Pifithrin- α Enhances the Survival of Transplanted Neural Stem Cells in Stroke Rats by Inhibiting p53 Nuclear Translocation

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SUMMARY

Aims: To examine a novel strategy to enhance the survival of grafted neural stem cells (NSCs) in stroke model. **Methods:** Using a cell counting kit-8 (CCK-8) and TUNEL assay to test the protective effects of p53 inhibitor, pifithrin- α (PFT- α), on oxygen glucose deprivation (OGD) in NSCs. We compared the effects of vehicle + NSCs and PFT- α + NSCs on the efficacy of transplantation in stroke rat model using behavioral analysis, immunohistochemistry, etc. **Results:** Pifithrin- α increased viability and decreased apoptosis in NSCs after OGD *in vitro*. By *in vivo* studies, we showed that the best recovery of neurological function in the stroke rats and the maximum survival of grafted NSCs were found in the PFT- α + NSCs group. Twelve hours after cell transplantation, p53 was localized to the nuclei of grafted NSCs in the vehicle + NSCs group but was primarily localized to the cytoplasm in the PFT- α + NSCs group. The p53-upregulated modulator of apoptosis (PUMA) was highly expressed among the grafted cells in the vehicle + NSCs group compared with that in the PFT- α + NSCs group. **Conclusion:** Our results indicate that PFT- α enhances the survival of grafted NSCs through the inhibition of p53 translocation into the nucleus.

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Introduction

Stroke is often a consequence of hypertension and is one of the major causes of disability in the adult population, and it is the third leading cause of death followed by cancer and myocardial infarction [1,2]. Unfortunately, the available therapeutic tools to treat stroke patients and to improve the recovery of their impaired functions are quite limited. In recent years, many studies have shown that neural stem cells (NSCs) have the capacity to differentiate into various cell types, such as neurons, astrocytes, and oligodendrocytes and can secrete various neurotrophic factors both *in vitro* and *in vivo* [3–6]. The transplantation of NSCs has been

proposed for the effective treatment of neurological diseases, such as stroke and Parkinson's disease [7,8]. However, poor cell viability after transplantation poses a critical problem for clinical application. Although more than 90% of the cells are viable at the time of transplantation, the vast majority of these cells die after transplantation [9–12]. Therefore, the enhancement of graft survival is an essential strategy for improving the clinical efficacy of cell transplantation therapies.

Apoptosis is the most common morphological type of programmed cell death in neurons, which occurs under a variety of pathological conditions, such as neurodegenerative diseases and hypoxic-ischemic brain injury [13,14]. Several studies suggest

that the tumor suppressor protein, p53, is a key regulator of cell death under multiple physiological and pathological conditions. DNA-damaging agents, excitotoxic stimuli, oxidative stress, and hypoxia/ischemia all can induce p53-dependent cell death in the nervous system [15–17]. The role of p53 in neuronal cell apoptosis is relatively well understood; however, the function of p53 in the NSCs following transplantation in the stroke brain remains unclear. A number of studies have shown that neural progenitor cells (NPCs) and multipotent NSCs express relatively high levels of p53 [18,19]. In the mouse brain, p53 is critical for the induction of apoptosis in NPCs during the development of the central nervous system [20,21]. The neurospheres from the lateral ventricle wall of p53 null animals display an increased self-renewal, an enhanced cell proliferation, and a reduced cell apoptosis [19]. The administration of the p53 inhibitor enhances the survival, proliferation, and migration of subventricular zone NPCs and inhibits the expression of p53-dependent pro-apoptotic genes in animal stroke models [22]. Taken together, these data suggest that p53 may function as a regulator of survival/apoptosis in NSCs and NPCs under ischemic conditions. Therefore, we hypothesized that p53 inhibition might reduce apoptosis and enhance the survival of NSCs following transplantation in the stroke brain.

In this study, the p53 inhibitor, pifithrin- α (PFT- α), was used to pretreat NSCs and administered once to the stroke rats 30 min after cell transplantation; we subsequently evaluated whether this combined treatment could enhance the survival of grafted NSCs and improve recovery in the rat stroke model.

Materials and Methods

The Harbin Medical University Animal Supervision Committee approved all the experiments conducted in this study.

In Vitro Studies

Isolation and Culture of NSCs

Neural stem cells were obtained from the cortex and ganglionic eminence of 14-day-old Sprague–Dawley (SD) fetal rats as described previously [23,24]. Briefly, the brain tissue was dissected in PBS containing penicillin (50 U/mL; Invitrogen Corp, Carlsbad, CA, USA) and streptomycin (50 μ g/mL; Invitrogen Corp). Immediately after dissection, the tissue was triturated to a single-cell suspension using a transfer pipette. The cells were seeded and cultured in a 1:1 mixture of DMEM/F12 (Invitrogen Corp) supplemented with 20 ng/mL EGF, 20 ng/mL b-FGF (Pepro Tech Asia, Rehovot, Israel), 25 mM L-glutamine, 2% B27 (Invitrogen Corp), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C, 5% CO₂, 95% humidified atmosphere. The NSCs were cultured as free-floating neurospheres, and half of the medium was replaced by equal volume of fresh culturing medium every 3–4 days. The cells were passaged every 7–9 days.

Oxygen Glucose Deprivation and PFT- α Treatment of NSCs

To induce oxygen glucose deprivation (OGD), the NSCs (10⁴/well) were seeded in 96-well plates in 200 μ L of glucose-free NBM-B27

medium (Neurobasal glucose-free; Invitrogen) and incubated at 37°C, 5% CO₂, and 94% N₂ in an incubator under hypoxic conditions (Thermo Scientific, Marietta, OH, USA). Subsequently, the cell viability was determined (refer to below) by cell counting kit-8 (CCK-8; Beyotime, Shanghai, China) at 0, 1, 3, 6, 12, and 24 h, respectively. The cell viability of NSCs began to decrease with OGD exposure times of >1 h; therefore, we evaluated the protective effect of PFT- α (>98%; Beyotime) on NSCs at the time points of 3, 6, and 12 h. A final concentration of 0, 0.5, 1, 5, 10, 20, 40, and 80 μ M PFT- α was added, respectively, into the glucose-free NBM-B27 medium under OGD conditions.

Cell Viability Analysis

The cell viability of NSCs cultured in normal condition for 24 h was examined in the presence of 0–80 μ M PFT- α . We then evaluated the cell viability under the conditions of OGD or OGD + PFT- α treatment. Briefly, 20 μ L of CCK-8 solution was added to each well, and the cells were incubated at 37°C for 2 h. Subsequently, the absorbance of samples was measured on a microplate reader (TECAN, Männedorf, Switzerland) at a wavelength of 450 nm. The cell growth was expressed as relative cell viability (%) and was calculated using the following equations: $(A_{\text{OGD}} - A_{\text{Blank}}) / (A_{\text{Normoxia}} - A_{\text{Blank}}) \times 100\%$ or $(A_{\text{PFT-}\alpha} - A_{\text{Blank}}) / (A_{\text{Control}} - A_{\text{Blank}}) \times 100\%$, where A represents the absorbance values obtained under the indicated conditions. Each experiment was performed in triplicate.

Diameter Measurement of Neurospheres

Under an inverted phase contrast microscope, the passage 2 neurospheres (diameter of 80–90 μ m) were individually transferred into nonadherent 96-well plates (one neurosphere/well) in 200 μ L of medium using a sterile capillary tube. Then, the neurospheres were randomly divided into control + vehicle (DMSO) (culture in normal growth condition), control + PFT- α (10 μ M), OGD + vehicle (DMSO), and OGD + PFT- α (10 μ M) groups (n = 6 for each group). One hour later, OGD exposed and OGD exposed + PFT- α (10 μ M) neurospheres were transferred to normal growth conditions for 7 days. The diameter of the neurospheres was measured using a DP71 camera and Image-Pro Plus version 5.0.1 software (Media Cybernetics Inc. Shanghai, China) according to previously described methods [25,26]. The diameters for each treatment group were obtained from more than three independent experiments.

TUNEL Assay

The cells were divided into two groups: OGD + vehicle (DMSO) group and OGD + PFT- α (10 μ M) group. Each group was treated under OGD condition for 0, 1, 3, and 6 h, respectively, and then fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% TritonX-100 for 2 min on ice followed by staining with TUNEL reagent for 1 h at 37°C. The FITC-labeled TUNEL-positive cells were imaged using fluorescent microscopy (Olympus Fluoview 1000; Olympus, Tokyo, Japan) with 488 nm excitation and 530 nm emission wavelengths. The cells with green fluorescence were described as apoptotic cells. The

number of TUNEL-positive cells was counted on a computer screen obtained in 6 randomly selected fields from each group ($\times 200$). Two independent investigators quantified the number of TUNEL-positive cells.

In Vivo Study

Middle Cerebral Arterial Occlusion Surgery and Reperfusion

Male SD rats weighing 200–240 g were anesthetized with chloral hydrate (350 mg/kg-bw, i.p.). The middle cerebral artery was isolated through a skin incision, occluded with a nylon monofilament (0.24 mm diameter) suture with a rounded tip using an intraluminal vascular occlusion method [27,28]. Two hours after the occlusion, the filament was withdrawn for reperfusion. The body temperature of the rats was maintained at 37°C using a heating pad provided with a rectal probe during the entire procedure, and the rats were monitored postoperatively for 4 h after surgery. Twenty-four hours later, a CT scan was performed to confirm infarction occurred in right hemisphere of the Middle Cerebral Arterial Occlusion (MCAO) rats.

Experimental Groups

At 48 h after MCAO surgery, the rats were randomly divided into three groups: PFT- α + NSCs group ($n = 18$), vehicle (DMSO) + NSCs group ($n = 18$), and control group ($n = 6$). For the PFT- α + NSCs therapeutic group, the NSCs were pretreated with 10 μ M PFT- α for 30 min, and subsequently, 2×10^5 cells suspended in 2 μ L of PFT- α (10 μ M) in PBS were transplanted intracerebrally; at 30 min after transplantation, the MCAO rats were injected with PFT- α (2 mg/kg-bw, i.p.). The rats in the vehicle (DMSO) + NSCs group were transplanted with equal amounts of NSCs that were pretreated with vehicle (DMSO) and were administered vehicle alone (20 μ L DMSO in 1 mL saline, i.p.) injections. The control animals underwent the same procedure of injection with equal amounts of PBS but were injected with PFT- α (2 mg/kg-bw, i.p.) after transplantation.

PFT- α Pretreatment and Transplantation of NSCs

Under anesthesia with chloral hydrate (350 mg/kg-bw, i.p.), the rats were fixed in a stereotaxic frame (AlcBio, Shanghai, China) 48 h after MCAO. At the same time, the NSCs were labeled with the green fluorescent tracer 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) and were pretreated with PFT- α or vehicle (DMSO). Briefly, CMFDA at a dose of 1 μ g was loaded in 10 mL of NSCs (1×10^7) containing culturing medium and incubated at 37°C for 45 min; then, the CMFDA containing medium was removed and was replaced by 100 μ L of 10 μ M PFT- α or 100 μ L vehicle-PBS for 30 min. Two microliters of NSCs (2×10^5) containing PBS was transplanted in the right striatum with a 26-gauge Hamilton syringe at a flow rate of 0.5 μ L/min. Coordinates from the bregma were as follows: AP = -1.0 mm, ML = $+3.0$ mm, and DV = -5.0 mm. The control animals underwent the same procedure of injection using the same volume of PBS-vehicle. The needle was left in place for 5 min after

each injection and then removed slowly at a speed rate of 1 mm/min.

Evaluation of Neurological Function

Two unbiased investigators performed the neurological function testing without knowledge of the treatment information. The neurological function was evaluated at 2 h before and 1, 3, and 7 days after assessing cell transplantation using the neurological severity scores as previously modified [29]. The neurological function evaluation was graded on a scale of 0–18 (0 = normal score; 18 = maximal deficit score). We compared trends in the neurological function scores over the 7 days following transplantation. We also compared the differences between scores measured on days 3 and 1 and between scores measured on days 7 and 1 after transplantation.

Immunohistochemistry and Hematoxylin and Eosin (H&E) Staining

Rats were sacrificed using anesthetic chloral hydrate overdose at 12, 24 h, and 7 days after transplantation and were perfused transcardially with ice-cold PBS (pH 7.2). The brains were isolated and sliced into 5-mm-thick coronal slices, which involved needle entry site identifiable on the brain surface. Then, the brain slices were embedded in Tissue-Tek OCT compound (Sakura Seiki Fine-tek, Tokyo, Japan) and preserved at -80°C . At grafted point, a series of coronal sections (4 μ m/section) at 20- μ m intervals of every section according to the A-P axis were prepared. Brain sections were incubated with DAPI (1:1000) in 1% BSA for 10 min, then mounted on albumin-coated slides and sealed. To quantify the number of grafted cells (CMFDA labeled), six representative sections from each animal were blindly chosen and were analyzed with fluorescence microscopy. The CMFDA-positive cells were counted at 10 random fields (around injection point adjacent to ischemic border) on one slide, and the CMFDA-positive cell numbers from 10 fields of each slide and six sections from each animal were averaged, respectively. Two independent researchers blindly quantified the number of the CMFDA-positive cells.

Mouse monoclonal anti-p53 (diluted at 1:250; Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-p53 upregulated modulator of apoptosis (PUMA, diluted at 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to detect the expression of p53 and its targeting gene PUMA. Goat anti-mouse IgG and goat anti-rabbit IgG conjugated with Alexa 559 (diluted at 1:200; Molecular Probe, Eugene, OR, USA) were used to identify the primary antibodies, respectively. The control sections were incubated without primary antibody. To detect the injection site of NSCs in striatum, the brain sections (20 μ m) were stained with H&E staining.

Statistics

All data were expressed as the mean $\pm$ SD. Comparisons between different groups were made using the repeated measures one-way analysis of variance (ANOVA). When the F values were significant ($P < 0.05$), multiple comparisons were performed using the least significant difference test. The data were analyzed using the SPSS software (version 11.5.0; Chicago, IL, USA).

Results

PFT- α Exerts a Protective Effect on the OGD-induced Reduction in NSCs Viability and Growth Through Inhibition of Cellular Apoptosis

The cell viability of NSCs after the administration of OGD at the different time points was shown in Figure 1A. The results showed that the cell viability started to decrease as early as 1 h after OGD exposure ($P < 0.05$), followed by a substantial decrease at 3, 6, and 12 h with no further decrease at 24 h after OGD exposure ($P < 0.01$); however, as shown in Figure 1B, PFT- α attenuates

OGD-induced reduction in viability in NSCs with a dose-dependent manner; however, PFT- α treatment at dosages $>10 \mu\text{M}$ did not show an obvious enhancement of cell viability (data not shown). We have to point out that PFT- α at the concentrations up to $80 \mu\text{M}$ did not affect cell viability under the normal culturing condition in our studies (data not shown).

As shown in Figure 1C, D, there was no difference in the diameters of the neurospheres measured at 7 days in control + vehicle and control + PFT- α groups ($133.8 \pm 4.14 \mu\text{m}$ vs. $135.32 \pm 6.4 \mu\text{m}$). These results suggest that PFT- α does not affect the growth of neurospheres under normal culturing condition.

Figure 1 The protective effect of pifithrin- α (PFT- α) on oxygen glucose deprivation (OGD)-induced neural stem cells (NSCs) injury *in vitro*. **(A)** NSCs were exposed to OGD for 0–24 h as indicated. The viability of NSCs was reduced by OGD exposure in a time-dependent manner (* $P < 0.05$ and ** $P < 0.01$ vs. control, respectively); the OGD-induced reduction in viability reached plateau at 12 h. **(B)** OGD-induced reduction in cell viability was enhanced by PFT- α in a dose-dependent manner (* $P < 0.05$ and ** $P < 0.01$ vs. control, respectively). **(C)** Representative images of the neurospheres under the conditions as indicated (scale bar = $100 \mu\text{m}$; $n = 6$ neurospheres in each group). Ctr + vehicle and Ctr + PFT- α indicate that the neurospheres were cultured under the normal condition for 7 days in the presence of DMSO or PFT- α . OGD + vehicle and OGD + PFT- α indicate that the neurospheres were exposed to OGD for 1 h and subsequently were cultured under the normal condition for 7 days in the presence of DMSO or PFT- α . The diameter of neurospheres was measured on day 7 for each experiment group. **(D)** The growth of neurospheres was significantly inhibited by OGD exposure; PFT- α attenuated the inhibitory effect of OGD on neurosphere growth (* $P < 0.01$ compared with Ctr + vehicle; # $P < 0.01$ compared with Ctr + PFT- α ; \$ $P < 0.01$ vs. OGD + vehicle). **(E, F)** PFT- α attenuates OGD-induced apoptosis of NSCs. **(E)** Representative TUNEL staining fields were acquired from different experimental groups as indicated. The NSCs were exposed to OGD for 0, 1, 3, or 6 h in the absence or in the presence of PFT- α ($10 \mu\text{M}$). The cells undergoing apoptosis were positively stained with TUNEL reagent (shown in green; scale bar = $20 \mu\text{m}$). **(F)** Quantitative analysis of TUNEL-positive cells from different groups ($n = 3$ independent experiments). TUNEL-positive cells were counted from 10 images obtained in randomly selected fields from each group (## $P < 0.01$ vs. OGD + vehicle).

However, when exposing neurospheres to OGD for 1 h followed by normal culturing condition for 7 days (OGD + vehicle), it appeared that the diameter of neurospheres ($100.26 \pm 6.19 \mu\text{m}$) was significantly smaller compared with either control + vehicle and control + PFT- α group ($P < 0.01$). In comparison, in OGD + PFT- α group, the diameter of neurospheres was significantly larger than that of OGD + vehicle ($117.78 \pm 5.73 \mu\text{m}$ vs. 100.26 ± 6.19 ; $P < 0.01$), albeit was much smaller than that of either control + vehicle group and control + PFT- α group ($P < 0.01$). These results suggest that the growth of neurospheres was significantly inhibited by 1-h OGD treatment and that attenuated growth of neurospheres brought about by OGD was significantly rescued by PFT- α ($10 \mu\text{M}$).

The result of TUNEL staining assays shows that the number of apoptotic NSCs (FITC-positive cells) was significantly increased after OGD exposure for 1, 3, and 6 h; however, the abundance of apoptotic NSCs was greatly reduced after the administration of $10 \mu\text{M}$ of PFT- α (Figure 1E, F). These results suggest that PFT- α exhibits a protective effect on the OGD-induced reduction in NSC viability through the inhibition of cellular apoptosis *in vitro*.

The Combined Application of PFT- α and NSCs Transplantation Improves the Recovery of Neurological Function in MCAo Rats

The trend analysis showed that the neurological severity scores were significantly declined 3 days after transplantation in the vehicle + NSCs and PFT- α + NSCs groups compared with the control group ($P < 0.05$) (Figure 2A); however, there was no significant difference between these two cell transplantation groups. At 7 days after transplantation, the neurological severity scores continued to decline in the PFT- α + NSCs group compared with those either in the vehicle + NSCs group ($\#P < 0.05$) or in the control group ($P < 0.01$) (Figure 2A). The differences (changes in neurological severity scores) from scores measured on day 1 after cell transplantation were significantly greater on day 3 and day 7 in both cell transplant groups compared with the control group ($P < 0.01$) (Figure 2B). More importantly, on day 7 post-trans-

plantation, the differences from scores measured on day 1 after transplantation in the PFT- α + NSCs group were further improved compared with the vehicle + NSCs group ($\#P < 0.05$; Figure 2B). These results together suggest that the degree of recovery in neurological function of stroke rats was more significant in the PFT- α + NSCs group.

PFT- α Enhances the Survival of Implanted NSCs

To evaluate the reproducibility and stability of MCAo model, all the rats were examined by a CT scan 24 h after establishing MCAo model. The results showed that we were able to establish MCAo model with a consistent formation and size of infarction in right hemisphere of the rats (Figure 3A–C). CMFDA-labeled NSCs were transplanted into the medial part of the striatum, adjacent to the stroke-induced lesion (Figure 3D). The grafted NSCs were detected by immunohistochemistry (Figure 3E) and H&E staining (Figure 3F) at the injection site ($20\text{-}\mu\text{m}$ coronal sections, $\times 100$) 7 days after transplantation. The number of surviving cells (CMFDA-positive cell) was estimated from the six representative coronal sections ($4 \mu\text{m}$ along A–P axis, $\times 600$) where the NSCs were transplanted. As shown in Figure 3G, H, more surviving cells were observed in the PFT- α + NSCs group (16.70 ± 2.69 cells/field) compared with the vehicle + NSCs group (10.55 ± 2.47 cells/field, $P < 0.01$).

PFT- α Inhibits the p53 Nuclear Translocation and the Expression of p53 Target Gene, PUMA

The expression of p53 in the implanted NSCs showed that a large amount of p53 was accumulated in grafted NSCs at 12 h in both cell transplantation groups; however, it was hardly to find the expression of p53 in grafted NSCs at 24 h in both cell transplantation groups (data not shown). The cellular localization of p53 was observed using the amplification of the local area in Figure 4A, B (bottom panels) and shows that p53 was predominantly present in the nucleus in the vehicle + NSCs group but mainly localized in the cytoplasm in the PFT- α + NSCs group.

Figure 2 (A) Neurological severity scores were evaluated at 2 h before and 1, 3, or 7 days after cell transplantation. Three days after transplantation, the neurological severity scores were declined in the vehicle + neural stem cells (NSCs) and pifithrin- α (PFT- α) + NSCs groups at the same degree ($*P < 0.05$ compared with the control group). On day 7, the neurological severity scores in the PFT- α + NSCs group were significantly declined compared with those in control group ($**P < 0.001$) and in the vehicle + NSCs group ($\#P = 0.024$). (B) The differences of neurological severity scores (difference between scores measured on days 1 and 3 or days 1 and 7 after transplantation) were improved significantly in cell-transplanted rats on days 3 and 7, with further improvement in the PFT- α + NSCs group on days 7 compared with the vehicle + NSCs group ($**P < 0.01$ vs. control; $\#P < 0.05$ compared with vehicle + NSCs; $n = 6$ rats in each group).

Figure 3 Extent of striatal damage and location of the graft. (A–C) The CT scan images demonstrate the areas of the stroke lesion. The low condensed portions in right hemisphere, where indicated by the white dashed lines, represent the infarction regions. (D) The white dashed line formed circle indicates the graft point of neural stem cells (NSCs; the white arrow head), which is in the vicinity of the stroke-induced lesion area. (E, F) The grafted NSCs were detected by immunohistochemistry, hematoxylin and eosin staining at the injection site 7 days after transplantation; the brain sections were sliced 20 μ m each ($\times 100$). (G, H) Seven days after transplantation, six sections (the brain sections were sliced 4 μ m each; $\times 600$) from the vehicle + NSCs and pifithrin- α (PFT- α) + NSCs groups, respectively, were examined by fluorescence microscopy to observe chloromethylfluorescein diacetate (CMFDA)-labeled NSCs. The white arrow heads indicate CFMDA-positive cells. (H) More surviving cells (CMFDA-positive) were observed in the PFT- α + NSCs group compared with those in the vehicle + NSCs group (16.70 ± 2.69 cells/field vs. 10.55 ± 2.47 cells/field, respectively; $**P < 0.01$; scale bar = 10 μ m).

Figure 4 (A, B) Localization and expression profile of p53 in the grafted neural stem cells (NSCs). Cellular expression and localization of p53 were determined at 12 h after transplantation and viewed by confocal microscopy. In the vehicle + NSCs group, p53 was predominantly localized in nuclei of grafted NSCs; in contrast, p53 was mainly localized in the cytoplasm of grafted NSCs in the pifithrin- α (PFT- α) + NSCs group. The images shown in lower panels indicate the amplified fields as indicated by the white frames in the top panels. (C, D) Expression profile of p53-upregulated modulator of apoptosis (PUMA) in grafted NSCs. (C) Immunofluorescence staining was used to detect the expression of PUMA in the grafted NSCs at 12 h after cell transplantation. The white arrow heads indicate the PUMA-positive grafted NSCs. Scale bar = 10 μ m. (D) The ratio of PUMA-positive grafted NSCs was significantly reduced in the PFT- α + NSCs group compared with the vehicle + NSCs group ($29.4 \pm 5.6\%$ vs. $38.9 \pm 4.3\%$; $**P < 0.01$).

As shown in Figure 4C, PUMA-positive grafted NSCs were found in both cell transplantation groups. Six sections from each cell transplantation group were used to calculate the rate of PUMA-positive/CMFDA-positive cells in 10 random fields. As shown in Figure 4D, the ratio of PUMA-positive/CMFDA-positive NSCs was significantly reduced in the PFT- α + NSCs group compared with the vehicle + NSCs group ($29.4 \pm 5.6\%$ vs. $38.9 \pm 4.3\%$; $**P < 0.01$).

Discussion

Our data show, for the first time, that PFT- α significantly increases the survival of NSCs under the OGD conditions *in vitro* and under the ischemic environment *in vivo*. A combination of PFT- α and NSCs transplantation promotes the recovery of neurological function in rat stroke models.

The standard model of OGD is based on the lack of both glucose and oxygen [30]. To mimic the hostile environment of the ischemic brain *in vitro*, we used 1% O₂ and glucose deprivation as the OGD model in the *in vitro* study; nevertheless, under the OGD condition, we observed a significant decrease in cell viability and enhanced induction of apoptosis in NSCs. As the OGD-induced injury is closely related to the activation of p53, we also examined whether treatment with the p53 inhibitor, PFT- α , could attenuate OGD-induced injury in NSCs. We observed that in the presence of PFT- α , the NSCs exhibited an increased viability and a reduced apoptosis under the conditions of OGD. These results are consistent with the notions that PFT- α protects hippocampal neurons against OGD injury [31] and p53-null neural stem/progenitor cells showed an increased number of neurosphere formations and increased sphere size [32].

In the process of implantation and after implantation in the stroke brain, the transplanted cells were exposed to hypoxia/ischemia damage, which could lead to the transplanted cell death or to reduce cell viability. Numerous methods have been developed to improve grafted cell survival [33–36], including the cytoprotection of donor cells or modulation of the host tissue environment. PFT- α is a small molecular weight lipophilic compound that can pass through the blood–brain barrier when administered systemically [37]; therefore, it is amenable to systemic administration to animals undergoing transplantation. However, the lack of blood flow in ischemic brain tissues could reduce the concentration of the drug; thus, we pretreated NSCs with PFT- α before transplantation and subsequently administered an intraperitoneal injection of PFT- α to stroke rats at 30 min after transplantation. Our results indicate that this novel strategy significantly improved the survival of exogenous NSCs grafts. Although it has been reported that apoptosis in grafts lasts for a week after transplantation [38], p53-dependent programmed cell death occurs during early-stage ischemia [16]. The effectiveness of p53 inhibition was only observed when the inhibitor was adminis-

tered before or shortly after MCAo surgery; therefore, it is possible to protect the grafted NSCs through the pretreatment of implanted NSCs with PFT- α and/or post-transplantation injection of PFT- α (2 mg/kg/bw, i.p.) in MCAo rats. It has been shown that the early recovery (before 14 days) of the host animal is largely due to the trophic effect of the grafted cells rather than to the replenishment of lost cells [39]. Therefore, when the survival rate of grafted cells is high, there would be more trophic factors released by these cells, which could explain why we observed increased behavioral recovery in the PFT- α + NSCs group compared with the NSCs transplantation group.

The mechanisms by which p53 induces cell death have been extensively studied in both cerebral ischemic animal models *in vivo* and under hypoxic conditions *in vitro* [16,31,40]. Death stimuli, such as oxidative stress and ischemia injury, cause p53 nuclear accumulation and activation of the expression of downstream pro-apoptotic genes including PUMA, Noxa, and/or Bax [15]. Among these pro-apoptotic genes, PUMA plays a critical role in p53-dependent cell death. Gene knockout studies revealed that DNA damage-induced p53-dependent apoptosis was severely diminished in PUMA-deficient cells *in vitro* [41]. PUMA-deficient neurons are resistant to p53-induced neuronal apoptosis [42]. The deletion of PUMA attenuated cell death and improved physiological functions after ischemic–reperfusion injury [43]. These studies suggest that reducing the expression of PUMA would lead to an increase in cell survival. Similarly, in our study, we observed that the survival of exogenous NSC grafts in stroke rats was also significantly enhanced through the inhibition of p53 and its target gene PUMA. These results suggest that PFT- α promotes the survival of NSCs, at least in part, through inhibiting the activation of p53 and the expression of PUMA.

In conclusion, our results demonstrate that a combination of PFT- α and NSCs transplantation enhances the survival of NSCs following transplantation in a rat stroke model. More importantly, this method promotes the recovery of neurological function in MCAo rats. Clinically, this combined treatment might provide a simple, safe, and economical method of enhancing the efficacy of cell transplantation therapy.

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Conflict of Interest

The authors declare no conflict of interest.

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