery rate and differentiation rate of the HUMSCs.

Given the observation that HUMSC transplantation is an effective and safety method for the treatment of PD in rodent models, it still has a long way to go as a clinical therapeutic alternative. Clinical trials indicate that no long-term improvement of the neurologic condition and survival has been consistently achieved after the transplantation of human MSCs in stroke and other neurodegenerative diseases [59]. Clinical trials are quite different from the experimental animals, and the pathogeneses of human diseases are much more complicated than the lesion induced by experimental agents. However, it is appreciated that BM stem cells began their application in hematopoietic malignancies only 8 years after hematopoietic stem cells showed their treatment effectiveness in animal models. Further confirmation needs to be done in the effectiveness, safety, and underlying mechanism of long-term HUMSCs transplanted to nonhuman primates.

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Authorship Statement

N.X., X.C., Z.Z., Z.L., and T.W. designed the study. N.X, X.C, Z.Z., and J.H. collected and analyzed the results, and wrote the paper. N.X., X.C., Z.Z., C.C., M.J., and J.X. contributed to the performance of the research. N.X. and Z.L. contributed new reagents and analytic tools. X.C., Z.L., S.S., and T.W. reviewed and approved the manuscript. The authors declare no conflict of interest.

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