Stem Cells International

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Editorial Board

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Hindawi
Stem Cells International
Volume 2018, Article ID 4243102, 2 pages
https://doi.org/10.1155/2018/4243102

Editorial

Application of Stem Cells and Advanced Materials in Nerve Tissue Regeneration

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Received 29 July 2018; Accepted 29 July 2018; Published 16 September 2018

Tissue engineering is a field that combines engineering and life science principles to bring about a betterment to humanity through cellular or tissue function reconstitution. With this in mind, two main components of materials and cell sources are usually included in optimizing tissue engineering constructs. Engineered neural graft integrates advanced biomaterials (including various bioactive factors), and suitable stem cells can be orchestrated to facilitate nerve tissue repair and regeneration. This special issue addresses the role of stem cells and biomaterials (including growth factors) in neural tissue regeneration. The themes include scaffold design, signaling pathway study, different stem cell assessments, and various nerve tissue regeneration applications, such as brain injury repair, cerebral ischemia-reperfusion injury repair, and spinal cord injury repair. From 10 submissions, 6 papers are published in this special issue. Each paper was reviewed by at least two reviewers and revised according to reviewers' comments. The papers cover the following neural repair and regeneration strategies: seeding of appropriate stem cells only and integration of biomaterials (including synthetic biopolymers, hydrogels, and growth factors) and stem cells.

During the last decade, tremendous progress has been achieved in stem cell biology and its application in treating various disorders of neural systems. In particular, the multipotency and easy access to large quantity of specific target cell offer unique opportunities for neural tissue regeneration. In this special issue, prospects and emerging challenges of using different stem cell-based therapies on common neurological diseases were discussed. Dental pulp stem cells (DPSCs) is one kind of mesenchymal stem cells (MSCs) and possess MSC-like characteristics such as the ability for self-renewal and multilineage differentiation. In addition, DPSCs which originated from the cranial neural crest express neuron-related markers even before being induced to neuronal differentiation. Taken together, these unique properties make DPSCs an excellent candidate for stem cell-related therapies in nerve diseases. In L. Luo et al.'s review article, the authors summarized the neuronal differentiation potential, neurotrophic, angiogenic, and immunomodulatory properties of DPSCs in the pathological and injured nervous system. This paper reports that DPSCs have the biological properties of MSCs and possess a considerable capacity to differentiate into neuron-like cells and secrete neuron-related trophic factors due to their cranial neural crest origin. DPSCs also can express neuronal markers without preinduced differentiation. Meanwhile, DPSCs can both directly and indirectly stimulate formation of new blood vessels and enhance blood flow to injury sites because of its vascularization and immunomodulatory properties. Thus, both nondifferentiated and differentiated DPSCs are emerging as new cell sources for the treatment of nervous system deficits associated with SCI, stroke, AD, PD, and long gaps of peripheral nerve injury.

In Y. Liu et al.'s paper, menstrual blood-derived endometrial stem cells (MenSCs) were successfully isolated and applied to possible nerve tissue regeneration. Firstly, MenSCs were characterized to show their multipotency and immunophenotype. An upregulation of N-cadherin (N-cad)

expression at mRNA and protein levels was detected after transdifferentiation into glial-like cells, when MenSCs were cultured and induced *in vitro*. Moreover, *in vivo* studies also clearly showed that the knockdown of N-cad by in utero electroporation perturbed the migration and maturation of mouse neural precursor cells (NPCs). Taken together, the paracrine effect of MenSCs on neuroprotection and their potential of transdifferentiation into glial-like cells were confirmed. This study implies that transgenic MenSC-based therapy could be a potential tool for peripheral nerve injury repair.

In H. Yang et al.'s paper, the authors explored the effects of multiple tail vein injections of NSC-CM (conditioned medium derived from neural stem cells) on cerebral ischemia-reperfusion (I/R) rat model. They found that NSC-CM significantly ameliorated neurological defects and reduced cerebral infarct volume, accompanied by preserved mitochondria ultrastructure. Significant cell apoptosis of NSC-CM in ischemic hemisphere via improving the expression of Bcl-2 (B-cell lymphoma-2) was found too. This work suggested that NSC-CM might be an alternative and effective therapeutic intervention for ischemic stroke.

Spinal cord injury (SCI) is an intractable challenge with limited treatments in the world. Induced pluripotent stem cell-derived neural stem/progenitor cell (iPSC-NS/PCs) transplantation has been shown as a promising treatment for SCI; however, these applications are still vulnerable which may lead to tumorigenicity. Tumorigenicity of iPSC-NS/PCs is comprised of two aspects: teratoma and true tumor formation. Recent studies revealed that manifold factors had participated into the process of tumorigenesis including the use of tumor-inducing reprogramming factors and residual undifferentiated cells. Of note, the change of epigenetics played a pivotal role in iPSC-NS/PCs tumorogenesis. In J. Deng et al.'s review article, the authors summarized the recent studies regarding iPSC-NS/PC tumorigenicity in the treatment of SCI. Two different routes of tumorigenicity (teratomas and true tumors) and underlying mechanisms behind them were discussed. Finally, possible solutions to circumvent them were briefly introduced as well.

As compared to using pure stem cell-based therapeutic approach to facilitate neural regeneration, engineered biomaterial scaffolds exhibit a better prospective strategy, where grafted scaffold could provide a matrix support for cell penetration, proliferation, differentiation, and guidance to the target area. In L. Luo et al.'s another paper, the authors use DPSCs as the seeded cells in nerve tissue engineering and evaluate the effects of heparin-poloxamer (HP) hydrogel as a scaffold containing bFGF and DPSCs on the neural regeneration and functional recovery after spinal cord injury (SCI). The authors claimed that DPSCs combined with bFGF in HP-hydrogel (DPSCs-bFGF-HP) could enhance motor and sensory functional recovery after SCI than those of the other experimental groups. Increased expression of Bcl-2 and reduced expression of Bax and Caspase-3 to inhibit neuron apoptosis in the early process of recovery were also found. DPSCs-bFGF-HP could promote the expression of MBP and GAP43 during nerve repair process after SCI. As a result, the combination of HP hydrogel, DPSCs, and bFGF could be applied as an effective treatment for neuron regeneration and tissue restoration after SCI in the future. In L. Zhou et al.'s paper, poly(lactic-co-glycolic acid) (PLGA) scaffold was utilized to support mesenchymal stem cell (MSC) and neuron growth *in vitro* and *in vivo*. In particular, the effect of PLGA scaffold on cell proliferation and differentiation of MSC towards neurogenesis were assessed. This study suggested that MSCs-PLGA complex may be used as tissue engineering material for brain injury.

In this interdisciplinary research field of neural tissue regeneration, these papers show us exciting outcomes and insightful perspectives with state-of-the-art in rising topics. We hope that this special issue would be of great concern to the interested readers. We also would like to express our great appreciation to all the authors, reviewers, and the editors for their support in making this special issue possible.

Conflicts of Interest

The authors would like to declare that there is no conflict of interest for the submission of this special issue.

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Ming Su

Hindawi
Stem Cells International
Volume 2018, Article ID 5024175, 8 pages
https://doi.org/10.1155/2018/5024175

Research Article

Combining PLGA Scaffold and MSCs for Brain Tissue Engineering: A Potential Tool for Treatment of Brain Injury

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Received 2 March 2018; Revised 26 June 2018; Accepted 12 July 2018; Published 5 August 2018

Academic Editor: Andrzej Lange

Nerve tissue engineering is an important strategy for the treatment of brain injuries. Mesenchymal stem cell (MSC) transplantation has been proven to be able to promote repair and functional recovery of brain damage, and poly (lactic-co-glycolic acid) (PLGA) has also been found to have the capability of bearing cells. In the present study, to observe the ability of PLGA scaffold in supporting the adherent growth of MSCs and neurons in vivo and vitro and to assess the effects of PLGA scaffold on proliferation and neural differentiation of MSCs, this study undertakes the following steps. First, MSCs and neurons were cultured and labeled with green fluorescent protein (GFP) or otherwise identified and the PLGA scaffold was synthesized. Next, MSCs and neurons were inoculated on PLGA scaffolds and their adhesion rates were investigated and the proliferation of MSCs was evaluated by using MTT assay. After MSCs were induced by a neural induction medium, the morphological change and neural differentiation of MSCs were detected using scanning electron microscopy (SEM) and immunocytochemistry, respectively. Finally, cell migration and adhesion in the PLGA scaffold in vivo were examined by immunohistochemistry, nuclear staining, and SEM. The experimental results demonstrated that PLGA did not interfere with the proliferation and neural differentiation of MSCs and that MSCs and neuron could grow and migrate in PLGA scaffold. These data suggest that the MSC-PLGA complex may be used as tissue engineering material for brain injuries.

1. Introduction

In recent years, the development of tissue engineering has provided a new strategy for the repair of tissue injuries. The core of tissue engineering is to construct new tissue substitutes composed of biological materials and cells for promoting the recovery and maintenance of biological functions [1, 2]. Biological materials not only offer three-dimensional space for cell adhesion, growth, and migration but also form adjustable microenvironments for the nutrition obtainment and waste excretion of cells [3]. Biological materials used for neural tissue engineering can be mainly divided into 5 categories: artificial synthetic nonbiodegradable materials, nondegradable composite ducts, natural biological materials, biodegradable composites, and biodegradable polymer materials.

Poly (lactic-co-glycolic acid) (PLGA) is one biodegradable polymer material and the degradation time of PLGA can be adjusted simply by altering the ratio of lactic acid and glycolic acid in its copolymer for particular applications. PLGA with a ratio of 75 : 25 of PLA : PGA showed great stability in body fluids (pH 7.2) with an optimum degradation rate (9% to 12% or so), and axons could regenerate into the implanted PLGA scaffolds in rats subjected to thoracic spinal cord transection injury [4]. Mesenchymal stem cells (MSCs) could differentiate into neuron-like cells under

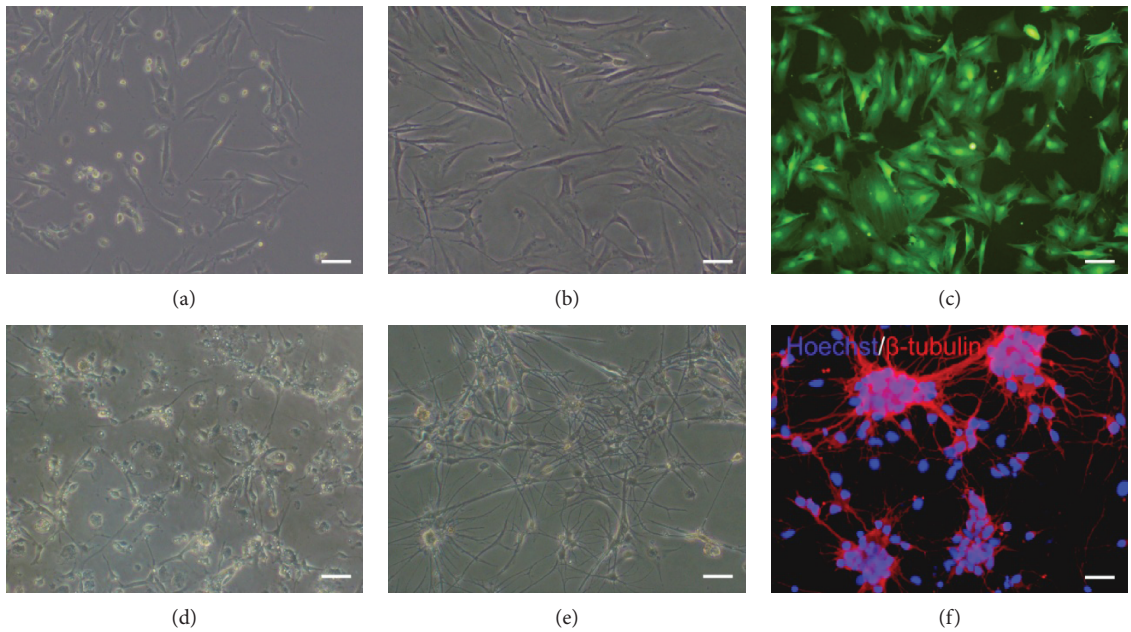


Figure 1: Morphologic characteristics of MSCs and neurons. (a) The primary MSCs were cultured for 4 days. (b) The 3rd-passage MSCs were cultured for 2 days. (c) The MSCs infected with adenovirus for 2 days were lighted green fluorescence. The primary neurons were cultured for (d) 3 days and (e) 7days. (f) The identification of neurons by immunostaining with β-tubulin. Bar = 50 μm.

specific culture conditions and had some electrophysiological properties of neurons [5–7], which makes them a kind of seed cells for the treatment of nerve tissue injuries. The aim of this study is to evaluate whether the MSC-PLGA scaffold complex is a potential tool for the treatment of brain injuries.

2. Materials and Methods

2.1. Preparation and Labeling of MSCs. Two-month-old adult and 1-day-old newborn Sprague Dawley (SD) rats (Animal House Center, Southwest Medical University) were used in this study. The procedure to use the animals was in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China. Bone marrow was obtained from femoral marrow cavities of 2-month-old rats. The MSCs were isolated and purified from bone marrow by density gradient centrifugation and adherent culture methods, and they were cultured by using alpha-minimum essential medium (α-MEM) (HyClone) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco). When the cultured cells became confluent, they were resuspended and subcultured.

To facilitate the observation of the growth and migration of MSCs in the PLGA scaffold or a brain, MSCs were labeled with green fluorescent protein (GFP) as previously described [8]. Briefly, we amplified the previously frozen adenovirus (pAdEasy-1-pAdTrack cytomegalovirus) that contained the GFP gene. When the third passage MSCs grew to 70–80% confluence, the adenovirus solution was added, which was followed by incubation for 2 days in an incubator (37°C, 5% CO2), and MSCs showed green fluorescence under fluorescent microscopy. Then the cells were harvested for follow-up experiments.

2.2. Preparation and Identification of Neurons. Cortical neurons were prepared from 1-day-old newborn rats as previously described [9]. Newborn rats were decapitated, and cerebral cortexes were transferred to PBS. After the removal of the meninges and blood vessels, tissues were cut into small pieces, followed by incubation in 0.25% trypsin-EDTA solution (Beyotime) at 37°C for 20 minutes. Then LG-DMEM with 20% fetal bovine serum was added to terminate the incubation. After centrifugation at 800 rpm for 10 minutes, the cells were collected for follow-up experiments.

Cortical neurons were dispersed with a neuronal medium (Sciencell) with 1% (vol/vol) neuronal growth supplement (Sciencell), and then they were seeded at a density of 5×10^5/ml onto coverslips precoated with poly-L-lysine in 6-well plates (Corning). The medium was replaced once every 3 days, and after 7 days, the cells were fixed with 4% formaldehyde and used for the identification of neurons by immunocytochemistry.

2.3. Fabrication of PLGA Scaffold. The PLGA was synthesized by the room temperature molding/particle leaching method as previous described [8]. In short, 75% lactic acid and 25% glycolic acid were dissolved in dichloromethane and blended with sieved sodium chloride particles ranging from 80 to 120 μm. The mixture was poured in molds to form discs (5 cm × 5 cm × 0.2 cm). After molding for 24 hours under pressure, the discs were taken out and immersed in deionized water to release the sodium

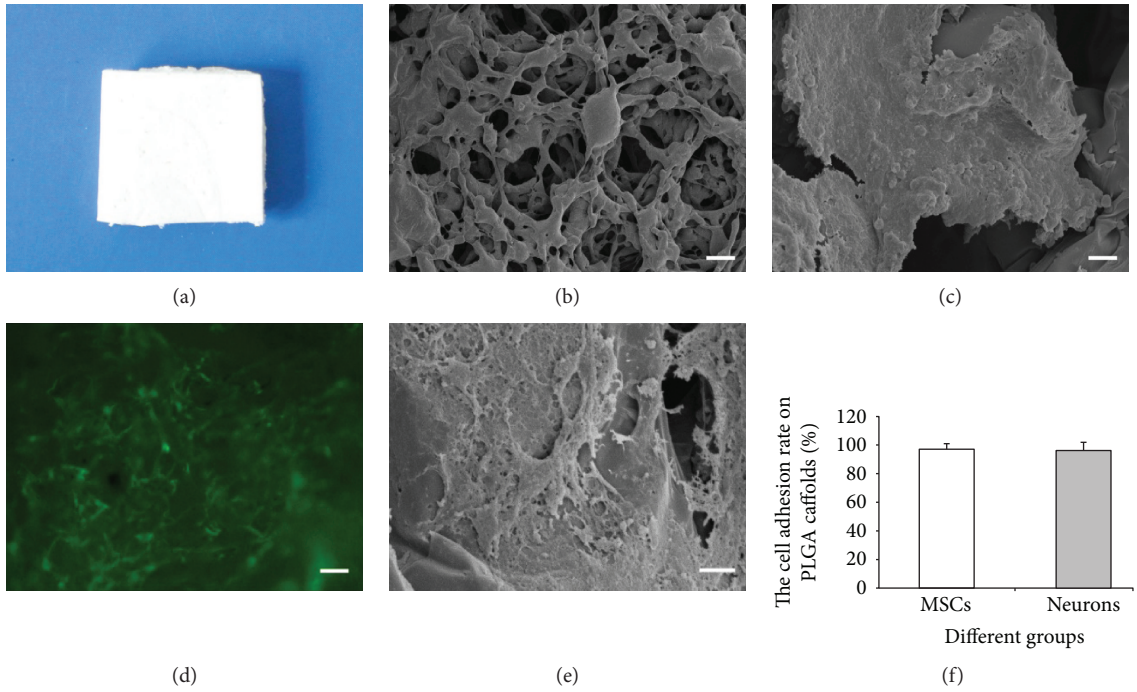


Figure 2: PLGA scaffold and cell adhesion. (a) PLGA scaffold. (b) SEM imaging of the PLGA scaffold. Bar = 100 μm. (c) SEM imaging of the neurons on the PLGA scaffold. Bar = 10 μm. (d) MSCs planted on the PLGA scaffold were lighted green fluorescence. Bar = 50 μm. (e) SEM imaging of MSCs on the PLGA scaffold. Bar = 10 μm. (f) The adhesion rates of MSCs and neurons on the PLGA scaffold.

chloride particles and the scaffolds were desiccated and kept in a vacuum plastic bag before use.

2.4. Cell Adhesion on PLGA Scaffold. The PLGA scaffold was cut into pieces (1 cm × 1 cm × 0.2 cm). The latter were dipped in 75% alcohol for 2 hours and then washed 3 times with sterile water and dried in clean bench; after that, the sterile PLGA scaffolds were placed in a 24-well culture plate. MSCs and neurons were seeded on the scaffolds at a density of 1×10^5 cells per scaffold in the corresponding medium under standard cell culture conditions. After 3 days, the culture mediums were removed and the cells on the scaffolds and wells were collected by trypsin digestion and counted as n_{PLGA} and n_{well}, respectively. The adhesion rates of MSCs and neurons on scaffolds were calculated as follows:

$$r = \frac{n_{\text{PLGA}}}{n_{\text{PLGA}} + n_{\text{well}}} \times 100\%. \quad (1)$$

2.5. MTT Assay. The effect of the PLGA on the proliferation of MSCs in vitro was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay based on the instruction manual of the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime). Briefly, MSCs were seeded on PLGA scaffolds at a seeding concentration of 1×10^5 cells per scaffold (1 cm × 1 cm × 0.2 cm) per well of the 24-well culture plate and cultured for 48 hours. The control group was identically processed, except that the PLGA scaffolds were omitted. The culture medium was replaced with fresh medium for 24 hours. Then the MTT reaction solution was added to each well, and next, the plate was incubated for 4 hours at 37°C. After the mediums of all wells were removed, formazan dissolving solution was added into each well and incubated at 37°C for 4 hours. The supernatants of all wells were transferred into a 96-well culture plate, and the absorbance of each well was measured at 570 nm by a microplate reader.

2.6. Neuronal Induction of MSCs. MSCs were seeded on coverslips and PLGA scaffolds in a 24-well culture plate in α-MEM supplemented with 10% FBS (HyClone). After 3 days, the medium was replaced with a preinduction solution composed of α-MEM with 10% FBS and 1 mmol/l β-mercaptoethanol (β-ME) for 24 hours. This was followed by a neuronal induction medium that consisted of α-MEM with 1 mmol/l β-ME, 2% dimethyl sulfoxide (DMSO), and 1 μmol/l all-trans retinoic acid (RA) (Sigma) for 6 hours. Five coverslips and 5 PLGA scaffolds were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline, and the scaffolds were cut into 15 μm sections. The coverslips and sections were used to observe the effect of the PLGA scaffold on the differentiation of MSCs via immunocytochemistry. Five scaffolds were applied for transplantation and 5 scaffolds fixed with 4% glutaraldehyde were utilized for scanning electron microscopy (SEM).

2.7. PLGA Scaffold Transplantation. The traumatic brain injury (TBI) model was generated as previously described [10]. In brief, a 2-month-old rat was anesthetized with 1% pentobarbital sodium (40 mg/kg) via intraperitoneal administration and then fixed on a stereotaxic frame (Stoelting) in the prone position. Following the scalp incision, a piece of right parietal bone was removed by drilling. A dual incision

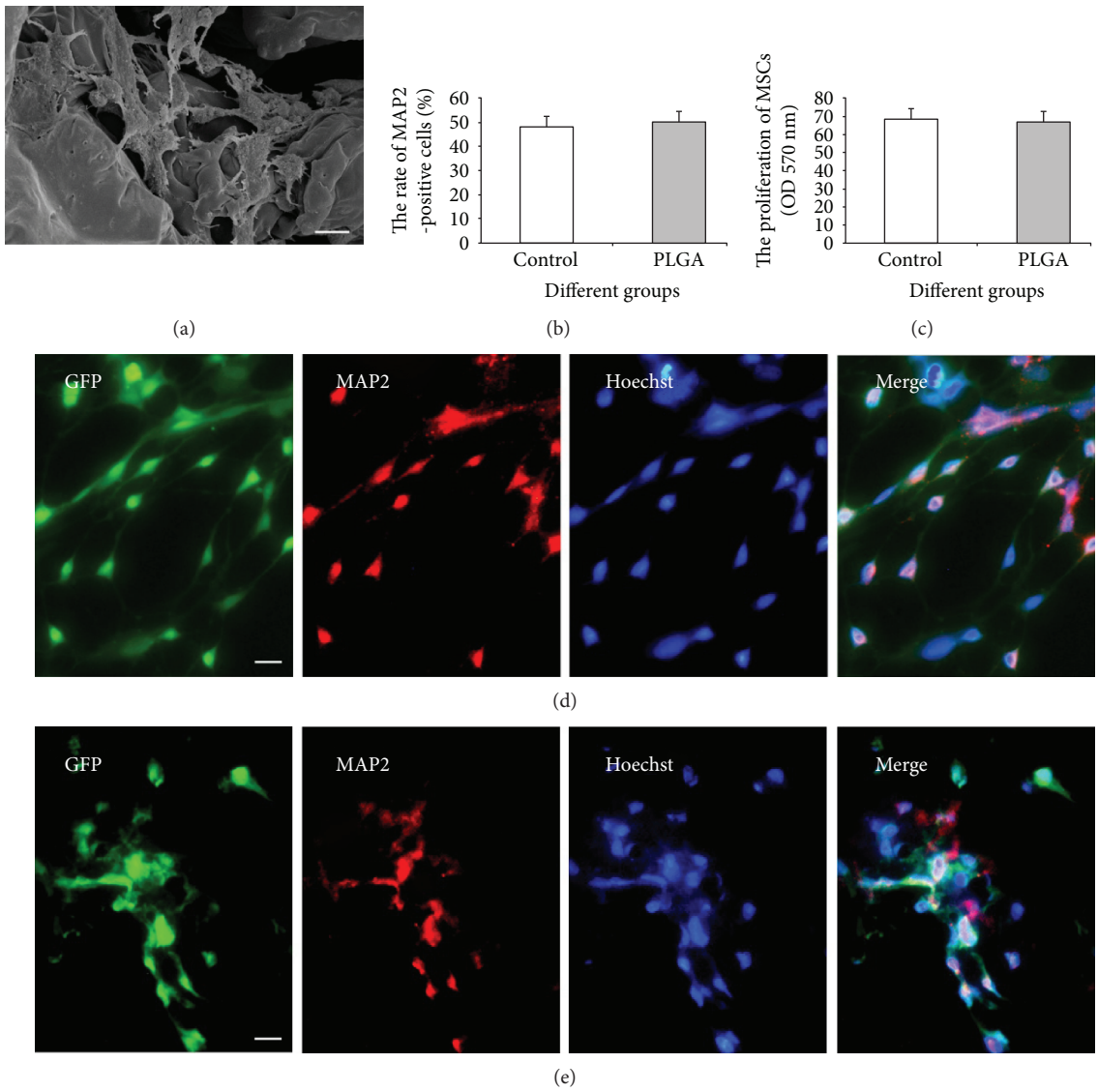


Figure 3: The effect of the PLGA scaffold on the differentiation and proliferation of MSCs in vitro. (a) SEM imaging of induced MSCs planted on the PLGA scaffold. Bar = 10 μm. (b) The rate of MAP2-positive cells among the MSCs after neural induction. (c) The proliferation of MSCs on the coverslip and PLGA scaffold. The control group (d) and the PLGA scaffold group (e): green fluorescence showed MSCs in vitro. Neurons (MAP2 positive) were stained with red fluorescence. Yellow fluorescence showed the colocalization of green and red, thus indicating the differentiation of MSCs. Bar =50 μm.

was made to expose the forebrain, and a defect area (3 mm × 3 mm × 2 mm) was created with a scalpel in the brain. The PLGA scaffold or MSC-PLGA scaffold complex was inserted in the brain, and the wound was sutured. On day 14 after TBI, rats (5/group) were killed by deep anesthesia and their brains were removed and then PLGA scaffolds and MSC-PLGA scaffold complex were taken for SEM. The other rats were perfused transcardially with 0.9% saline followed by ice-cold 4% PFA. The brains were taken out and postfixed in 4% PFA for 24 h. Then they were dehydrated with 30% sucrose solution, embedded in tissue-freezing medium, and cut into serial coronal sections (15 μm thickness) with a freezing microtome (Leica) for nuclear staining and immunohistochemical staining.

2.8. Scanning Electron Microscopy. Scanning electron microscopy (S-3400N, Hitachi, Japan) was used to observe the characteristics of the PLGA scaffold and the morphologies of the cells attached to it. Prior to imaging, cells that were cultured or grown on the scaffolds were fixed with 4% glutaraldehyde and dehydrated through a graded acetone series and then sputter coated with gold. Samples were examined at an accelerating voltage of 10 kV.

2.9. Immunohistochemistry. The slides of cells or frozen sections were treated with 0.3% Triton X-100 and then blocked with 8% goat serum. After being incubated with primary antibodies including β-tubulin (1 : 100 dilution, Abcam), microtubule-associated protein-2 (MAP2) (1 : 100 dilution,

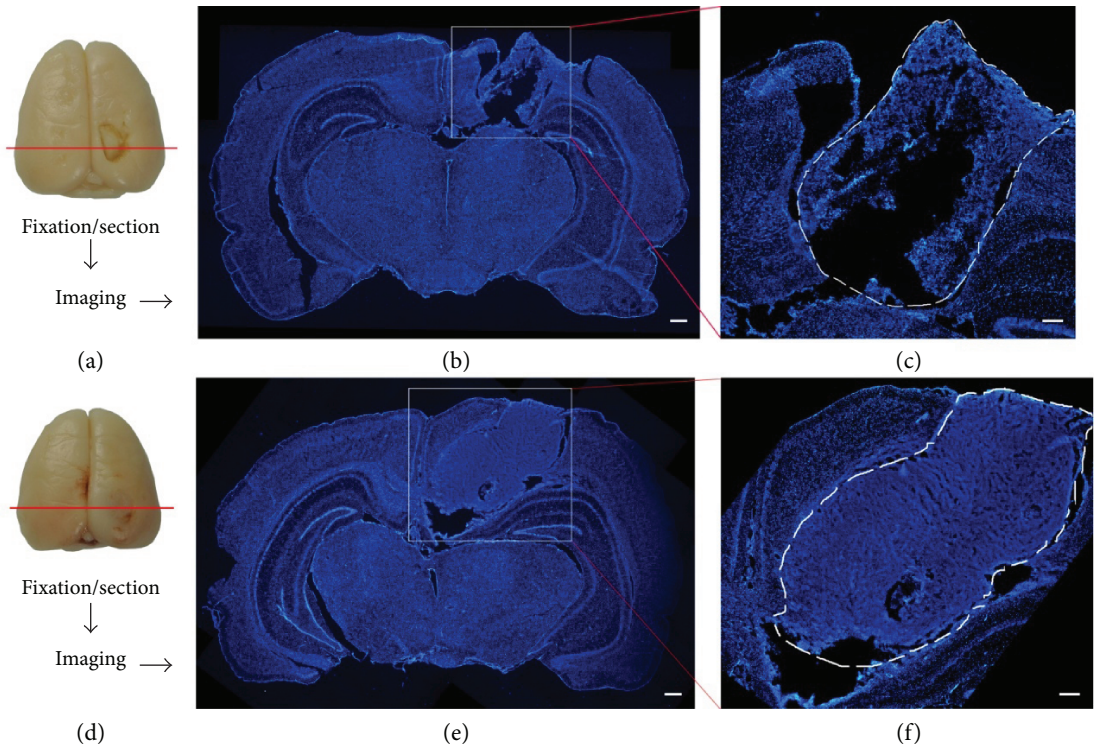


Figure 4: The structural change of the PLGA scaffold in brains with TBI. (a) Procedure to observe PLGA scaffold in the brain with TBI. (b) Nuclear staining shows the planted PLGA scaffold in the brain. Bar = 500 μm. (c) The magnifying picture of the square frame in (b). The PLGA scaffold in the outlined region (dashed line). Bar = 200 μm. (d) Procedure to observe the MSC-PLGA scaffold complex in the brain with TBI. (e) Nuclear staining shows the planted MSC-PLGA scaffold complex in the brain. Bar = 500 μm. (f) The magnified picture of the square frame in (e). The MSC-PLGA scaffold complex in the outlined region (dashed line). Bar = 200 μm.

Abcam), or glial fibrillary acidic protein (GFAP) (1 : 200 dilution, Sigma) overnight at 4°C, the samples were treated with anti-rabbit/mouse IgG (Alexa Fluor® 488/Fluor 594 Conjugate) (1200 dilution; Cell Signaling Technology) for 30 min at 37°C and then stained with Hoechst dye. Negative controls were identically processed, except that the primary antibodies were omitted.

2.10. Statistical Analysis. All cell experiments in vitro were repeated three times and the analyses were performed using the SPSS 18.0 software for Windows. The data are presented as the means ± standard error (SE), and the statistical comparisons were performed using one-way ANOVA. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Morphologic Characteristics of Cultured Cells. The primary MSCs began to adhere within 12 hours and presented round, polygon, or spindle shapes after 3-4 days (Figure 1(a)). The 3rd passage of MSCs displayed obvious uniformity (Figure 1(b)), and they were infected by the adenovirus-lighted green fluorescence under fluorescence microscope (Figure 1(c)). The primary cortical neurons showed fewer and shorter protuberances within 3 days (Figure 1(d)). Then many neurites appeared, which formed many neural networks on the seventh day (Figure 1(e)), and presented positive β-tubulin staining via immunocytochemistry (Figure 1(f)).

3.2. Attachment of MSCs and Neurons on PLGA Scaffolds. The volume of porosity of the PLGA scaffold approached 90% by using the liquid replacement approach, and the interior pores of the PLGA scaffold that were directly visualized by SEM were intercommunicated (Figures 2(a) and 2(b)). After culturing of the MSCs and neurons for 5 days, a large number of cells were found adhered and extended on the surface of the PLGA scaffold and the cells were flat and connected to each other (Figures 2(c)–2(e)). On the 3rd day after inoculation, the adhesion rates of MSCs and neurons on the PLGA scaffold were 97.4% and 96.5%, respectively, and there was no significant difference between the two groups (Figure 2(f)).

3.3. The Effect of PLGA Scaffold on Differentiation and Proliferation of MSCs In Vitro. After induction, the MSCs presented neuron-like morphology with swollen cell bodies and long thin processes, and intercellular boundaries became manifested (Figure 3(a)). The induced MSCs could express the marker of neurons (MAP2), and the rate of both $MAP2^+$ MSCs in the PLGA scaffold group was not significantly different compared with that in the control group (Figures 3(b), 3(d), and 3(e)). Furthermore, MTT assay was used to evaluate the proliferation of the MSCs cultured on the PLGA scaffold and the MSCs showed similar

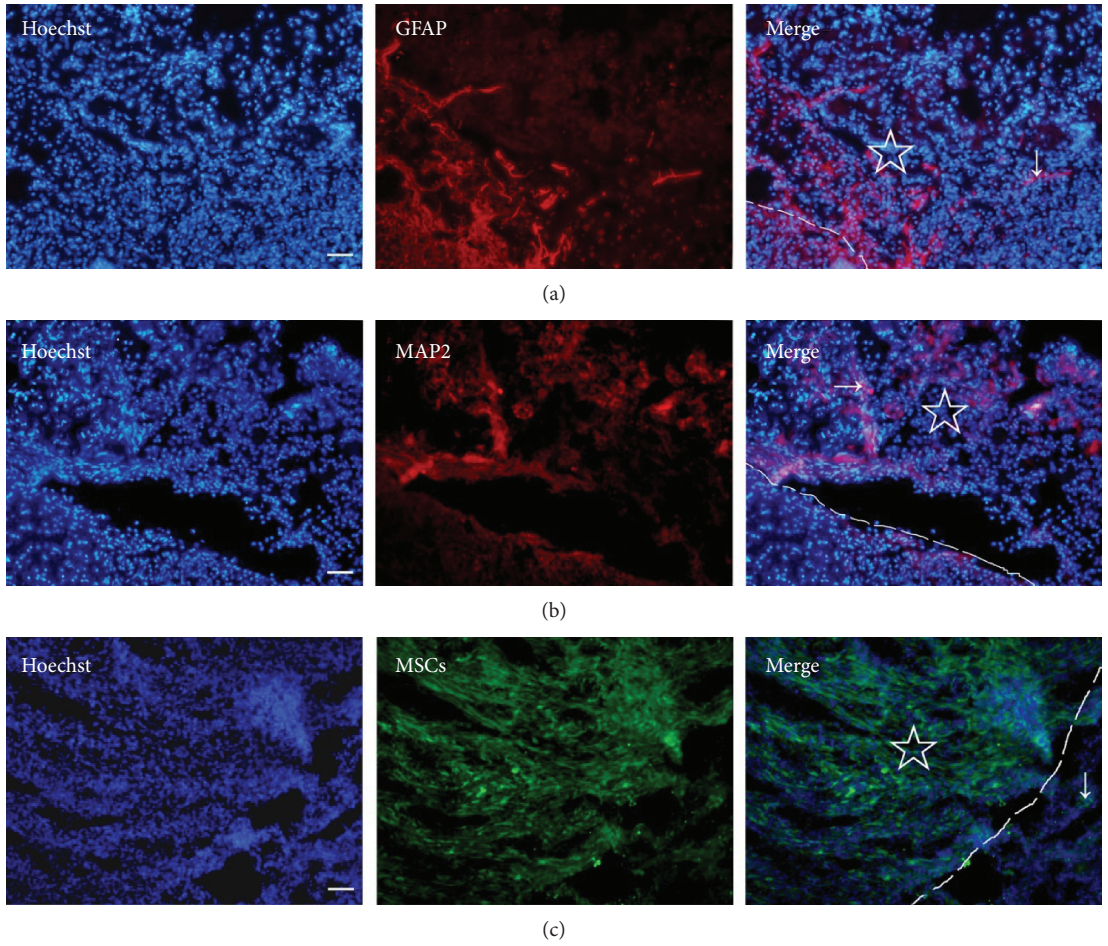


Figure 5: Cell migrations in the PLGA scaffold in the brain with TBI. (a) Astrocytes (arrow) stained with anti-GFAP (red) migrated in the PLGA scaffold. (b) Neurons (arrow) stained with anti-MAP2 (red) migrated in the PLGA scaffold. (c) MSCs (green, arrow) migrated out of the MSC-PLGA scaffold complex. "☆" shows the PLGA scaffold or the MSC-PLGA scaffold complex in the brain, and the dashed line indicates the boundary of the scaffold. Bar = 50 μm.

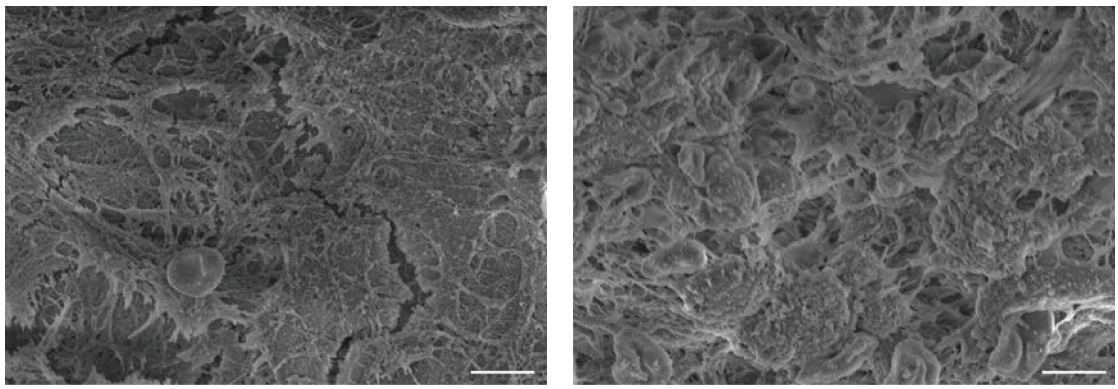
absorbance in the PLGA and control groups after incubation for 7 days ($P < 0.05$) (Figure 3(c)). These results suggest that the PLGA scaffold did not interfere with the proliferation and neuronal differentiation of MSCs in vitro.

3.4. The Structure of PLGA In Vivo. The structure of the PLGA in brains was assessed by morphological observation with nuclear staining at 14 days after TBI. Under a microscope, the tissue organization of the transplanted PLGA scaffold was distinctive between the PLGA scaffold group (Figures 4(a)–4(c)) and the MSC-PLGA scaffold group (Figures 4(d)–4(f)). In the former, the tissue structure was looser and the interstitial space was larger, while a more compact structure and smaller spaces could be observed in the latter.

3.5. Cell Migration and Adhesion in PLGA Scaffold In Vivo. After the transplantation of the PLGA scaffold into the brain at 14 days after TBI, some cells migrated into the PLGA, including glial cells (Figure 5(a)) and neurons (Figure 5(b)). When the MSC-PLGA scaffold complex was implanted in the brain, the MSCs could migrate out to the adjacent brain area (Figure 5(c)). In addition, we found that cells could adhere better on the MSC-PLGA scaffold complex than the PLGA scaffold (Figures 6(a) and 6(b)). The results suggest that the PLGA-mixed MSCs are more beneficial to cell adhesion and migration, and thus, the combination of the brain tissue and scaffold is closer.

4. Discussion

Compatibility between the biomaterial scaffold and seed cells is a core issue to construct engineered tissues and organs. The biomaterial scaffold as the cell carrier and exogenous graft should have a positive effect or no palpable side effect on cell growth and differentiation, and it should be accompanied by good biocompatibility and easy degradation in vivo after implantation [11, 12]. PLGA has been served as a drug carrier because of its biocompatibility and biodegradation [13, 14]. Seed cells derived from autologous tissue used to construct engineered tissue are the preferable choice. MSCs have received extensive attention because they can be obtained



(a) (b)

Figure 6: Cells' adhesion on the PLGA scaffold in the brain with TBI. (a) SEM imaging of cells' adhesion on the PLGA scaffold. (b) SEM imaging of cells' adhesion on the MSC-PLGA scaffold complex. Bar = 5 μm.

from autologous tissue and they were easily isolated, expanded in vitro, and further induced to differentiate into neuronal cells, adipocytes, osteoblasts, myocytes, and other cell types [15–18]. In this study, we found that PLGA did not interfere with the proliferation and neural differentiation of MSCs.

Cell adhesion and migration properties will influence the proliferation, differentiation, and function of cells [19, 20]. To achieve the function of the cell scaffold, the cell carrier should ensure good adhesion, growth, and reproduction of seed cells and host cells. In the present work, the advantages show that PLGA scaffolds are capable of supporting 3D growth for MSCs and neurons in vivo and in vitro and neural-induced MSCs can still adhere to the PLGA scaffold. Previous studies have shown that MSCs can secrete various extracellular matrixes, neurotrophic factors, and cell adhesion factors [21, 22] and the transplantation of MSCs can promote the repair of central nervous system injuries [23, 24]. We found that the transplantation of the MSC-PLGA complex made the impaired brain more complete than that of the simple PLGA scaffold. In summary, our results suggested that MSC-PLGA may be used as suitable graft for nerve tissue engineering, but its biological properties in vivo merit further study.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no competing interests.

Authors' Contributions

Ling Zhou and Jiangyi Tu contributed equally to the article.

Acknowledgments

This work is supported by Research Project of Science Foundation of Sichuan Province Educational Commission of China (18ZA0516) and the joint research project of the Department of Science and Technology of Sichuan Province and Luzhou Municipal People's Government and Luzhou Medical College (14JC0140). The authors thank Yansheng Liao for the suggestion on experimental methods.

References

[1] E. Avolio, V. V. Alvino, M. T. Ghorbel, and P. Campagnolo, "Perivascular cells and tissue engineering: current applications and untapped potential," *Pharmacology & Therapeutics*, vol. 171, no. 171, pp. 83–92, 2017.

[2] R. Kurimoto, K. Kanie, K. Uto et al., "Combinational effects of polymer viscoelasticity and immobilized peptides on cell adhesion to cell-selective scaffolds," *Analytical Sciences*, vol. 32, no. 11, pp. 1195–1202, 2016.

[3] A. Haider, K. C. Gupta, and I. K. Kang, "Morphological effects of HA on the cell compatibility of electrospun HA/PLGA composite nanofiber scaffolds," *BioMed Research International*, vol. 2014, Article ID 308306, 11 pages, 2014.

[4] B. K. Chen, A. M. Knight, G. C. W. de Ruiter et al., "Axon regeneration through scaffold into distal spinal cord after transection," *Journal of Neurotrauma*, vol. 26, no. 10, pp. 1759–1771, 2009.

[5] J. L. Munoz, S. J. Greco, S. A. Patel et al., "Feline bone marrow-derived mesenchymal stromal cells (MSCs) show similar phenotype and functions with regards to neuronal differentiation as human MSCs," *Differentiation*, vol. 84, no. 2, pp. 214–222, 2012.

[6] Y. Zeng, M. Rong, Y. Liu et al., "Electrophysiological characterisation of human umbilical cord blood-derived mesenchymal stem cells induced by olfactory ensheathing cell-conditioned medium," *Neurochemical Research*, vol. 38, no. 12, pp. 2483–2489, 2013.

[7] K. J. Cho, K. A. Trzaska, S. J. Greco et al., "Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter

substance P by interleukin-1α," *Stem Cells*, vol. 23, no. 3, pp. 383–391, 2005.

[8] Y. S. Liao, L. Deng, X. Q. Gao, and C. X. Yang, "Three-dimensional culture and neural differentiation of bone marrow mesenchymal stem cells on PLGA scaffolds," in *Proceedings of the 2015 International Conference (2015 International Conference on Medicine and Biopharmaceutical), vol. 15-16*, pp. 54–62, Guangzhou, China, 2015.

[9] C. Yang, L. Zhou, X. Gao et al., "Neuroprotective effects of bone marrow stem cells overexpressing glial cell line-derived neurotrophic factor on rats with intracerebral hemorrhage and neurons exposed to hypoxia/reoxygenation," *Neurosurgery*, vol. 68, no. 3, pp. 691–704, 2011.

[10] W. Shi, C. J. Huang, X. D. Xu et al., "Transplantation of RADA16-BDNF peptide scaffold with human umbilical cord mesenchymal stem cells forced with CXCR4 and activated astrocytes for repair of traumatic brain injury," *Acta Biomater*, vol. 45, pp. 247–261, 2016.

[11] R. J. O'Keefe and J. Mao, "Bone tissue engineering and regeneration: from discovery to the clinic—an overview," *Tissue Engineering Part B: Reviews*, vol. 17, no. 6, pp. 389–392, 2011.

[12] X. Miao, D. M. Tan, J. Li, Y. Xiao, and R. Crawford, "Mechanical and biological properties of hydroxyapatite/tricalcium phosphate scaffolds coated with poly (lactic-co-glycolic acid)," *Acta Biomaterialia*, vol. 4, no. 3, pp. 638–645, 2008.

[13] B.-S. Lee, C.-C. Lee, Y.-P. Wang et al., "Controlled-release of tetracycline and lovastatin by poly(D,L-lactide-co-glycolide acid)-chitosan nanoparticles enhances periodontal regeneration in dogs," *International Journal of Nanomedicine*, vol. 11, pp. 285–297, 2016.

[14] C. Fornaguera, A. Dols-Perez, G. Calderó, M. J. García-Celma, J. Camarasa, and C. Solans, "PLGA nanoparticles prepared by nano-emulsion templating using low-energy methods as efficient nanocarriers for drug delivery across the blood-brain barrier," *Journal of Controlled Release*, vol. 211, pp. 134–143, 2015.

[15] R. Shokohi, M. Nabiuni, S. Irian, and J. A. Miyan, "In vitro effects of wistar rat prenatal and postnatal cerebrospinal fluid on neural differentiation and proliferation of mesenchymal stromal cells derived from bone marrow," *Cell Journal*, vol. 19, no. 4, pp. 537–544, 2018.

[16] J. Stachecka, A. Walczak, B. Kociucka, B. Ruszczycki, G. Wilczyński, and I. Szczerbal, "Nuclear organization during in vitro differentiation of porcine mesenchymal stem cells (MSCs) into adipocytes," *Histochemistry and Cell Biology*, vol. 149, no. 2, pp. 113–126, 2018.

[17] A. Nemoto, N. Chosa, S. Kyakumoto et al., "Water-soluble factors eluated from surface pre-reacted glass-ionomer filler promote osteoblastic differentiation of human mesenchymal stem cells," *Molecular Medicine Reports*, vol. 17, no. 3, pp. 3448–3454, 2018.

[18] S. Khajeniazi, M. Solati, Y. Yazdani, M. Soleimani, and A. Kianmehr, "Synergistic induction of cardiomyocyte differentiation from human bone marrow mesenchymal stem cells by interleukin 1β and 5-azacytidine," *Biological Chemistry*, vol. 397, no. 12, pp. 1355–1364, 2016.

[19] K. Anselme, "Osteoblast adhesion on biomaterials," *Biomaterials*, vol. 21, no. 7, pp. 667–681, 2000.

[20] N. Zhang and D. H. Kohn, "Using polymeric materials to control stem cell behavior for tissue regeneration," *Birth Defects Research, Part C Embryo Today*, vol. 96, no. 1, pp. 63–81, 2012.

[21] U. T. Arasu, R. Kärnä, K. Härkönen et al., "Human mesenchymal stem cells secrete hyaluronan-coated extracellular vesicles," *Matrix Biology*, vol. 64, pp. 54–68, 2017.

[22] L. Junyi, L. Na, and J. Yan, "Mesenchymal stem cells secrete brain-derived neurotrophic factor and promote retinal ganglion cell survival after traumatic optic neuropathy," *The Journal of Craniofacial Surgery*, vol. 26, no. 2, pp. 548–552, 2015.

[23] J. Qu and H. Zhang, "Roles of mesenchymal stem cells in spinal cord injury," *Stem Cells International*, vol. 2017, Article ID 5251313, 12 pages, 2017.

[24] D. Chen, W. Fu, W. Zhuang, C. Lv, F. Li, and X. Wang, "Therapeutic effects of intranigral transplantation of mesenchymal stem cells in rat models of Parkinson's disease," *Journal of Neuroscience Research*, vol. 95, no. 3, pp. 907–917, 2017.

Hindawi
Stem Cells International
Volume 2018, Article ID 1731289, 15 pages
https://doi.org/10.1155/2018/1731289

Review Article

Potential Roles of Dental Pulp Stem Cells in Neural Regeneration and Repair

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Received 7 November 2017; Accepted 22 March 2018; Published 7 May 2018

Academic Editor: Tao-Sheng Li

This review summarizes current advances in dental pulp stem cells (DPSCs) and their potential applications in the nervous diseases. Injured adult mammalian nervous system has a limited regenerative capacity due to an insufficient pool of precursor cells in both central and peripheral nervous systems. Nerve growth is also constrained by inhibitory factors (associated with central myelin) and barrier tissues (glial scarring). Stem cells, possessing the capacity of self-renewal and multicellular differentiation, promise new therapeutic strategies for overcoming these impediments to neural regeneration. Dental pulp stem cells (DPSCs) derive from a cranial neural crest lineage, retain a remarkable potential for neuronal differentiation, and additionally express multiple factors that are suitable for neuronal and axonal regeneration. DPSCs can also express immunomodulatory factors that stimulate formation of blood vessels and enhance regeneration and repair of injured nerve. These unique properties together with their ready accessibility make DPSCs an attractive cell source for tissue engineering in injured and diseased nervous systems. In this review, we interrogate the neuronal differentiation potential as well as the neuroprotective, neurotrophic, angiogenic, and immunomodulatory properties of DPSCs and its application in the injured nervous system. Taken together, DPSCs are an ideal stem cell resource for therapeutic approaches to neural repair and regeneration in nerve diseases.

1. Introduction

Traumatic events, iatrogenic injuries, and neurodegenerative diseases can lead to axonal degeneration, inflammation, neuron death, and cytoarchitectural malformation in both the peripheral nervous system (PNS) and central nervous system (CNS) [1–6]. Conventional medical therapies have limited efficacy in supporting functional recovery from nervous damage since the mature nervous system lacks the necessary precursor cells to generate new neurons and glial cells [7]. Recently, stem cell-based strategies in combination with novel technologies (e.g., precisely controlled hydrogels) have heralded potential new therapeutic approaches for addressing nerve regeneration and repair [8–11].

Mesenchymal stem cells (MSCs) harvested from adult tissues are potentially an important therapeutic cell source for treatment of CNS and PNS perturbations since they possess the capacity for both neuronal and glial differentiation. MSCs also express numerous anti-inflammatory and neurotrophic factors supporting nerve repair [8–14]. These multipotent stem cells are present in bone marrow [15, 16], adipose tissue [17, 18], umbilical cord [19, 20], and dental

tissue [21–25]. Dental pulp stem cells (DPSCs) can readily be obtained from the third molars, usually discarded as medical waste. DPSCs have MSC-like characteristics such as the ability for self-renewal and multilineage differentiation. These dental pulp-derived MSCs avoid ethical concerns when sourced from other tissue, and they can be obtained without unnecessary invasive procedures, for example, MSCs collected from bone marrow or adipose tissue [9, 26–28]. DPSCs can differentiate into neuron-like cells and secrete neurotrophic factors such as neurotrophin (NT) [29, 30]. In addition, DPSCs express neuron-related markers even before being induced to neuronal differentiation [29, 31, 32]. Taken together, these unique properties make DPSCs an excellent candidate for stem cell-related therapies in nerve diseases.

2. Dental Pulp Stem Cells (DPSCs)

2.1. The Characteristics of DPSCs. The basic tooth structure consists of an outer enamel layer, a middle dentin layer, and an inner dental pulp layer. It develops from both cranial neural crest-derived mesenchymal stem cells (MSCs) and oral-derived epithelial stem cells in the early stages of embryogenesis [33–35]. Dental pulp, a soft connective tissue containing blood vessels, nerves, and mesenchymal tissue, has a central role in primary and secondary tooth development and ongoing maintenance for instance in reaction to caries [36, 37]. Stem cells can be isolated from the dental pulp tissue and possess MSC-like characteristics including self-renewal and multipotency [21, 38–40]. The first dental pulp-related stem cells were isolated from the third molar dental pulp by Gronthos et al. in 2000 [21]. Subsequently, it was reported that DPSCs could also be isolated from other dental pulps including human exfoliated deciduous teeth [22], human permanent and primary teeth [41], and supernumerary teeth [42]. Meanwhile, they are featured by high-proliferative capacity [43–47]. Most importantly, compared with collection procedures of other tissue-derived stem cells, the collection of DPSCs involves none harm to the donor or invasive surgical procedures [27, 40].

There are currently no specific biomarkers that uniquely define DPSCs. They express MSC-like phenotypic markers such as CD27, CD29, CD44, CD73, CD90, CD105, CD146, CD166, CD271, and STRO-1. Yet they do not express CD34, CD45, CD14, or CD19 and HLA-DR surface molecules [38, 39, 48]. Similar to embryonic stem cells, DPSCs express stemness-related markers such as Oct-4, Nanog, and Sox-2, as well as the cytoskeleton-related markers (Nestin and Vimentin) [29, 49, 50]. In addition, DPSCs express other cranial neural crest cell-related neural markers such as glial fibrillary acidic protein (GFAP), β-III tubulin, and microtubule-associated protein-2 (MAP-2) [29, 50, 51].

DPSCs are multipotent and can be induced to differentiate into cells for osteogenesis [52], chondrogenesis [53], adipogenesis [53], neurogenesis [54], dentinogenesis [53], odontogenesis [55], and myogenic lineages [56] (Figure 1). Using classic reprogramming factors (e.g., Oct3/4, Sox2, Klf4, and c-MYC), human DPSCs can be converted into induced pluripotent stem cells (iPSCs) [57, 58]. iPSCs exhibit the characteristics of embryonic stem cells and can differentiate into all three germ layers [59, 60]. Human DPSCs have a higher reprogramming efficiency than human dermal fibroblasts because they have a rapid proliferation rate and endogenously express high levels of the reprogramming factors c-MYC and Klf4 [57]. Therefore, DPSCs are potentially an important patient-specific cell source of iPSCs for clinical applications, regenerative medicine, and tissue engineering.

2.2. Neuronal Differentiation of DPSCs. DPSCs arise from the cranial neural crest and possess neuron-like characteristics that facilitate their *in vitro* induction into functional neurons. Numerous protocols have been developed to differentiate DPSCs into neurons. Typically, such methods rely on growth factors and various small molecules including basic fibroblast growth factor (bFGF) [61, 62], epidermal growth factor (EGF) [63], nerve growth factor (NGF) [62, 64], brain-derived neurotrophic factor (BDNF) [65], glial cell line-derived neurotrophic factor (GDNF) [66], sonic hedgehog [66], neurotrophin 3 (NT-3) [61], retinoic acid (RA) [63], forskolin [50, 67], and heparin [66] as well as culture supplements such as B27 [61], insulin-transferrin-sodium selenite (ITS) [54], nonessential amino acids [66], and N2 [61, 66]. Under controlled *in vitro* conditions (e.g., spheroid suspension culture in serum-free media), it is possible to differentiate DPSCs into neural lineages that expressed numerous neural markers [61, 63, 64, 68]. Chun et al. have demonstrated that DPSCs could be differentiated into dopaminergic neural cells by the formation of neurosphere [69]. However, huge variations exist in the neural differentiation of DPSCs due to alterations made to the culture of neurosphere, which indicates a delicate regulatory approach is necessary to achieve target differentiation. It is controversial on the timing of neurosphere formation. The study of Gervois et al. showed that it formed in the initial phase during a neural induction [61], whereas studies of Karbanova et al. observed that the neurosphere formed in a rather late phase during the differentiation [70].

Nevertheless, it is possible to bypass neurosphere formation by using endogenous environmental cues and directly differentiate DPSCs into motor and dopaminergic neuronal sublineages [65, 71]. Studies of Chang et al. reported that DPSCs could be directly differentiated into motor neurons by growth factors and small molecules, for example, BDNF and all-trans retinoic acid [71]. Gnanasegaran et al. demonstrated that DPSCs could be induced to differentiate into dopaminergic-like cells by multistage inductive protocols [72]. The study of Singh et al. showed that DPSCs are induced by a two-step method to generate functional dopaminergic neurons: FGF2 first with an addition of BDNF on 9th day. Furthermore, when induced, DPSCs showed much more distinct neuronal characteristics comparing to the other tissue-derived MSCs, for example, bone marrow and adipose tissue [73]. In addition, DPSCs could be differentiated into spiral ganglion neuron-like cells by treating with BDNF, NT-3, and GDNF [74].

Typically, a successful neuronal differentiation of DPSCs is confirmed by the increased expression of neuronal markers such as NeuN [61], neurofilament-200 [54], MAP-2 [61, 75],

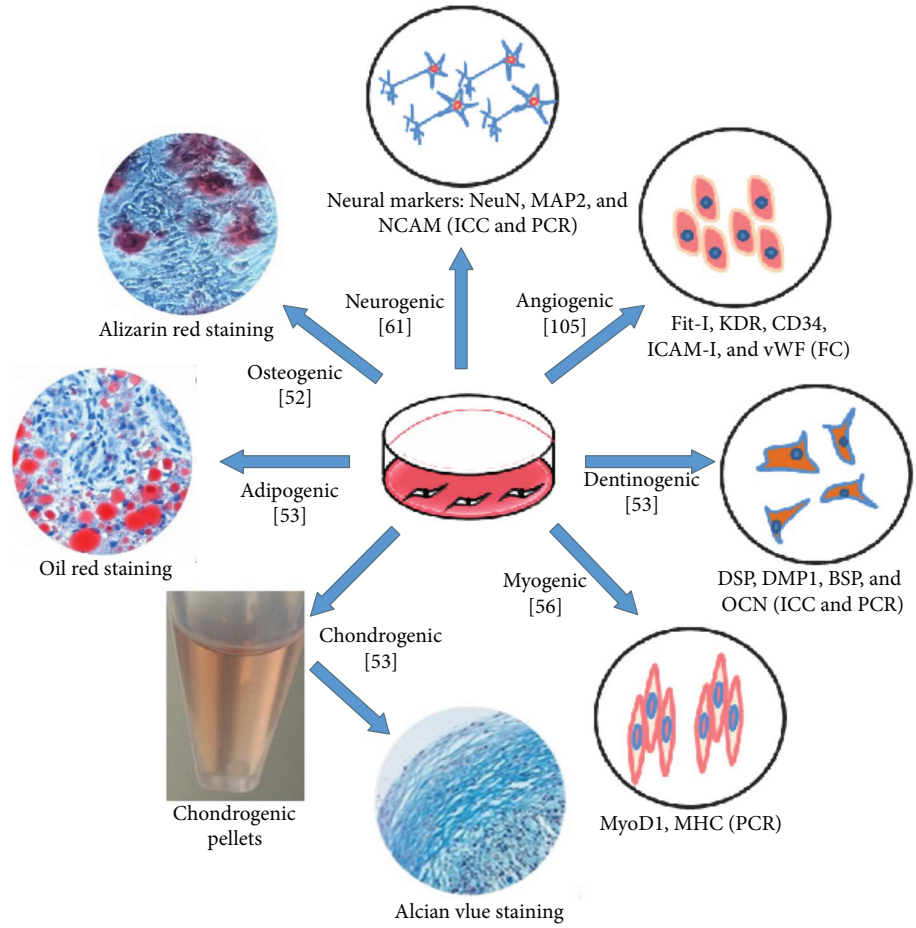

Figure 1: Multidifferentiation potential of DPSCs. DPSCs possess MSC-like properties and are multipotent. *NCAM*: neural cell adhesion molecule; MAP2: microtubule-associated protein 2; NeuN: neuron-specific nuclear protein; Fit-I: VEGF receptor 1; KDR: VEGF receptor 2; CD34: cluster of differentiation 34; ICAM-I: intercellular cell adhesion molecule-1; vWF: von Willebrand factor, DSP: dentin sialoprotein, DMP1: dentin matrix acidic phosphoprotein 1, BSP: bone sialoprotein, OCN: osteocalcin, MyoD1: myoblast determination protein 1; MHC: major histocompatibility complex; PCR: polymerase chain reaction; FC: flow cytometry; ICC: immunocytochemical.

synaptophysin [61], and neural cell adhesion molecules [76]. Few studies have used ultrastructural and/or electrophysiological analyses to confirm the state of differentiation [61]. Previous studies focus on differentiation directions: DPSCs could be differentiated into either neuronal precursor cells (rather than mature neurons capable of generating action potentials) or immature Schwann cells and oligodendrocytes that can support nerve regeneration [77–80] (Figure 2). Recently, research has evolved into in-depth studies on functional and mechanism of DPSC-differentiated neurons. A series of studies have explored the functional activities of DPSC-differentiated neurons in voltage-gated sodium and potassium channels as well the neuronal marker expressions, indicating a successful differentiation is active and functional new neurons have emerged [50, 54, 67]. Further, these predifferentiated DPSCs have been traced and proved well integrated into the central nervous tissue when transplanted in animal models [54, 67]. In summary, versatile differentiations of DPSCs depend on inductive protocols. They can be differentiated into neurons, dopaminergic-like cells, Schwann cells, and oligodendrocytes. Thus, DPSCs are an attractive cell source for stem cell therapy to treat the nervous diseases.

2.3. Neuroprotective and Neurotrophic Properties of DPSCs. The efficacy of stem cell therapies in nervous diseases is strongly influenced by trophic factors, for example, BDNF, GDNF, NGF, NT-3, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) [29, 30]. The expression of these trophic factors by DPSCs is remarkably higher than those of MSCs derived from bone marrow (BMSCs) and adipose tissue [9, 30]. Further *in vivo* study also demonstrates a more efficient secretion of BDNF and GDNF than BMSCs [81]. These findings confirm that in comparison to other MSCs, DPSCs exhibit superior neuroprotective and neural supportive properties in response to injuries and pathologies of the nervous system. DPSCs have the ability to reduce neurodegeneration in the early stages of neuronal apoptosis and promote motor and sensory neuron survival in spinal cord injury (SCI) by the secretion of BDNF and NGF [82, 83]. Furthermore, trophic factors secreted by DPSCs promoted axon regeneration despite the presence of axon growth inhibitors in the completely transected spinal cord model of SCI [84, 85]. DPSCs also provided both direct and indirect protections against cell death by secreting cytoprotective factors in an ischemic astrocyte model of injury

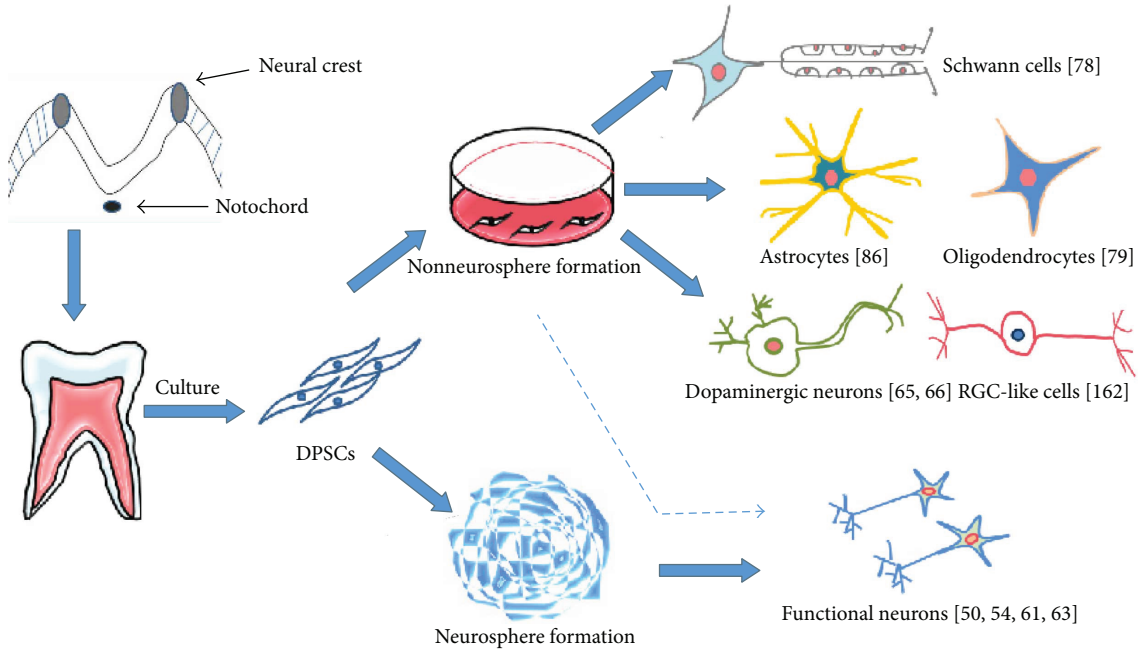

Figure 2: Neural differentiation potential of DPSCs. DPSCs can be induced to differentiate into neural cell lineages including Schwann cells, astrocytes, and dopaminergic neurons.

[86, 87]. Compared with other stem cells (DFSCs, SCAP, and BMSCs), DPSCs have shown a higher cytokine expression facilitating neuronal differentiations [88].

2.4. Angiogenic Properties of DPSCs. In general, the human body needs abundant nutrition and blood supply in order to maintain its tissues and organs in a healthy condition. The sprouting of new capillaries from existing blood vessels during inflammation and hypoxic conditions depends on the expression and secretion of specific angiogenic trophic factors [89, 90]. Some MSCs are able to promote therapeutic angiogenesis by the secretion of angiogenic growth factors and by differentiating into endothelial cells [91–93]. In particular, DPSCs have been found to secrete and produce abundant angiogenic factors, for example, colony-stimulating factor, interleukin-8, angiogenin, endothelin-1, angiopoietin-1, and insulin-like growth factor binding protein-3 [94–96]. DPSCs also secrete and express other stimulatory growth factors such as VEGF, PDGF, bFGF, and NGF [19, 30, 97]. Synergistically, these factors can promote proliferation and survival of vascular endothelial cells [98, 99] as well as endothelial tubulogenesis [100]. Both the formation and function of new blood vessels are improved by either injection of DPSCs into neuronal disease models or transplantation of DPSCs into ischemia and myocardial infarction animal models [101, 102]. Moreover, Nam et al. observed that by coinjection of DPSCs and HUVECs into immunodeficient mice, microvessel-like structures would be formed, which illustrated that DPSCs could perform as perivascular cells for *in vivo* angiogenesis [103]. DPSCs also have the ability to differentiate into endothelial-like cells. When incubated with VEGF, the expression of VEGFR1, VEGFR2, von Willebrand factor, and CD54 is increased [104, 105]. These VEGF-induced DPSCs exhibited endothelial features and formed capillary-like structures when cultured on a fibrin clot [105]. More recently, a structured dentin-/pulp-like tissue with vasculatures has been created using DPSCs via 3D print technique, suggesting a new direction for customized application for individual design of defect repair [106].

2.5. Immunomodulatory Properties of DPSCs. MSCs exhibit some immunomodulatory and anti-inflammatory factors, for example, interleukin-10 (IL-10) [107], hepatocyte growth factor (HGF), [108], transforming growth factor-β (TGF-β) [109], and prostaglandin E2 [110]. MSCs can act as an immunosuppressive agent by modulating the immune response in inflammatory or autoimmune diseases [111, 112]. DPSCs also have immunomodulatory properties associated with expression of soluble factors that inhibit T cell function. For instance, it has been reported that DPSCs express interleukin-8 (IL-8), interleukin-6 (IL-6), and TGF-β via Toll-like receptor (TLR) 4 during neuroinflammation in neurodegenerative diseases [8, 113]. An upregulated expression of TLR4 appeared to increase the expression of IL-8 in DPSCs [114], particularly in SCI crush injury where IL-8 preserves axon integrity and decreases cavitation [115, 116]. DPSCs also express TGF-β, HGF, and indoleamine 2,3-dioxygenase (IDO) without prior activation [117, 118] and suppress the proliferation of peripheral blood mononuclear cells and the activation of T cells [119, 120]. Coculture of DPSCs and T cells promoted T cell secretion of human leukocyte antigen-G, vascular adhesion molecule-1, intracellular adhesion molecule-1, IL-6, TGF-β, HGF, and IL-10, while it downregulated proinflammatory cytokines such as IL-2, IL-6 receptor, IL-12, IL-17A, and tumor necrosis factor-α (TNF-α) [121]. It was reported that the proliferation

of T cells was inhibited by over 90% when cocultured with DPSCs *in vitro* [8, 122]. In addition, recent studies demonstrated that human and rat DPSCs were able to induce FasL-mediated apoptosis of IL-17 T-helper cells, and rat DPSCs exhibited a very strong ameliorating effect on DSS-induced colitis in mice [123, 124]. The study of Hong et al. reported that DPSCs could modulate immune tolerance by increasing CD4+CD25+FoxP3+ regulatory T cells. The results of the intraperitoneal injection of DPSCs into Balb/c(H-2^d) mice demonstrated that DPSCs had a meaningful effect on mixed lymphocyte reaction [125]. Studies of Kwack et al. demonstrated that DPSCs could inhibit acute allogeneic immune responses by the release of TGF-β as a result of allogeneic stimulation of T lymphocytes and provide a novel insight for the allogeneic transplantation of DPSCs in future clinical use [120]. Recent animal studies conclude that DPSCs could modulate immune tolerance and influence apoptosis via T cells and lymphocytes.

3. Dental Pulp Stem Cells (DPSCs) and Central Nervous System Diseases

Traumatic damage to the brain and spinal cord leading to a CNS dysfunction, stroke, Parkinson's disease, Alzheimer's disease, and retinal injury is a common central nervous system disease. The CNS typically has a poor ability to repair and regenerate new neurons because of its limited pool of precursor cells [126, 127], expression of myelin-associated growth inhibitory factors [128], and the inherent propensity of resident glial cells to form scar tissue [129]. At present, it is very difficult to treat CNS diseases with conventional clinical therapies. Some studies have suggested that stem cell treatment may offer a novel therapeutic strategy for CNS disease [127, 130]. The hope is that the application of exogenous stem cells (particularly DPSCs) will lead to both regeneration of new neural precursor cells and their enhanced neuronal and glial differentiation as well as to survival and maintenance of existing neural cells through secretion of trophic factors [29, 30, 40].

3.1. DPSCs and SCI. SCI in humans can cause partial or complete loss of motor and sensory function that reduces the quality of an individual's life and leads to an economic burden on society [124, 131]. SCI involves an initial primary tissue disruption (e.g., mechanical damage to nerve cells and blood vessels) and then a secondary injury caused by neuroinflammatory responses (e.g., excitotoxicity, blood-brain barrier disruption, oxidative stress, and apoptosis) [132, 133]. Because of their neural crest lineage, DPSCs have championed as preferred stem cells for SCI therapies supported by growing evidence of DPSCs differentiating into neuron-like and oligodendrocyte-like cells that may promote axonal regeneration and tissue repair after SCI [28, 127, 134, 135]. DPSCs also reduce secondary inflammatory injury, which facilitates axonal regeneration and reduces progressive hemorrhagic necrosis associated with interleukin-1β (IL-1β), ras homolog gene family member A (RhoA), and sulfonylurea receptor1 (SUR1) expression [136]. When transplanted together with artificial scaffolds such as chitosan, DPSCs promoted motor functional recovery and inhibited cell apoptosis after SCI by secreting BDNF, GDNF, and NT-3 and reducing the expression of active-caspase 3 [8, 137].

3.2. DPSCs and Stroke. Stroke is an ischemic cerebrovascular condition that leads to brain damage, long-term disability, and even death [138]. Due to prolonged period of insufficient blood supply and poor oxygen perfusion, damages on affected brain are irreversible. There are unfortunately few effective strategies that can reverse the damage effect on the brain or restore one's function to prestrike level [139]. Recent studies indicate that stem cell therapy may present a novel strategy for stroke treatment due to the multipotency, immunomodulatory, and neuroprotective and angiogenic properties of these cells [140, 141]. Some *in vivo* studies have shown that transplantation of DPSCs into the ischemic areas of middle cerebral artery occlusion (MCAO) in Sprague-Dawley (SD) rats promoted locomotor functional recovery and decreased infarct areas by their differentiation into dopaminergic neurons and secretion of neurotrophic factors [102, 142]. DPSC transplantation into ischemic areas of focal cerebral ischemia in rats led to expression of proangiogenic factors that supported dense capillary formation and renormalization of blood flow [143]. Intracerebral transplantation of DPSCs into regions of focal cerebral ischemia in rodent models promoted forelimb sensory and motor functional recovery at 4 weeks posttreatment [140]. DPSCs also provided cytoprotection for astrocytes by reducing reactive gliosis and preventing free radical and IL-1β secretion within *in vitro* ischemic models [86]. Thus, DPSCs may play an immunomodulatory role to promote functional recovery after ischemic stroke.

3.3. DPSCs and Parkinson's Disease. Parkinson's disease (PD) is a progressive neurodegenerative condition associated with loss of nigrostriatal dopaminergic (DA) neurons that leads to muscle rigidity, bradykinesia, resting tremor, and postural instability [144]. Stem cell-based therapies hold some promise as a novel strategy for PD treatment [145]. DPSCs can be induced to differentiate into dopamine expressing DA neuron-like cells *in vitro* by using experimental cell induction media [65]. Intrathecal transplantation of DPSCs into the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) induced old-aged mouse model of PD promoted recovery of behavioral deficits, restored DA functions, and attenuated MPTP-induced damage by reducing the secretion of proinflammatory factors such as IL-1α, IL-1β, IL6, IL8, and TNF-α and by upregulating the expression levels of anti-inflammatory factors such as IL2, IL4, and TNF-β [146]. DPSCs also showed neuroimmunomodulatory activity in an *in vitro* model of PD by reducing MPTP-induced deficits associated with reactive oxygen species, DNA damage, and nitric oxide release [146, 147]. DPSCs also promoted survival of DA neurons and enhanced nigrostriatal tract functional recovery in a 6-hydroxydopamine- (6-OHDA-) induced PD rat model by 6 weeks post-transplantation [148]. Some studies have also shown that DPSCs reduced 6-OHDA-induced damage in the *in vitro*

model of PD [69, 145]. The clinical use of DPSCs may be a promising approach for treating PD in the future.

3.4. DPSCs and Alzheimer's Disease. Alzheimer's disease (AD) is a progressive neurodegenerative condition caused by the loss of neurons, intracellular neurofibrillary tangles, and deposition of insoluble β-amyloid peptides in the brain [149, 150]. Clinical symptoms of AD include memory loss, cognitive deficits, and linguistic disorders [150]. Recently, several studies reported that stem cell-based therapies in both *in vitro* and *in vivo* models of AD improved AD-induced pathologies and behavioral deficits [151–153]. DPSCs promoted neuronal repair and regeneration by restoring cytoskeletal structure, protecting microtubule stability, and reducing tau phosphorylation in the okadaic acid- (OA-) induced cellular model of AD [154]. DPSCs can also reduce amyloid beta (Aβ) peptide-induced cytotoxicity and apoptosis in the AD cellular model by secreting higher levels of VEGF, fractalkine, RANTES, fms-related tyrosine kinase 3, and monocyte chemotactic protein 1 [155, 156]. These results suggest that DPSCs are a promising cell source for secretome-based treatment of AD.

3.5. DPSCs and Retinal Injury. The retina is a part of the CNS and is composed of photoreceptors, bipolar cells, and retinal ganglion cells (RGCs) [43, 157]. Head injuries can cause traumatic optic neuropathy (TON) while ocular chronic degenerative diseases such as glaucoma lead to the slow loss of RGCs [158]. Retinal and optic nerve injuries have a limited capacity to repair and regenerate because of axon growth inhibitory molecules and reduced production of neurotrophic growth factors [7, 159]. One study reported that DPSC transplantation into the vitreous of optic nerve injury rat model could promote axonal regeneration and RGC survival by a neurotrophin-mediated mechanism [83]. This same study revealed that DPSCs were more beneficial for axonal regeneration than BMSCs because of their higher secretion of neurotrophin factors. A subsequent report showed that intravitreal transplantation of DPSCs in an animal model of glaucoma maintained visual function up to 35 days after treatment by preventing RGC death [160]. Although not assessed *in vivo*, some *in vitro* studies have reported that DPSCs can be induced to differentiate into both RGC-like and photoreceptor cells [161, 162]. Taken together, these results suggest that DPSCs may become an important cell source for stem cell-based therapies in ocular diseases.

4. DPSCs and Peripheral Nerve Injury

Peripheral nerve injury caused by traumatic accidents and iatrogenic damage often accompanies physical disability and neuropathic pain. There are many current clinical treatments including direct end-to-end nerve suturing, nerve grafts, and nerve conduits containing growth-stimulatory biomaterials to repair and regenerate injured peripheral nerves [163–165]. Among them, autologous nerve grafting is the gold standard therapy for the long gap of peripheral nerve deficits [166, 167]. However, there are some disadvantages which restrict the clinical use of autografting, such as donor nerve availability and morphometric mismatching [168–171]. With the development of nerve tissue engineering and stem cell-related therapy, various novel nerve conduits in combination with stem cells are providing alternate strategies and approaches for the treatment of peripheral nerve injury [165, 172]. Some studies suggest that DPSC-embedded biomaterial nerve conduits such as polylactic glycolic acid tubes have the ability to promote regeneration of injured facial nerve and to improve functional recovery comparable to that of autografts [173]. Collagen conduits loaded with Schwann-like cells induced from DPSCs *in vitro* have facilitated repair and regeneration of 15 mm sciatic nerve defects [174]. In another report, differentiated DPSCs combined with collagen scaffolds exhibited Schwann cell-related properties and promoted axonal outgrowth and myelination in 2D or 3D culture conditions of an *in vitro* model [78]. Moreover, DPSCs transfected with oligodendrocyte lineage transcription factor 2 differentiated into functional oligodendrocytes *in vitro* and promoted injured peripheral nerve repair and regeneration in a mouse model [175]. DPSCs transplanted into diabetic rats secreted various cytokines that modulated the proportions of M1/M2 macrophages and provided beneficial anti-inflammatory effects in diabetic polyneuropathy [176].

In summary, DPSCs have the capacity to differentiate into Schwann-like and oligodendrocyte-like cells and they secrete neurotrophic factors that provide neuroprotection and modulate the immune response. These cells are poised to become a promising cell source for peripheral nerve injury treatment in the future.

5. Conclusions and Future Insights

This review summarizes the neuronal differentiation potential, neuroprotective features, and neurotrophic, angiogenic, and immunomodulatory properties of DPSCs in the pathological and injured nervous system. DPSCs have the biological properties of MSCs and possess a considerable capacity to differentiate into neuron-like cells and secrete neuron-related trophic factors due to their cranial neural crest origin. DPSCs are able to express neuronal markers without preinduced differentiation. Thus, both nondifferentiated and differentiated DPSCs are emerging as new cell sources for the treatment of nervous system deficits associated with SCI, stroke, AD, PD, and long gaps of peripheral nerve injury. DPSCs have several advantages over other exogenous stem cells for nervous system therapies because they are easily harvested without highly invasive surgery, have low immunogenicity, and arise from a neural crest origin that facilitates their neural differentiation. Moreover, after lentiviral transfection with Lin28, Nanog, Oct4, and Sox2 or retroviral transfection with Oct3/4, Sox2, and Klf4, DPSCs can be reprogrammed to generate an embryoid body of iPSCs. The DPSC-derived iPSCs have ability to differentiate into β-III tubulin neuron-like cells and tyrosine hydroxylase-positive dopaminergic neuron-like cells and may become another DPSC-related cell sources for the treatment of nervous system diseases in the future.

Because of the vascularization and immunomodulatory properties of DPSCs, these cells can both directly and indirectly stimulate formation of new blood vessels and enhance

Table 1: Examples for the beneficial of DPSCs on the central nervous system (CNS) diseases and the peripheral nervous system (PNS) diseases.

Type of diseases	Author	Differentiated status of DPSCs	Delivery method	Function of DPSCs	References
The central nervous system (CNS) diseases					
Spinal cord injury (SCI)	Yamamoto et al.	Undifferentiated	DPSC transplantation	DPSCs inhibited massive SCI-induced apoptosis, preserved neural fibers and myelin, regenerated transected axons, and replaced damaged cells by differentiating into oligodendrocytes	[134]
	Yang et al.	Undifferentiated	DPSCs transplanted with cell pellets	DPSCs reduced inflammatory injury, promoted axonal regeneration, and reduced progressive hemorrhagic necrosis after SCI by inhibiting IL-1β, RhoA, and SUR1 expression	[136]
	Zhang et al.	Undifferentiated	DPSCs transplanted with chitosan-scaffold	DPSCs promoted motor functional recovery and inhibited cell apoptosis after SCI through secreting BDNF, GDNF, NT-3 and reducing the expression of active-caspase 3	[137]
Stroke	Song et al.	Undifferentiated	DPSCs cocultured with the conditioned medium *in vitro*	DPSCs conferred superior cytoprotection against cell death by reducing reactive gliosis and suppressing free radical and proinflammatory cytokine expression	[86]
	Song et al.	Undifferentiated	Intravenous DPSC injection	DPSCs reduced the infarct volume of SD rats after middle cerebral artery occlusion (MCAO) due to high angiogenesis and neurogenic differentiation and reduction of reactive gliosis	[87]
	Sugiyama et al.	Dental pulp-derived CD31(−)/CD146(−) side population (SP) stem cells	CD31(−)/CD146(−) SP cells transplantation	DPSCs promoted migration and differentiation of the endogenous neuronal progenitor cells and induced vasculogenesis and ameliorated ischemic brain injury of SD rats after transient middle cerebral artery occlusion (TMCAO)	[102]
	Yang et al.	Dental pulp-derived neuronal stem cells (tNSCs)	tNSC transplantation	Transplanted tNSC promoted function recovery after MCAO because of possessing hypoimmunogenic properties and immune modulation abilities	[142]
	Leong et al.	Undifferentiated	Intracerebral DPSC transplantation	DPSCs enhanced the recovery of poststroke sensorimotor deficits owing to differentiation into astrocytes and mediation through DPSC-dependent paracrine effects	[143]
Parkinson's disease (PD)	Kanafi et al.	Dopaminergic cell-type differentiated	DPSCs were induced *in vitro*	DPSCs showed efficient propensity towards functional dopaminergic cell type	[65]
	Chun et al.	Dopaminergic neurons differentiated	DPSCs were treated with the dopaminergic neuron differentiation kit *in vitro*	DPSCs could differentiate into dopaminergic neural cells under experimental cell differentiation conditions	[69]

Table 1: Continued.

Type of diseases	Author	Differentiated status of DPSCs	Delivery method	Function of DPSCs	References
	Gnanasegaran et al.	Undifferentiated	Intrathecal DPSC transplantation into a mouse model of PD *in vitro*	DPSCs could treat the PD by regulating inflammatory mediators such as reducing the secretions of proinflammatory factors (IL-1α, IL-1β, IL6, IL8, and TNF-α) and upregulating the expression levels of anti-inflammatory factors (IL2, IL4, and TNF-β)	[146]
	Gnanasegaran et al.	DAergic-like cells differentiated	DPSCs were cultured in a system which consists of neuron and microglia *in vitro*	DPSCs were shown to have immunomodulatory capacities to reduce 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) induced deficits such as reactive oxygen species, DNA damages, and nitric oxide release	[147]
Alzheimer's disease (AD)	Wang et al.	Undifferentiated	DPSCs cocultured with okadaic acid- (OA-) induced cellular model of AD *in vitro*	DPSC-treated cells had the morphology of restored neurons, elongated dendrites, densely arranged microfilaments, and thickened microtubular fibrils	[154]
	Ahmed et al.	Undifferentiated	DPSCs cocultured with amyloid beta (Aβ) peptide-induced cellular model of AD *in vitro*	DPSCs secreted and produced numerous vascular endothelial growth factor (VEGF), fractalkine, RANTES, fms-related tyrosine kinase 3 (FLT-3), and monocyte chemotactic protein 1 (MCP-1)	[155]
Retinal injury	Mead et al.	Undifferentiated	Intravitreal DPSC transplantation	DPSCs produced and secreted lots of neurotrophins in order to promote neuritogenesis/axogenesis of retinal cells	[83]
	Mead et al.	Undifferentiated	Intravitreal DPSC transplantation	DPSC provided protection from retinal ganglion cell (RGC) loss and retinal nerve fiber layer thickness (RNFL) thinning and preserved RGC function	[160]
	Bray et al.	Undifferentiated	DPSCs cocultured with the conditioned media which were obtained from organotypic explants from damaged rat retinas *in vitro*	DPSCs had ability to promote neurodifferentiation and expression of retinal neuronal markers in order to cure the rat retinas	[161]
The peripheral nervous system (PNS) diseases					
Facial nerve defect	Sasaki et al.	Undifferentiated	DPSCs transplanted with poly-DL-lactide-coglycolide (PLGA) and collagen gel	DPSCs promoted the axon regeneration and myelinated nerve formation	[173]
Sciatic nerve defect	Sanen et al.	Schwann cell-type differentiated	DPSCs transplanted with NeuraWrap™ conduits	DPSCs promoted in growing neurites, myelinated nerve, and newly blood vessel formation and survival	[174]
Sciatic nerve defect	Askari et al.	Oligodendrocyte progenitor cell- (OPC-) type differentiated	DPSC-induced OPC transplantation	DPSCs could be differentiated into functional oligodendrocytes	[175]
Sciatic nerve defect	Omi et al.	Undifferentiated	DPSC transplantation	DPSCs increased the gene expression of interleukin-10 and promoted macrophages polarization towards anti-inflammatory M2 phenotypes	[176]

blood flow to injury sites. In addition to their roles in regeneration and repair of injured neural tissue (Table 1), therapies using DPSCs are emerging as a promising novel strategy for treating other brain conditions and syndromes such as traumatic brain injury, multiple sclerosis, and autism spectrum disorders.

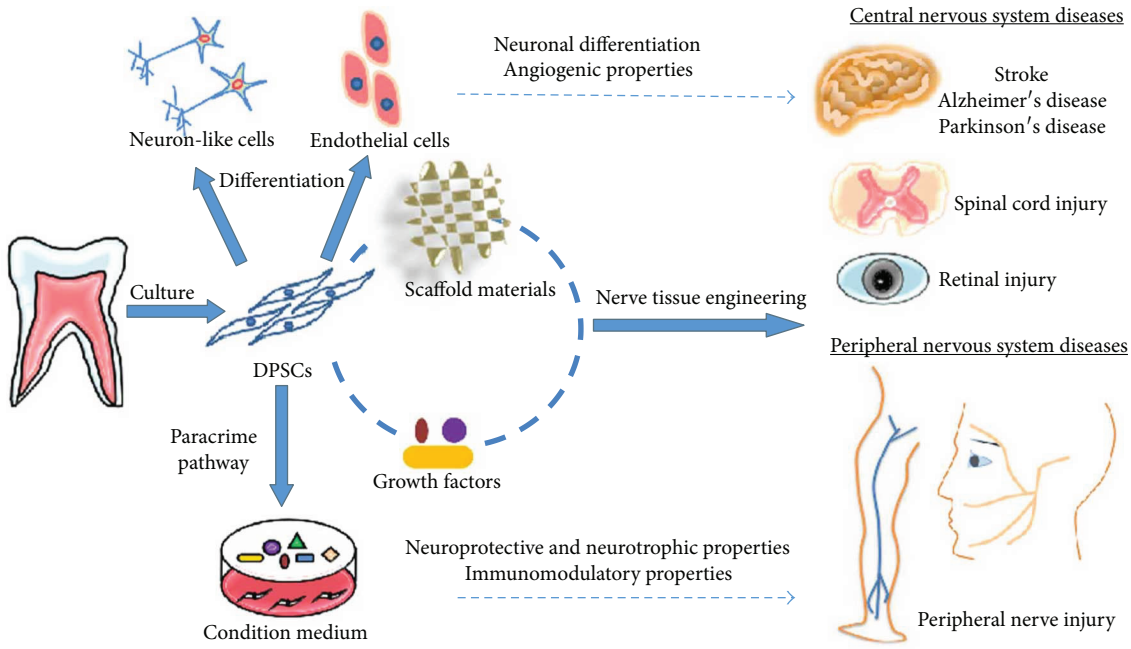

Figure 3: Tissue-engineered constructs of DPSCs, scaffolds, and growth factors and their applications in nervous system diseases. In the constructs, scaffolds can provide biomimetic environments and structural support for cell survival and proliferation. Growth factors can promote neuronal cell proliferation and survival *in vivo* and *in vitro*. DPSCs can enhance neuronal regeneration and repair due to their neuronal differentiation potential and their neurotrophic, neuroprotective, angiogenic, and immunomodulatory properties.

However, despite the functional advantages of using DPSCs for the treatment of nervous system injuries and diseases, there remain significant roadblocks with respect to overcoming the nervous system's seemingly inherent and immutable resistance to regeneration and repair. Nerve tissue engineering approaches are now beginning to adopt combinatorial strategies that involve simultaneous manipulations to cells, growth factors, and scaffolds in order to circumvent the recalcitrant nature of the nervous system (Figure 3). In particular, novel scaffolds such as hydrogels have a 3D porous structure and good cytocompatibility that can be used to provide an *in vivo*-like microenvironment and structural support for cell adhesion, proliferation, and growth. Scaffolds can be designed to embed biological important macromolecules such as bFGF and NGF and to precisely tune their diffusion rate and enzymatic degradation. Seed cells such as DPSCs have beneficial effects on neural regeneration and repair associated with their neural differentiation potential and their neurotrophic, angiogenic, and immunomodulatory properties. Therefore, the spatiotemporal combination of DPSCs, scaffolds, and growth factors provides a promising strategy for treating nervous system-related diseases and injuries in future clinical approaches.

Conflicts of Interest

The authors declare that they have no competing interests regarding the publication of this paper.

Authors' Contributions

Lihua Luo and Yan He contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81601626 to Huaqiong Li), the UQDVCR (610709 to Qingsong Ye), the Wenzhou Medical University (QTJ16026 to Lihua Luo), and Research and Development Program Project of Xiangyang (20270268020 to Lihua Luo).

References

[1] K. Kahveci, M. Dincer, C. Doger, and A. K. Yarici, "Traumatic brain injury and palliative care: a retrospective analysis of 49 patients receiving palliative care during 2013–2016 in Turkey," *Neural Regeneration Research*, vol. 12, no. 1, pp. 77–83, 2017.

[2] P. Ciaramitaro, M. Mondelli, F. Logullo et al., "Traumatic peripheral nerve injuries: epidemiological findings, neuropathic pain and quality of life in 158 patients," *Journal of the Peripheral Nervous System*, vol. 15, no. 2, pp. 120–127, 2010.

[3] Y. Esquenazi, S. H. Park, D. G. Kline, and D. H. Kim, "Surgical management and outcome of iatrogenic radial nerve injection injuries," *Clinical Neurology and Neurosurgery*, vol. 142, pp. 98–103, 2016.

[4] J. Baumard, M. Lesourd, C. Remigereau et al., "Tool use in neurodegenerative diseases: planning or technical reasoning?," *Journal of Neuropsychology*, 2017.

[5] A. Rolfe and D. Sun, "Stem cell therapy in brain trauma: implications for repair and regeneration of injured brain in experimental TBI models," in *Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects*, F. H. Kobeissy, Ed., pp. 587–596, CRC Press/Taylor & Francis, Boca Raton, FL, USA, 2015.

[6] H. Hong, B. S. Kim, and H. I. Im, "Pathophysiological role of neuroinflammation in neurodegenerative diseases and psychiatric disorders," *International Neurourology Journal*, vol. 20, Supplement 1, pp. S2–S7, 2016.

[7] M. Berry, Z. Ahmed, B. Lorber, M. Douglas, and A. Logan, "Regeneration of axons in the visual system," *Restorative Neurology and Neuroscience*, vol. 26, no. 2-3, pp. 147–174, 2008.

[8] J. Bianco, P. De Berdt, R. Deumens, and A. des Rieux, "Taking a bite out of spinal cord injury: do dental stem cells have the teeth for it?," *Cellular and Molecular Life Sciences*, vol. 73, no. 7, pp. 1413–1437, 2016.

[9] A. R. Caseiro, T. Pereira, G. Ivanova, A. L. Luís, and A. C. Maurício, "Neuromuscular regeneration: perspective on the application of mesenchymal stem cells and their secretion products," *Stem Cells International*, vol. 2016, Article ID 9756973, 16 pages, 2016.

[10] B. Mead, M. Berry, A. Logan, R. A. H. Scott, W. Leadbeater, and B. A. Scheven, "Stem cell treatment of degenerative eye disease," *Stem Cell Research*, vol. 14, no. 3, pp. 243–257, 2015.

[11] H. Ghasemi Hamidabadi, Z. Rezvani, M. Nazm Bojnordi et al., "Chitosan-intercalated montmorillonite/poly(vinyl alcohol) nanofibers as a platform to guide neuronlike differentiation of human dental pulp stem cells," *ACS Applied Materials & Interfaces*, vol. 9, no. 13, pp. 11392–11404, 2017.

[12] L. Ferroni, C. Gardin, I. Tocco et al., "Potential for neural differentiation of mesenchymal stem cells," *Advances in Biochemical Engineering/Biotechnology*, vol. 129, pp. 89–115, 2012.

[13] V. Neirinckx, C. Coste, B. Rogister, and S. Wislet-Gendebien, "Concise review: adult mesenchymal stem cells, adult neural crest stem cells, and therapy of neurological pathologies: a state of play," *Stem Cells Translational Medicine*, vol. 2, no. 4, pp. 284–296, 2013.

[14] F. G. Teixeira, M. M. Carvalho, N. Sousa, and A. J. Salgado, "Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration?," *Cellular and Molecular Life Sciences*, vol. 70, no. 20, pp. 3871–3882, 2013.

[15] B. M. Abdallah and M. Kassem, "Human mesenchymal stem cells: from basic biology to clinical applications," *Gene Therapy*, vol. 15, no. 2, pp. 109–116, 2008.

[16] M. Ohishi and E. Schipani, "Bone marrow mesenchymal stem cells," *Journal of Cellular Biochemistry*, vol. 109, no. 2, pp. 277–282, 2010.

[17] H. Jin, Y. Bae, M. Kim et al., "Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy," *International Journal of Molecular Sciences*, vol. 14, no. 9, pp. 17986–18001, 2013.

[18] M. H. Lim, W. K. Ong, and S. Sugii, "The current landscape of adipose-derived stem cells in clinical applications," *Expert Reviews in Molecular Medicine*, vol. 16, article e8, 2014.

[19] J. Ribeiro, A. Gartner, T. Pereira et al., "Chapter four - perspectives of employing mesenchymal stem cells from the Wharton's jelly of the umbilical cord for peripheral nerve repair," *International Review of Neurobiology*, vol. 108, pp. 79–120, 2013.

[20] S. Frausin, S. Viventi, L. Verga Falzacappa et al., "Wharton's jelly derived mesenchymal stromal cells: biological properties, induction of neuronal phenotype and current applications in neurodegeneration research," *Acta Histochemica*, vol. 117, no. 4-5, pp. 329–338, 2015.

[21] S. Gronthos, M. Mankani, J. Brahim, P. G. Robey, and S. Shi, "Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13625–13630, 2000.

[22] M. Miura, S. Gronthos, M. Zhao et al., "SHED: stem cells from human exfoliated deciduous teeth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 5807–5812, 2003.

[23] W. Sonoyama, Y. Liu, T. Yamaza et al., "Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study," *Journal of Endodontics*, vol. 34, no. 2, pp. 166–171, 2008.

[24] B. M. Seo, M. Miura, S. Gronthos et al., "Investigation of multipotent postnatal stem cells from human periodontal ligament," *The Lancet*, vol. 364, no. 9429, pp. 149–155, 2004.

[25] C. Morsczeck, W. Gotz, J. Schierholz et al., "Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth," *Matrix Biology*, vol. 24, no. 2, pp. 155–165, 2005.

[26] P. Y. Collart-Dutilleul, F. Chaubron, J. De Vos, and F. J. Cuisinier, "Allogenic banking of dental pulp stem cells for innovative therapeutics," *World Journal of Stem Cells*, vol. 7, no. 7, pp. 1010–1021, 2015.

[27] Y. W. Geng, Z. Zhang, M. Y. Liu, and W. P. Hu, "Differentiation of human dental pulp stem cells into neuronal by resveratrol," *Cell Biology Interenational*, vol. 41, no. 12, pp. 1391–1398, 2017.

[28] L. Hidalgo San Jose, P. Stephens, B. Song, and D. Barrow, "Microfluidic encapsulation supports stem cell viability, proliferation, and neuronal differentiation," *Tissue Engineering Part C: Methods*, vol. 24, no. 3, pp. 158–170, 2018.

[29] K. Sakai, A. Yamamoto, K. Matsubara et al., "Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms," *The Journal of Clinical Investigation*, vol. 122, no. 1, pp. 80–90, 2012.

[30] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells," *PLoS One*, vol. 9, no. 10, article e109305, 2014.

[31] D. Foudah, M. Monfrini, E. Donzelli et al., "Expression of neural markers by undifferentiated mesenchymal-like stem cells from different sources," *Journal of Immunology Research*, vol. 2014, Article ID 987678, 16 pages, 2014.

[32] I. Ullah, J. M. Park, Y. H. Kang et al., "Transplantation of human dental pulp-derived stem cells or differentiated neuronal cells from human dental pulp-derived stem cells identically enhances regeneration of the injured peripheral nerve," *Stem Cells and Development*, vol. 26, no. 17, pp. 1247–1257, 2017.

[33] Y. Chai, X. Jiang, Y. Ito et al., "Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis," *Development*, vol. 127, no. 8, pp. 1671–1679, 2000.

[34] S. Shi and S. Gronthos, "Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp," *Journal of Bone and Mineral Research*, vol. 18, no. 4, pp. 696–704, 2003.

[35] E. Karaoz, P. C. Demircan, O. Saglam, A. Aksoy, F. Kaymaz, and G. Duruksu, "Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells," *Histochemistry and Cell Biology*, vol. 136, no. 4, pp. 455–473, 2011.

[36] W. Martens, A. Bronckaers, C. Politis, R. Jacobs, and I. Lambrichts, "Dental stem cells and their promising role in neural regeneration: an update," *Clinical Oral Investigations*, vol. 17, no. 9, pp. 1969–1983, 2013.

[37] R. Kuang, Z. Zhang, X. Jin et al., "Nanofibrous spongy microspheres for the delivery of hypoxia-primed human dental pulp stem cells to regenerate vascularized dental pulp," *Acta Biomaterialia*, vol. 33, pp. 225–234, 2016.

[38] S. Gronthos, J. Brahim, W. Li et al., "Stem cell properties of human dental pulp stem cells," *Journal of Dental Research*, vol. 81, no. 8, pp. 531–535, 2002.

[39] N. Kawashima, "Characterisation of dental pulp stem cells: a new horizon for tissue regeneration?," *Archives of Oral Biology*, vol. 57, no. 11, pp. 1439–1458, 2012.

[40] L. Xiao, R. Ide, C. Saiki, Y. Kumazawa, and H. Okamura, "Human dental pulp cells differentiate toward neuronal cells and promote neuroregeneration in adult organotypic hippocampal slices in vitro," *International Journal of Molecular Sciences*, vol. 18, no. 8, p. 1745, 2017.

[41] E. Karaoz, B. N. Dogan, A. Aksoy et al., "Isolation and in vitro characterisation of dental pulp stem cells from natal teeth," *Histochemistry and Cell Biology*, vol. 133, no. 1, pp. 95–112, 2010.

[42] A. H.-C. Huang, Y.-K. Chen, L.-M. Lin, T.-Y. Shieh, and A. W.-S. Chan, "Isolation and characterization of dental pulp stem cells from a supernumerary tooth," *Journal of Oral Pathology & Medicine*, vol. 37, no. 9, pp. 571–574, 2008.

[43] S. Rai, S. Kaur, M. Kaur, and S. P. Arora, "Redefining the potential applications of dental stem cells: an asset for future," *Indian Journal of Human Genetics*, vol. 18, no. 3, pp. 276–284, 2012.

[44] R. Kabir, M. Gupta, A. Aggarwal, D. Sharma, A. Sarin, and M. Z. Kola, "Imperative role of dental pulp stem cells in regenerative therapies: a systematic review," *Nigerian Journal of Surgery*, vol. 20, no. 1, pp. 1–8, 2014.

[45] P. Torkzaban, A. Saffarpour, M. Bidgoli, and S. Sohilifar, "In vitro evaluation of isolation possibility of stem cells from intra oral soft tissue and comparison of them with bone marrow stem cells," *Journal of Dentistry*, vol. 9, no. 1, pp. 1–6, 2012.

[46] P. Stanko, K. Kaiserova, V. Altanerova, and C. Altaner, "Comparison of human mesenchymal stem cells derived from dental pulp, bone marrow, adipose tissue, and umbilical cord tissue by gene expression," *Biomedical Papers*, vol. 158, no. 3, pp. 373–377, 2014.

[47] E. P. Chalisserry, S. Y. Nam, S. H. Park, and S. Anil, "Therapeutic potential of dental stem cells," *Journal of Tissue Engineering*, vol. 8, 2017.

[48] A. Palmieri, F. Pezzetti, A. Graziano et al., "Comparison between osteoblasts derived from human dental pulp stem cells and osteosarcoma cell lines," *Cell Biology International*, vol. 32, no. 7, pp. 733–738, 2008.

[49] P.-H. Cheng, B. Snyder, D. Fillos, C. C. Ibegbu, A. Huang, and A. W. S. Chan, "Postnatal stem/progenitor cells derived from the dental pulp of adult chimpanzee," *BMC Cell Biology*, vol. 9, no. 1, p. 20, 2008.

[50] M. Kiraly, B. Porcsalmy, A. Pataki et al., "Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons," *Neurochemistry International*, vol. 55, no. 5, pp. 323–332, 2009.

[51] X. Feng, J. Xing, G. Feng et al., "Age-dependent impaired neurogenic differentiation capacity of dental stem cell is associated with Wnt/β-catenin signaling," *Cellular and Molecular Neurobiology*, vol. 33, no. 8, pp. 1023–1031, 2013.

[52] L. Liu, J. Ling, X. Wei, L. Wu, and Y. Xiao, "Stem cell regulatory gene expression in human adult dental pulp and periodontal ligament cells undergoing odontogenic/osteogenic differentiation," *Journal of Endodontics*, vol. 35, no. 10, pp. 1368–1376, 2009.

[53] K. Iohara, L. Zheng, M. Ito, A. Tomokiyo, K. Matsushita, and M. Nakashima, "Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis," *Stem Cells*, vol. 24, no. 11, pp. 2493–2503, 2006.

[54] A. Arthur, G. Rychkov, S. Shi, S. A. Koblar, and S. Gronthos, "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues," *Stem Cells*, vol. 26, no. 7, pp. 1787–1795, 2008.

[55] Y. X. Wang, Z. F. Ma, N. Huo et al., "Porcine tooth germ cell conditioned medium can induce odontogenic differentiation of human dental pulp stem cells," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 5, no. 5, pp. 354–362, 2011.

[56] W. Zhang, X. F. Walboomers, S. Shi, M. Fan, and J. A. Jansen, "Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation," *Tissue Engineering*, vol. 12, no. 10, pp. 2813–2823, 2006.

[57] X. Yan, H. Qin, C. Qu, R. S. Tuan, S. Shi, and G. T. J. Huang, "iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin," *Stem Cells and Development*, vol. 19, no. 4, pp. 469–480, 2010.

[58] N. Tamaoki, K. Takahashi, T. Tanaka et al., "Dental pulp cells for induced pluripotent stem cell banking," *Journal of Dental Research*, vol. 89, no. 8, pp. 773–778, 2010.

[59] N. Nordin, M. I. Lai, A. Veerakumarasivam et al., "Induced pluripotent stem cells: history, properties and potential applications," *The Medical Journal of Malaysia*, vol. 66, no. 1, pp. 4–9, 2011.

[60] N. Malhotra, "Induced pluripotent stem (iPS) cells in dentistry: a review," *International Journal of Stem Cells*, vol. 9, no. 2, pp. 176–185, 2016.

[61] P. Gervois, T. Struys, P. Hilkens et al., "Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons," *Stem Cells and Development*, vol. 24, no. 3, pp. 296–311, 2015.

[62] J. Zhang, M. Lian, P. Cao et al., "Effects of nerve growth factor and basic fibroblast growth factor promote human dental pulp stem cells to neural differentiation," *Neurochemical Research*, vol. 42, no. 4, pp. 1015–1025, 2017.

[63] T. Osathanon, C. Sawangmake, N. Nowwarote, and P. Pavasant, "Neurogenic differentiation of human dental pulp stem cells using different induction protocols," *Oral Diseases*, vol. 20, no. 4, pp. 352–358, 2014.

[64] L. Xiao and T. Tsutsui, "Characterization of human dental pulp cells-derived spheroids in serum-free medium: stem

cells in the core," *Journal of Cellular Biochemistry*, vol. 114, no. 11, pp. 2624–2636, 2013.

[65] M. Kanafi, D. Majumdar, R. Bhonde, P. Gupta, and I. Datta, "Midbrain cues dictate differentiation of human dental pulp stem cells towards functional dopaminergic neurons," *Journal of Cellular Physiology*, vol. 229, no. 10, pp. 1369–1377, 2014.

[66] C. C. Chang, K. C. Chang, S. J. Tsai, H. H. Chang, and C. P. Lin, "Neurogenic differentiation of dental pulp stem cells to neuron-like cells in dopaminergic and motor neuronal inductive media," *Journal of the Formosan Medical Association*, vol. 113, no. 12, pp. 956–965, 2014.

[67] M. Kiraly, K. Kadar, D. B. Horvathy et al., "Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo," *Neurochemistry International*, vol. 59, no. 3, pp. 371–381, 2011.

[68] N. Fatima, A. A. Khan, and S. K. Vishwakarma, "Immunophenotypic and molecular analysis of human dental pulp stem cells potential for neurogenic differentiation," *Contemporary Clinical Dentistry*, vol. 8, no. 1, pp. 81–89, 2017.

[69] S. Y. Chun, S. Soker, Y. J. Jang, T. G. Kwon, and E. S. Yoo, "Differentiation of human dental pulp stem cells into dopaminergic neuron-like cells in vitro," *Journal of Korean Medical Science*, vol. 31, no. 2, pp. 171–177, 2016.

[70] J. Karbanova, T. Soukup, J. Suchanek, R. Pytlik, D. Corbeil, and J. Mokry, "Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium," *Cells, Tissues, Organs*, vol. 193, no. 6, pp. 344–365, 2011.

[71] Y. Lu, X. Yuan, Y. Ou et al., "Autophagy and apoptosis during adult adipose-derived stromal cells differentiation into neuron-like cells in vitro," *Neural Regeneration Research*, vol. 7, no. 16, pp. 1205–1212, 2012.

[72] N. Gnanasegaran, V. Govindasamy, P. Kathirvaloo, S. Musa, and N. H. Abu Kasim, "Effects of cell cycle phases on the induction of dental pulp stem cells toward dopaminergic-like cells," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 12, no. 2, pp. e881–e893, 2018.

[73] M. Singh, A. Kakkar, R. Sharma et al., "Synergistic effect of BDNF and FGF2 in efficient generation of functional dopaminergic neurons from human mesenchymal stem cells," *Scientific Reports*, vol. 7, no. 1, article 10378, 2017.

[74] T. Gonmanee, C. Thonabulsombat, K. Vongsavan, and H. Sritanaudomchai, "Differentiation of stem cells from human deciduous and permanent teeth into spiral ganglion neuron-like cells," *Archives of Oral Biology*, vol. 88, pp. 34–41, 2018.

[75] X. Feng, X. Lu, D. Huang et al., "3D porous chitosan scaffolds suit survival and neural differentiation of dental pulp stem cells," *Cellular and Molecular Neurobiology*, vol. 34, no. 6, pp. 859–870, 2014.

[76] M. Ustiashvili, D. Kordzaia, M. Mamaladze, M. Jangavadze, and L. Sanodze, "Investigation of functional activity human dental pulp stem cells at acute and chronic pulpitis," *Georgian Medical News*, vol. 9, no. 234, pp. 19–24, 2014.

[77] B. C. Heng, L. W. Lim, W. Wu, and C. Zhang, "An overview of protocols for the neural induction of dental and oral stem cells *in vitro*," *Tissue Engineering Part B: Reviews*, vol. 22, no. 3, pp. 220–250, 2016.

[78] W. Martens, K. Sanen, M. Georgiou et al., "Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct *in vitro*," *The FASEB Journal*, vol. 28, no. 4, pp. 1634–1643, 2014.

[79] N. Askari, M. M. Yaghoobi, M. Shamsara, and S. Esmaeili-Mahani, "Human dental pulp stem cells differentiate into oligodendrocyte progenitors using the expression of olig2 transcription factor," *Cells, Tissues, Organs*, vol. 200, no. 2, pp. 93–103, 2014.

[80] S. Goorha and L. T. Reiter, "Culturing and neuronal differentiation of human dental pulp stem cells," *Current Protocols in Human Genetics*, vol. 92, pp. 21.6.1–21.6.10, 2017.

[81] Y. H. Li, J. E. Li, and W. W. Liu, "Comparing the effect of neurotrophic factor induced MSCs (BMSC and DPSC) on the expression of myelin proteins Nogo-A and OMgp in a glaucoma rat model," *International Journal of Clinical and Experimental Medicine*, vol. 10, no. 3, pp. 4705–4713, 2017.

[82] I. V. Nosrat, J. Widenfalk, L. Olson, and C. A. Nosrat, "Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons *in vitro*, and rescue motoneurons after spinal cord injury," *Developmental Biology*, vol. 238, no. 1, pp. 120–132, 2001.

[83] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 12, pp. 7544–7556, 2013.

[84] A. Arthur, S. Shi, A. C. W. Zannettino, N. Fujii, S. Gronthos, and S. A. Koblar, "Implanted adult human dental pulp stem cells induce endogenous axon guidance," *Stem Cells*, vol. 27, no. 9, pp. 2229–2237, 2009.

[85] M. K. Kolar, V. N. Itte, P. J. Kingham, L. N. Novikov, M. Wiberg, and P. Kelk, "The neurotrophic effects of different human dental mesenchymal stem cells," *Scientific Reports*, vol. 7, no. 1, article 12605, 2017.

[86] M. Song, S. S. Jue, Y. A. Cho, and E. C. Kim, "Comparison of the effects of human dental pulp stem cells and human bone marrow-derived mesenchymal stem cells on ischemic human astrocytes in vitro," *Journal of Neuroscience Research*, vol. 93, no. 6, pp. 973–983, 2015.

[87] M. Song, J. H. Lee, J. Bae, Y. Bu, and E. C. Kim, "Human dental pulp stem cells are more effective than human bone marrow-derived mesenchymal stem cells in cerebral ischemic injury," *Cell Transplantation*, vol. 26, no. 6, pp. 1001–1016, 2017.

[88] A. Kumar, V. Kumar, V. Rattan, V. Jha, and S. Bhattacharyya, "Secretome cues modulate the neurogenic potential of bone marrow and dental stem cells," *Molecular Neurobiology*, vol. 54, no. 6, pp. 4672–4682, 2017.

[89] P. Carmeliet, "Mechanisms of angiogenesis and arteriogenesis," *Nature Medicine*, vol. 6, no. 4, pp. 389–395, 2000.

[90] J. Folkman, "Tumor angiogenesis: therapeutic implications," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[91] A. Giordano, U. Galderisi, and I. R. Marino, "From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells," *Journal of Cellular Physiology*, vol. 211, no. 1, pp. 27–35, 2007.

[92] P. J. Psaltis, A. C. W. Zannettino, S. G. Worthley, and S. Gronthos, "Concise review: mesenchymal stromal cells: potential for cardiovascular repair," *Stem Cells*, vol. 26, no. 9, pp. 2201–2210, 2008.

[93] D. P. Sieveking and M. K. Ng, "Cell therapies for therapeutic angiogenesis: back to the bench," *Vascular Medicine*, vol. 14, no. 2, pp. 153–166, 2009.

[94] P. Hilkens, Y. Fanton, W. Martens et al., "Pro-angiogenic impact of dental stem cells *in vitro* and *in vivo*," *Stem Cell Research*, vol. 12, no. 3, pp. 778–790, 2014.

[95] A. Bronckaers, P. Hilkens, Y. Fanton et al., "Angiogenic properties of human dental pulp stem cells," *PLoS One*, vol. 8, no. 8, article e71104, 2013.

[96] J. Ratajczak, A. Bronckaers, Y. Dillen et al., "The neurovascular properties of dental stem cells and their importance in dental tissue engineering," *Stem Cells International*, vol. 2016, Article ID 9762871, 17 pages, 2016.

[97] L. Tran-Hung, P. Laurent, J. Camps, and I. About, "Quantification of angiogenic growth factors released by human dental cells after injury," *Archives of Oral Biology*, vol. 53, no. 1, pp. 9–13, 2008.

[98] A. M. F. Aranha, Z. Zhang, K. G. Neiva, C. A. S. Costa, J. Hebling, and J. E. Nör, "Hypoxia enhances the angiogenic potential of human dental pulp cells," *Journal of Endodontics*, vol. 36, no. 10, pp. 1633–1637, 2010.

[99] K. Iohara, L. Zheng, H. Wake et al., "A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp," *Stem Cells*, vol. 26, no. 9, pp. 2408–2418, 2008.

[100] L. Tran-Hung, S. Mathieu, and I. About, "Role of human pulp fibroblasts in angiogenesis," *Journal of Dental Research*, vol. 85, no. 9, pp. 819–823, 2006.

[101] C. Gandia, A. Armiñan, J. M. García-Verdugo et al., "Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction," *Stem Cells*, vol. 26, no. 3, pp. 638–645, 2008.

[102] M. Sugiyama, K. Iohara, H. Wakita et al., "Dental pulp-derived $CD31^-/CD146^-$ side population stem/progenitor cells enhance recovery of focal cerebral ischemia in rats," *Tissue Engineering Part A*, vol. 17, no. 9-10, pp. 1303–1311, 2011.

[103] H. Nam, G. H. Kim, Y. K. Bae et al., "Angiogenic capacity of dental pulp stem cell regulated by SDF-1α-CXCR4 axis," *Stem Cells International*, vol. 2017, Article ID 8085462, 10 pages, 2017.

[104] R. d'Aquino, A. Graziano, M. Sampaolesi et al., "Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation," *Cell Death and Differentiation*, vol. 14, no. 6, pp. 1162–1171, 2007.

[105] C. Marchionni, L. Bonsi, F. Alviano et al., "Angiogenic potential of human dental pulp stromal (stem) cells," *International Journal of Immunopathology and Pharmacology*, vol. 22, no. 3, pp. 699–706, 2009.

[106] P. Hilkens, A. Bronckaers, J. Ratajczak, P. Gervois, E. Wolfs, and I. Lambrichts, "The angiogenic potential of DPSCs and SCAPs in an *in vivo* model of dental pulp regeneration," *Stem Cells International*, vol. 2017, Article ID 2582080, 14 pages, 2017.

[107] T. Deuse, M. Stubbendorff, K. Tang-Quan et al., "Immunogenicity and immunomodulatory properties of umbilical cord lining mesenchymal stem cells," *Cell Transplantation*, vol. 20, no. 5, pp. 655–667, 2011.

[108] J. W. Kang, H. C. Koo, S. Y. Hwang et al., "Immunomodulatory effects of human amniotic membrane-derived mesenchymal stem cells," *Journal of Veterinary Science*, vol. 13, no. 1, pp. 23–31, 2012.

[109] P. Comite, L. Cobianchi, M. A. Avanzini et al., "Immunomodulatory properties of porcine, bone marrow-derived multipotent mesenchymal stromal cells and comparison with their human counterpart," *Cellular and Molecular Biology*, vol. 57, no. 2, 2011Supplement, 2011.

[110] E. Soleymaninejadian, K. Pramanik, and E. Samadian, "Immunomodulatory properties of mesenchymal stem cells: cytokines and factors," *American Journal of Reproductive Immunology*, vol. 67, no. 1, pp. 1–8, 2012.

[111] A. Pourgholaminejad, N. Aghdami, H. Baharvand, and S. M. Moazzeni, "The effect of pro-inflammatory cytokines on immunophenotype, differentiation capacity and immunomodulatory functions of human mesenchymal stem cells," *Cytokine*, vol. 85, pp. 51–60, 2016.

[112] M. P. De Miguel, S. Fuentes-Julián, A. Blázquez-Martínez et al., "Immunosuppressive properties of mesenchymal stem cells: advances and applications," *Current Molecular Medicine*, vol. 12, no. 5, pp. 574–591, 2012.

[113] I. R. Rajput, A. Hussain, Y. L. Li et al., "*Saccharomyces boulardii* and *bacillus subtilis* B10 modulate TLRs mediated signaling to induce immunity by chicken BMDCs," *Journal of Cellular Biochemistry*, vol. 115, no. 1, pp. 189–198, 2014.

[114] W. He, T. Qu, Q. Yu et al., "LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells," *International Endodontic Journal*, vol. 46, no. 2, pp. 128–136, 2013.

[115] A. Heiman, A. Pallottie, R. F. Heary, and S. Elkabes, "Toll-like receptors in central nervous system injury and disease: a focus on the spinal cord," *Brain, Behavior, and Immunity*, vol. 42, pp. 232–245, 2014.

[116] L. Guth, Z. Zhang, N. A. DiProspero, K. Joubin, and M. T. Fitch, "Spinal cord injury in the rat: treatment with bacterial lipopolysaccharide and indomethacin enhances cellular repair and locomotor function," *Experimental Neurology*, vol. 126, no. 1, pp. 76–87, 1994.

[117] S. Tomic, J. Djokic, S. Vasilijic et al., "Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists," *Stem Cells and Development*, vol. 20, no. 4, pp. 695–708, 2011.

[118] A. T. Ozdemir, R. B. Ozgul Ozdemir, C. Kirmaz et al., "The paracrine immunomodulatory interactions between the human dental pulp derived mesenchymal stem cells and CD4 T cell subsets," *Cellular Immunology*, vol. 310, pp. 108–115, 2016.

[119] S. Sugita, H. Kamao, Y. Iwasaki et al., "Inhibition of T-cell activation by retinal pigment epithelial cells derived from induced pluripotent stem cells," *Investigative Ophthalmology & Visual Science*, vol. 56, no. 2, pp. 1051–1062, 2015.

[120] K. H. Kwack, J. M. Lee, S. H. Park, and H. W. Lee, "Human dental pulp stem cells suppress alloantigen-induced immunity by stimulating T cells to release transforming growth factor beta," *Journal of Endodontics*, vol. 43, no. 1, pp. 100–108, 2017.

[121] P. C. Demircan, A. E. Sariboyaci, Z. S. Unal, G. Gacar, C. Subasi, and E. Karaoz, "Immunoregulatory effects of human dental pulp-derived stem cells on T cells: comparison of transwell co-culture and mixed lymphocyte reaction systems," *Cytotherapy*, vol. 13, no. 10, pp. 1205–1220, 2011.

[122] L. Pierdomenico, L. Bonsi, M. Calvitti et al., "Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp," *Transplantation*, vol. 80, no. 6, pp. 836–842, 2005.

[123] Y. Zhao, L. Wang, Y. Jin, and S. Shi, "Fas ligand regulates the immunomodulatory properties of dental pulp stem cells," *Journal of Dental Research*, vol. 91, no. 10, pp. 948–954, 2012.

[124] A. Földes, K. Kádár, B. Kerémi et al., "Mesenchymal stem cells of dental origin-their potential for antiinflammatory and regenerative actions in brain and gut damage," *Current Neuropharmacology*, vol. 14, no. 8, pp. 914–934, 2016.

[125] J. W. Hong, J. H. Lim, C. J. Chung et al., "Immune tolerance of human dental pulp-derived mesenchymal stem cells mediated by $CD4^+CD25^+FoxP3^+$ regulatory T-Cells and induced by TGF-β1 and IL-10," *Yonsei Medical Journal*, vol. 58, no. 5, pp. 1031–1039, 2017.

[126] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Concise review: dental pulp stem cells: a novel cell therapy for retinal and central nervous system repair," *Stem Cells*, vol. 35, no. 1, pp. 61–67, 2017.

[127] G. Varga and G. Gerber, "Mesenchymal stem cells of dental origin as promising tools for neuroregeneration," *Stem Cell Research & Therapy*, vol. 5, no. 2, p. 61, 2014.

[128] M. E. Schwab, "Myelin-associated inhibitors of neurite growth and regeneration in the CNS," *Trends in Neurosciences*, vol. 13, no. 11, pp. 452–456, 1990.

[129] C. C. Stichel and H. W. Muller, "The CNS lesion scar: new vistas on an old regeneration barrier," *Cell and Tissue Research*, vol. 294, no. 1, pp. 1–9, 1998.

[130] P. Gervois, E. Wolfs, Y. Dillen et al., "Paracrine maturation and migration of SH-SY5Y cells by dental pulp stem cells," *Journal of Dental Research*, vol. 96, no. 6, pp. 654–662, 2017.

[131] Z. Khazaeipour, A. Norouzi-Javidan, M. Kaveh, F. Khanzadeh Mehrabani, E. Kazazi, and S. H. Emami-Razavi, "Psychosocial outcomes following spinal cord injury in Iran," *The Journal of Spinal Cord Medicine*, vol. 37, no. 3, pp. 338–345, 2014.

[132] H. Huang, H.-Q. Chen, J. Gul, and R.-H. Yul, "Comparative study of hyperbaric oxygen therapy and conventional drug treatment on spinal cord injury at different therapeutic windows," *Scientific Research and Essays*, vol. 6, no. 5, pp. 1117–1122, 2011.

[133] Y. Jiang, F. L. Gong, G. B. Zhao, and J. Li, "Chrysin suppressed inflammatory responses and the inducible nitric oxide synthase pathway after spinal cord injury in rats," *International Journal of Molecular Sciences*, vol. 15, no. 7, pp. 12270–12279, 2014.

[134] A. Yamamoto, K. Matsubara, F. Kano, and K. Sakai, "Analysis of the neuroregenerative activities of mesenchymal stem cells in functional recovery after rat spinal cord injury," *Methods in Molecular Biology*, vol. 1213, pp. 321–328, 2014.

[135] A. Yamamoto, K. Sakai, K. Matsubara, F. Kano, and M. Ueda, "Multifaceted neuro-regenerative activities of human dental pulp stem cells for functional recovery after spinal cord injury," *Neuroscience Research*, vol. 78, pp. 16–20, 2014.

[136] C. Yang, X. Li, L. Sun, W. Guo, and W. Tian, "Potential of human dental stem cells in repairing the complete transection of rat spinal cord," *Journal of Neural Engineering*, vol. 14, no. 2, article 026005, 2017.

[137] J. Zhang, X. Lu, G. Feng et al., "Chitosan scaffolds induce human dental pulp stem cells to neural differentiation: potential roles for spinal cord injury therapy," *Cell and Tissue Research*, vol. 366, no. 1, pp. 129–142, 2016.

[138] M. E. Sughrue, A. Mehra, E. S. Connolly Jr, and A. L. D'Ambrosio, "Anti-adhesion molecule strategies as potential neuroprotective agents in cerebral ischemia: a critical review of the literature," *Inflammation Research*, vol. 53, no. 10, pp. 497–508, 2004.

[139] K. A. Hossmann, "Pathophysiology and therapy of experimental stroke," *Cellular and Molecular Neurobiology*, vol. 26, no. 7-8, pp. 1055–1081, 2006.

[140] W. K. Leong, M. D. Lewis, and S. A. Koblar, "Concise review: preclinical studies on human cell-based therapy in rodent ischemic stroke models: where are we now after a decade?," *Stem Cells*, vol. 31, no. 6, pp. 1040–1043, 2013.

[141] R. Lemmens and G. K. Steinberg, "Stem cell therapy for acute cerebral injury: what do we know and what will the future bring?," *Current Opinion in Neurology*, vol. 26, no. 6, pp. 617–625, 2013.

[142] K. L. Yang, M. F. Chen, C. H. Liao, C. Y. Pang, and P. Y. Lin, "A simple and efficient method for generating Nurr1-positive neuronal stem cells from human wisdom teeth (tNSC) and the potential of tNSC for stroke therapy," *Cytotherapy*, vol. 11, no. 5, pp. 606–617, 2009.

[143] W. K. Leong, T. L. Henshall, A. Arthur et al., "Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms," *Stem Cells Translational Medicine*, vol. 1, no. 3, pp. 177–187, 2012.

[144] W. Dauer and S. Przedborski, "Parkinson's disease: mechanisms and models," *Neuron*, vol. 39, no. 6, pp. 889–909, 2003.

[145] Y. Wang, S. Chen, D. Yang, and W. D. Le, "Stem cell transplantation: a promising therapy for Parkinson's disease," *Journal of Neuroimmune Pharmacology*, vol. 2, no. 3, pp. 243–250, 2007.

[146] N. Gnanasegaran, V. Govindasamy, C. Simon et al., "Effect of dental pulp stem cells in MPTP-induced old-aged mice model," *European Journal of Clinical Investigation*, vol. 47, no. 6, pp. 403–414, 2017.

[147] N. Gnanasegaran, V. Govindasamy, V. Mani, and N. H. Abu Kasim, "Neuroimmunomodulatory properties of DPSCs in an in vitro model of Parkinson's disease," *IUBMB Life*, vol. 69, no. 9, pp. 689–699, 2017.

[148] H. Fujii, K. Matsubara, K. Sakai et al., "Dopaminergic differentiation of stem cells from human deciduous teeth and their therapeutic benefits for Parkinsonian rats," *Brain Research*, vol. 1613, pp. 59–72, 2015.

[149] M. Citron, "Alzheimer's disease: strategies for disease modification," *Nature Reviews Drug Discovery*, vol. 9, no. 5, pp. 387–398, 2010.

[150] Y. Huang and L. Mucke, "Alzheimer mechanisms and therapeutic strategies," *Cell*, vol. 148, no. 6, pp. 1204–1222, 2012.

[151] M. Li, K. Guo, and S. Ikehara, "Stem cell treatment for Alzheimer's disease," *International Journal of Molecular Sciences*, vol. 15, no. 10, pp. 19226–19238, 2014.

[152] J. Y. Shin, H. J. Park, H. N. Kim et al., "Mesenchymal stem cells enhance autophagy and increase β-amyloid clearance in Alzheimer disease models," *Autophagy*, vol. 10, no. 1, pp. 32–44, 2014.

[153] C. Apel, O. V. Forlenza, V. J. R. de Paula et al., "The neuroprotective effect of dental pulp cells in models of Alzheimer's and Parkinson's disease," *Journal of Neural Transmission*, vol. 116, no. 1, pp. 71–78, 2009.

[154] F. Wang, Y. Jia, J. Liu et al., "Dental pulp stem cells promote regeneration of damaged neuron cells on the cellular model of Alzheimer's disease," *Cell Biology International*, vol. 41, no. 6, pp. 639–650, 2017.

[155] N. E.-M. B. Ahmed, M. Murakami, Y. Hirose, and M. Nakashima, "Therapeutic potential of dental pulp stem cell secretome for Alzheimer's disease treatment: an in vitro study," *Stem Cells International*, vol. 2016, Article ID 8102478, 11 pages, 2016.

[156] T. Mita, Y. Furukawa-Hibi, H. Takeuchi et al., "Conditioned medium from the stem cells of human dental pulp improves cognitive function in a mouse model of Alzheimer's disease," *Behavioural Brain Research*, vol. 293, pp. 189–197, 2015.

[157] T. A. Reh and A. J. Fischer, "Retinal stem cells," *Methods in Enzymology*, vol. 419, pp. 52–73, 2006.

[158] Y. Munemasa and Y. Kitaoka, "Autophagy in axonal degeneration in glaucomatous optic neuropathy," *Progress in Retinal and Eye Research*, vol. 47, pp. 1–18, 2015.

[159] P. M. Richardson, U. M. McGuinness, and A. J. Aguayo, "Axons from CNS neurones regenerate into PNS grafts," *Nature*, vol. 284, no. 5753, pp. 264-265, 1980.

[160] B. Mead, L. J. Hill, R. J. Blanch et al., "Mesenchymal stromal cell–mediated neuroprotection and functional preservation of retinal ganglion cells in a rodent model of glaucoma," *Cytotherapy*, vol. 18, no. 4, pp. 487–496, 2016.

[161] A. F. Bray, R. R. Cevallos, K. Gazarian, and M. Lamas, "Human dental pulp stem cells respond to cues from the rat retina and differentiate to express the retinal neuronal marker rhodopsin," *Neuroscience*, vol. 280, pp. 142–155, 2014.

[162] R. Roozafzoon, A. Lashay, M. Vasei et al., "Dental pulp stem cells differentiation into retinal ganglion-like cells in a three dimensional network," *Biochemical and Biophysical Research Communications*, vol. 457, no. 2, pp. 154–160, 2015.

[163] B. Battiston, S. Geuna, M. Ferrero, and P. Tos, "Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair," *Microsurgery*, vol. 25, no. 4, pp. 258–267, 2005.

[164] T. Matsuyama, M. Mackay, and R. Midha, "Peripheral nerve repair and grafting techniques: a review," *Neurologia Medico-Chirurgica*, vol. 40, no. 4, pp. 187–199, 2000.

[165] B. J. Pfister, T. Gordon, J. R. Loverde, A. S. Kochar, S. E. Mackinnon, and D. K. Cullen, "Biomedical engineering strategies for peripheral nerve repair: surgical applications, state of the art, and future challenges," *Critical Reviews™ in Biomedical Engineering*, vol. 39, no. 2, pp. 81–124, 2011.

[166] T. Tamaki, M. Hirata, N. Nakajima et al., "A long-gap peripheral nerve injury therapy using human skeletal muscle-derived stem cells (Sk-SCs): an achievement of significant morphological, numerical and functional recovery," *PLoS One*, vol. 11, no. 11, article e0166639, 2016.

[167] V. Pertici, J. Laurin, F. Feron, T. Marqueste, and P. Decherchi, "Functional recovery after repair of peroneal nerve gap using different collagen conduits," *Acta Neurochirurgica*, vol. 156, no. 5, pp. 1029–1040, 2014.

[168] S. K. Lee and S. W. Wolfe, "Peripheral nerve injury and repair," *The Journal of the American Academy of Orthopaedic Surgeons*, vol. 8, no. 4, pp. 243–252, 2000.

[169] O. Alluin, C. Wittmann, T. Marqueste et al., "Functional recovery after peripheral nerve injury and implantation of a collagen guide," *Biomaterials*, vol. 30, no. 3, pp. 363–373, 2009.

[170] L. G. Dai, G. S. Huang, and S. H. Hsu, "Sciatic nerve regeneration by cocultured Schwann cells and stem cells on microporous nerve conduits," *Cell Transplantation*, vol. 22, no. 11, pp. 2029–2039, 2013.

[171] A. Faroni, S. A. Mobasseri, P. J. Kingham, and A. J. Reid, "Peripheral nerve regeneration: experimental strategies and future perspectives," *Advanced Drug Delivery Reviews*, vol. 82-83, pp. 160–167, 2015.

[172] A. Gärtner, I. Amorim, A. Almeida et al., "Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-ε-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: *in vitro* and *in vivo* analysis," *BioMed Research International*, vol. 2014, Article ID 302659, 17 pages, 2014.

[173] R. Sasaki, S. Aoki, M. Yamato et al., "PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 5, no. 10, pp. 823–830, 2011.

[174] K. Sanen, W. Martens, M. Georgiou, M. Ameloot, I. Lambrichts, and J. Phillips, "Engineered neural tissue with Schwann cell differentiated human dental pulp stem cells: potential for peripheral nerve repair?," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 11, no. 12, pp. 3362–3372, 2017.

[175] N. Askari, M. M. Yaghoobi, M. Shamsara, and S. Esmaeili-Mahani, "Tetracycline-regulated expression of *OLIG2* gene in human dental pulp stem cells lead to mouse sciatic nerve regeneration upon transplantation," *Neuroscience*, vol. 305, pp. 197–208, 2015.

[176] M. Omi, M. Hata, N. Nakamura et al., "Transplantation of dental pulp stem cells suppressed inflammation in sciatic nerves by promoting macrophage polarization towards anti-inflammation phenotypes and ameliorated diabetic polyneuropathy," *Journal of Diabetes Investigation*, vol. 7, no. 4, pp. 485–496, 2016.

Hindawi
Stem Cells International
Volume 2018, Article ID 4659159, 7 pages
https://doi.org/10.1155/2018/4659159

Research Article

Neural Stem Cell-Conditioned Medium Ameliorated Cerebral Ischemia-Reperfusion Injury in Rats

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Received 9 July 2017; Revised 22 September 2017; Accepted 10 October 2017; Published 3 April 2018

Academic Editor: Adam Ye

Introduction. Our previous study suggested that NSC-CM (neural stem cell-conditioned medium) inhibited cell apoptosis *in vitro*. In addition, many studies have shown that neurotrophic factors and microparticles secreted into a conditioned medium by NSCs had neuroprotective effects. Thus, we hypothesized that NSC-CM had the capacity of protecting against cerebral I/R injury. *Methods*. Adult male Sprague-Dawley rats receiving middle cerebral artery occlusion surgery as an animal model of cerebral I/R injury were randomly assigned to two groups: the control group and NSC-CM-treated group. 1.5 ml NSC-CM or PBS (phosphate buffer saline) was administrated slowly by tail vein at 3 h, 24 h, and 48 h after ischemia onset. *Results*. NSC-CM significantly ameliorated neurological defects and reduced cerebral infarct volume, accompanied by preserved mitochondrial ultrastructure. In addition, we also found that NSC-CM significantly inhibited cell apoptosis in the ischemic hemisphere via improving the expression of Bcl-2 (B-cell lymphoma-2). *Conclusion*. NSC-CM might be an alternative and effective therapeutic intervention for ischemic stroke.

1. Introduction

Ischemic stroke is considered to the major cause of disability and the second cause of death in the world [1]. To date, tissue plasminogen activator (tPA) is still the only effective treatment. But, narrow treatment window (4.5 h) and relative high risk of intracerebral hemorrhage limited the clinical application of tPA [1]. In addition, neuroprotective agents tested in patients were proved to fail in clinical trials because of side effects and/or low efficacy although all such agents were demonstrated to decrease neuronal cell death and infarct size in cell culture and animal stroke model, respectively [1]. Thus, we need to find new therapy strategy to treat ischemic stroke.

Recently, stem cell-based therapy, especially neural stem cell (NSC) therapy, gained more and more attracts in treating ischemic stroke. Previous studies proved that NSCs were able to promote neurological recovery via direct action (neuronal replacement) [2] and indirect bystander actions secreting BDNF [3] (brain-derived neurotrophic factor), inhibiting the process of inflammation and enhancing endoneurogenesis [4]). But, the original resource, low survival, and neuronal differentiation rates [5] as well as the potential tumor formation of NSCs [6] limited their clinical application. Thus, stem cell-based treatment is not an ideal therapeutic intervention for ischemic stroke. However, it has been reported that NSCs released many neurotrophic factors, such as BDNF [7], GDNF (glial cell line-derived neurotrophic factor), NT-3 (neurotrophin-3) [8], and other soluble factors, as well as microvesicles (MVs) [9] in a culture-conditioned medium. In addition, there are also unknown neurotrophic factors and nondegraded mitotic factors, such as: bFGF (basic fibroblast growth factor) and EGF (epidermal growth factor). Especially, low circulating concentration of BDNF

is associated with poor long-term functional outcome of ischemic stroke [10] while intranasal BDNF protected against cerebral I/R injury [11]. In addition, GDNF and NT-3 have been proved to exert neuroprotection against cerebral I/R injury [12, 13].

MVs (microvesicles) are nanosized, membrane-bound vesicles released from cells that can transport cargo—including DNA, RNA, and proteins—between cells as a form of intercellular communication. MVs, released in the culture medium by NSCs during development, were confirmed to play a protective role in nerve damage, modulate neuronal activity, and play an important role in the development and function of the nervous system [9, 14, 15]. These genetic information in MVs from stem cells were confirmed to repair damaged tissues without directly replacing cells [16]. It has been demonstrated that there were a negative correlation between lesion volumes and NSC-derived MVs in the ischemic stroke patients [17], which suggested that transplantation of NSC-derived MVs could decrease cerebral infarct volume. In addition, a previous study also demonstrated that MVs from human NSCs were able to attenuate neuroinflammation and preserve host neuronal morphology in the irradiated brain [18]. In addition, transplantation of NSC-CM into injured mouse brains not only caused expansion of the NSC population in the subventricular zone but also enhanced the formation of new neurons that migrated to the damaged site [19]. NSC-CM was confirmed to have the capacity of inducing mesenchymal stem cells into neural stem cell-like cells *in vitro* [20], which further suggested that NSC-CM might enhance endoneurogenesis. More importantly, NSC-CM could significantly attenuate neuron apoptosis after spinal cord injury in rats [21]. Our previous study also suggested that NSC-CM significantly inhibited cell apoptosis and enhanced neuronal differentiation of RA-differentiated SH-SY5Y cells *in vitro* [22]. Therefore, we hypothesized that NSC-CM might have the capacity of protecting against cerebral I/R injury.

Thus, in the current study, we tested whether intravenously injected NSC-CM could improve the neurological functional recovery and reduction of cerebral infarction volume caused by cerebral ischemia/reperfusion (I/R) injury in rats. Furthermore, we tried to investigate the possible neuroprotective mechanisms of NSC-CM on cerebral I/R injury in rats.

2. Materials and Methods

2.1. NSC-CM (Neural Stem Cell-Conditioned Medium) Preparation. All animal protocols and procedures in the current study were reviewed and approved by the guidelines of the Ethical Committee for Animal Experiments of Shandong University. E15–18 pregnant Sprague-Dawley (SD) rats were purchased from the Animal Research Center of Shandong Traditional Medicine University (Jinan, China) and used for isolating neural stem cells (NSCs). The method of isolating and culturing NSCs was performed according to the protocol by Kim et al. [23], and the detailed method of collecting NSC-CM was performed according to the protocol we previously provided [22]. In brief, the cortex region of the E15–18 SD rats was isolated, and the meninges was peeled off on the clean bench. The cortexes were transferred to a 15 ml conical tube containing 3 ml HBSS (Hanks balance salt solution) for 5mins, then dissociated into small pieces using a 1 ml pipette tip. 3 ml HBSS containing small pieces of cortexes was filtered by 100 nm filters and centrifuged at 1000 RPM $*$ 10 minutes at RT (room temperature) to get single cells. After that, the cells were resuspend in a completed culture medium including DMEM/F12 (Invitrogen, CA, USA), human recombinant epidermal growth factor (EGF; 20 ng/ml) and basic fibroblast growth factor (bFGF; 20 ng/ml) (R&D Systems, Minneapolis, MN, USA), B27 (serum-free medium supplements formulated to provide optimal growth condition for NSC expansion, 1 : 50; Invitrogen), heparin (5 μg/ml; Sigma, St Louis, MO, USA), 2 mM L-glutamine, and an antibiotic-antimycotic mixture (1 : 100; Invitrogen, 10,000 u/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B). The number of viable cells in the suspension was assessed using trypan blue, and cell density of the suspension was adjusted to $2 * 10^5$ cells/ml. Then, the cell suspensions were seeded into a T-75 flask containing a 15 ml completed culture medium at 37°C in a 5% CO_2-humidified incubation chamber (Fisher, Pittsburgh, PA, USA) for 4days. After 4days, the single cells were cloned into NSC spheroids, and the medium was changed completely with a fresh completed culture medium. A 7.5 ml fresh completed culture medium was changed every 3 days. Every 2 weeks, we cut the larger neurospheroids into small spheroids under the observation of the microscope (Olympus, Japan). At the time of each medium change, we collected the rat NSC conditioned medium by filtering through a membrane with a pore size of 0.4 μm in diameter (Millipore, Billerica, MA, USA). The filtered conditioned mediums were centrifuged at 1000 RPM $*$ 10 minutes at RT. After that, we observed the medium under the microscope to make sure that there was no cell contamination. The neural stem cell-conditioned medium was kept at 4°C for 7 days.

2.2. Animal Model Preparation and Treatment. Male Sprague-Dawley rats ($n = 40$) weighing 150–200 g were purchased from the Animal Research Center of Shandong Traditional Medicine University (Jinan, China). The animals were kept under standard laboratory conditions, maintained in temperature and humidity controlled rooms on a 12 h/12 h light/dark cycle, and had free access to food and water. The cerebral ischemia/reperfusion (I/R) injury induced middle cerebral artery occlusion (MCAO) as previously described by our group [24]. Briefly, the rats were anaesthetized by 10% of chloral hydrate (3 ml/kg BW, i.p.) and the right carotid bifurcation, the right common carotid artery (CCA), the right internal carotid artery (ICA), and the right external carotid artery (ECA) were exposed to the performer by a ventral neck incision. The monofilament nylon with a silicone-beaded tip (Sunbio Biotech, Beijing), 0.28 mm in diameter, was labeled at 18 mm to the silicone-beaded tip before inserting into the right ICA. The monofilament nylon was inserted into the right ICA until resistance was felt at 16–20 mm from the bifurcation of the right CCA. The monofilament nylon was then fixed and carefully withdrawn after

90 min of middle cerebral artery occlusion to permit reperfusion. Throughout the procedure, body temperature was maintained at 37 ± 0.5°C with a thermostatically controlled infrared lamp. The cerebral ischemia/reperfusion rats were randomly divided into 2 groups. One group was slowly administrated with 1.5 ml NSC-CM by tail vein injection at 3 h, 24 h, and 48 h after ischemia onset. The other group was slowly administrated with 1.5 ml PBS (phosphate buffer saline) by tail vein injection at 3 h, 24 h, and 48 h after ischemia onset.

2.3. Neurological Defect Score and Cerebral Infarct Volume Measurement. The blind examiner assessed the neurological defect scores at the 3rd day after ischemia onset. The 21-point behavioral scale (normal and maximum score, 21) was used to evaluate neurologic defects according to the previous report [25]. A lower score correlated with the worst neurological defects. Data of 10 rats from each group were averaged, expressed as the mean ± SEM, and compared between two groups.

After assessment of the neurological defect score, rats ($n = 4$ per group) were killed using overdose of 10% of chloral hydrate (4 ml/kg BW, i.p.) for the isolation of the brains to measure cerebral infarct volumes using TTC (2, 3, 5-triphenyltetrazolium chloride) staining as previously described by us [24]. The isolated brains were stored at −20°C for 20 min; then, five coronal sections were dissected and incubated in 2% TTC (Sigma, USA) at 37°C for 30 min. After incubation, all sections were fixed in 4% PFA (paraformaldehyde buffer) for 24 h; the IPP6.0 system (Media Cybernetics, USA) was used to calculate the cerebral ischemic volume. The total ischemic volume was expressed as a percentage of cerebral ischemic volume in the hemisphere ipsilateral to the lesion. Data from 4 rats from each group were averaged, expressed as the mean ± SEM, and compared between two groups.

2.4. TUNEL Staining. After assessment of the neurological defect score, rats ($n = 5$ per group) were anesthetized with 10% chloral hydrate (3 ml/kg BW, i.p.) and perfused transcardially with ice PBS followed by 4% PFA (paraformaldehyde in PBS, pH = 7.4). After that, the brains were removed and dehydrated in 30% and 20% sucrose solution. The brains were frozen in Tissuse-Tek embedding compound (Sakura Finetek, Japan) and cryosectioned on a cryostat (Leica CM1850, Germany). The sections of the brains were used for further terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) staining. TUNEL staining (Boster, Wuhan, China) was performed to detect apoptotic cells in the ischemic hemisphere and applied according to the manufacturers' instructions. In brief, five serial sections with an interval of 50 μm were randomly obtained from each rat. After incubating in 0.025% 3, 3-diaminobenzidine (DAB, Boster, Wuhan, China) plus 0.033% H_2O_2 in PBS for 10 min, the sections were counterstained with hematoxylin. After that, the sections were dehydrated, covered with neutral balsam, and examined with a light microscope (Olympus, Japan). IPP6.0 was supplied to calculate TUNEL staining-positive cells. Five regions within the cortex and penumbra per section were randomly selected for cell counting on the cerebral ischemia hemisphere at 20× magnification. The total cell numbers and TUNEL-positive cells were obtained in each region. The percentage of TUNEL-positive cells is described as the percentage of the numbers of TUNEL-positive cells to the total numbers of cells in each region. Data from five regions of ten sections were averaged, expressed as the mean ± SEM, and compared between two groups.

2.5. Western Blot Analysis. After assessment of neurological defect score, rats ($n = 5$ per group) were killed by overdose of 10% chloral hydrate (4 ml/kg BW, i.p.) and the ischemic hemisphere was isolated for further Western blot analysis. Each ischemic hemisphere was centrifuged at 12,000 rpm for 30 minutes at 4°C. The supernatant was collected, and protein concentration was measured using a BCA protein assay kit (Beyotime, Shanghai, China). Protein extract and sample buffer were mixed and boiled 5 minutes at 100°C before loading onto 15% polyacrylamide gels. We performed Western blot analysis using standard techniques with an ECL Plus detection kit (Millipore, Billerica, MA). The antibodies used in Western blot analysis were rabbit anti-Bcl-2 (Boster, Wuhan, China, 1 : 200) and mouse β-actin (1 : 1000; ZSGB-Bio). Every sample was repeated 3 times for Western blot analysis. Bands were normalized to β-actin levels, and the density of the band was measured using ImageJ analysis software (NIH, Bethesda, MD). Data were averaged, expressed as the mean 6 SEM, and compared between 2 groups.

2.6. Electron Microscopy. After assessment of the neurological defect score, rats ($n = 4$ per group) were killed by overdose of 10% chloral hydrate (4 ml/kg BW, i.p.) and rapidly isolated 1 mm^3 ischemic hemisphere cortex for the next electron microscopy analysis. The above isolated ischemic hemisphere cortexes were immediately postfixed in 3% glutaraldehyde (3% in 0.1 M cacodylate buffer, pH = 7.4) at 4°C overnight. Following rinsing three times with PBS, the ischemic hemisphere cortexes were then osmicated in 1% osmium tetroxide in PBS for 2 h, dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 15 min and embedded in Epon 812 resin. Ultrathin sections (0.06 μm) were sliced and stained with uranyl acetate and lead citrate, then examined with JEM-1200 EX electron microscope by a blind examiner.

2.7. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Data were expressed as means with SEM. Two-sample t test was used for the data analysis. Significance was set at $P < 0.05$.

3. Results

3.1. NSC-CM Significantly Protected against the Neurological Defect Caused by Cerebral Ischemia/Reperfusion Injury. To evaluate whether NSC-CM exert neuroprotective effect on cerebral I/R injury in rats, we measured the neurological defect scores and cerebral infarct volumes on the 3rd day after cerebral ischemia onset. We applied a 21-point scale to assess the neurological defect scores of rats on the 3rd

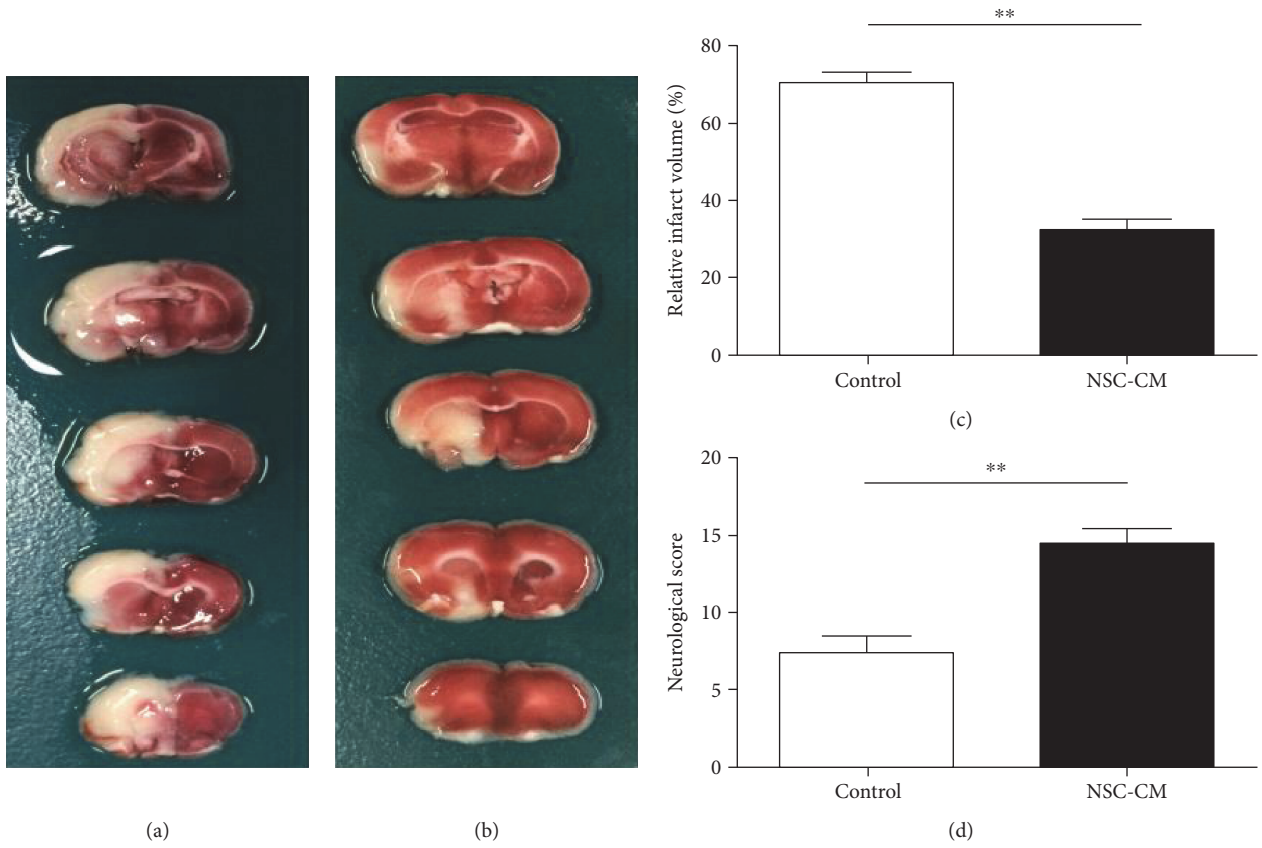

Figure 1: The effect of NSC-CM on the neurological defect score and cerebral infarct volume of cerebral ischemia/reperfusion injury. Representative TTC staining image on the 3rd day after cerebral ischemia onset of the control group (a) and the NSC-CM group (b). The bar graph showing that the percentage of cerebral ischemic volume (c) in the hemisphere ipsilateral to the lesion on the 3rd day after cerebral ischemia onset was significantly decreased by posttreatment with NSC-CM compared with the control group ($n = 5$ each group). $^{**}P < 0.01$, two-sample t test. The bar graph showing that NSC-CM (d) significantly improved the neurological defect score according to a 21-score point, compared to control. $^{**}P < 0.01$, two-sample t test.

day after cerebral ischemia onset. A lower score correlated with the worst neurological defects. As demonstrated in Figure 1(d), we found that the neurological defect scores of rats receiving NSC-CM tail vein injection were significantly higher than the control group ($P < 0.01$). In addition, we also performed TTC staining to measure the cerebral infarct volumes. As illustrated in Figures 1(a), 1(b), and 1(c), we observed that NSC-CM vein tail injection significantly reduced the cerebral infarct volumes, compared to control group ($P < 0.01$). Thus, these data indicated that NSC-CM had the capacity of protecting the rats against cerebral I/R injury.

3.2. NSC-CM Significantly Attenuated Cell Apoptosis via Improving the Expression of Bcl-2. To investigate the possible mechanisms of the neuroprotective effects of NSC-CM on cerebral I/R injury in rats, we applied TUNEL staining to measure the number of apoptosis cells in the ischemic hemisphere. As demonstrated in Figures 2(a), 2(b), and 2(f), NSC-CM significantly reduced the number of apoptotic cells in the ischemic hemisphere, compared to the control group. To further clarify how NSC-CM inhibit cell apoptosis, we applied Western blot to measure the expression of Bcl-2 in the cerebral hemisphere. As illustrated in Figures 2(c) and 2(g), NSC-CM significantly increased the expression of Bcl-2 in the ischemic hemisphere ($P < 0.01$). Thus, these data suggested that the capacity of NSC-CM to improve the expression of Bcl-2 in the ischemic hemisphere might contribute to inhibit cell apoptosis.

3.3. NCS-CM Significantly Preserved Mitochondrial Ultrastructure in the Cerebral Ischemia Hemisphere. To further clarify whether NSC-CM have the capacity of preserving mitochondria, we used electron microscopy to observe the mitochondrial ultrastructure. As illustrated in Figure 2(d), the mitochondria in the cerebral I/R injury rats became swollen. Especially, mitochondrial cristae almost disappeared or appeared disintegrated. But, in the NSC-CM tail vein injection group, we still observed some normal mitochondria with integrated cristae although most mitochondria became swollen and appeared disintegrated cristae (Figure 2(e)). More importantly, the mitochondria in the NSC-CM tail vein injection group were less severely swollen than the control group. Thus, the above data indicated that preserved mitochondrial ultrastructure caused by NSC-CM contributed to neuroprotection against cerebral I/R injury.

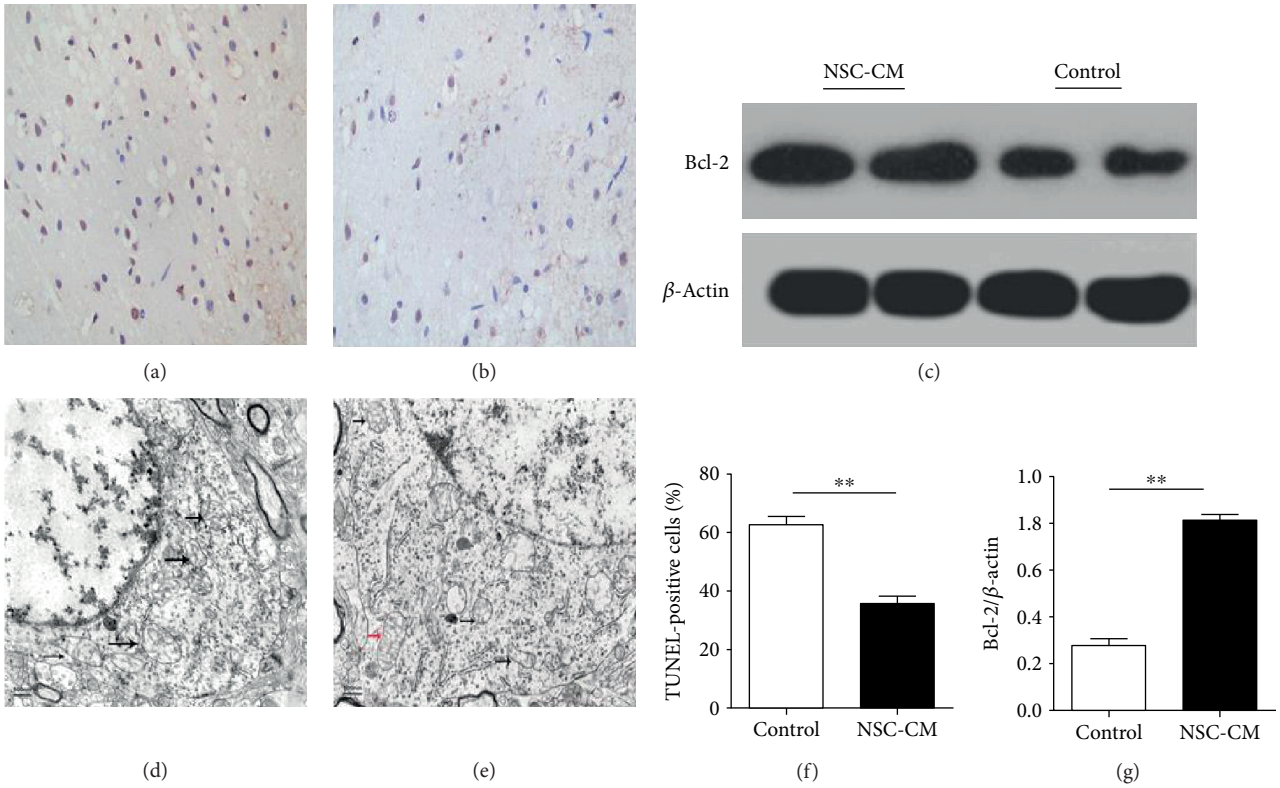

Figure 2: The effect of NSC-CM on cell apoptosis and mitochondrial ultrastructure in the ischemic sphere. Representative TUNEL staining image on the 3rd day after cerebral ischemia onset of the control group (a) and the NSC-CM group (b). The brown particle is the TUNEL-positive cells whereas the blue particle is the normal cells (c). The protein expression of Bcl-2 in the cerebral ischemia penumbra of 2 groups assessed by Western blot. Control: the group receiving PBS by tail vein injection; NSC-CM: the group receiving NSC-CM by tail vein injection. Representative mitochondrial ultrastructures of the control group (d) and the NSC-CM group (e) in neuron. The black arrows indicated swollen mitochondria with disintegrated or disappeared cristase. The red arrows indicated normal mitochondria with integrated cristase. Bar: 500 nm (f). The bar graph showing that NSC-CM significantly decreased the number of TUNEL-positive cells in cerebral ischemic hemisphere, compared to the control group. $^{**}P < 0.01$, two-sample t test (g). The bar graph showing that NSC-CM significantly increased the expression of Bcl-2 in cerebral ischemic hemisphere, compared to control group. $^{**}P < 0.01$, Two-sample t test.

4. Discussion

Despite the promising effect of using NSCs to improve the recovery of cerebral I/R injury, issues like ethics, immunorejection, potential tumor formation, and low survival and differentiation rates in the affected brain still limit the clinical application of NSCs [5]. NSC-CM is always discarded as the waste because NSCs produce possible harmful materials in NSC-CM during cell division *in vitro*. But, NSC-CM has been receiving more and more attentions in recent years because bystander actions of NSCs in vivo are extensively investigated, especially MVs released by NSCs. NSC-CM was demonstrated to exert antiapoptotic effect in vitro [22] and in vivo [21]. To circumvent some potential confounders and find a novel therapeutic strategy for ameliorating cerebral I/R injury, we for the first time explored the effects of multiple tail vein injections of NSC-CM in a cerebral I/R rat model and found that NSC-CM effectively ameliorated cerebral I/R injury in rats.

As expected, our data suggested that continuous administration of NSC-CM significantly reduced the cerebral infarct volume and improved the neurological defect scores (Figures 1(b) and 1(c)). Ischemic stroke is considered to be caused by decreased blood flow to brain tissue, followed by the activation of the ischemic cascade which leads to cell death and severe neuronal damage [26], which includes apoptosis, oxidative stress, inflammation, and calcium overload. It has been demonstrated that apoptosis plays an important role in cerebral ischemic pathogenesis in rats [27]. NSC-CM include neurotrophic factors, like GDNF and BDNF, while BDNF or GDNF was confirmed to exert antiapoptotic effect after transient middle cerebral artery occlusions in rats [28, 29]. In addition, NSC-CM was also confirmed to inhibit the cell apoptosis of SH-SY5Y cell caused by RA (retinoic acid) in vitro [22]. Thus, to dissect the potential mechanisms underlying these beneficial neuroprotective effects of NSC-CM, we performed TUNEL staining to observe cell apoptosis. Consistent with the above two researches [22, 29], NSC-CM significantly reduced the number of TUNEL-postive cells in the ischemic hemisphere (Figures 2(a), 2(b), and 2(f)), which indicated that NSC-CM had the capacity of inhibiting cell apoptosis in vivo.

It is not new that Bcl-2 has a protective role after focal ischemia [30], and Bcl-2, as the one of antiapoptotic proteins,

was one of the reduced genes most characterized after focal ischemia [30]. In addition, it also has been demonstrated that BDNF was able to protect against cerebral I/R injury via improving the expression of Bcl-2 [28]. MVs secreted by NSCs were confirmed to have the capacity of attending the process of inflammation [18] while inflammation contributed to the cell apoptosis in the cerebral I/R injury [1]. Thus, we guessed that NSC-CM had the capacity of inhibiting cell apoptosis. To further explore the mechanisms of NSC-CM against apoptosis, we selected Bcl-2 as the key antiapoptotic protein. Our data suggested that NSC-CM significantly improved the expression of Bcl-2 in the ischemic hemisphere (Figures 2(c) and 2(g), $P < 0.01$). More and more evidences suggested that Bcl-2 halted apoptosis by stabilizing mitochondrial integrity [27]. Mitochondria, as important components of ischemic neuronal death for several decades, is demonstrated to become swollen when cerebral I/R injury occurs [31]. More importantly, the earliest manifestations of ischemic neuronal demise were caused by the loss of mitochondrial cristae [31]. We still observed some normal mitochondria with integrated cristae although most of mitochondria became swollen and appeared disintegrated cristae in receiving the NSC-CM group (Figure 2(e)), which indicated that NSC-CM had the capacity of preserving mitochondrial ultrastructure. Thus, we concluded that preserved mitochondrial ultrastructure contributed to the increased expression of Bcl-2. It has been demonstrated that MVs secreted by NSCs play important roles in the development of the nervous system, enhancing the endoneurogenesis and inhibiting the process of inflammation [18]. It is well known that the integrity of BBB is broken during cerebral I/R injury, which is beneficial to penetrate BBB for some factors [32]. The components of NSC-CM are complicated, containing those already known neurotrophic factors, that is, BDNF [7], GDNF, and NT-3 [8], together with many other unknown soluble factors released from NSCs, as well as microparticles [9] in the CM. Although single application of BDNF, GDNF, and NT-3 can penetrate BBB and protect against cerebral I/R injury as demonstrated in previous studies by others, there are no researches about the effect of combinational application of neurotrophic factors on cerebral I/R injury. In the current preliminary studies, however, we did not focus on the detailed mechanisms of NSC-CM. Further studies are warranted to identify those soluble factors and calculate the proportions by global proteomic analysis of NSC-CM contents in the future, which may help elucidate the mechanism of NSC-CM on cerebral I/R injury. And, we in the future also will isolate microparticles using ultracentrifugation to testify their effects on cerebral I/R injury.

In conclusion, we showed that multiple tail vein injection of NSC-CM significantly ameliorated cerebral I/R injury via inhibiting cell apoptosis and preserving the mitochondrial ultrastructure. In another word, our data suggested that NSC-CM is another promising cell-free strategy for ischemic stroke.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

HongNa Yang designed and performed the experiment. HongNa Yang and Tingyu Qu wrote the manuscript. Cuilan Wang and Hui Chen performed the animal experiment. Lan Li and Shuang Ma performed TTC staining, Western blot, and electron microscopy. Hao Wang performed statistical analysis.

Acknowledgments

This research is supported by grants of (1) Boothroyd Foundation, USA, (2) Program of 5150 in Jinan City, (3) Taishan Overseas Scholars (tshw201502002), (4) Key Research and Development Plan (2016ZDJQ0104) in Shandong Province, China, to Tingyu Qu, and (5) the National Natural Science Foundation of China (no. 81701057) to HongNa Yang.

References

[1] D. Y.-W. Fann, S.-Y. Lee, S. Manzanero, P. Chunduri, C. G. Sobey, and T. V. Arumugam, "Pathogenesis of acute stroke and the role of inflammasomes," *Ageing Research Reviews*, vol. 12, no. 4, pp. 941–966, 2013.

[2] Y. Cheng, J. Zhang, L. Deng et al., "Intravenously delivered neural stem cells migrate into ischemic brain, differentiate and improve functional recovery after transient ischemic stroke in adult rats," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 3, pp. 2928–2936, 2015.

[3] M. Z. Ratajczak, M. Kucia, T. Jadczyk et al., "Pivotal role of paracrine effects in stem cell therapies in regenerative medicine: can we translate stem cell-secreted paracrine factors and microvesicles into better therapeutic strategies?," *Leukemia*, vol. 26, pp. 1166–1173, 2011.

[4] S. Ryu, S. H. Lee, S. U. Kim, and B. W. Yoon, "Human neural stem cells promote proliferation of endogenous neural stem cells and enhance angiogenesis in ischemic rat brain," *Neural Regeneration Research*, vol. 11, pp. 298–304, 2016.

[5] Y.-H. Tang, Y.-Y. Ma, Z.-J. Zhang, Y.-T. Wang, and G.-Y. Yang, "Opportunities and challenges: stem cell-based therapy for the treatment of ischemic stroke," *CNS Neuroscience & Therapeutics*, vol. 21, pp. 337–347, 2015.

[6] T. R. Doeppner and D. M. Hermann, "Stem cell-based treatments against stroke: observations from human proof-of-concept studies and considerations regarding clinical applicability," *Frontiers in Cellular Neuroscience*, vol. 8, 2014.

[7] T. D. Aumann, R. Talaverón, E. R. Matarredona, R. R. de la Cruz, and A. M. Pastor, "Neural progenitor cell implants modulate vascular endothelial growth factor and brain-derived neurotrophic factor expression in rat axotomized neurons," *PLoS One*, vol. 8, article e54519, 2013.

[8] P. Lu, L. L. Jones, E. Y. Snyder, and M. H. Tuszynski, "Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury," *Experimental Neurology*, vol. 181, pp. 115–129, 2003.

[9] S. M. Schindler, J. P. Little, and A. Klegeris, "Microparticles: a new perspective in central nervous system disorders," *BioMed Research International*, vol. 2014, Article ID 756327, 17 pages, 2014.

[10] T. M. Stanne, N. D. Aberg, S. Nilsson et al., "Low circulating acute brain-derived neurotrophic factor levels are associated

with poor long-term functional outcome after ischemic stroke," *Stroke*, vol. 47, pp. 1943–1945, 2016.

[11] Y. Jiang, N. Wei, T. Lu, J. Zhu, G. Xu, and X. Liu, "Intranasal brain-derived neurotrophic factor protects brain from ischemic insult via modulating local inflammation in rats," *Neuroscience*, vol. 172, pp. 398–405, 2011.

[12] U. Kilic, E. Kilic, G. P. Dietz, and M. Bahr, "Intravenous TAT-GDNF is protective after focal cerebral ischemia in mice," *Stroke*, vol. 34, pp. 1304–1310, 2003.

[13] J. Zhang, Q. Shi, P. Yang et al., "Neuroprotection of neurotrophin-3 against focal cerebral ischemia/reperfusion injury is regulated by hypoxia-responsive element in rats," *Neuroscience*, vol. 222, pp. 1–9, 2012.

[14] E. M. Kramer-Albers and W. Ping Kuo-Elsner, "Extracellular vesicles: goodies for the brain?," *Neuropsychopharmacology*, vol. 41, pp. 371-372, 2016.

[15] C. Porro, T. Trotta, and M. A. Panaro, "Microvesicles in the brain: biomarker, messenger or mediator?," *Journal of Neuroimmunology*, vol. 288, pp. 70–78, 2015.

[16] G. Camussi, M. C. Deregibus, and C. Tetta, "Paracrine/endocrine mechanism of stem cells on kidney repair: role of microvesicle-mediated transfer of genetic information," *Current Opinion in Nephrology and Hypertension*, vol. 19, no. 1, pp. 7–12, 2010.

[17] G. Chiva-Blanch, R. Suades, J. Crespo et al., "Microparticle shedding from neural progenitor cells and vascular compartment cells is increased in ischemic stroke," *PLoS One*, vol. 11, no. 1, article e0148176, 2016.

[18] J. E. Baulch, M. M. Acharya, B. D. Allen et al., "Cranial grafting of stem cell-derived microvesicles improves cognition and reduces neuropathology in the irradiated brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 17, pp. 4836–4841, 2016.

[19] Y. H. Rhee, S. H. Yi, J. Y. Kim et al., "Neural stem cells secrete factors facilitating brain regeneration upon constitutive Raf-Erk activation," *Scientific Reports*, vol. 6, article 32025, 2016.

[20] K. Ma, L. Fox, G. Shi et al., "Generation of neural stem cell-like cells from bone marrow-derived human mesenchymal stem cells," *Neurological Research*, vol. 33, pp. 1083–1093, 2011.

[21] P. Liang, J. Liu, J. Xiong et al., "Neural stem cell-conditioned medium protects neurons and promotes propriospinal neurons relay neural circuit reconnection after spinal cord injury," *Cell Transplantation*, vol. 23, Supplement 1, pp. S45–S56, 2014.

[22] H. Yang, J. Wang, J. Sun, X. Liu, W. M. Duan, and T. Qu, "A new method to effectively and rapidly generate neurons from SH-SY5Y cells," *Neuroscience Letters*, vol. 610, pp. 43–47, 2016.

[23] M.-Y. Kim, B.-S. Moon, and K.-Y. Choi, "Isolation and maintenance of cortical neural progenitor cells in vitro," *Methods in Molecular Biology*, vol. 1018, pp. 3–10, 2013.

[24] H. Yang, Y. Lu, S. Ma et al., "Targeted delivery of extracellular matrix protected against neurologic defects after focal ischemia reperfusion in rats," *Journal of Stroke and Cerebrovascular Diseases*, vol. 24, pp. 154–162, 2015.

[25] A. J. Hunter, J. Hatcher, D. Virley et al., "Functional assessments in mice and rats after focal stroke," *Neuropharmacology*, vol. 39, no. 5, pp. 806–816, 2000.

[26] A. Chamorro, A. Meisel, A. M. Planas, X. Urra, D. van de Beek, and R. Veltkamp, "The immunology of acute stroke," *Nature Reviews. Neurology*, vol. 8, no. 7, pp. 401–410, 2012.

[27] J. Miao, L. Wang, X. Zhang et al., "Protective effect of aliskiren in experimental ischemic stroke: up-regulated p-PI3K, p-AKT, Bcl-2 expression, attenuated Bax expression," *Neurochemical Research*, vol. 41, pp. 2300–2310, 2016.

[28] W. R. Schabitz, C. Sommer, W. Zoder, M. Kiessling, M. Schwaninger, and S. Schwab, "Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia," *Stroke*, vol. 31, pp. 2212–2217, 2000.

[29] J. Shang, K. Deguchi, T. Yamashita et al., "Antiapoptotic and antiautophagic effects of glial cell line-derived neurotrophic factor and hepatocyte growth factor after transient middle cerebral artery occlusion in rats," *Journal of Neuroscience Research*, vol. 88, pp. 2197–2206, 2010.

[30] X.-L. Hu, T. Olsson, I.-M. Johansson, T. Brannstrom, and P. Wester, "Dynamic changes of the anti- and pro-apoptotic proteins Bcl-w, Bcl-2, and Bax with Smac/Diablo mitochondrial release after photothrombotic ring stroke in rats," *European Journal of Neuroscience*, vol. 20, no. 5, pp. 1177–1188, 2004.

[31] P. Baxter, Y. Chen, Y. Xu, and R. A. Swanson, "Mitochondrial dysfunction induced by nuclear poly(ADP-ribose) polymerase-1: a treatable cause of cell death in stroke," *Translational Stroke Research*, vol. 5, no. 1, pp. 136–144, 2014.

[32] J. Liang, Z. Qi, W. Liu et al., "Normobaric hyperoxia slows blood-brain barrier damage and expands the therapeutic time window for tissue-type plasminogen activator treatment in cerebral ischemia," *Stroke*, vol. 46, pp. 1344–1351, 2015.

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Stem Cells International
Volume 2018, Article ID 2398521, 13 pages
https://doi.org/10.1155/2018/2398521

Research Article

Effects of Transplanted Heparin-Poloxamer Hydrogel Combining Dental Pulp Stem Cells and bFGF on Spinal Cord Injury Repair

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Received 8 November 2017; Accepted 30 January 2018; Published 27 March 2018

Academic Editor: Cinzia Marchese

Spinal cord injury (SCI) is one of serious traumatic diseases of the central nervous system and has no effective treatment because of its complicated pathophysiology. Tissue engineering strategy which contains scaffolds, cells, and growth factors can provide a promising treatment for SCI. Hydrogel that has 3D network structure and biomimetic microenvironment can support cellular growth and embed biological macromolecules for sustaining release. Dental pulp stem cells (DPSCs), derived from cranial neural crest, possess mesenchymal stem cell (MSC) characteristics and have an ability to provide neuroprotective and neurotrophic properties for SCI treatment. Basic fibroblast growth factor (bFGF) is able to promote cell survival and proliferation and also has beneficial effect on neural regeneration and functional recovery after SCI. Herein, a thermosensitive heparin-poloxamer (HP) hydrogel containing DPSCs and bFGF was prepared, and the effects of HP-bFGF-DPSCs on neuron restoration after SCI were evaluated by functional recovery tests, western blotting, magnetic resonance imaging (MRI), histology evaluation, and immunohistochemistry. The results suggested that transplanted HP hydrogel containing DPSCs and bFGF had a significant impact on spinal cord repair and regeneration and may provide a promising strategy for neuron repair, functional recovery, and tissue regeneration after SCI.

1. Introduction

Spinal cord injury (SCI) is a common disease of the central nervous system, resulting in partial or complete loss of motor and sensory functions [1]. The pathological process of SCI can be divided into two phases: the first phase contains a primary mechanical damage of spinal cord which leads to direct damage and loss of axons, neuronal cells, and blood vessels and the second phase includes a secondary injury of neuroinflammatory response which results in excitotoxicity, blood-brain barrier disruption, oxidative stress, and apoptosis [2, 3]. This complicated pathophysiology prevents spinal cord tissue from regeneration and repair. Meanwhile, functional recovery after SCI by conventional therapies has been rarely promoted to a satisfactory level due to the limitation of necessary precursor cells which are crucial in neuron renewal and glial cell regeneration [4, 5]. Therefore, the development of a promising strategy to promote functional recovery after SCI remains to be a significant challenge.

There are some studies suggesting that stem cell transplantation will provide an effective approach for SCI treatment due to their neural differentiation potential. This potential could offer new neural cells to replace dying cells and produce numerous trophic factors, for example, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and neurotrophin 3 (NT-3), in order to promote neural survival and regeneration [6]. Dental stem cells (DSCs), for

instance, dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), stem cells from the apical papilla (SCAPs), dental follicle stem cells (DFSCs), and periodontal ligament stem cells (PDLSCs), are considered to be an attractive source of mesenchymal stem cells (MSCs) and exhibit stem cell characteristics such as self-renewal and multilineage differentiation potential [7, 8]. DPSCs, the first dental-related stem cells, were isolated from the third molars in 2000 by Dr. Gronthos [9]. DPSCs have been found to possess the characteristics of MSCs such as multidifferentiation potential and neuroprotective and immunomodulatory properties and to express MSC-like markers such as CD73, CD90, CD105, CD146, CD166, and STRO-1 [10, 11]. Furthermore, originating from the cranial neural crest, DPSCs can also express neural markers such as nestin, β-tubulin III, glial fibrillary acidic protein (GFAP), and microtubule-associated protein-2 (MAP-2) [6, 12, 13]. Studies suggest that when induced, DPSCs can differentiate into neuronal-like and oligodendrocyte-like cells, which results in axonal regeneration and repair after SCI [14, 15]. Evidence also demonstrates that DPSC transplantation can promote motor functional recovery by secreting BDNF, GDNF, and NT-3 after SCI. Therefore, DPSCs have the ability to provide beneficial strategy for SCI treatment due to their neuronal differentiation potential and neuroprotective and neurotrophic properties [16, 17]. However, it is unlikely to achieve a complete restoration of neuron function after SCI by DPSC-alone strategy because it is difficult to guarantee an effective cellular density and growth in injured site. Recently, researchers and clinicians propose that the tissue engineering technology including scaffolds, cells, and growth factors will provide a promising strategy for SCI treatment [18, 19].

Human basic fibroblast growth factor (bFGF) is a member of the fibroblast growth factor family, having the ability to mediate cell proliferation and survival in vitro [20]. Besides, bFGF is highly expressed in nervous system and it has been confirmed that bFGF can provide neuroprotection for injured axons and neurons facilitating functional recovery after SCI [19, 21]. However, as a biological macromolecular protein, it is difficult for bFGF to pass through the blood spinal cord barrier, and bFGF is disadvantaged by its rapid diffusion and short half-life time [22]. To overcome these shortcomings, an in situ delivery system should be designed to locally diffuse bFGF in injured site of SCI.

Biomaterial scaffold is one of the key components of tissue engineering to provide a platform for cell adhesion and transplantation and to permit delivery of growth factors to ensure cell survival and proliferation [23]. In addition, some studies indicate that biomaterial scaffold interacting with seeded cells has an ability to modulate cellular functions and behaviors. For instance, substrate stiffness, matrix topology, structure, mechanical force, and biochemical property of biomaterial scaffolds form different tissue-specific microenvironments regulating cell growth, proliferation, migration, and differentiation [24–27]. Therefore, ideal scaffolds should have low/nontoxicity, good cytocompatibility, and tissue compatibility, structural stability and mimic 3D biological microenvironment [23]. Recently, hydrogels have been recognised as attractive scaffolds for tissue engineering due to similar 3D network structure to the natural extracellular matrix, the ability to accommodate cells and to deliver bioactive molecules, and the capability to maintain the structure and porosity of scaffold [28–30].

In this work, we designed a novel thermosensitive heparin-poloxamer (HP) hydrogel containing bFGF and DPSCs, which could be delivered to injured site in spinal cord in order to ensure high density of DPSCs and sustained effect of bFGF during recovery. Our previous studies indicated that HP had a characteristic of controlled phase transition by the variation of temperature, presenting a solution state at 4°C and becoming a hydrogel state at human body temperature [31]. Moreover, HP has a high affinity to growth factors (GFs) such as nerve growth factor (NGF) and acidic fibroblast growth factor (aFGF) and is also able to protect GFs from degradation of protease [32, 33]. In addition, HP hydrogel shows a 3D porous structure and has great cytocompatibility [30, 34]. Thus, in this study, we used HP as the engineered tissue scaffold and transplanted HP hydrogel containing bFGF and DPSCs into the injured site of the spinal cord in order to investigate the effects of DPSCs combined with bFGF on neural regeneration and functional recovery after SCI.

2. Materials and Methods

2.1. Isolation and Culture of DPSCs. Impacted third molars with no caries, periodontal disease, or periapical disease were collected from healthy volunteers (18–30 years old) at the Department of Oral and Maxillofacial Surgery, Stomatological Hospital of Wenzhou Medical University, Wenzhou, China. Tooth surfaces were sterilized by 75% alcohol and the dental pulp tissues were removed by dental handpiece, then cut into small pieces (approximately 1 mm × 1 mm × 1 mm) and washed three times with 2.5% antibiotics of phosphate-buffered saline (PBS). The pulp tissues were put into 1.5 mL EP tube and digested with 3 mg/mL collagenase type I (Gibco, USA) and 4 mg/mL dispase (Sigma, Germany) for 30 minutes at 37°C. Then cell suspension and pulp tissue were cultured at 37°C in 5% CO_2 humidified atmosphere with α-modified Eagle's medium (α-MEM, Gibco, USA) supplemented with 20% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA). The culture medium was replaced after the first 5 days culture, and then the culture medium was replaced every 3 days routinely.

2.2. Flow Cytometry. The characteristics of DPSCs were identified by flow cytometry and the identification procedure of DPSCs was described as previous method [35]. Briefly, after 90% confluence, the second passage of DPSCs was characterized by flow cytometry using the antibodies of human CD73 (BD Pharmingen™, USA), CD90 (BD Pharmingen, USA), CD166 (BD Pharmingen, USA), CD19 (BioLegend, USA), CD14 (BioLegend, USA), and HLA-DR (BioLegend, USA) according to standard protocols. The data were evaluated with CytoFLEX flow cytometers (Beckman Coulter, California, USA).

2.3. Multilineage Differentiation. The multidifferentiation potential of DPSCs was analyzed by osteogenic, adipogenic, chondrogenic, and neurogenic differentiations. The process of differentiation was described as follows:

Osteogenic differentiation: After 60%–70% confluence, the culture medium was carefully aspirated from each well and replaced with 2 mL of OriCell TM mesenchymal stem cell osteogenic differentiation medium (Cyagen, USA), and the medium was changed every 3 days. After 2 weeks of differentiation, cells were fixed with 4% formaldehyde for 30 minutes and stained with alizarin red S for 5 minutes. Finally, the cells were visualized and analyzed under light microscope (TS100, Nikon, Japan).

Adipogenic differentiation: When cells were approximately 100% confluent or postconfluent, growth medium was carefully aspirated off and OriCell TM mesenchymal stem cells adipogenic differentiation medium (Cyagen, USA) was added according to the manufacturers' instructions. By the end of differentiation, cells were fixed with 4% formaldehyde for 30 minutes and stained with oil red O for 30 minutes. The cells were visualized and analyzed by light microscope (TS100, Nikon, Japan).

Chondrogenic differentiation: DPSCs were collected with the density of 2.5×10^5 cells per well and centrifuged at 1000 rpm for 5 minutes at room temperature. Then the pellets were incubated with chondrogenic medium at 37°C, 5% CO_2 humidified atmosphere. The chondrogenic medium was changed every 3 days. After 30 days, chondrogenic pellets were harvested, fixed with 4% formaldehyde and stained with Alcian blue, and then visualized and analyzed by light microscope (TS100, Nikon, Japan).

Neurogenic differentiation: DPSCs were plated onto cover slips in 6-well plates with the density of 4×10^4 cells per well and incubated at 37°C in 5% CO_2 humidified atmosphere. After 1 day, the cell culture medium was changed to neuronal induced medium with serum-free DMEM-high glucose containing 10^{-7} M dexamethasone, 50 μg/mL ascorbic acid-2-phosphate, 50 μM indomethacin, 10 μg/mL insulin, and 0.45 mM 3-isobutyl-1-methyl-xanthine for 6 days, and then the neuronal induced medium was changed every 3 days. Finally, the cells were evaluated by immunofluorescence staining with Nestin (1 : 1000, *Sigma-Aldrich,* USA), NeuN (1 : 200, Thermo Fisher, USA), GFAP (1 : 200, *Sigma-Aldrich,* USA), and β-tubulin III (1 : 2000, *Sigma-Aldrich,* USA).

2.4. CCK-8 Assay. To determine the efficiency of bFGF affecting DPSCs in vitro, cell proliferation was evaluated by CCK-8 assay (Dojindo, Kumamoto, Japan) as the following method [36]. Human basic fibroblast growth factor (bFGF) was synthesized and provided by the Key Laboratory of Biotechnology and Pharmaceutical Engineering, Wenzhou Medical University. DPSCs were plated into 96-well plates with the density of 2.0×10^3 cells per well and cultured in α-MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, which was used as the control group. The experimental group was the culture medium containing bFGF and the final concentration of bFGF was set at 20 ng/mL [37]. The cell culture medium was changed every other day. After 1, 3, 4, 5, 6, and 7 days' incubation, 10 μL of CCK-8 solution was added into each well and incubated for another 1 hour in 5% CO_2 at 37°C. The OD values were measured photometrically at 450 nm by an absorbance microplate reader (Varioskan LUX, Thermo Fisher, USA).

2.5. Fabrication of HP-bFGF and HP-bFGF-DPSC Hydrogels. Poloxamer 407 was obtained from Badische Anilin Soda Fabrik Ga (Shanghai, China). The preparation of heparin-poloxamer (HP) was described in our previous work as follows [31]. Firstly, poloxamer 407 and diaminoethylene solutions were used to prepare monoamine-terminated poloxamer (MATP). Then, MATP was reacted with heparin salt by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) in 4-morpholine ethane sulfonic acid (MES) buffer for 1 day in order to form the amide bonds through the amine groups of poloxamer 407 coupling with the carboxyl ones of heparin. Finally, the mixture was dialyzed with deionized water about 72 hours and lyophilized by freeze dryer in order to obtain the final product.

HP-bFGF hydrogel was prepared according to the cold method as previously described [38]. Briefly, lyophilized HP powder was mixed with bFGF solution at 4°C under modest stirring for 2 hours, and then the mixture was stored at 4°C overnight to obtain a transparent solution, resulting in HP loaded with bFGF.

For the preparation of HP/HP-bFGF loaded with DPSC hydrogel, DPSCs were detached and collected by centrifuging at 1000 rpm for 5 minutes and resuspended in complete α-MEM medium. Then the DPSC suspensions were added into HP/HP-bFGF solution and thoroughly mixed at 4°C, kept at 37°C, 5% CO_2 incubator to obtain HP-DPSC and HP-bFGF-DPSC hydrogels.

2.6. Morphology of HP and HP-bFGF Hydrogels. The morphology of HP and HP-bFGF hydrogels were observed by scanning electron microscope (SEM, H-7500, Hitachi, Japan) with 15 kV as the accelerating voltage. HP and HP-bFGF hydrogels were wiped on copper meshes and frozen in liquid nitrogen. Then, the hydrogels were dried at critical point by vacuum freeze dryer for 48 hours. The surfaces of specimens were sputter-coated with gold for SEM observation. The average apparent pore size (*dpore*) was measured from the SEM images by a high-resolution imaging treatment system (HLPAS-1000, Wenzhou Medical University, Wenzhou, China).

2.7. Live/Dead Assay. The percentage of viable DPSCs in hydrogels was evaluated by a Live/Dead Viability/Cytotoxicity Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. After DPSCs cocultured with HP and HP-bFGF hydrogels for 48 hours, the samples were incubated with reagents at 37°C for 15 minutes and then observed by fluorescence microscope (Eclipse 80i, Nikon, Japan).

2.8. Animal Model of SCI and HP-bFGF-DPSC Hydrogels Application. 72 Sprague–Dawley female rats (200–250 g) were purchased from the Animal Center of Chinese Academy of Science (Shanghai, China) and housed in the animal

care facility for 14 days prior to surgery. All experiments were performed according to the Guide of Chinese National Institutes of Health and the Animal Care and Use Committee of Wenzhou Medical University. The experiments were divided into 6 groups, including HP, HP-bFGF, HP-DPSCs, HP-bFGF-DPSCs, SCI, and sham control groups. The working concentration of bFGF was set at 3 μg/μL [39]. Animals were anesthetized by intraperitoneal administration of 10% chloralic hydras at a dose of 3.5 mL per kg body weight. After anesthesia, back hair was shaved and the skin was sanitized with 70% alcohol solution. After incision extended along the middle of back and exposed the vertebral column, a laminectomy was performed at T9 segmental level vertebrae. The spinal cord was completely exposed and a moderate injury was created by a vascular clip clipping for 2 minutes (30 g forces, Oscar, China). After SCI, 10 μL (containing 3 μg/μL bFGF wherever applicable) of HP, HP-bFGF, HP-DPSC, and HP-bFGF-DPSC hydrogels was in situ injected by a microsyringe. Animals in SCI model group received the same surgical procedures and were injected with sterile saline solutions (10 μL per animal). The sham control group received the same surgical procedures but the spinal cord was not injured by the vascular clip. Postoperative animals were conventionally housed and the bladder was manually emptied twice a day.

2.9. Functional Recovery Analysis. The functional recovery of all animals was evaluated by Basso, Beattie, and Bresnahan (BBB) scoring method, inclined plane test, Reuter scoring method, and footprint test as described previously [29, 40, 41]. The BBB scoring method, ranging from 0 (no movement) to 21 (normal gait), was performed to assess motor functional improvement at day 1, 3, 7, 14, 21, and 28. For the inclined plane test, all animals were performed at the same time points. The maximum angle of the inclined plane on which the animal stayed for 5 seconds was recorded for each rat, and the average score was used for each group. The Reuter scoring method, ranging from 0 (normal) to 11 (no sensory), was performed to assess the sensory functional recovery at the same time as above, including stretch reflex, pain retraction reflex, back feeling, muscle tension, and muscle strength. The footprint assay was performed at day 28 by staining the hind paws of animals with red dye. All functional assays were recorded by two independent observers blinded to the experimental protocol.

2.10. Western Blot Analysis. For western blot analysis of in vivo proteins collected at day 7 and 21, the spinal cord segments (0.5 cm in length) at the contusion epicenter were dissected and stored at –80°C as soon as possible. According to protein extraction, the spinal cord tissues were homogenized in modified RIPA buffer including protease inhibitor cocktail (10 μL/mL, GE Healthcare Biosciences, PA, USA) and centrifuged at 12,000 rpm. Supernatant was collected for protein analysis. The extracts were quantified with bicinchoninic acid reagents (BCA, Thermo Scientific Pierce, USA). Proteins (80 μg) were added to electrophoresis in 10% SDS-PAGE gels and then transferred onto the poly(vinylidene difluoride) membranes (PVDF, Millipore, Germany). The membranes were blocked with 5% (*w*/*v*) milk (BD, USA) in tris-buffered saline with 0.05% Tween-20 (TBST) for 90 minutes and incubated with the following primary antibodies at 4°C for 16 hours: Bcl-2 (1 : 300, Santa, USA), Bax (1 : 1000, CST, USA), Caspase-3 (1 : 1000, Abcam, Britain), MBP (1 : 300, Santa, USA), and GAP-43 (1 : 1000, Abcam, Britain). Then the horseradish peroxidase-conjugated secondary antibodies were added to the membranes for another 1 hour at room temperature. Detection of target proteins were performed by ChemiDoc XRS$^+$ imaging system (Bio-Rad). All tests were performed in triplicate.

2.11. Magnetic Resonance Imaging (MRI), Histology Evaluation, and Immunohistochemistry Analysis. After 28 days, animals were anesthetized and analyzed by MRI in order to observe the regeneration of spinal cord. All animal experiments were performed using GE Signa HDxT 3.0T superconducting MRI imager (GE Medical Systems, USA) in the Second Affiliated Hospital, Wenzhou Medical University. High-resolution sagittal images were obtained from each animal using a spin-echo T2-weighted MRI sequence (TR/TE: 2560/92 ms, FOV: 9 cm, acquisition matrix: 320 × 256, NEX: 4.0, slice thickness: 1.5 mm, band width: 41.67 kHz). During MRI scanning process, animals were put in a thermostat-heated cradle to maintain the body temperature at 37°C.

For histology evaluation after 28 days, animals were euthanized and tissue of interest was perfused with 4% paraformaldehyde in 0.01 M PBS (pH = 7.4). Sections of spinal cord at T8–T10 were fixed with 4% paraformaldehyde for 6 hours, dehydrated with increasing concentrations of ethanol and embedded in paraffin, cut into segments with thickness of 4–6 mm, and stained with hematoxylin-eosin (HE). HE-stained tissue samples at section T8–T10 were observed by light microscope (TS100, Nikon, Japan). As to immunohistochemistry, the sections were treated with primary antibody of GAP-43 (1 : 300, Abcam, Britain) overnight at 4°C and incubated with horseradish peroxidase-conjugated secondary antibodies for another 2 hours at 37°C. Then the sections were blocked with 3,3′-diaminobenzidine (DAB). All sections were observed by fluorescence microscope (Eclipse 80i, Nikon, Japan).

2.12. Statistical Analysis. All data were presented in mean ± standard error. Independent samples *t*-test was used in Figures 1(b), 2(b), and 2(d). Otherwise, one-way ANOVA was used. $P < 0.05$ was considered as statistically significant. Statistical analysis was performed using SPSS 19.0 (SPSS, Chicago, IL).

3. Results

3.1. DPSCs Culture and Identification. DPSCs were one kind of MSCs and possessed the properties of MSCs such as expressing MSC-like markers and having multidifferentiation potential. Figure 3(a) showed that DPSCs displayed a typical fibroblast-like morphology and began to proliferate at day 4 and covered the whole T-25 cm^2 flask at day 8. The first passage of DPSCs (P_1) also had great proliferation and

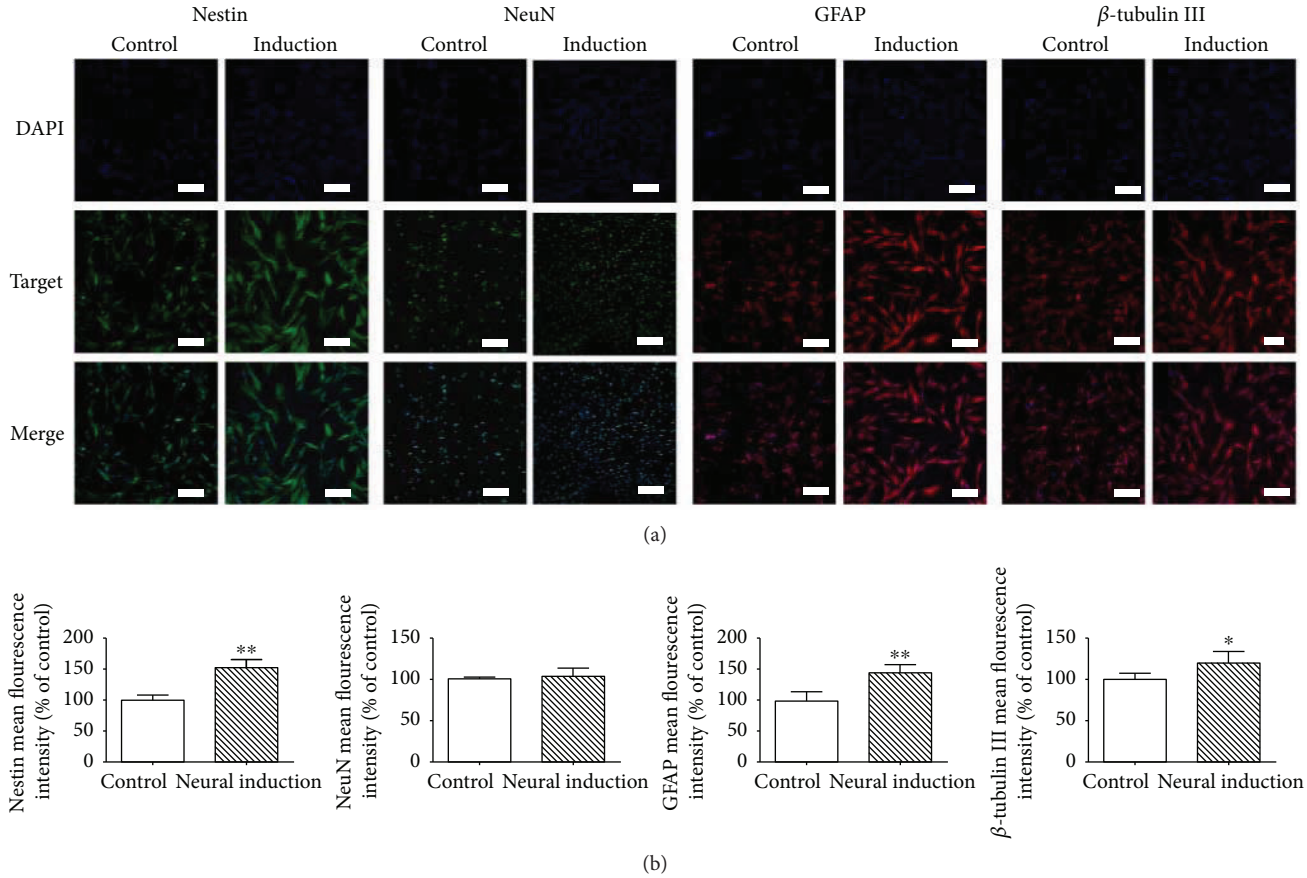

Figure 1: The neurogenic differentiation potential of DPSCs. (a) The expressions of Nestin, NeuN, GFAP, and β-tubulin III. Scale bar: 200 μm. (b) Quantification of the fluorescence intensity of Nestin, NeuN, GFAP, and β-tubulin III. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group.

survival rate. For the identification of DPSCs, flow cytometry and multilineage differentiation were performed to investigate the MSC-like characteristics of DPSCs. As shown in Figure 3(b), the results of flow cytometric analysis indicated that DPSCs positively expressed MSC-like phenotypic markers, for instance, CD73, CD90, and CD166, but negatively expressed the surface antigen of hematopoietic stem cells such as CD14, CD19, and HLA-DR. As a result of multilineage differentiation (Figure 3(c)), DPSCs had the ability to form mineralized nodules with alizarin red S staining in osteogenic inductive medium, and lipid droplets were observed and stained by oil red O in adipogenic differentiation. Moreover, DPSCs also showed positive results in terms of chondrogenic differentiation with Alcian blue staining.

For neurogenic differentiation, DPSCs were determined by the expression of neural surface markers such as Nestin, NeuN, GFAP, and β-tubulin III. The results suggested that DPSCs were positive for Nestin, NeuN, GFAP, and β-tubulin III (Figure 1(a)). There was significant difference between the control group and the neurogenic-induced group with the fluorescence intensity of β-tubulin III ($^{*}P < 0.05$), Nestin, and GFAP ($^{**}P < 0.01$), but NeuN showed no difference between the groups (Figure 1(b)).

3.2. bFGF Promoted the Proliferation of DPSCs In Vitro. The proliferation of DPSCs cocultured with bFGF in vitro was evaluated by CCK-8 assay (Figure 4). The results showed that the cells were growing up from day 1 to day 7 in both bFGF and control groups. The viability of DPSCs demonstrated no significant difference between control group and bFGF group at day 1 and day 3. However, from day 4 to day 7, the cell proliferation of bFGF group was much higher than that of control group. On the fourth day, the cellular proliferation rate of bFGF group reached the highest level compared to the rest groups.

3.3. Morphology of HP and HP-bFGF Hydrogels and Cytocompatibility of Hydrogels with DPSCs. The micromorphology of hydrogels and cytocompatibility of hydrogels with DPSCs were evaluated by SEM and Live/Dead assay, respectively. In Figure 2(a), SEM images of HP and HP-bFGF hydrogels showed porous structure, and the inner pores of hydrogels were interconnected. Meanwhile, the pore size of HP-bFGF hydrogel was smaller than that of HP hydrogel yet more uniform. And the number of pores in HP-bFGF hydrogel was also higher than that of in HP hydrogel (Figure 2(b)). As shown in Figure 2(c), Live/Dead assay results indicated that hydrogels had good cytocompatibility

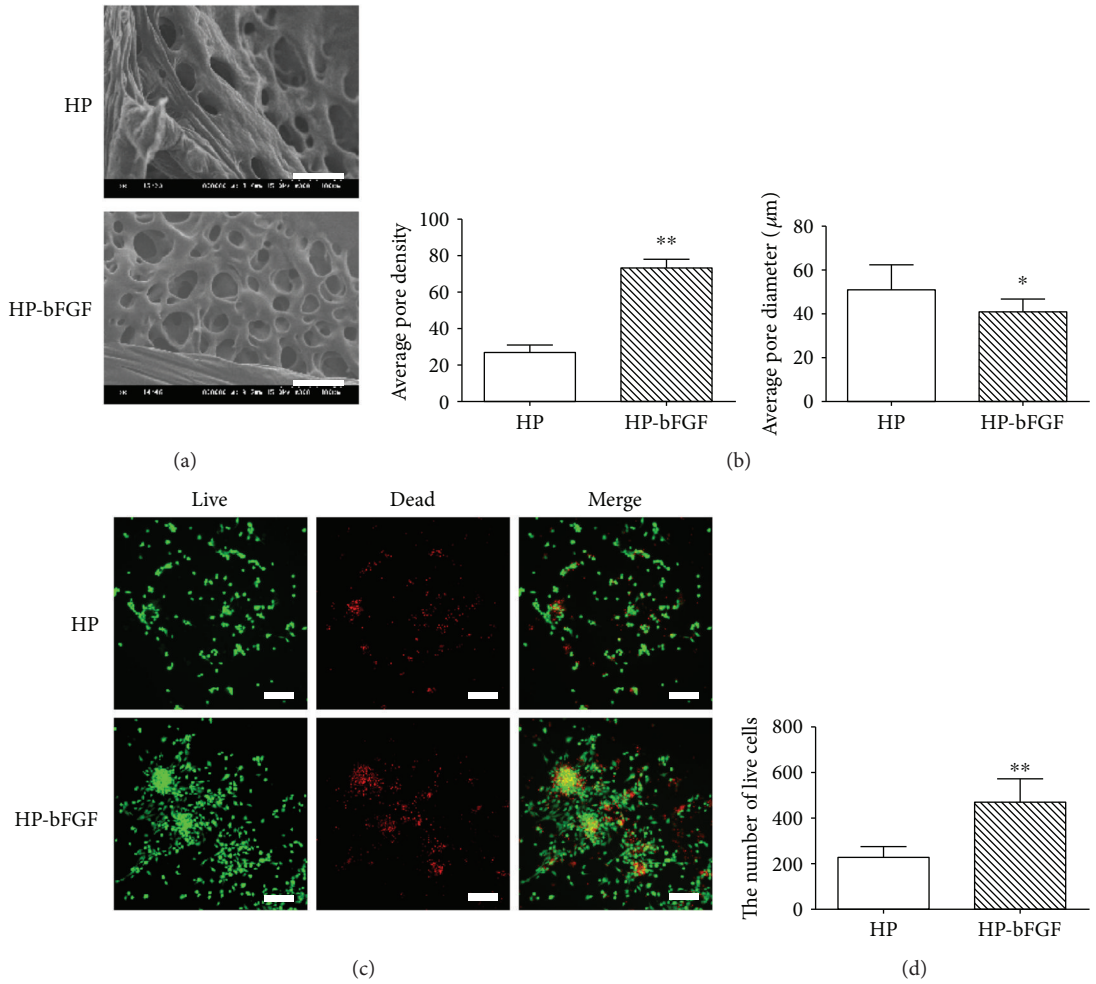

Figure 2: Morphology of HP and HP-bFGF hydrogels and cytocompatibility of hydrogels with DPSCs. (a) SEM images of HP and HP-bFGF hydrogel. Scale bar: 100 μm. (b) Quantification of pore density and pore size. (c) Live/Dead staining of cells in HP and HP-bFGF hydrogels at 48 hours. Scale bar: 200 μm. (d) Quantification of live cells in HP and HP-bFGF hydrogels by Image J. Data were displayed in mean ± standard error from 3 rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus HP group.

with DPSCs, and numerous DPSCs were stained in green, which were regarded as alive. In addition, the number of viable DPSCs in HP-bFGF hydrogel was more than that of in HP hydrogel (Figure 2(d)).

3.4. DPSCs Combined with bFGF Enhanced Motor and Sensory Functional Recovery after SCI. The motor and sensory functional recovery after SCI were evaluated by BBB rating scale, inclined plane test, footprint test, and Reuter rating scale, respectively. The hind legs of all animals lost functions and had no movement immediately after SCI. According to BBB scores, inclined plane test scores, and Reuter scores (Figures 5(a), (b), and (c)), the function of hind legs in all experimental groups had no improvement at day 1. From day 3 to day 28, motor functional scores of BBB and inclined plane test were gradually increasing, and decrease of the sensory functional scores of Reuter was observed, which indicated the recovery of sensory function. The functional recovery of hind legs was: HP-bFGF-DPSCs group > HP-DPSCs group > HP-bFGF group > HP group > SCI group. HP-bFGF-DPSCs group showed the strongest beneficial impact on the motor and sensory functional recovery after SCI and had significant difference compared to those of the other experimental groups at day 21 or before (Supplementary Table 1). Meanwhile, the promotion of functional recovery after SCI had no significant difference between HP-bFGF-DPSC group and HP-DPSCs group and showed similar effect at day 28. As shown in footprint test (Figure 5(d)), the results echoed experiment observations of BBB, inclined plane, and Reuter tests, suggesting that HP-bFGF-DPSCs group had the best effect on the functional restoration of hind leg at day 28. The animals in HP-bFGF-DPSCs group showed coordinated crawling with tails raised up, similar to what being observed in the sham control group. On contrast, animals were paralyzed and dragging hind legs in the SCI and HP groups.

3.5. Protein Expression of Apoptotic-Related Factors and Neuronal Markers after SCI. In this work, the protein expression of apoptotic-related factors (e.g., Bax, Bcl-2, and Caspase-3) and neuronal markers (e.g., MBP and GAP-43) were detected by western blotting at day 7 and 21,

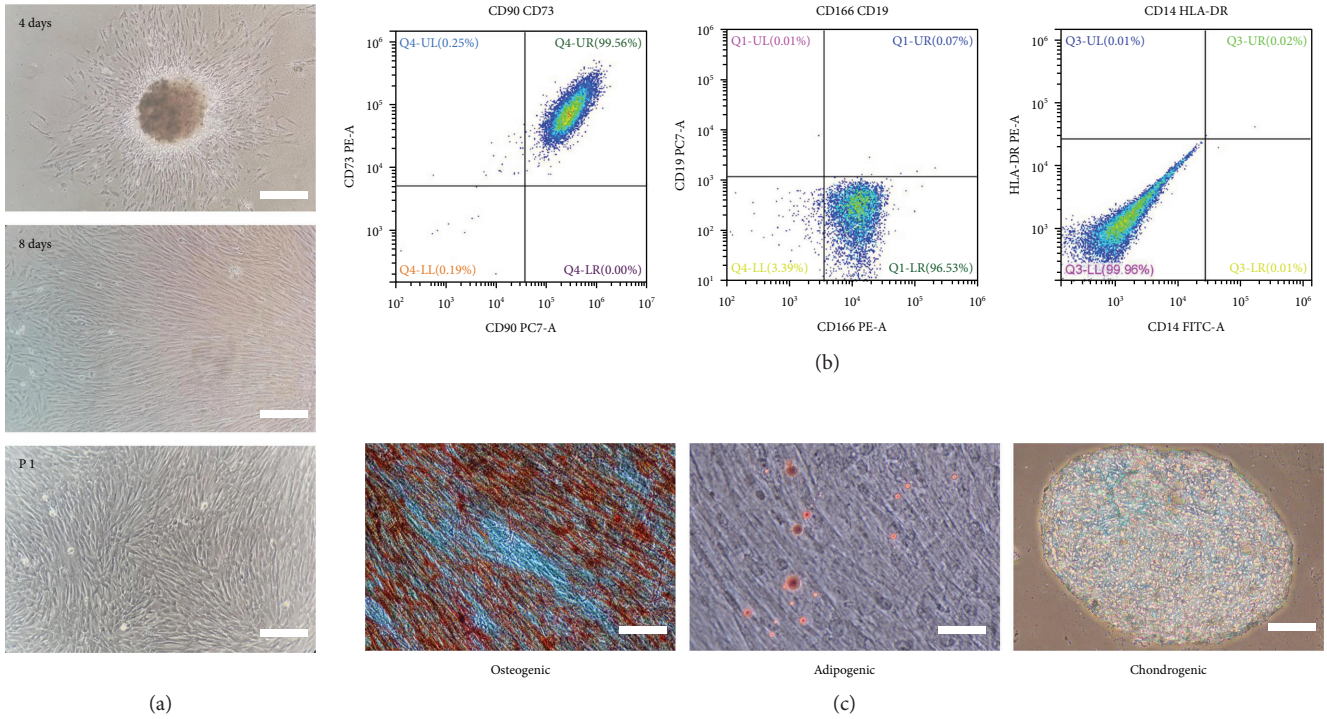

FIGURE 3: Isolation, culture, and identification of DPSCs. (a) DPSCs culture at day 4, day 8, and the first passage. Scale bar: 200 μm. (b) The expression of surface markers of DPSCs. (c) The osteogenic/adipogenic/chondrogenic differentiation potential of DPSCs. Scale bar: 100 μm.

respectively. In all experimental groups, the results showed that the proteins of Bax (proapoptotic factor) and Caspase-3 (the main apoptotic protein) were expressed the most in SCI model group but the least in HP-bFGF-DPSC group which was close to those of the control group. Remarkably, the antiapoptotic factor of Bcl-2 presented the opposite results in the protein expression profile in aforementioned groups (Figure 6(a)). Furthermore, HP-DPSC group also displayed higher expression of Bcl-2 and lower expression of Bax and Caspase-3, similar to those of HP-bFGF-DPSC group. The results of Bcl-2, Bax, and Caspase-3 expression also indicated that HP-bFGF-DPSC group had significant differences compared to the other experimental groups excluding HP-DPSC group (Figure 6(b)). As shown in Figure 6(c), the protein expression levels of MBP and GAP-43 in all experimental groups were HP-bFGF-DPSC group > HP-DPSC group > HP-bFGF group > HP group > SCI group. Almost reaching to a comparable level as the control group, HP-bFGF-DPSC group showed the highest protein expression, followed by HP-DPSC group, whereas the SCI model group had minimum protein expression (Figure 6(d)). Taken together, transplantation of HP hydrogel possessing DPSCs and bFGF could prevent apoptosis and promote new neuron regeneration at both early and later postoperative stages of SCI.

3.6. MRI, Histology Evaluation, and Immunohistochemistry. According to the results of functional recovery analysis and western blotting, the order of effect on neuronal repair and regeneration after SCI in all experimental groups was HP-bFGF-DPSC group > HP-DPSCs group > HP-bFGF group > HP group > SCI model group. Therefore, we chose experimental groups of HP-bFGF-DPSCs, HP-DPSCs, and HP-bFGF, which had better impact on rehabilitation of neurons after SCI, to perform the following experimental analyses: MRI, histology evaluation and immunohistochemistry. According to the MRI results, spinal cord repair and regeneration after SCI could be easily observed. In Figure 7(a), some defects were shown on injured area of spinal cord in HP-bFGF and HP-DPSC groups, which

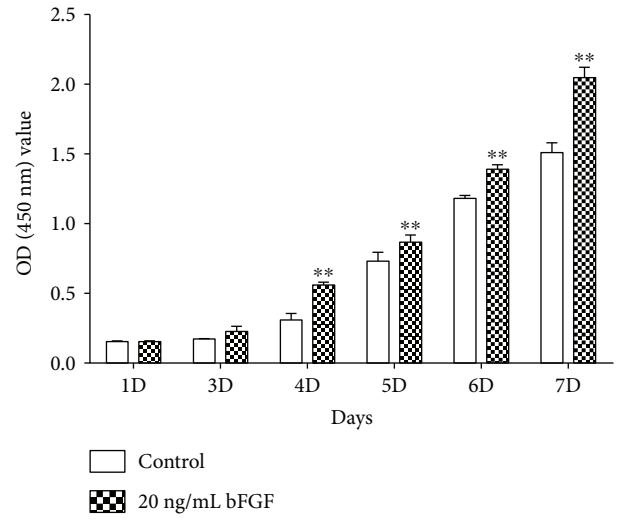

FIGURE 4: The cell proliferation of DPSCs after bFGF promotion at day 1, 3, 4, 5, 6, and 7. $^{**}P < 0.01$ versus the control group.

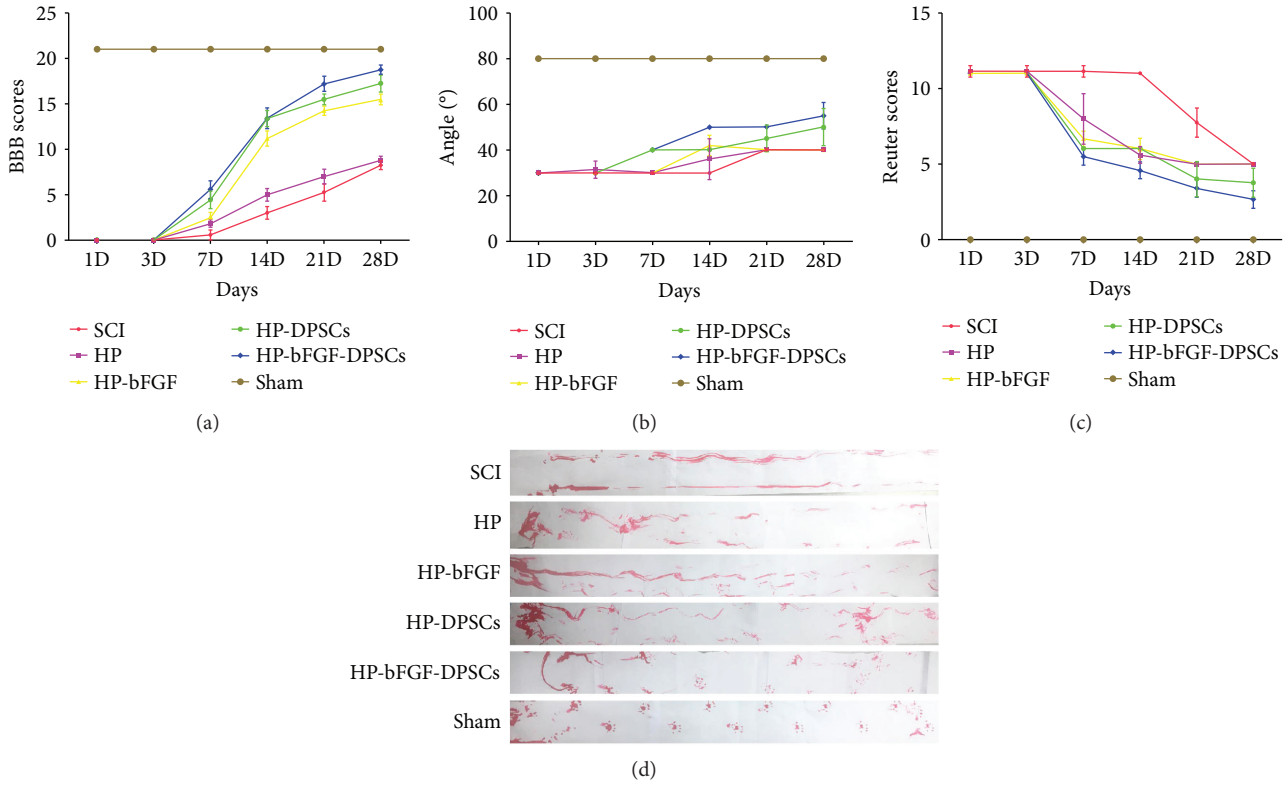

Figure 5: Motor and sensory functional recovery after SCI. (a) The BBB locomotion scores of different groups. (b) The inclined plane test scores of different groups. (c) The Reuter scores of different groups. (d) Footprint analysis of different groups.

became smaller than that of at the SCI modeling stage. Almost identical to control group, defect was hardly seen on injured area of spinal cord in HP-bFGF-DPSC group, suggesting great tissue restoration had occurred. This indicated that HP-bFGF-DPSCs had the ability to support neuron regeneration and tissue repair after SCI. As shown in Figure 7(b), HE staining suggested that the structure of gray and white matter had been damaged in the experimental groups. Compared to the control group, large-scale destruction of gray matter was observed in HP-bFGF group, yet ventral motor neurons (VMNs) and a few blood vessel regeneration were observed in the damaged areas. In HP-bFGF-DPSC group, abundant blood vessels and VMNs were formed and the scale of the damaged areas was decreased, indicating good effects on tissue repair and regeneration. Quantification of the number of ventral motor neurons (VMNs) and the percent of preserved tissue in the gray matter of spinal cord depicted that HP-bFGF-DPSCs group had similar amount of tissue compared to the control group and more than those of the HP-DPSC group (Figure 7(c)).

According to the immunohistochemical staining of GAP-43, positive expressions were frequently observed in the experimental groups (Figure 7(d)). The intensity of GAP-43 positive regions was HP-bFGF-DPSC group > HP-DPSC group > HP-bFGF group. And the difference between HP-bFGF-DPSC group and control group was statistically insignificant (Figure 7(e)). The results were consistent with previous data, indicating that the combination of HP, DPSCs, and bFGF had provided a promising strategy and beneficial effect on promoting the neuron regeneration and tissue repair after SCI.

4. Discussion

Spinal cord injury (SCI), accompanying with motor and sensory dysfunction and disability, which is often caused by traumatic damages, leads to an increase in the socioeconomical costs and compromised quality of life of the injured [1]. It is very difficult to promote neuronal repair and regeneration after SCI because of limitation in necessary precursor cells and secretion of inflammatory cytokines [4]. Dental pulp stem cells (DPSCs) originate from cranial neural crest and display neural-related characteristics including (1) expression of neural-related markers without preinduced differentiation [6, 42]; (2) possession of MSC-like biological properties; and (3) having great capability to differentiate into neuron-like cells and to secrete numerous neurotrophic factors (NFs) in order to provide functional neurons and neuroprotection promoting nerve growth and regeneration [6, 9, 12, 14]. Meanwhile, DPSCs also have vascularization and immunomodulatory properties to enhance blood flow and to improve neural regeneration, respectively [43, 44]. In this work, DPSCs showed the typical MSC-like morphology, for example, fibroblastic and spindle shape and expressed the neural-related markers, for example, Nestin, NeuN, GFAP, and β-tubulin III. After differentiation, DPSCs demonstrated an enhanced expression of neural markers and had significant difference in expression of

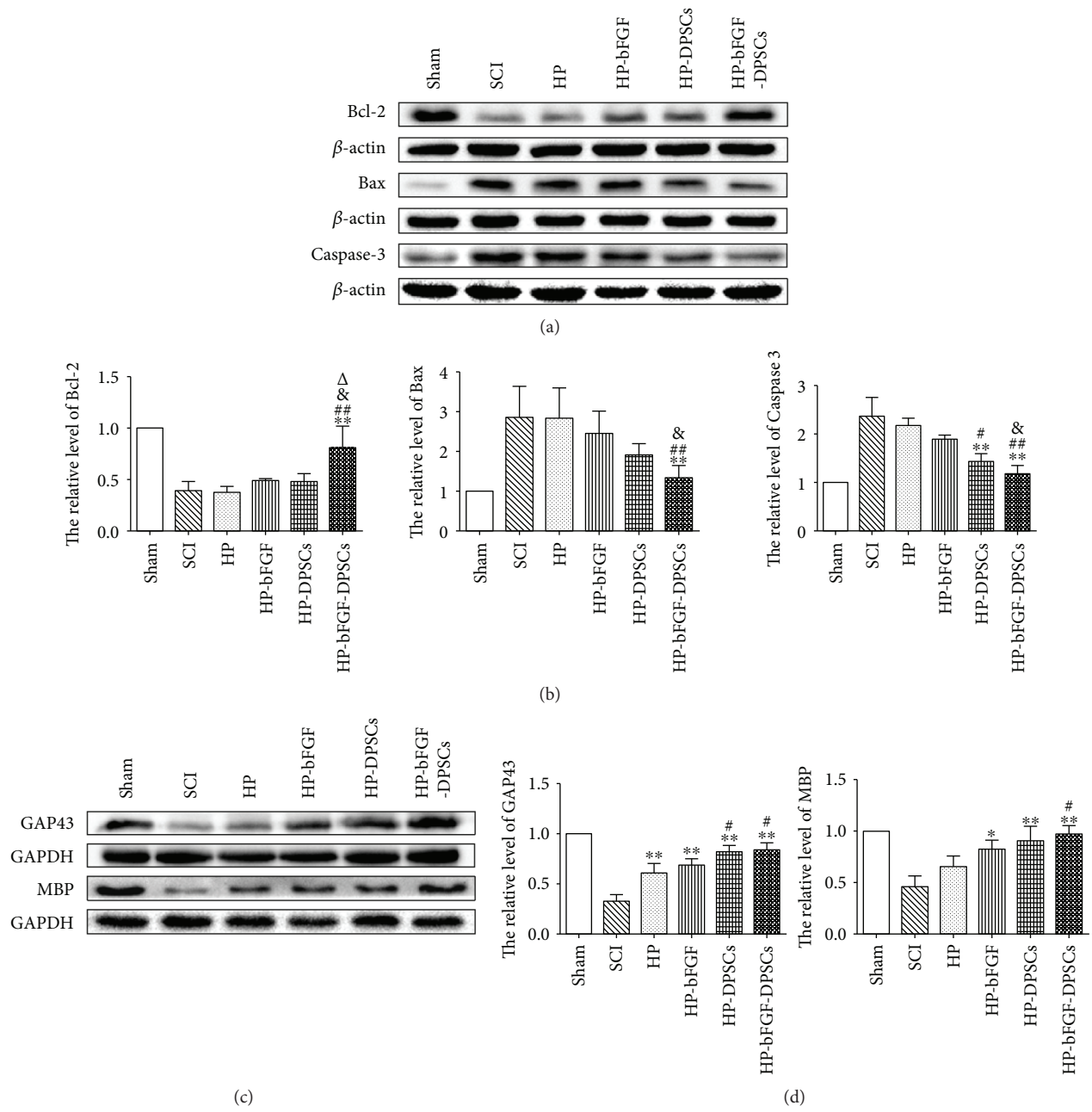

Figure 6: The protein expression of apoptotic-related factors and neuronal markers after SCI. (a) The expressions of Bcl-2, Bax, and Caspase-3 at day 7 after SCI. (b) Quantification of Bcl-2, Bax, and Caspase-3 expression levels. (c) The expressions of MBP and GAP43 at day 21 after SCI. (d) Quantification of MBP and GAP43 expression levels. $^{*}P < 0.05$, $^{**}P < 0.01$ versus SCI group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HP group; $^{\&}P < 0.05$ versus HP-bFGF group; $^{\Delta}P < 0.05$ versus HP-DPSCs group.

β-tubulin III ($^{*}P < 0.05$), Nestin, and GFAP ($^{**}P < 0.01$) (Figures 1 and 3(a)). The results suggested that DPSCs could be a promising cell source for the treatment of defects in nerve system such as SCI. Although DPSCs have innate advantages in the therapy of SCI compared to stem cells from other sources, for example, good availability, low immunogenicity, and noninvasiveness, it is unlikely to restore the motor and sensory function of SCI using a single DPSCs strategy because the cellular retention and survival rate around injured site cannot be guaranteed. A tissue-engineered construct containing cells, growth factors, and scaffolds could provide a combined strategy promoting axon and neuron repair and regeneration after SCI.

Human basic fibroblast growth factor (bFGF), a growth-promoting stimulus, displays multiple biological functions such as promoting cell proliferation, cell differentiation, and self-renewal [45, 46]. In our study, in vitro studies have been confirmed that bFGF could provide DPSCs beneficial effect on survival and proliferation. Compared to control group, cell proliferation rate of bFGF group reached the highest level at the fourth day and gradually decreased from day 5 to day 7. This phenomenon of decrease could be largely attributed to

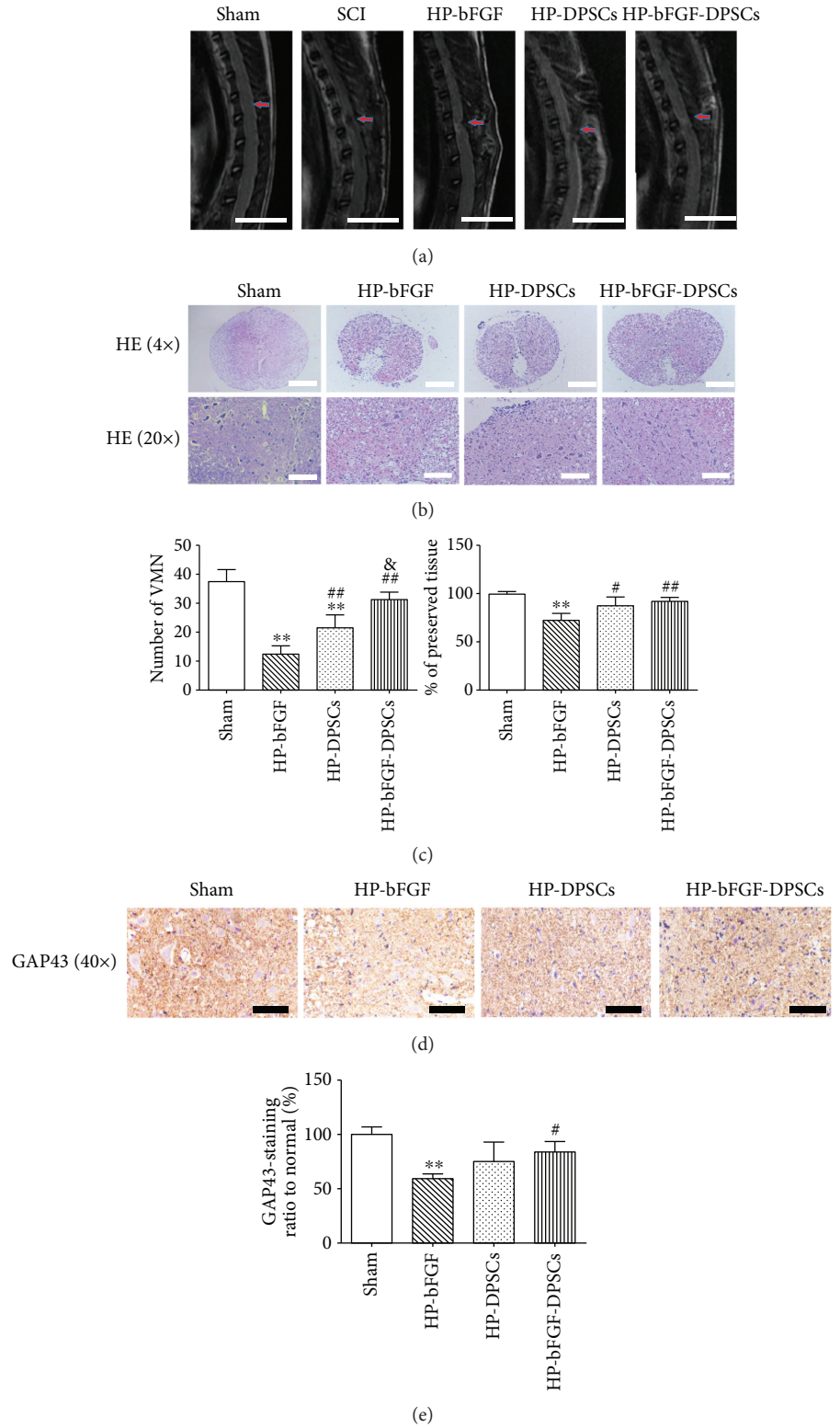

Figure 7: The analyses of MRI, histology evaluation, and immunohistochemistry. (a) MRI images of HP-bFGF, HP-DPSC, and HP-bFGF-DPSC groups at day 28, and SCI modeling stage at 6 hours. Arrows indicated the segmental SCI. Scale bar: 1 cm. (b) Representative images of HE staining at day 28. Scale bar: 500 μm (4×) and 100 μm (20×). (c) Quantification of the number of ventral motor neurons (VMNs) and the percent of preserved tissue in the gray matter of spinal cord. (d) Representative images of GAP43 from immunohistochemisty. Scale bar: 50 μm. (e) Quantification of the GAP43 positive staining ratio to normal in spinal cord. The quantification results obtained by Image J. Data were presented as mean ± standard error from 3 rats in each group. $^{**}P < 0.01$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HP-bFGF group; $^{\&}P < 0.05$ versus HP-DPSC group.

the fact that DPSCs were approximately 90%–100% confluence around day 4, and the bottom of culture dish was completely covered with monolayer cells. The continuation of cell culture after a full confluence had a negative effect on cell growth for the existed inhibition of cellular contact, which resulted in the drop of cell proliferation rate in bFGF group in day 5–7 [47]. However, the cell proliferation of bFGF group was always higher than that of control group in in vitro assay. Moreover, studies indicated that bFGF had the capability to promote axon regeneration, to provide neuroprotection, and to improve behavioral effects in vivo after SCI [19, 21]. Other experiments also showed that bFGF could enhance the survival of neurons and prohibit apoptosis in injured site of SCI [48, 49]. As a macromolecule protein that has very short half-life, bFGF is usually cleaned from the tissue very fast through enzymatic degradation and diffusion [22]. Therefore, new delivery system such as nanoliposomes and biomaterial scaffolds can provide promising strategies to overcome these limitations [30, 33].

Hydrogel has been considered as an attractive biomaterial scaffold for tissue engineering owing to its unique structure to mimic the natural extracellular matrix, to control release bioactive molecules, and to accommodate seeded cells [23]. 3D network of hydrogel scaffold is suitable for cell adhesion, growth, and proliferation. Meanwhile, hydrogel possesses the biomimetic environment that can be used to load and encapsulate biological macromolecules preventing from rapid diffusion and enzymatic degradation [30, 33, 34]. Therefore, hydrogel can act as a substitute to extracellular matrix to provide an engineered scaffold for tissue regeneration after SCI [30]. In this study, we developed HP thermosensitive hydrogel which was nontoxic and had 3D porous structure. The HP hydrogel was prepared from heparin and poloxamer and had a high affinity with growth factors through the heparin-SH group. According to our previous studies [29], HP hydrogel had the ability to provide protective agents for loading and delivering biological macromolecules such as aFGF and NGF, promoting axon regeneration and new blood vessel formation in injured site after SCI. Moreover, HP had good biocompatibility with neural cell lines such as PC12 in vitro. Thus, in this research, HP hydrogel had been used as a scaffold to load and deliver bFGF and DPSCs for in situ administration after SCI because of its 3D network structure, good biocompatibility, and high affinity.

Live/Dead assay was performed to investigate the cytocompatibility of HP hydrogel with DPSCs in vitro in our study. Because of its nontoxicity and mild nature, HP hydrogel has been shown to possess good compatibility with DPSCs. The effects of transplanted HP hydrogel containing bFGF and DPSCs on axon regeneration and neuron repair after SCI were analyzed by functional recovery tests, Western blot, MRI, HE, and immunohistochemistry. The functional recovery results showed that animals which were treated with HP-bFGF, HP-DPSCs, and HP-bFGF-DPSCs had better outcome than the HP and SCI groups. Meanwhile, the degree of locomotor and sensory recovery of HP-bFGF-DPSC group was the best among the experimental groups, indicating that application of DPSCs combined with bFGF had a stronger impact on restoration and regeneration of neuronal function than bFGF-alone and DPSC-alone applications. Bcl-2 as an antiapoptotic factor as well as Bax and Caspase-3 as proapoptotic factors regulate the cellular survival and proliferation. Studies suggested that bFGF could prevent apoptosis by upregulating the expression of Bcl-2 and downregulating the expression of Bax in order to promote the cell proliferation [50, 51]. DPSCs had been found to regulate the expression of Caspase-3 by secreting and producing many immunomodulatory factors [52]. In our study, HP-bFGF-DPSC group demonstrated the highest expression of Bcl-2 and the lowest expression of Bax and Caspase-3, which was consistent with the outcome of functional recovery analysis. Furthermore, the neural-related markers of GAP-43 and MBP also had the highest expression in HP-bFGF-DPSC group.

MRI results were used to reflect the degree of repair in spinal cord after SCI in vivo. HP-bFGF-DPSC showed the best impact on the spinal cord regeneration. The damaged area of HP-bFGF-DPSCs almost disappeared and was replaced by newly regenerated tissue, which was similar to the control group. HE staining showed that HP-bFGF-DPSC group had more newly regenerated cells and blood vessels than HP-bFGF and HP-DPSC group did. And the injured area of HP-bFGF-DPSCs group was the smallest compared to the other experimental groups, similar to the control group. Immunohistochemistry of GAP-43 staining suggested that HP-bFGF-DPSCs group had the most GAP-43 positive expression, indicating that DPSCs combined with bFGF had stronger promotion of neuronal regeneration than single bFGF or DPSCs strategy. Taken together, all results indicated that the combination of HP hydrogel, DPSCs, and bFGF had more impact on neuronal regeneration, functional recovery, and tissue repair than transplanted HP with bFGF-alone or DPSC-alone strategies, which can be a promising strategy to promote neuron regeneration and tissue repair after SCI.

5. Conclusions

This study for the first time identified an optimal combination of scaffold, cell, and growth factor for neuronal regeneration as well as functional recovery after SCI. Our results clearly demonstrated that transplanted HP hydrogel containing DPSCs and bFGF resulted in remarkably beneficial effects on the treatment of SCI. Therefore, the study provided a novel therapeutic strategy for unmet clinical needs in neuron repair, function restoration, and tissue regeneration after SCI.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Lihua Luo, Abdullkhaleg Ali Albashari, and Xiaoyan Wang contributed equally to this work.

Acknowledgments

This work was supported by the following funding bodies and grants. Lihua Luo: the Wenzhou Medical University grant (QTJ16026), China. Jian Xiao: the National Natural Science Foundation of China (81372112), Zhejiang Provincial Program for the Cultivation of High-level Innovative Health talents, China. Qingsong Ye: UQDVCR grant (610709), the University of Queensland, Australia.

Supplementary Materials

The supplementary material provides the results of pairwise statistical analysis of functional behavioral score between groups. BBB scores: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Reuter scores: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; Angle scores: $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$. *(Supplementary Materials)*

References

[1] Z. Khazaeipour, A. Norouzi-Javidan, M. Kaveh, F. Khanzadeh Mehrabani, E. Kazazi, and S. H. Emami-Razavi, "Psychosocial outcomes following spinal cord injury in Iran," *The Journal of Spinal Cord Medicine*, vol. 37, no. 3, pp. 338–345, 2014.

[2] H. Huang, H.-q. Chen, J. Gu, and R.-h. Yu, "Comparative study of hyperbaric oxygen therapy and conventional drug treatment on spinal cord injury at different therapeutic windows," *Scientific Research and Essays*, vol. 6, no. 5, pp. 1117–1122, 2011.

[3] Y. Jiang, F.-L. Gong, G.-B. Zhao, and J. Li, "Chrysin suppressed inflammatory responses and the inducible nitric oxide synthase pathway after spinal cord injury in rats," *International Journal of Molecular Sciences*, vol. 15, no. 12, pp. 12270–12279, 2014.

[4] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Concise review: dental pulp stem cells: a novel cell therapy for retinal and central nervous system repair," *Stem Cells*, vol. 35, no. 1, pp. 61–67, 2017.

[5] M. E. Schwab, "Myelin-associated inhibitors of neurite growth and regeneration in the CNS," *Trends in Neurosciences*, vol. 13, no. 11, pp. 452–456, 1990.

[6] K. Sakai, A. Yamamoto, K. Matsubara et al., "Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms," *The Journal of Clinical Investigation*, vol. 122, no. 1, pp. 80–90, 2012.

[7] J. Ratajczak, A. Bronckaers, Y. Dillen et al., "The neurovascular properties of dental stem cells and their importance in dental tissue engineering," *Stem Cells International*, vol. 2016, 17 pages, 2016.

[8] X. Chen, T. Zhang, J. Shi et al., "Notch1 signaling regulates the proliferation and self-renewal of human dental follicle cells by modulating the G1/S phase transition and telomerase activity," *PLoS One*, vol. 8, no. 7, article e69967, 2013.

[9] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells," *PLoS One*, vol. 9, no. 10, article e109305, 2014.

[10] S. Gronthos, J. Brahim, W. Li et al., "Stem cell properties of human dental pulp stem cells," *Journal of Dental Research*, vol. 81, no. 8, pp. 531–535, 2002.

[11] N. Kawashima, "Characterisation of dental pulp stem cells: a new horizon for tissue regeneration?," *Archives of Oral Biology*, vol. 57, no. 11, pp. 1439–1458, 2012.

[12] M. Kiraly, B. Porcsalmy, A. Pataki et al., "Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons," *Neurochemistry International*, vol. 55, no. 5, pp. 323–332, 2009.

[13] X. Feng, J. Xing, G. Feng et al., "Age-dependent impaired neurogenic differentiation capacity of dental stem cell is associated with Wnt/β-catenin signaling," *Cellular and Molecular Neurobiology*, vol. 33, no. 8, pp. 1023–1031, 2013.

[14] A. Arthur, G. Rychkov, S. Shi, S. A. Koblar, and S. Gronthos, "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues," *Stem Cells*, vol. 26, no. 7, pp. 1787–1795, 2008.

[15] N. Askari, M. M. Yaghoobi, M. Shamsara, and S. Esmaeili-Mahani, "Human dental pulp stem cells differentiate into oligodendrocyte progenitors using the expression of olig2 transcription factor," *Cells Tissues Organs*, vol. 200, no. 2, pp. 93–103, 2014.

[16] I. V. Nosrat, J. Widenfalk, L. Olson, and C. A. Nosrat, "Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons *in vitro*, and rescue motoneurons after spinal cord injury," *Developmental Biology*, vol. 238, no. 1, pp. 120–132, 2001.

[17] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 12, pp. 7544–7556, 2013.

[18] J. Zhang, X. Lu, G. Feng et al., "Chitosan scaffolds induce human dental pulp stem cells to neural differentiation: potential roles for spinal cord injury therapy," *Cell and Tissue Research*, vol. 366, no. 1, pp. 129–142, 2016.

[19] Q. Shi, W. Gao, X. Han et al., "Collagen scaffolds modified with collagen-binding bFGF promotes the neural regeneration in a rat hemisected spinal cord injury model," *Science China Life Sciences*, vol. 57, no. 2, pp. 232–240, 2014.

[20] E. Shaulian, D. Resnitzky, O. Shifman et al., "Induction of Mdm2 and enhancement of cell survival by bFGF," *Oncogene*, vol. 15, no. 22, pp. 2717–2725, 1997.

[21] H. Y. Zhang, Z. G. Wang, F. Z. Wu et al., "Regulation of autophagy and ubiquitinated protein accumulation by bFGF promotes functional recovery and neural protection in a rat model of spinal cord injury," *Molecular Neurobiology*, vol. 48, no. 3, pp. 452–464, 2013.

[22] L. B. Ye, X. C. Yu, Q. H. Xia et al., "Regulation of caveolin-1 and junction proteins by bFGF contributes to the integrity of blood–spinal cord barrier and functional recovery," *Neurotherapeutics*, vol. 13, no. 4, pp. 844–858, 2016.

[23] I. M. El-Sherbiny and M. H. Yacoub, "Hydrogel scaffolds for tissue engineering: progress and challenges," *Global Cardiology Science and Practice*, vol. 2013, no. 3, p. 38, 2013.

[24] H. Yang, K. T. Nguyen, D. T. Leong, N. S. Tan, and C. Y. Tay, "Soft material approach to induce oxidative stress in mesenchymal stem cells for functional tissue repair," *ACS Applied Materials & Interfaces*, vol. 8, no. 40, pp. 26591–26599, 2016.

[25] W. S. Leong, S. C. Wu, M. Pal et al., "Cyclic tensile loading regulates human mesenchymal stem cell differentiation into neuron-like phenotype," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 6, Supplement 3, pp. s68–s79, 2012.

[26] C. Y. Tay, C. G. Koh, N. S. Tan, D. T. Leong, and L. P. Tan, "Mechanoregulation of stem cell fate via micro-/nano-scale manipulation for regenerative medicine," *Nanomedicine*, vol. 8, no. 4, pp. 623–638, 2013.

[27] C. Y. Tay, Y. L. Wu, P. Cai et al., "Bio-inspired micropatterned hydrogel to direct and deconstruct hierarchical processing of geometry-force signals by human mesenchymal stem cells during smooth muscle cell differentiation," *NPG Asia Materials*, vol. 7, no. 7, article e199, 2015.

[28] J. Zhu and R. E. Marchant, "Design properties of hydrogel tissue-engineering scaffolds," *Expert Review of Medical Devices*, vol. 8, no. 5, pp. 607–626, 2011.

[29] Q. Wang, Y. He, Y. Zhao et al., "A thermosensitive heparin-poloxamer hydrogel bridges aFGF to treat spinal cord injury," *ACS Applied Materials & Interfaces*, vol. 9, no. 8, pp. 6725–6745, 2017.

[30] G. Perale, F. Rossi, E. Sundstrom et al., "Hydrogels in spinal cord injury repair strategies," *ACS Chemical Neuroscience*, vol. 2, no. 7, pp. 336–345, 2011.

[31] J. L. Tian, Y. Z. Zhao, Z. Jin et al., "Synthesis and characterization of poloxamer 188-grafted heparin copolymer," *Drug Development and Industrial Pharmacy*, vol. 36, no. 7, pp. 832–838, 2010.

[32] B. Chen, J. He, H. Yang et al., "Repair of spinal cord injury by implantation of bFGF-incorporated HEMA-MOETACL hydrogel in rats," *Scientific Reports*, vol. 5, no. 1, p. 9017, 2015.

[33] S. Lindsey, J. H. Piatt, P. Worthington et al., "Beta hairpin peptide hydrogels as an injectable solid vehicle for neurotrophic growth factor delivery," *Biomacromolecules*, vol. 16, no. 9, pp. 2672–2683, 2015.

[34] S. M. Derkaoui, T. Avramoglou, C. Barbaud, and D. Letourneur, "Synthesis and characterization of a new polysaccharide-*graft*-polymethacrylate copolymer for three-dimensional hybrid hydrogels," *Biomacromolecules*, vol. 9, no. 11, pp. 3033–3038, 2008.

[35] C. Del Angel-Mosqueda, Y. Gutiérrez-Puente, A. P. López-Lozano et al., "Epidermal growth factor enhances osteogenic differentiation of dental pulp stem cells *in vitro*," *Head & Face Medicine*, vol. 11, no. 1, p. 29, 2015.

[36] Z. Yin, Q. Wang, Y. Li, H. Wei, J. Shi, and A. Li, "A novel method for banking stem cells from human exfoliated deciduous teeth: lentiviral TERT immortalization and phenotypical analysis," *Stem Cell Research & Therapy*, vol. 7, no. 1, p. 50, 2016.

[37] A. Gilardino, S. Farcito, P. Zamburlin, C. Audisio, and D. Lovisolo, "Specificity of the second messenger pathways involved in basic fibroblast growth factor-induced survival and neurite growth in chick ciliary ganglion neurons," *Journal of Neuroscience Research*, vol. 87, no. 13, pp. 2951–2962, 2009.

[38] C. S. Yong, J. S. Choi, Q. Z. Quan et al., "Effect of sodium chloride on the gelation temperature, gel strength and bioadhesive force of poloxamer gels containing diclofenac sodium," *International Journal of Pharmaceutics*, vol. 226, no. 1-2, pp. 195–205, 2001.

[39] H. L. Xu, F. R. Tian, C. T. Lu et al., "Thermo-sensitive hydrogels combined with decellularised matrix deliver bFGF for the functional recovery of rats after a spinal cord injury," *Scientific Reports*, vol. 6, no. 1, p. 38332, 2016.

[40] F. Y. He, W. Z. Feng, J. Zhong, W. Xu, H. Y. Shao, and Y. R. Zhang, "Effects of propofol and dexmedetomidine anesthesia on Th1/Th2 of rat spinal cord injury," *European Review for Medical and Pharmacological Sciences*, vol. 21, no. 6, pp. 1355–1361, 2017.

[41] M. Hayashibe, T. Homma, K. Fujimoto et al., "Locomotor improvement of spinal cord-injured rats through treadmill training by forced plantar placement of hind paws," *Spinal Cord*, vol. 54, no. 7, pp. 521–529, 2016.

[42] E. Karaoz, P. C. Demircan, O. Saglam, A. Aksoy, F. Kaymaz, and G. Duruksu, "Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells," *Histochemistry and Cell Biology*, vol. 136, no. 4, pp. 455–473, 2011.

[43] L. Tran-Hung, P. Laurent, J. Camps, and I. About, "Quantification of angiogenic growth factors released by human dental cells after injury," *Archives of Oral Biology*, vol. 53, no. 1, pp. 9–13, 2008.

[44] W. He, T. Qu, Q. Yu et al., "LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells," *International Endodontic Journal*, vol. 46, no. 2, pp. 128–136, 2013.

[45] S. Tsutsumi, A. Shimazu, K. Miyazaki et al., "Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF," *Biochemical and Biophysical Research Communications*, vol. 288, no. 2, pp. 413–419, 2001.

[46] J. S. G. Yeoh and G. de Haan, "Fibroblast growth factors as regulators of stem cell self-renewal and aging," *Mechanisms of Ageing and Development*, vol. 128, no. 1, pp. 17–24, 2007.

[47] E. H. Cho and Y. Dai, "SIRT1 controls cell proliferation by regulating contact inhibition," *Biochemical and Biophysical Research Communications*, vol. 478, no. 2, pp. 868–872, 2016.

[48] X.-t. Meng, C. Li, Z.-y. Dong et al., "Co-transplantation of bFGF-expressing amniotic epithelial cells and neural stem cells promotes functional recovery in spinal cord-injured rats," *Cell Biology International*, vol. 32, no. 12, pp. 1546–1558, 2008.

[49] H. Y. Zhang, X. Zhang, Z. G. Wang et al., "Exogenous basic fibroblast growth factor inhibits ER stress–induced apoptosis and improves recovery from spinal cord injury," *CNS Neuroscience & Therapeutics*, vol. 19, no. 1, pp. 20–29, 2013.

[50] W. Rios-Munoz, I. Soto, M. V. Duprey-Diaz, J. Blagburn, and R. E. Blanco, "Fibroblast growth factor 2 applied to the optic nerve after axotomy increases Bcl-2 and decreases Bax in ganglion cells by activating the extracellular signal-regulated kinase signaling pathway," *Journal of Neurochemistry*, vol. 93, no. 6, pp. 1422–1433, 2005.

[51] J. Huang, L. Wu, S.-i. Tashiro, S. Onodera, and T. Ikejima, "Fibroblast growth factor-2 suppresses oridonin-induced L929 apoptosis through extracellular signal-regulated kinase-dependent and phosphatidylinositol 3-kinase-independent pathway," *Journal of Pharmaceutical Sciences*, vol. 102, no. 3, pp. 305–313, 2006.

[52] P. C. Demircan, A. E. Sariboyaci, Z. S. Unal, G. Gacar, C. Subasi, and E. Karaoz, "Immunoregulatory effects of human dental pulp-derived stem cells on T cells: comparison of transwell co-culture and mixed lymphocyte reaction systems," *Cytotherapy*, vol. 13, no. 10, pp. 1205–1220, 2011.

Hindawi
Stem Cells International
Volume 2018, Article ID 3250379, 10 pages
https://doi.org/10.1155/2018/3250379

Research Article

N-Cadherin Upregulation Promotes the Neurogenic Differentiation of Menstrual Blood-Derived Endometrial Stem Cells

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Received 9 August 2017; Revised 19 November 2017; Accepted 4 December 2017; Published 5 March 2018

Academic Editor: Heinrich Sauer

Peripheral nerve injuries are typically caused by either trauma or medical disorders, and recently, stem cell-based therapies have provided a promising treatment approach. Menstrual blood-derived endometrial stem cells (MenSCs) are considered an ideal therapeutic option for peripheral nerve repair due to a noninvasive collection procedure and their high proliferation rate and immunological tolerance. Here, we successfully isolated MenSCs and examined their biological characteristics including their morphology, multipotency, and immunophenotype. Subsequent in vitro studies demonstrated that MenSCs express high levels of neurotrophic factors, such as NT3, NT4, BDNF, and NGF, and are capable of transdifferentiating into glial-like cells under conventional induction conditions. Moreover, upregulation of N-cadherin (N-cad) mRNA and protein expression was observed after neurogenic differentiation. In vivo studies clearly showed that N-cad knockdown via in utero electroporation perturbed the migration and maturation of mouse neural precursor cells (NPCs). Finally, a further transfection assay also confirmed that N-cad upregulation in MenSCs results in the expression of S100. Collectively, our results confirmed the paracrine effect of MenSCs on neuroprotection as well as their potential for transdifferentiation into glial-like cells and demonstrated that N-cad upregulation promotes the neurogenic differentiation of MenSCs, thereby providing support for transgenic MenSC-based therapy for peripheral nerve injury.

1. Introduction

Schwann cells (SCs) play a key role in the development, function, and regeneration of peripheral nerves. However, clinical application of SC transplantation is limited due to the intrinsic shortcomings of SCs, such as the invasive isolation requirements, their limited proliferation capacity in vitro, and their high immunogenicity [1, 2]. Recently, increasing evidence has suggested that adult stem cell (ASC) transplantation is an ideal alternative to SC transplantation and may be promising in the clinic [2–4]. Transplantation of undifferentiated ASCs has been demonstrated to be beneficial for peripheral nerve repair, most likely via the paracrine production of neurotrophic factors rather than the direct transdifferentiation into SCs. Therefore, to improve the benefit of ASC-based therapy for peripheral nerve repair, several studies have attempted to predifferentiate ASCs into Schwann-like cells in vitro prior to transplantation [5–7]. The main purpose of predifferentiation is to improve the survival of the predifferentiated ASCs residing at the injury site and promote their fusion with endogenous SCs while simultaneously reducing the possibility of differentiation of the transplanted cells into undesired cell types.

As a class of newly defined ASCs, menstrual blood-derived endometrial stem cells (MenSCs) show promise for clinical application. MenSCs are mesenchymal-like stem cells

that can be harvested from human menstrual blood shed from the endometrium every month and have a high proliferation and differentiation capability under specific differentiation conditions. The convenience of obtaining MenSCs without invasive surgical intervention or hospitalization and the absence of any ethical issues associated with their isolation provide reasonable advantages for the clinical application of MenSCs [8–10]. In addition to being positive for classical mesenchymal stem cell markers (CD29, CD44, CD73, CD90, and CD105) and negative for hematopoietic cell surface markers (CD34, CD45, and CD133), MenSCs also express several pluripotency markers, including OCT-4, SOX2, and SSEA-4 [11]. Differentiation of MenSCs into adipocytes, chondrocytes, osteocytes, hepatocytes, cardiomyocytes, and pancreatic cells has been demonstrated previously. The promising therapeutic potential of MenSCs has been demonstrated in central nerve repair (using experimental mouse models of stroke and Parkinson's disease), and the safety of MenSC administration has been evaluated [10, 12, 13].

N-Cadherin (N-cad) is primarily expressed in neural tissues and plays a critical role in the development of the nervous system. N-cad is responsible for regulating maintenance, proliferation, and differentiation of neural precursor cells (NPCs) during development [14–16]. Based on earlier studies, predifferentiation of MenSCs into Schwann-like cells may be more beneficial for peripheral nerve repair; however, the toxicity of chemical factors and the high cost of biological factors for the predifferentiation process vastly limit its clinical application. Therefore, our study aims at first confirming the paracrine effect of MenSCs on neuroprotection and their potential for transdifferentiation into glial-like cells and, subsequently, at examining alterations in the level of N-cad during predifferentiation of MenSCs to explore an alternative source of genetically engineered MenSCs for treating peripheral nerve injury.

2. Materials and Methods

2.1. Plasmids, Cells, and Animals. The plasmids (pCAG-MCS-EGFP and human pCAG-N-cad-EGFP) were constructed in our laboratory. The MenSCs used in this study were harvested with informed consent from female donors, and this study was approved by the Ethics Committee of Xinxiang Medical University. Eight-week-old BALB/c mice (20–25 g) were purchased from Vital River Laboratories (Beijing, China) and bred and housed in a pathogen-free environment with a 12-hour light-dark cycle in the animal care facility. All experimental protocols were approved by the Animal Research Committee of Xinxiang Medical University according to the Chinese Council on Animal Care guidelines.

2.2. Isolation and Culture of MenSCs. Samples of menstrual blood were collected from healthy female donors ($n = 5$, 30 ± 5 years of age) with menstrual cups during the first three days of menses and quickly mixed with equal volumes of PBS containing 0.25 mg/ml amphotericin B, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM EDTA. Once the microbial contaminants were eliminated, MenSCs were isolated within 72 h using the standard Ficoll method as previously described [8], suspended in growth medium [high-glucose DMEM (HyClone, USA) supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin], and seeded in T25 flasks at 37°C, 5% CO_2. After 2 days of incubation, the nonadherent cells were washed away, and the growth medium was replaced every 3 days. When the cells reached 80–90% confluence (passage 0, P0), the cells were detached with trypsin and subcultured to new flasks at a ratio of 1 : 3.

2.3. Identification of MenSCs. The classical characteristics of adult stem cells, including in vitro multilineage differentiation potential and the typical immunophenotype, were routinely examined in P3 MenSCs, and each examination was repeated separately on MenSCs isolated from 3 donors ($n = 3$). In brief, the morphology of MenSCs (P0, P3, and P9) was determined by imaging; the expression of surface markers, such as CD29, CD44, CD73, CD90, CD105, HLA-ABC, HLA-DR, CD34, and CD45, was tested using FACS. Adipogenic, osteogenic, and chondrogenic differentiation was induced and identified as follows: adipogenic differentiation medium (growth medium + 1 μmol/l dexamethasone + 10 μg/ml recombinant human insulin + 200 μM indomethacin + 0.5 mM IBMX), for 14 days; osteogenic differentiation medium (growth medium + 0.1 μmol/l dexamethasone + 0.05 mmol/l ascorbic acid + 10 mM β-glycerophosphate), for 21 days; and chondrogenic differentiation medium (growth medium + 0.1 μmol/l dexamethasone + 0.2 mmol/l ascorbic acid + 1% insulin-transferrin-selenic acid + 10 ng/ml TGF-β3), for 21 days. At the end of the induction period, the cells were washed and fixed. Adipogenic differentiation was confirmed by Oil red O staining, osteogenic differentiation was confirmed by Alizarin red staining, and chondrogenic differentiation was confirmed by Alcian blue staining.

2.4. In Vitro Neurogenic Differentiation Assay. The P3 MenSCs ($n = 3$, the MenSCs were isolated from 3 donors) were suspended in growth medium and seeded at a density of 2×10^4 cells/well in a 6-well plate until confluence. Then, the growth medium was changed to glial differentiation medium (growth medium + 1 mM β-mercaptoethanol, for 1 day; growth medium + 35 ng/ml all-trans-retinoic acid, for 3 days; growth medium + 5 ng/ml platelet-derived growth factor + 10 ng/ml basic fibroblast growth factor + 14 μM forskolin + 126 ng/ml glial growth factor-2, for 14 days). The control cells were cultured in growth medium, and the medium was replaced every 3 days.

2.5. RT-PCR. Total RNA was isolated from cells with or without neurogenic differentiation (2×10^6), and cDNA was prepared from 2 μg of total RNA using PrimeScript RT Master Kit according to the manufacturer's instructions. The primers used to amplify the target genes were synthesized as listed in Table 1; the housekeeping gene (GAPDH) was used as an internal control. The PCR products were amplified using 2× Taq Master Mix (Cwbiotech, China) and analyzed by electrophoresis on 2% agarose gels and with

Table 1: Primer sequences.

Gene		Oligonucleotide sequence (5′-3′)	Size (bp)	Reference
NT3	Sense	TACGCGGAGCATAAGAGTCAC	333	[17]
	Antisense	GGCACACACACAGGACGTGTC		
NT4	Sense	CTTTCGGGAGTCAGCAGGTGC	399	[17]
	Antisense	CAGGCAGTGTCAATTCGAATCC		
BDNF	Sense	TTCCACCAGGTGAGAAGAGT	474	[18]
	Antisense	ACTAATACTGTCACACACGC		
CNTF	Sense	TGGCTAGCAAGGAAGATTCGT	519	[19]
	Antisense	AATATAATGGCTCCCACGTGC		
NGF	Sense	CACACTGAGGTGCATAGCGT	389	[18]
	Antisense	TGATGACCGCTTGCTCCTGT		
LIF	Sense	ATGTCACAACAACCTCATGAA	466	[19]
	Antisense	GATCTGCTTATACTTCCCCAG		
	Antisense	AATGGTGATCCGGTTCTCCTC		
CNPase	Sense	AAGGACTTCCTGCCGCTCTA	466	[20]
	Antisense	TGTCCACATCACTCGGCCAC		
S100	Sense	ATGTCTGAGCTGGAGAAGG	338	[21]
	Antisense	CTGTCTGCTTTCTTGCATG		
GFAP	Sense	GTGGTACCGCTCCAAGTTTGCAG	376	[17]
	Antisense	AATGGTGATCCGGTTCTCCTC		
N-cad	Sense	TGTTTGACTATGAAGGCAGTGG	151	[22]
	Antisense	TCAGTCATCACCTCCACCAT		
GAPDH	Sense	GAAGGTGAAGGTCGGAGT	226	[23]
	Antisense	GAAGATGGTGATGGGATTTC		

ethidium bromide staining. The final data were normalized against the intensity (gray value) of the GAPDH signal and are expressed as mRNA expression relative to that of GAPDH.

2.6. Immunofluorescence. The cells were fixed in 4% PFA for 20 min and permeabilized with 0.05% Triton X-100 for 10 min; nonspecific binding was blocked with 5% goat serum for 30 min. Anti-S100, anti-GFAP, and anti-N-cad (Abcam, USA) antibodies were separately added, and the cells were incubated at 4°C overnight. Alexa Fluor 488- and Cy3-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Life Technologies, USA) were incubated with the cells at 37°C for 1 h. Cell nuclei were stained with DAPI. Finally, the cells were observed and imaged under an inverted fluorescence microscope (Leica, Germany). After neurogenic differentiation, the percentages of GFAP-/S100-/N-cad-positive cells in MenSCs were quantified based on the 10 images collected randomly.

2.7. In Utero Electroporation. The protocol for in utero electroporation (IUE) was followed as previously described [24]. The pGPU6-GFP-neo-shRNA-N-cad (plasmid-based shRNA) was ordered from Kangwei (Abgent, Jiangsu, China) based on the specific shRNA sequence for mouse N-cad (5′-GCCTATGAAGGAACCACATGA-3′), and pGPU6-GFP-neo-shRNA-Control served as the negative control. To strengthen the visible effect, an 8 : 1 ratio of the plasmid-based shRNA and pCAG-MCS-EGFP plasmid was used due to the weak intensity of GFP in mouse embryos induced by plasmid-based shRNA. Solutions (1 μl each) containing pCAG-MCS-EGFP plasmid (0.5 μg) and pGPU6-GFP-neo-shRNA-N-cad or pGPU6-GFP-neo-shRNA-Control (4 μg) were injected through the uterine wall into the lateral ventricle of the embryos at E15 (embryonic day 15). After injection, paddle-type electrodes were placed on either side of the head of the embryo, and five 60 ms square pulses with 600 ms intervals at 35 V were applied with an electroporator (NAPA gene, CUY21, Japan). The electroporated embryos were further incubated for five days, and GFP-positive brains ($n = 3$) were collected for investigation at E20 using a stereo fluorescence microscope (Leica M205FA, Germany). Brain cryosections were prepared according to a previously described protocol, and sections were imaged under a fluorescence microscope (Nikon ECLIPSE 80i, Japan) equipped with digital camera (Leica DFC300FX, Germany).

2.8. Transfection. MenSCs ($n = 3$, the MenSCs were isolated from 3 donors) were transfected with Lipofectamine™ 3000 reagent (Invitrogen Co. Ltd., USA) following the manufacturer's protocol. In brief, MenSCs were seeded at a density of 1×10^5 cells/well into a 24-well plate 24 h before transfection. Subsequently, a final volume of 50 μl of serum free DMEM containing 500 ng of plasmid (pCAG-MCS-EGFP or human pCAG-N-cad-EGFP), 1 μl P3000™ reagent, and 1.5 μl Lipofectamine 3000 reagent was added to each well.

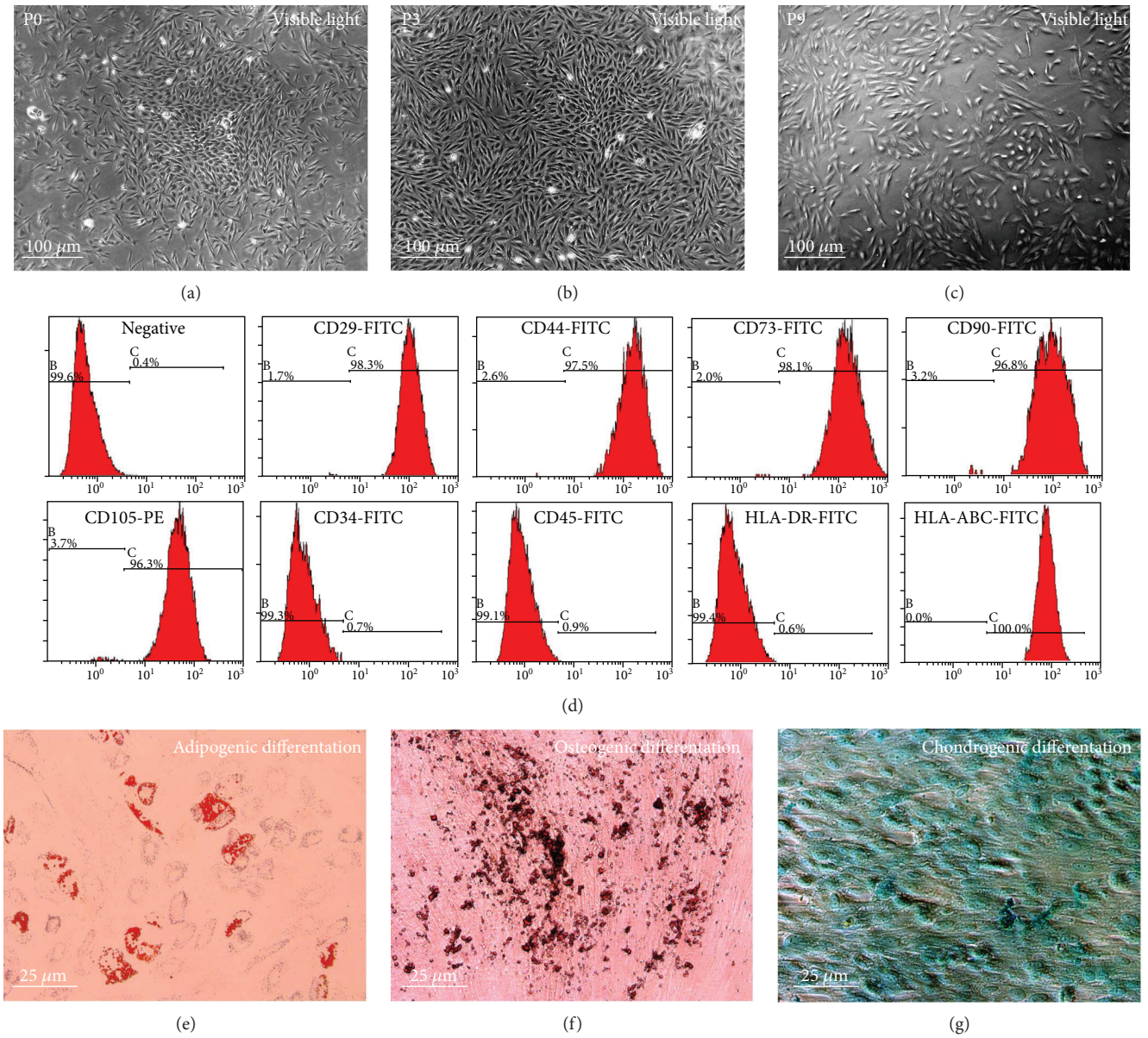

Figure 1: Isolation and identification of MenSCs. Phase contrast images showing the morphology of primary MenSCs at different passages: (a) P0, (b) P3, and (c) P9. (d) The phenotype of MenSCs. To determine the immunophenotype of the cells, P3 MenSCs were stained with the indicated conjugated antibodies and analyzed via FACS. The P3 MenSCs were positive for classical ASC markers (CD29, CD44, CD73, CD90, and CD105) and HLA-ABC and negative for hematopoietic stem cell markers (CD34 and CD45) and HLA-DR. (e) Adipogenic, (f) osteogenic, and (g) chondrogenic differentiation was conventionally induced, and the results were assessed with positive Oil red O, Alizarin red, and Alcian blue staining, respectively.

The medium was replaced with fresh medium (high-glucose DMEM supplemented with 2% FBS) every 3 days, and GFP fluorescence was detected with an inverted fluorescence microscope. After being cultured for 14 days, the cells were harvested and analyzed via immunofluorescence.

2.9. Statistical Analysis. The data are presented as the mean ± SD; all experiments were repeated at least in duplicate on MenSCs isolated from 3 donors; and the figures presented in the manuscript show representative images. Student's *t*-test was used to determine statistical significance, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and Identification of MenSCs. After isolation using the standard Ficoll method, a colony-like morphology was clearly observed in the primary cultures of MenSCs (Figure 1(a)), and the subcultured MenSCs demonstrated growth characteristics typical of ASCs (spindle fibroblast-like morphology with a radial or helical growth pattern). Moreover, the identified phenotype was stable throughout the subculture period from P0 to P9 (Figures 1(a)–1(c)). Subsequently, flow cytometric analysis of P3 MenSCs demonstrated that the cultured cells were positive for CD29, CD44,

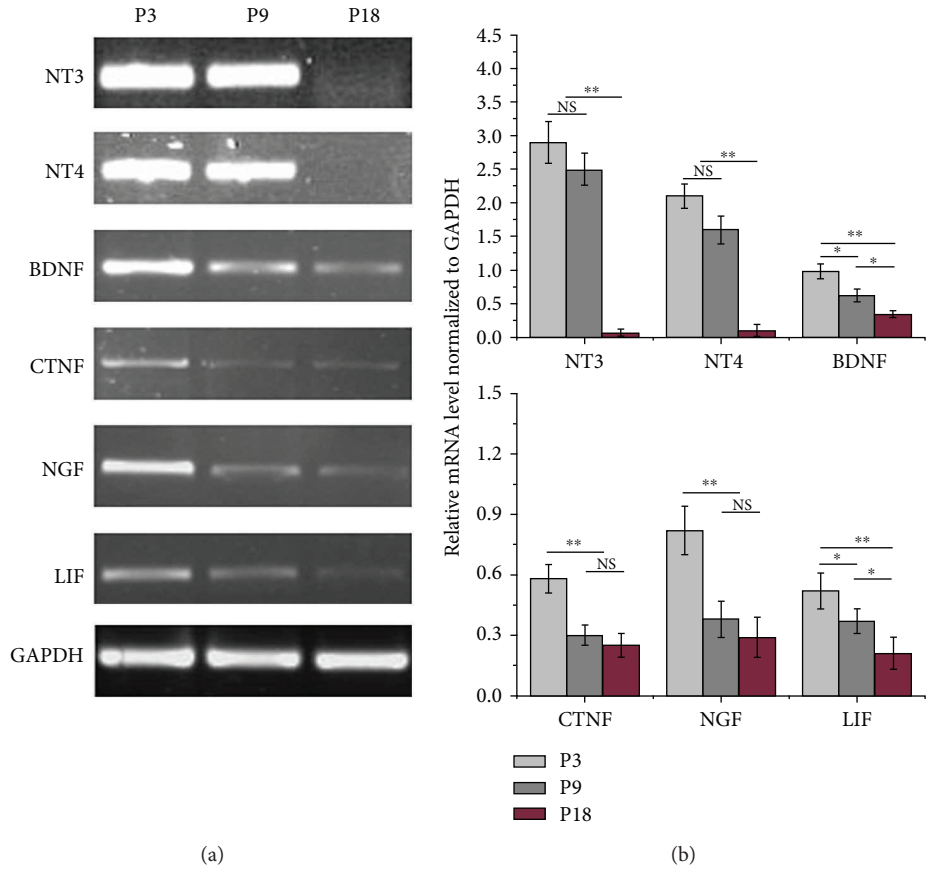

Figure 2: Expression of neurotrophic genes at different passages (P3, P9, and P18) of MenSCs cultured in vitro. (a) RT-PCR analysis of neurotrophic factors expressed at different passages in MenSCs. (b) Quantitative analysis of neurotrophic factors expressed at different passages in MenSCs. The data were normalized against the intensity (gray value) of GAPDH expression and are expressed as the expression level of mRNA relative to that of GAPDH. $^{*}p < 0.05$; $^{**}p < 0.01$.

CD73, CD90, CD105, and HLA-ABC but negative for CD34, CD45, and HLA-DR (Figure 1(d)). Finally, multilineage differentiation assays also confirmed that the MenSCs could undergo adipogenic (Figure 1(e)), osteogenic (Figure 1(f)), and chondrogenic (Figure 1(g)) differentiation after being treated with specific induction media.

3.2. Paracrine Effect of MenSCs on Neuroprotection. RT-PCR analysis of neurotrophic factors (Figure 2) revealed a high level of NT3, NT4, BDNF, and NGF expressions compared with the level of CTNF and LIF in the P3 MenSCs cultured in vitro. However, with an increase in culture time, the expression of the neurotrophic factors mentioned above was significantly decreased in the MenSCs, especially at P18, which suggested that the MenSCs passaged earlier were optimal for peripheral nerve repair.

3.3. Differentiation of MenSCs into Glial-like Cells. In accordance with previous reports, the neurogenic differentiation potential of MenSCs was confirmed in this study. After induction of neural differentiation, the morphology of the MenSCs was transformed from the original spindle fibroblast-like morphology into a glial-like morphology (Figures 3(a) and 3(b)). Subsequent immunofluorescence analysis demonstrated that the differentiated MenSCs were positive for GFAP and S100 (>80%, Figures 3(c), 3(d), 3(g), and 3(h)), which are well-recognized markers of glial cells, and RT-PCR results showed increased mRNA levels of CNPase, S100, and GFAP (Figures 3(e) and 3(f)).

3.4. Neurogenic Differentiation of MenSCs Promotes N-cad Expression. The immunofluorescence and RT-PCR results demonstrated the expression of N-cad in the MenSCs after neurogenic differentiation (Figures 4(a)–4(e)). Compared to the undifferentiated MenSCs, the expression of N-cad was significantly increased in the differentiated MenSCs at both the mRNA and protein levels, which suggests that N-cad plays a role during neurogenic differentiation of MenSCs.

3.5. Inhibition of N-cad Expression Affects the Migration of Neural Progenitor Cells. Equal amounts of pGPU6-GFP-neo-shRNA-N-cad and pGPU6-GFP-neo-shRNA-Control were transfected into the center of the cerebral hemisphere to target the lateral ventricle at E15 by IUE. Then, the GFP-positive embryos and brains were collected and imaged under a stereo fluorescence microscope at E20. As shown in Figure 4(f), compared with the negative control group, the intensity of GFP in the pGPU6-GFP-neo-shRNA-N-cad-transfected embryos and brains was visibly decreased, and the subsequently prepared cryosections clearly showed that

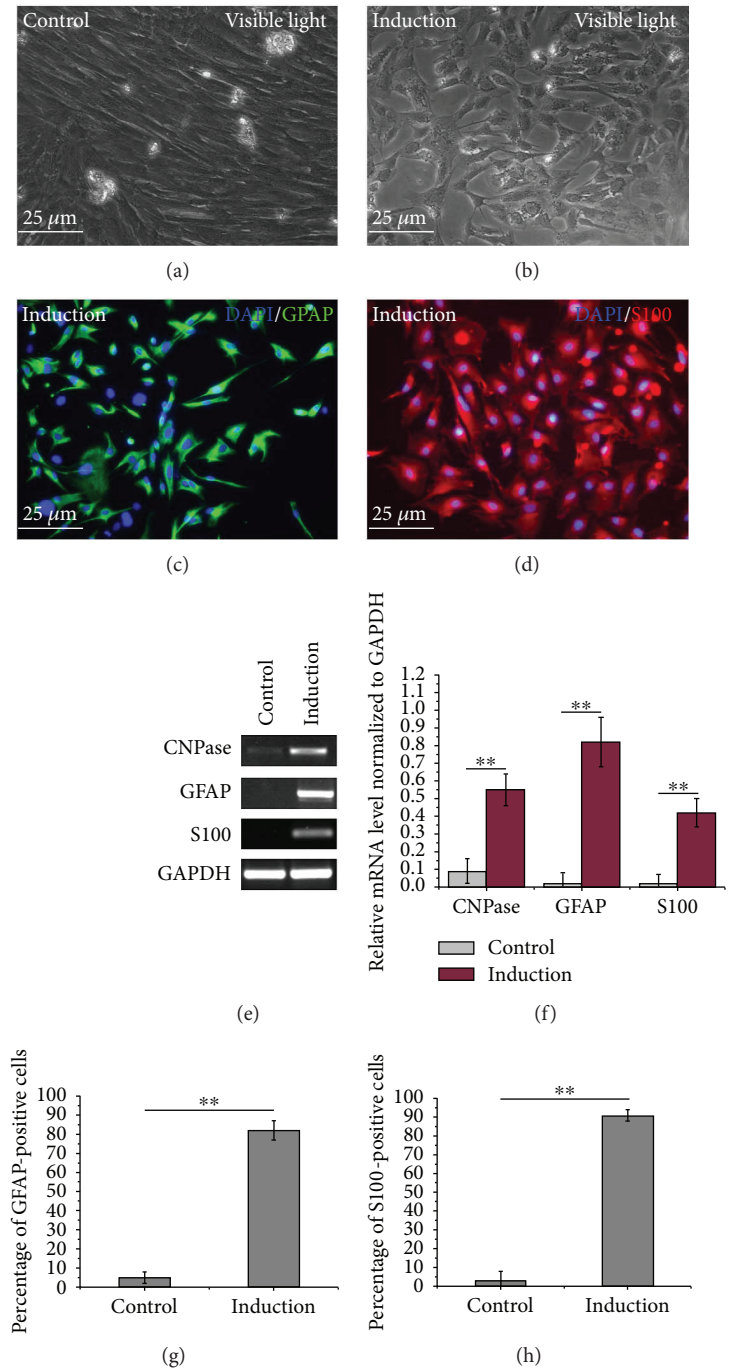

Figure 3: Differentiation of MenSCs into glial-like cells. (a, b) Morphology of P3 MenSCs with or without neurogenic induction. (c, d) Neurogenic differentiation of P3 MenSCs was induced, and the results were assessed by examining the positive expression of glial cell markers (GFAP and S100). (e, f) RT-PCR analysis of CNPase, GFAP, and S100 in MenSCs with or without neurogenic induction. (g, h) The percentage of GFAP- or S100-positive cells after neurogenic differentiation. $^{**}p < 0.01$.

the decrease in GFP was caused by abnormal retention of GFP-positive NPCs at the ventricular zone (VZ), which suggests that inhibition of N-cad expression significantly influences NPC migration and maturation.

3.6. Upregulation of N-cad Promotes Neurogenic Differentiation of MenSCs. To determine whether upregulation of N-cad could promote neurogenic differentiation of ASCs, MenSCs were transiently transfected with the N-cad overexpression vector (human pCAG-N-cad-EGFP), and the immunofluorescence results are shown in Figure 5. After transfection, EGFP-positive MenSCs in the human pCAG-N-cad-EGFP-treated group were positive for N-cad (Figure 5(g)) and S100 (Figure 5(o)) expressions, but the cells in the pCAG-MCS-EGFP-treated group were negative for N-cad (Figure 5(c)) and S100 (Figure 5(k)) expressions.

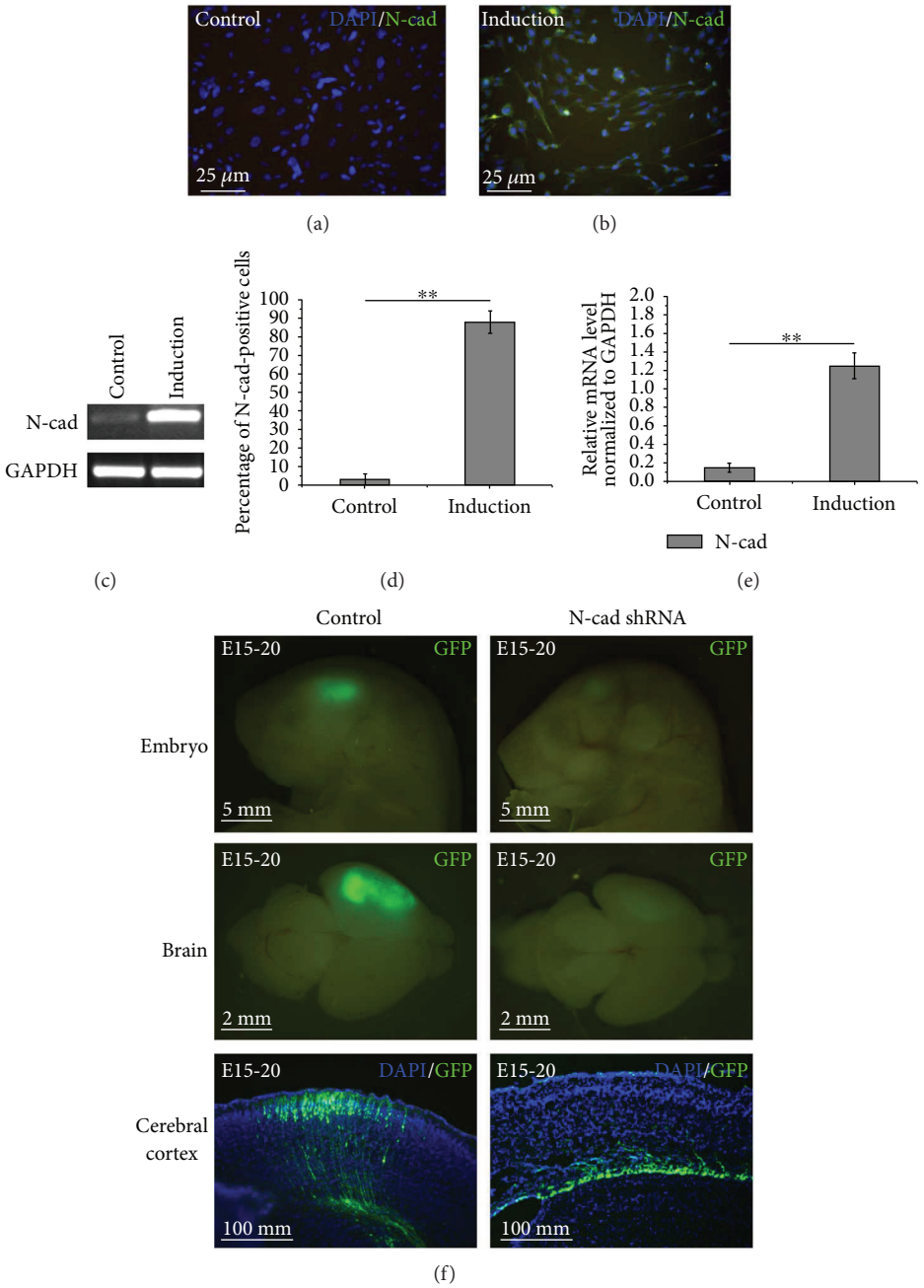

Figure 4: (a-e) Neurogenic differentiation of MenSCs promotes N-cad expression. Immunofluorescence staining (a, b) and RT-PCR (c) were used to determine the expression of N-cad in MenSCs with or without neurogenic induction. The percentage of N-cad-positive cells (d) and the relative mRNA expression levels of N-cad (e) in MenSCs after neurogenic differentiation. (f) Inhibition of N-cad expression affects the migration of NPCs in vivo. The GFP-positive embryos and brains were collected and imaged under a stereo fluorescence microscope at E20, and the subsequently prepared cryosections clearly showed that the decrease in GFP intensity was caused by abnormal retention of GFP-positive NPCs at the ventricular zone. $^{**}p < 0.01$.

4. Discussion

The high incidence of peripheral nerve injury represents a major economic and physiological burden for patients due to poor functional recovery [25]. Fortunately, ASC-based therapies are being established via experimental and clinical studies, and considerable progress has been made in the past decades, which offers hope for diseases that lack effective treatments [26, 27]. MenSCs, a newly identified class of ASCs, have potential for peripheral nerve repair because of essential advantages such as abundant sources, noninvasive isolation procedures, lack of ethical controversy, and high neurogenic transdifferentiation potency [8–13]. Our in vitro studies not only analyzed the paracrine effect of MenSCs on neuroprotection (Figure 2) but also confirmed that MenSCs could be predifferentiated

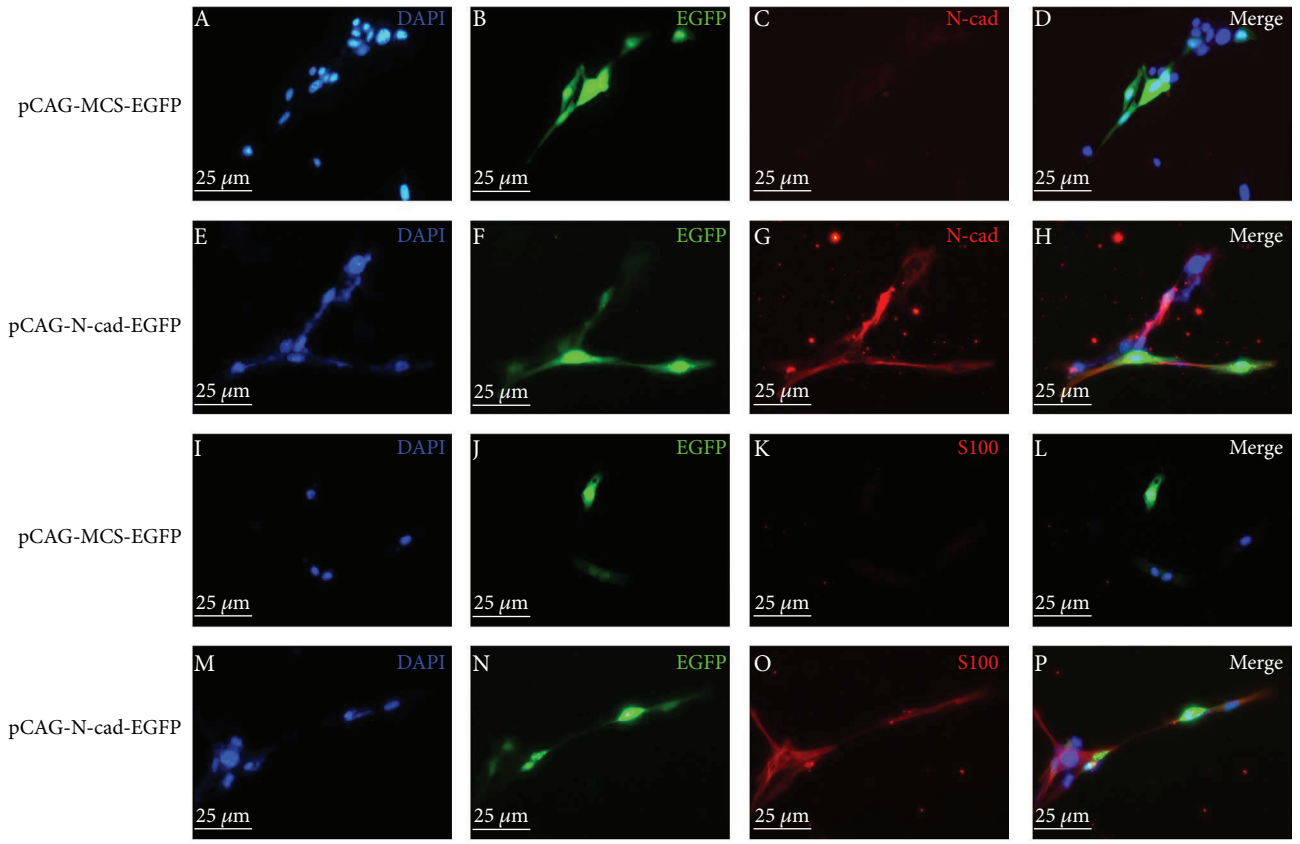

Figure 5: The promotive effect of N-cad upregulation on neurogenic differentiation of MenSCs. MenSCs were transfected with different plasmids, and the medium was replaced with fresh medium every 3 days. After cells were cultured for 14 days, immunofluorescence was performed. (a-d) and (i-l) EGFP-positive MenSCs in the pCAG-MCS-EGFP-treated group were negative for N-cad and S100 expressions. (e-h) and (m-p) EGFP-positive MenSCs in the human pCAG-N-cad-EGFP-treated group were positive for N-cad and S100 expressions.

into Schwann-like cells with conventional induction protocols (chemicals combined with biological factors), which demonstrated the neurogenic differentiation capacity of MenSCs and suggested their potential to transdifferentiate into Schwann-like cells in vivo. The above results showed the therapeutic potential of MenSCs transplantation for peripheral nerve regeneration.

Furthermore, several studies have reported that compared with transplantation of naïve ASCs, transplantation of predifferentiated ASCs is more promising and could improve the survival of the transplanted cells at the injury site and promote their fusion with endogenous SCs, while simultaneously reducing the possibility of differentiation of the transplanted cells into undesired cell types [5–7]. However, to maintain the differentiation of ASCs into Schwann-like cells in vitro, chemical inducers combined with a rather expensive mixture of biological factors are often used, and the induction procedure is extremely complex and time-consuming. Although, coculture with SCs is a simple way to differentiate ASCs into Schwann-like cells, culturing SCs in vitro is difficult because they are often contaminated with fibroblasts present in the axolemma [3, 28–30]. Consequently, predifferentiation of ASCs to Schwann-like cells through a transgenic technique may be a promising alternative approach.

N-cad-based adherens junctions (AJs) are known to be involved in various neural development processes, such as neurulation, migration of neurons, elongation and guidance of axons, and synaptogenesis, due to the contribution of AJs to cell-cell adhesion between NPCs and neurons [14]. As research in this field continues, apart from the contribution to the integrity of AJs and apicobasal polarity of NPCs, N-cad is believed to be responsible for regulating maintenance, proliferation, and differentiation of NPCs during nervous system development [15, 16]. A substantial amount of data has suggested that the onset of neurogenic differentiation is characterized by downregulation of N-cad in various NPCs. It has been reported that the N-cad downregulation promotes detachment of apical processes from the VZ of the spinal cord of chick embryo and an abnormal persistence of N-cad expression inhibits the withdrawal of the apical process and cell cycle exit in prospective neurons [31, 32]. Furthermore, experiments using zebra fish models have shown that downregulation of N-cad is a trigger for differentiation of NPCs during rostral migration [33]. However, in contrast to neurogenic differentiation of NPCs, immediate expression of N-cad in induced pluripotent stem cells (iPSCs) substantially enhances the efficiency of neurogenic differentiation, which suggests that early activation of N-cad determines its potent neurogenic differentiation-promoting

effect; subsequent knockdown experiments also confirmed that inhibition of N-cad expression by shRNA can block neurogenic differentiation [34].

Therefore, based on the results of previous studies and our preliminary findings, we examined the changes in N-cad expression after neurogenic differentiation of MenSCs, and the results showed upregulation of N-cad at both the mRNA and protein levels, suggesting the potential role of N-cad during neurogenic differentiation. In addition, our in vivo study confirmed that knockdown of N-cad by IUE perturbed the migration and maturation of mouse NPCs, which is in accordance with previous reports indicating that N-cad is needed to orient the migration of multipolar cells toward the cortical plate and that the potential mechanism is regulated by Jossin et al [35]. A further transfection assay confirmed that upregulation of N-cad in MenSCs indeed results in the expression of the glial cell marker S100, which indicated the potent neurogenic differentiation-promoting effect of upregulating N-cad in ASCs. Based on published data, the potent neurogenic differentiation-promoting effect of N-cad is likely mediated by β-catenin and Notch signaling pathways, which have been summarized in detail in several excellent reviews and papers [15, 16, 36]. Additionally, N-cad regulates the distribution and degradation of β-catenin, and GSK3β/Akt signal transduction may play critical roles during this regulation [15, 37]. Therefore, we postulate that the expression level of N-cad is likely to play an important role in neurogenic differentiation of MenSCs by regulating the structure of the cytoskeleton, which will indirectly influence the polarity and biomechanics of cells, and consequently promote neurogenic differentiation of MenSCs.

In conclusion, our results confirmed the paracrine effect of MenSCs on neuroprotection and their potential of transdifferentiation into glial-like cells. Moreover, we demonstrated that upregulation of N-cad could promote neurogenic differentiation of MenSCs. Future studies will be performed to validate the potential mechanisms and effect of transgenic MenSCs-based therapy in promoting peripheral nerve injury repair.

Conflicts of Interest

The authors have no competing interests to declare.

Authors' Contributions

Yanli Liu and Fen Yang contributed equally to this work.

Acknowledgments

This work was supported by Natural Science Foundation of China (81771226), Henan Province University Key Scientific Research Foundation (15A180009), Henan Province Natural Science Foundation (162300410214), Xinxiang City Foundation (CXRC16003 and ZD17008), and Xinxiang Medical University Foundation (20172DCG-03).

References

[1] S. Madduri and B. Gander, "Schwann cell delivery of neurotrophic factors for peripheral nerve regeneration," *Journal of the Peripheral Nervous System*, vol. 15, no. 2, pp. 93–103, 2010.

[2] A. D. Widgerow, A. A. Salibian, S. Lalezari, and G. R. D. Evans, "Neuromodulatory nerve regeneration: adipose tissue-derived stem cells and neurotrophic mediation in peripheral nerve regeneration," *Journal of Neuroscience Research*, vol. 91, no. 12, pp. 1517–1524, 2013.

[3] P. J. Kingham, D. F. Kalbermatten, D. Mahay, S. J. Armstrong, M. Wiberg, and G. Terenghi, "Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro," *Experimental Neurology*, vol. 207, no. 2, pp. 267–274, 2007.

[4] J. T. Oliveira, K. Mostacada, S. de Lima, and A. M. B. Martinez, "Chapter three—bone marrow mesenchymal stem cell transplantation for improving nerve regeneration," *International Review of Neurobiology*, vol. 108, pp. 59–77, 2013.

[5] A. Faroni, G. Terenghi, and A. J. Reid, "Chapter five—adipose-derived stem cells and nerve regeneration: promises and pitfalls," *International Review of Neurobiology*, vol. 108, pp. 121–136, 2013.

[6] V. I. Zemelko, I. V. Kozhucharova, Z. V. Kovaleva et al., "Brain-derived neurotrofic factor (BDNF) secretion of human mesenchymal stem cells isolated from bone marrow, endometrium and adipose tissue," *Cell and Tissue Biology*, vol. 8, no. 4, pp. 283–291, 2014.

[7] K. Tomita, T. Madura, C. Mantovani, and G. Terenghi, "Differentiated adipose-derived stem cells promote myelination and enhance functional recovery in a rat model of chronic denervation," *Journal of Neuroscience Research*, vol. 90, no. 7, pp. 1392–1402, 2012.

[8] X. Meng, T. E. Ichim, J. Zhong et al., "Endometrial regenerative cells: a novel stem cell population," *Journal of Translational Medicine*, vol. 5, no. 1, p. 57, 2007.

[9] M. Khoury, F. Alcayaga-Miranda, S. E. Illanes, S. E. Illanes, and F. E. Figueroa, "The promising potential of menstrual stem cells for antenatal diagnosis and cell therapy," *Frontiers in Immunology*, vol. 5, p. 205, 2014.

[10] D. Ulrich, R. Muralitharan, and C. E. Gargett, "Toward the use of endometrial and menstrual blood mesenchymal stem cells for cell-based therapies," *Expert Opinion on Biological Therapy*, vol. 13, no. 10, pp. 1387–1400, 2013.

[11] C. E. Gargett, K. E. Schwab, and J. A. Deane, "Endometrial stem/progenitor cells: the first 10 years," *Human Reproduction Update*, vol. 22, no. 2, pp. dmv051–dmv163, 2016.

[12] C. V. Borlongan, Y. Kaneko, M. Maki et al., "Menstrual blood cells display stem cell-like phenotypic markers and exert neuroprotection following transplantation in experimental stroke," *Stem Cells and Development*, vol. 19, no. 4, pp. 439–452, 2010.

[13] M. C. Rodrigues, L. E. Glover, N. Weinbren et al., "Toward personalized cell therapies: autologous menstrual blood cells for stroke," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 194720, 7 pages, 2011.

[14] K. Chalasani and R. M. Brewster, "N-cadherin–mediated cell adhesion restricts cell proliferation in the dorsal neural tube," *Molecular Biology of the Cell*, vol. 22, no. 9, pp. 1505–1515, 2011.

[15] Y. Miyamoto, F. Sakane, and K. Hashimoto, "N-cadherin-based adherens junction regulates the maintenance, proliferation, and differentiation of neural progenitor cells during development," *Cell Adhesion & Migration*, vol. 9, no. 3, pp. 183–192, 2015.

[16] F. Sakane and Y. Miyamoto, "N-cadherin regulates the proliferation and differentiation of ventral midbrain dopaminergic progenitors," *Developmental Neurobiology*, vol. 73, no. 7, pp. 518–529, 2013.

[17] V. I. Zemelko, I. B. Kozhukharova, L. L. Alekseenko et al., "Neurogenic potential of human mesenchymal stem cells isolated from bone marrow, adipose tissue and endometrium: a comparative study," *Cell and Tissue Biology*, vol. 7, no. 3, pp. 235–244, 2013.

[18] M. Yamamoto, G. Sobue, K. Yamamoto, S. Terao, and T. Mitsuma, "Expression of mRNAs for neurotrophic factors (NGF, BDNF, NT-3, and GDNF) and their receptors ($p75^{NGFR}$, TrkA, TrkB, and TrkC) in the adult human peripheral nervous system and nonneural tissues," *Neurochemical Research*, vol. 21, no. 8, pp. 929–938, 1996.

[19] Y. Ito, M. Yamamoto, N. Mitsuma, M. Li, N. Hattori, and G. Sobue, "Expression of mRNAs for ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and their receptors (CNTFRα, LIFRβ, IL-6Rα, and gp130) in human peripheral neuropathies," *Neurochemical Research*, vol. 26, no. 1, pp. 51–58, 2001.

[20] H. J. Lee, K. S. Kim, J. Ahn, H. M. Bae, I. Lim, and S. U. Kim, "Human motor neurons generated from neural stem cells delay clinical onset and prolong life in ALS mouse model," *PLoS One*, vol. 9, no. 5, article e97518, 2014.

[21] J. Ji, L. Zhao, X. Wang et al., "Differential expression of S100 gene family in human esophageal squamous cell carcinoma," *Journal of Cancer Research and Clinical Oncology*, vol. 130, no. 8, pp. 480–486, 2004.

[22] N. R. Alexander, N. L. Tran, H. Rekapally, C. E. Summers, C. Glackin, and R. L. Heimark, "N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1," *Cancer Research*, vol. 66, no. 7, pp. 3365–3369, 2006.

[23] C. Tricarico, P. Pinzani, S. Bianchi et al., "Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies," *Analytical Biochemistry*, vol. 309, no. 2, pp. 293–300, 2002.

[24] Y. Liu, S. Fu, R. Niu, C. Yang, and J. Lin, "Transcriptional activity assessment of three different promoters for mouse in utero electroporation system," *Plasmid*, vol. 74, pp. 52–58, 2014.

[25] J. Scheib and A. Höke, "Advances in peripheral nerve regeneration," *Nature Reviews Neurology*, vol. 9, no. 12, pp. 668–676, 2013.

[26] S. Hombach-Klonisch, S. Panigrahi, I. Rashedi et al., "Adult stem cells and their trans-differentiation potential—perspectives and therapeutic applications," *Journal of Molecular Medicine*, vol. 86, no. 12, pp. 1301–1314, 2008.

[27] O. Y. Bang, E. H. Kim, J. M. Cha, and G. J. Moon, "Adult stem cell therapy for stroke: challenges and progress," *Journal of Stroke*, vol. 18, no. 3, pp. 256–266, 2016.

[28] D. X. Qian, H. T. Zhang, X. Ma, X. D. Jiang, and R. X. Xu, "Comparison of the efficiencies of three neural induction protocols in human adipose stromal cells," *Neurochemical Research*, vol. 35, no. 4, pp. 572–579, 2010.

[29] F. Azedi, S. Kazemnejad, A. H. Zarnani et al., "Differentiation potential of menstrual blood- versus bone marrow-stem cells into glial-like cells," *Cell Biology International*, vol. 38, no. 5, pp. 615–624, 2014.

[30] Y. Liu, Z. Zhang, Y. Qin et al., "A new method for Schwann-like cell differentiation of adipose derived stem cells," *Neuroscience Letters*, vol. 551, pp. 79–83, 2013.

[31] D. L. Rousso, C. A. Pearson, Z. B. Gaber et al., "Foxp-mediated suppression of N-cadherin regulates neuroepithelial character and progenitor maintenance in the CNS," *Neuron*, vol. 74, no. 2, pp. 314–330, 2012.

[32] R. M. Das and K. G. Storey, "Apical abscission alters cell polarity and dismantles the primary cilium during neurogenesis," *Science*, vol. 343, no. 6167, pp. 200–204, 2014.

[33] Y. Yagita, T. Sakurai, H. Tanaka, K. Kitagawa, D. R. Colman, and W. Shan, "N-cadherin mediates interaction between precursor cells in the subventricular zone and regulates further differentiation," *Journal of Neuroscience Research*, vol. 87, no. 15, pp. 3331–3342, 2009.

[34] H. Su, L. Wang, W. Huang et al., "Immediate expression of Cdh2 is essential for efficient neural differentiation of mouse induced pluripotent stem cells," *Stem Cell Research*, vol. 10, no. 3, pp. 338–348, 2013.

[35] Y. Jossin and J. A. Cooper, "Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex," *Nature Neuroscience*, vol. 14, no. 6, pp. 697–703, 2011.

[36] S. M. Hansen, V. Berezin, and E. Bock, "Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-cadherin," *Cellular and Molecular Life Sciences*, vol. 65, no. 23, pp. 3809–3821, 2008.

[37] C. Liu, Y. Li, M. Semenov et al., "Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism," *Cell*, vol. 108, no. 6, pp. 837–847, 2002.

Hindawi
Stem Cells International
Volume 2018, Article ID 5653787, 7 pages
https://doi.org/10.1155/2018/5653787

Review Article

Cell Transplantation for Spinal Cord Injury: Tumorigenicity of Induced Pluripotent Stem Cell-Derived Neural Stem/Progenitor Cells

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Received 16 July 2017; Accepted 21 December 2017; Published 4 February 2018

Academic Editor: Ming Su

Spinal cord injury (SCI) is an intractable and worldwide difficult medical challenge with limited treatments. Neural stem/progenitor cell (NS/PC) transplantation derived from fetal tissues or embryonic stem cells (ESCs) has demonstrated therapeutic effects via replacement of lost neurons and severed axons and creation of permissive microenvironment to promote repair of spinal cord and axon regeneration but causes ethnical concerns and immunological rejections as well. Thus, the implementation of induced pluripotent stem cells (iPSCs), which can be generated from adult somatic cells and differentiated into NS/PCs, provides an effective alternation in the treatment of SCI. However, as researches further deepen, there is accumulating evidence that the use of iPSC-derived NS/PCs shows mounting concerns of safety, especially the tumorigenicity. This review discusses the tumorigenicity of iPSC-derived NS/PCs focusing on the two different routes of tumorigenicity (teratomas and true tumors) and underlying mechanisms behind them, as well as possible solutions to circumvent them.

1. Background

Spinal cord injury is a devastating neurological condition, which results in the disruption of signals between the brain and body yielding severe physical, psychological, and social dysfunction [1, 2]. Patients who have suffered a SCI not only become increasingly dependent on others for daily life but are more likely to die prematurely and are at risk for social exclusion [1, 2]. What is worse is that, due to the complex pathophysiological processes, significant treatment for SCI has progressed slowly.

Originally, glucocorticoid drugs like methylprednisolone were regarded as the classic therapeutic treatment for SCI [3], as they had been found to stabilize the plasma membrane of damaged cells by inhibiting lipid peroxidation and hydrolysis [3]. However, their application gradually became controversial because they had serious side effects like mounting vulnerability to acute corticosteroid myopathy or serious infection [4, 5]. Other clinical approaches to SCI included early surgical interventions [6] and alternative pharmacological therapy (e.g., GM-1 [7] and thyrotropin-releasing hormone [8]). However, these methods either had their own side effects or demonstrated weakly therapeutic efficacy.

Recent progress in cell transplantation has opened up new opportunities to understand and treat SCI. Among the several types of candidate cells, NS/PC holds great therapeutic potential for SCI, as it can replace the lost neurons and glia as well as create a growth-promoting environment [9]. Nevertheless, the acquisition of NS/PCs can be a difficult task since they are usually located deep in the brain so their isolation is a highly invasive procedure. To bypass this problem, people have also used ESCs from which they can generate sufficient NS/PCs. Indeed, ESC-derived NS/PCs were initially reported to have optimistic effects on SCI [10, 11]. Unfortunately, the application of ESC-based strategy, accompanied by immune rejections and ethical concerns [12], was less likely to be transformed into clinical practice. Subsequently, the advent of iPSCs appears to signal the future of stem cell treatments for SCI. However, while the therapeutic effects of iPSCs on SCI have been discussed by many studies, the side effects are rarely mentioned and talked over exclusively,

especially the tumorigenicity of iPSCs. In this paper, we briefly summarized the application of iPSCs, elucidated the tumorigenicity in detail, and described possible strategies to address it.

2. Application of iPSCs in Spinal Cord Injury: An Overview

In 2006, Takahashi and Yamanaka showed that fibroblasts from mouse somatic cells could regain pluripotency after expressing four transcriptional factors [13], thus developing iPSCs. It stands to reason that iPSCs may have the greatest potential for regenerative medicine, because they have abilities to indefinitely self-renew and differentiate into most if not all cell types [13, 14]. Compared to ESCs, autologous iPSCs also circumvent the ethical issues associated with embryonic tissue harvesting and free patients of immunosuppression, which is critical since SCI patients are at high risk for infection [15].

Of late, an increasing number of research groups have applied iPSCs to SCI and achieved interesting results (Table 1). In 2010, Tsuji et al. managed to produce neurospheres from mouse iPSCs and showed that transplantation of these cells promoted functional improvement in mice with SCI [16]. As a proof of principle, they also used human iPSCs (hiPSCs) and demonstrated significant therapeutic effects like the better recovery of motor function, synapse formation between the grafts and hosts, and enhanced axonal regrowth [17]. Kobayashi et al. transplanted hiPSC-derived NS/PCs into a nonhuman primate following cervical SCI and revealed behavioral improvements consistent with rodent studies [18]. Lu et al. reported that not only can the derivatives of iPSCs extend axons over nearly the whole length of the rat CNS [19] but can also form extensive synaptic connections with the host. More recently, several studies have elucidated potential mechanisms underlying behavioral improvement from SCI following transplantation of iPSC derivatives [20, 21]. They speculated that iPSC derivatives exerted their effects on SCI by substitution of lost neural cells, promotion of axonal remyelination, and regrowth as well as tissue sparing through trophic support.

There are also some negative reports on iPSC approaches to SCI. Two reports revealed that despite the ability to differentiate into neural cells [19, 22], iPSC-derived NS/PCs did not show any substantial improvement in function. Besides, it takes a long time to generate and evaluate iPSCs [23], making it unrealistic for individualized iPSC-based therapy because the optimal time for stem cell transplantation is the subacute phase [24]. As a result, either iPSCs would have to be generated from donor tissue, missing out on the major factor that makes them attractive in the first place, or transplanted at more chronic phases of injury [25], which showed a poor result after transplantation into the chronic SCI model. More importantly, like ESCs, there are widely found issues with respect to safety of iPSCs, particularly the possible tumorigenicity [16, 21, 26].

3. Characteristics and Underlying Mechanisms of iPSC in Tumorigenicity

Tumorigenicity of any stem cell transplants remains a major concern for clinical applications, and there is an urgent need for it to be addressed before translation of iPSC techniques into SCI treatment. From several reports [26, 27], tumorigenicity of iPSCs can be classified into two distinct types: teratoma and true tumors due to their different features and developmental processes, which we will discuss further below (Figure 1).

3.1. Teratoma Formation. Teratoma is a relatively common potential risk in grafts of iPSCs especially when individual iPSC clones were preevaluated as unsafe [16, 17, 28]. While the mechanism is not fully understood, most reports share the idea that undifferentiated iPSCs lead to teratoma formation [26, 29]. Teratoma formation requires the ability to escape or silence the immune responses for the purpose of survival in the host. Tumor cells could take effective measures to avoid immune responses by alteration of MHC-I, mutations in Fas or Trail, and so forth [30]. These traits are well shared with undifferentiated iPSCs. Besides, like tumor cells, iPSCs possess a virtually unlimited proliferation potential, by which they are vulnerable to the formation of a cell mass. Therefore, we reasonably postulate that residual-undifferentiated cells contribute greatly to teratoma formation. Moreover, Miura et al. discovered that the presence or absence of c-Myc in iPSCs and drug selection for NANOG or Fbxo15 expression [28, 31], all of which are considered closely associated with tumorigenesis, showed no correlation with teratoma formation. Namely, the underlying mechanism of teratoma formation is different from that of tumor, as they do not correlate with these tumor makers.

It is still unclear why undifferentiated cells remain in iPSC grafts. However, iPSC derivatives of different origins do demonstrate different teratoma-forming propensity [16, 28]. For instance, iPSCs derived from tail-tip fibroblasts showed the highest propensity for teratoma formation while iPSCs from embryonic fibroblasts and gastric epithelial cells showed the lowest. Since iPSCs from different origins exhibited distinctive features, it is possible that epigenetic memory, the residual features of somatic tissues, plays a role in teratoma formation. And due to epigenetic memory, iPSCs from certain cell lines may be likely to redifferentiate back into their initial cell type [32, 33]. Therefore, we might as well hold the belief that if we created a certain type of microenvironment supporting certain iPSCs to differentiate into NS/PCs, those derived from any other cell lines except neural ones may not be able to well differentiate and have to maintain undifferentiated status under this unfavorable condition. Besides, the inefficient methods of purifying the contaminated undifferentiated cells also aggravate the situation.

3.2. Substantial Tumorigenesis. Several studies have found that even if all undifferentiated cells are purged [26, 34], iPSC derivatives remain tumorigenic, as substantial tumors were present instead of teratomas. Such cases can be much worse because they are usually malignant and able to progress,

Table 1: Some current studies of iPS cell transplantation in the SCI model. This table summarizes some of the experimental studies involving iPSC derivative transplantation into SCI models and its outcomes.

Reference	Grafts	SCI model	Outcomes
Tsuji et al. [16]	Mouse iPSC-derived neurospheres	Mouse contusive SCI at T-10 level	Preevaluated safe iPSC-derived cells promoted functional recovery without teratomas or other tumors, while the "unsafe" iPSC-derived cell resulted in teratoma forming and functional deterioration
Nori et al. [17]	Human iPSC-derived neurospheres	Mouse contusive SCI at T-10 level	Human iPSC-derived cells improved motor functional recovery without tumor formation but some pluripotent stem cells remained at 112d post-SCI
Kobayashi et al. [18]	Human iPSC-derived NS/PCs	Marmoset contusive SCI at C-5 level	Preevaluated safe iPSC-derived cells promoted functional recovery without tumors, whereas some undifferentiated cells were still presented after 16 weeks posttransplantation
Fujimoto et al. [20]	Human iPSC-derived neuroepithelial-like stem cells (hiPS-It-NESC)	Mouse contusive SCI at T9-10 level	hiPS-It-NESCs promote recovery of motor function and reconstruct neuronal circuity with no tumors up to 12 weeks after SCI
LiuTang et al. 2013	Human iPSC-derived NSCs	Rhesus monkey contusive SCI at T9 level	Human iPSC-derived NSCs migrated into damaged regions and showed functional recovery with no tumors after 30 days post-SCI
Lu et al. [19]	Human iPSC-NSCs	Rats and mice lateral spinal cord lesions at C5 level	Human iPSC-NSCs showed long-distance growth of human axons without obvious functional recovery
Salewski et al. 2015	Mouse iPSC-NSCs	Clip-compression spinal cord injuries at the T6 level	Wildtype-iPSC-NSCs improved neurobehavioral function while nonmyelinating Shiverer-iPSC-NSC did not
Oh et al. 2015	iPSC-NPCs from human disc	Mouse compressional SCI at T-11 level	iPSC-NPCs promoted functional and structural recovery with no tumor formation but undifferentiated cells still existed five weeks later
Pomeshchik et al. [22]	Human iPSC-NPCs	Mouse contusive SCI at T-10 level	Transplanted cells failed to improve functional recovery but no tumor formed and undifferentiated cells were not detected
Kawabata et al. 2016	Human iPSC-OPC-enriched NS/PCs	Mouse contusive SCI at T-10 level	Transplanted cells lead to robust remyelination and enhance functional recovery without tumorigenicity
Okubo et al [21]	Human iPSC-NS/PCs with γ-secretase inhibitor (GSI)	Mouse contusive SCI at T-10 level	GSI-treated hiPSC-NS/PCs exhibited motor functional recovery and decreased residual immature cells
Itakura et al [27]	Human-integrated iCaspase9-iPSC-NS/PCs with chemical inducers of dimerization (CIDs)	Mouse contusive SCI at T-10 level	Transplanted cells of the CID group exhibited continually functional recovery while the control groups showed functional decline due to teratomas

invade, and metastasize. As such, understanding the mechanisms behind tumorigenesis is imperative.

The exact mechanism underlying iPSC tumorigenesis is still not clearly defined, but several factors are thought to contribute to it. Collectively, genomic and epigenomic instability correlates largely with tumorigenicity of iPSCs [35, 36]. Many factors can account for genomic instability. For instance, several oncogenes (like c-Myc and KLF4) or genes sometimes associated with tumorigenesis (like SOX2 and Oct-4) are used in the reprogramming process. Additionally, retroviral or lentiviral gene delivery systems are used in the reprogramming process and can be integrated into the genome-disrupting tumor suppressor genes and pathways. For example, the activation of transgenic Oct-4 and KLF4 has been found to induce tumor formation of NS/PCs via the Wnt/β-catenin signaling pathway [34]. This pathway was found to be able to enhance stabilization of telomeres, a signature of tumorigenesis, by increasing TERT expression. Furthermore, the mature cells harvested for iPSC induction have themselves already undergone multiple rounds of division and might possess their own genetic instability before induction [37]. Also, the low-efficiency reprogramming process and incomplete suppression of transgenic factors result in some partially reprogramming cells, which take part in tumor forming.

On the other hand, epigenomic instability, especially DNA methylation, also plays a role in the formation of true tumors [26]. DNA methylation has been found to have strong association with tumorigenesis in cancer tissues [38]. For instance, if oncogenes possess hypomethylation in a cell sample, such cells may show a higher likelihood to form tumors and vice versa. Consistent with this idea, 253G1-hiPSCs as well as 253G1-iPSC-NS/PCs, which had DNA hypomethylation mainly in oncogenes and hypermethylation in tumor suppressor genes, were more likely to develop tumors when compared with 207B1-hiPSCs and NS/PCs, which did not. In addition, tumorigenicity can be enhanced as induced cells are passaged because the passage of iPSCs

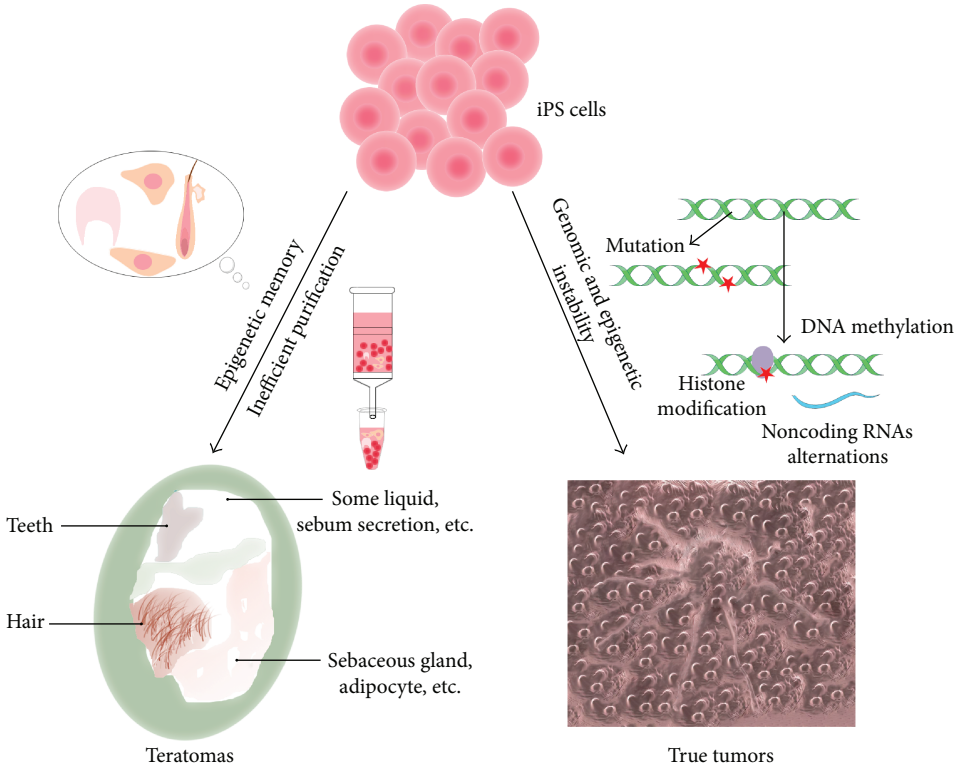

Figure 1: Two distinguished aspects of tumorigenicity and respective potential mechanisms. The tumorigenicity of iPSCs included two parts: one of them is teratoma formation and another is substantial tumor formation. The former is mostly attributed to epigenetic memory as well as inefficient purification, while the latter is ascribed to genomic instability and epigenetic changes.

and iPSC-derived NS/PCs further alters the epigenetic profiles via DNA methylation.

4. Possible Solutions to iPSC Tumorigenesis

4.1. Strategies to Prevent Teratoma Formation. As mentioned above, the formation of teratomas is largely attributed to undifferentiated cells. Based on this, some reports proposed various methods to address this problem including the following:

(1) Increased number of passages to weaken epigenetic memory. Several studies observed the loss of epigenetic memory with increased passage number [33, 39]. iPSCs at late passage and ESCs became indistinguishable and acquired similar ability of differentiation. Therefore, the undifferentiated cell correspondingly reduced when iPSCs were capable enough of differentiation into other cells. While the underlying mechanism is not quite clear, two possible aspects may account for this phenomenon: (i) most of the iPSCs will gradually erase somatic marks as those cells passaged and/or (ii) those rare, fully reprogrammed cells become superior and then are picked up step by step [39].

(2) Take advantage of epigenetic memory characteristics and use it to reprogram iPSCs away from a teratoma-inducing lineage. The propensity of iPSCs to differentiate bias into their starting cell lineage could be exploited to produce certain cell types. For example, to get more NS/PCs from iPSCs, we may ideally think of the utilization of neural cells. Some previous reports [40, 41] also confirmed that, in comparison with other cell lineages of origin, iPSCs from neural tissue are more likely and efficient to differentiate into NS/PCs. The more likely to differentiate into other cells, the less possibility of forming teratomas.

(3) Improve the ability to purify iPSC-NS/PCs. It is essential to better gain bona fide iPSC-NS/PCs, as the potential for contamination with undifferentiated iPSCs presents a big chance of forming teratomas. Therefore, scientists have tried many ways to achieve the common goal including finding more specific cell surface makers and diminishing residual undifferentiated cells like inhibiting DNA topoisomerase II or stearoyl-coA desaturase [21, 42]. Accordingly, it does help but it still urgently needs to pan for desired unique makers or proper methods of depleting undifferentiated cells.

(4) Transplant more mature cells instead of naive ones. It has been observed that teratomas formed from iPSC-derived NS/PCs were much smaller than those directly from iPSCs, indicating that predifferentiation of iPSCs can reduce certain aspects of tumorigenicity [43]. Consequently, grafting iPSCs directly in the treatment of SCI is not recommended.

Taken together, these ways to address undifferentiated cell contamination in iPSC-derived NS/PC transplants are, at least in part, currently effective, but it seems impossible for some of these methods to be translated into clinical application due to either the invasive operation or time-consumed culture to weaken epigenetic memory. And we had better transplanted relatively mature iPSC-derived NS/PCs instead of iPSC itself.

4.2. Strategies to Prevent True Tumors. As for substantial tumors, we also have several effective steps to reduce the risk including the following:

(1) Change the reprogramming methods into integration-free methods. Virally induced iPSCs with genomic integrations of transcriptional factors easily cause insertional mutagenesis and result in continual expression of residual factors in iPSCs [44]. Thus, instead of using integrative vectors like retrovirus or lentivirus, we need to pursue integration-free methods, not perturbing the genome. Episomal vector and Sender virus vector were once thought to be ideal nonintegrating methods, as the former works as extrachromosomal DNA in the nucleus while the latter is a method of transgene-free induction. But as the potential spontaneous integration by episomal vector and the involvement viral particles, both are limited to clinical applications. Subsequently, Woltjen et al. discovered that piggyBac transposons could be integrated into genomes of the host so the reprogramming factors that they carried were able to express continuously and stably [45]. Furthermore, the piggyBac transposons could be cut out of the genomes completely [45]. Afterwards, the advent of DNA-free and viral-free methods like recombinant proteins, messager RNA, and mature microRNA made iPSCs stride towards clinical use despite being technically challenging or inefficient. Of note, iPSCs of the first clinical trial were generated by the nonintegrative method of reprogramming with recombinant proteins [46].

(2) Avoid using transgenic factors of oncogenesis. The Yamanaka factors are competent enough to induce tumorigenesis playing important roles in the development and maintenance of cancer. It appears quite necessary to reduce reprogramming factors in order to decrease the possibility of tumor formation and hasten the clinical use. Nakagawa et al. initiated a series of experiments to test whether fewer factors are capable enough of inducing the stem cell. It was found that exogenous c-Myc was not necessarily needed to generate iPSCs [31]. They then found that exogenous Oct-4 together with KLF4 or SOX2 could produce iPSCs from NSC. Furthermore, they discovered that transcriptional factor Oct-4 alone is sufficient to acquire iPSCs [41]. Although the low-reprogramming efficiency of them limits their applications, their attempt provides us with new ideas.

(3) Reduce undesirable DNA methylation. Decreasing DNA methylation of tumor suppressor genes and increasing that of oncogenes can certainly reduce the rate of tumor formation from iPSCs. The application of knocking down the maintenance methyltransferase DNMT1 or the demethylating agent like 5-AZA can reduce residual methylation of resulting cells and convert them to authentic pluripotent cells [33]. Besides, Mikkelsen et al. found that demethylation appears passage dependent [47]. Some reports showed that DNA methylation could be gradually erased as the cells were passaged [33, 39]. Iida et al. [26], however, found that DNA methylation patterns became more unstable with cells passaged. Maybe, this can be accounted for the fact that the cell clones that they used were different indicating that the ability of passaging to gradually diminish methylation cannot be applicable to all clones.

(4) Establish reliable ways to distinguish the safe and unsafe cell clones. By virtue of the teratoma-forming activity of the iPSC derivatives after their transplantation [28], we are capable of differentiating the safe iPSC clones from all cultured cell clones. Pre-evaluated safe clones can show significant therapeutic effects without tumor formation [16–18], while pree-valuated unsafe clones demonstrate high rates of tumor formation. Iida discovered that methylation states of CAT and PSMD5 genes can be applied to discriminate between safe and unsafe hiPSC-NS/PCs [26].

In brief, across the entire process of iPSC generation and NS/PC differentiation, there are steps that can be taken to reduce nonteratoma tumor formation. These strategies mentioned above just provide some possible way to circumvent the tumorigenicity, but I am afraid that there is still a long way from clinical applications.

5. Conclusions

Despite numerous therapeutic discoveries in the laboratory, to our knowledge, faithfully effective treatment for spinal cord injury remains unavailable. iPSC transplantation for SCI is currently an unrealistic strategy, but we have already recognized the huge potential of iPSCs for SCI because of their ability to self-renew and differentiate into various types of neural cells. In addition, iPSCs also avoid the ethical issues associated with some transplant sources and importantly can be performed in an autologous manner removing the need for immune suppression.

However, although the Takahashi group claimed that they were warranted to restart their clinical trials on iPSCs, safety concerns, especially tumorigenicity, still seriously limit considerations for clinical application, at least on SCI [48]. They once carried out the first clinical application of iPSCs in 2014, but were required to halt for some reasons. In this review, we focused on the two different routes of tumorigenicity and underlying mechanisms behind them. We also

put forward some potential solutions to tumorigenesis. But in the current state, not enough is understood about underlying causes of tumor genesis from iPSC derivatives to completely elucidate the issue. More explorations and attempts need to be done in the future.

Abbreviations

ESCs: Embryonic stem cells
hiPSCs: Human iPSCs
iPSCs: Induced pluripotent stem cells
NS/PC: Neural stem cell or progenitor cell
SCI: Spinal cord injury.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Junhao Deng wrote the initial manuscript. Yiling Zhang, Yong Xie, and Licheng Zhang participated in drafting the manuscript. Peifu Tang revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors thank Xie Wu and their laboratory members for their dedicated work. They are supported by the projects of the international cooperation and exchanges of the National Natural Science Foundation of China (81520108017).

References

[1] A. Singh, L. Tetreault, S. Kalsi-Ryan, A. Nouri, and M. G. Fehlings, "Global prevalence and incidence of traumatic spinal cord injury," *Clinical Epidemiology*, vol. 6, pp. 309–331, 2014.

[2] World Health Organization, "International perspectives on SCI," November 2013, http://www.who.int/mediacentre/factsheets/fs384/en/.

[3] M. B. Bracken, M. J. Shepard, W. F. Collins et al., "A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury — results of the Second National Acute Spinal Cord Injury Study," *The New England Journal of Medicine*, vol. 322, no. 20, pp. 1405–1411, 1990.

[4] T. Qian, D. Campagnolo, and S. Kirshblum, "High-dose methylprednisolone may do more harm for spinal cord injury," *Medical Hypotheses*, vol. 55, no. 5, pp. 452-453, 2000.

[5] T. Qian, X. Guo, A. D. Levi, S. Vanni, and R. T. Shebert, "High-dose methylprednisolone may cause myopathy in acute spinal cord injury patients," *Spinal Cord*, vol. 43, no. 4, pp. 199–203, 2005.

[6] G. W. J. Hawryluk, J. Rowland, B. K. Kwon, and M. G. Fehlings, "Protection and repair of the injured spinal cord: a review of completed, ongoing, and planned clinical trials for acute spinal cord injury. A review," *Neurosurgical Focus*, vol. 25, no. 5, p. E14, 2008.

[7] B. A. Sabel, M. D. Slavin, and D. G. Stein, "GM1 ganglioside treatment facilitates behavioral recovery from bilateral brain damage," *Science*, vol. 225, no. 4659, pp. 340–342, 1984.

[8] T. Hashimoto and N. Fukuda, "Effect of thyrotropin-releasing hormone on the neurologic impairment in rats with spinal cord injury: treatment starting 24 h and 7 days after injury," *European Journal of Pharmacology*, vol. 203, no. 1, pp. 25–32, 1991.

[9] A. J. Mothe and C. H. Tator, "Review of transplantation of neural stem/progenitor cells for spinal cord injury," *International Journal of Developmental Neuroscience*, vol. 31, no. 7, pp. 701–713, 2013.

[10] S. Strauss, "Geron trial resumes, but standards for stem cell trials remain elusive," *Nature Biotechnology*, vol. 28, no. 10, pp. 989-990, 2010.

[11] G. Kumagai, Y. Okada, J. Yamane et al., "Roles of ES cell-derived gliogenic neural stem/progenitor cells in functional recovery after spinal cord injury," *PLoS One*, vol. 4, no. 11, article e7706, 2009.

[12] M. Nakamura and H. Okano, "Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells," *Cell Research*, vol. 23, no. 1, pp. 70–80, 2013.

[13] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[14] I. H. Park, R. Zhao, J. A. West et al., "Reprogramming of human somatic cells to pluripotency with defined factors," *Nature*, vol. 451, no. 7175, pp. 141–146, 2008.

[15] K. Hochedlinger and R. Jaenisch, "Nuclear reprogramming and pluripotency," *Nature*, vol. 441, no. 7097, pp. 1061–1067, 2006.

[16] O. Tsuji, K. Miura, Y. Okada et al., "Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 28, pp. 12704–12709, 2010.

[17] S. Nori, Y. Okada, A. Yasuda et al., "Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 40, pp. 16825–16830, 2011.

[18] Y. Kobayashi, Y. Okada, G. Itakura et al., "Pre-evaluated safe human iPSC-derived neural stem cells promote functional recovery after spinal cord injury in common marmoset without tumorigenicity," *PLoS One*, vol. 7, no. 12, article e52787, 2012.

[19] P. Lu, G. Woodruff, Y. Wang et al., "Long-distance axonal growth from human induced pluripotent stem cells after spinal cord injury," *Neuron*, vol. 83, no. 4, pp. 789–796, 2014.

[20] Y. Fujimoto, M. Abematsu, A. Falk et al., "Treatment of a mouse model of spinal cord injury by transplantation of human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem cells," *Stem Cells*, vol. 30, no. 6, pp. 1163–1173, 2012.

[21] T. Okubo, A. Iwanami, J. Kohyama et al., "Pretreatment with a γ-secretase inhibitor prevents tumor-like overgrowth in human iPSC-derived transplants for spinal cord injury," *Stem Cell Reports*, vol. 7, no. 4, pp. 649–663, 2016.

[22] Y. Pomeshchik, K. A. Puttonen, I. Kidin et al., "Transplanted human induced pluripotent stem cell-derived neural progenitor cells do not promote functional recovery of pharmacologically immunosuppressed mice with contusion spinal cord injury," *Cell Transplantation*, vol. 24, no. 9, pp. 1799–1812, 2015.

[23] M. Ozaki, A. Iwanami, N. Nagoshi et al., "Evaluation of the immunogenicity of human iPS cell-derived neural stem/progenitor cells in vitro," *Stem Cell Research*, vol. 19, pp. 128–138, 2017.

[24] S. Nishimura, A. Yasuda, H. Iwai et al., "Time-dependent changes in the microenvironment of injured spinal cord affects the therapeutic potential of neural stem cell transplantation for spinal cord injury," *Molecular Brain*, vol. 6, no. 1, p. 3, 2013.

[25] S. E. Nutt, E. A. Chang, S. T. Suhr et al., "Caudalized human iPSC-derived neural progenitor cells produce neurons and glia but fail to restore function in an early chronic spinal cord injury model," *Experimental Neurology*, vol. 248, pp. 491–503, 2013.

[26] T. Iida, A. Iwanami, T. Sanosaka et al., "Whole-genome DNA methylation analyses revealed epigenetic instability in tumorigenic human iPS cell-derived neural stem/progenitor cells," *Stem Cells*, vol. 35, no. 5, pp. 1316–1327, 2017.

[27] G. Itakura, S. Kawabata, M. Ando et al., "Fail-Safe system against potential tumorigenicity after transplantation of iPSC derivatives," *Stem Cell Reports*, vol. 8, no. 3, pp. 673–684, 2017.

[28] K. Miura, Y. Okada, T. Aoi et al., "Variation in the safety of induced pluripotent stem cell lines," *Nature Biotechnology*, vol. 27, no. 8, pp. 743–745, 2009.

[29] C. J. Parr, S. Katayama, K. Miki et al., "MicroRNA-302 switch to identify and eliminate undifferentiated human pluripotent stem cells," *Scientific Reports*, vol. 6, no. 1, article 32532, 2016.

[30] M. R. Simpson-Abelson, J. L. Loyall, H. K. Lehman et al., "Human ovarian tumor ascites fluids rapidly and reversibly inhibit T cell receptor-induced NF-κB and NFAT signaling in tumor-associated T cells," *Cancer Immunology Research*, vol. 13, no. 3, p. 14, 2013.

[31] M. Nakagawa, M. Koyanagi, K. Tanabe et al., "Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts," *Nature Biotechnology*, vol. 26, no. 1, pp. 101–106, 2008.

[32] R. Lister, M. Pelizzola, Y. S. Kida et al., "Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells," *Nature*, vol. 471, no. 7336, pp. 68–73, 2011.

[33] K. Kim, A. Doi, B. Wen et al., "Epigenetic memory in induced pluripotent stem cells," *Nature*, vol. 467, no. 7313, pp. 285–290, 2010.

[34] S. Nori, Y. Okada, S. Nishimura et al., "Long-term safety issues of iPSC-based cell therapy in a spinal cord injury model: oncogenic transformation with epithelial-mesenchymal transition," *Stem Cell Reports*, vol. 4, no. 3, pp. 360–373, 2015.

[35] C. E. Goldring, P. A. Duffy, N. Benvenisty et al., "Assessing the safety of stem cell therapeutics," *Cell Stem Cell*, vol. 8, no. 6, pp. 618–628, 2011.

[36] U. Ben-David and N. Benvenisty, "The tumorigenicity of human embryonic and induced pluripotent stem cells," *Nature Reviews Cancer*, vol. 11, no. 4, pp. 268–277, 2011.

[37] L. Warren, P. D. Manos, T. Ahfeldt et al., "Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA," *Cell Stem Cell*, vol. 7, no. 5, pp. 618–630, 2010.

[38] S. C. Mack, C. G. Hubert, T. E. Miller, M. D. Taylor, and J. N. Rich, "An epigenetic gateway to brain tumor cell identity," *Nature Neuroscience*, vol. 19, no. 1, pp. 10–19, 2015.

[39] J. M. Polo, S. Liu, M. E. Figueroa et al., "Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells," *Nature Biotechnology*, vol. 28, no. 8, pp. 848–855, 2010.

[40] J. B. Kim, H. Zaehres, M. J. Araúzo-Bravo, and H. R. Schöler, "Generation of induced pluripotent stem cells from neural stem cells," *Nature Protocols*, vol. 4, no. 10, pp. 1464–1470, 2009.

[41] J. B. Kim, V. Sebastiano, G. Wu et al., "Oct4-induced pluripotency in adult neural stem cells," *Cell*, vol. 136, no. 3, pp. 411–419, 2009.

[42] A. Bedel, F. Beliveau, I. Lamrissi-Garcia et al., "Preventing pluripotent cell teratoma in regenerative medicine applied to hematology disorders," *Stem Cells Translational Medicine*, vol. 6, no. 2, pp. 382–393, 2017.

[43] Z. Liu, Y. Tang, S. Lu et al., "The tumourigenicity of iPS cells and their differentiated derivates," *Journal of Cellular and Molecular Medicine*, vol. 17, no. 6, pp. 782–791, 2013.

[44] X. Kang, Q. Yu, Y. Huang et al., "Effects of integrating and non-integrating reprogramming methods on copy number variation and genomic stability of human induced pluripotent stem cells," *PLoS One*, vol. 10, no. 7, article e131128, p. e0131128, 2015.

[45] K. Woltjen, I. P. Michael, P. Mohseni et al., "piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells," *Nature*, vol. 458, no. 7239, pp. 766–770, 2009.

[46] M. Takahashi, "Retinal cell therapy using iPS cells," *Rinsho Shinkeigaku*, vol. 53, no. 11, article 1016, 2013.

[47] T. S. Mikkelsen, J. Hanna, X. Zhang et al., "Dissecting direct reprogramming through integrative genomic analysis," *Nature*, vol. 454, no. 7200, pp. 49–55, 2008.

[48] M. Mandai, A. Watanabe, Y. Kurimoto et al., "Autologous induced stem-cell-derived retinal cells for macular degeneration," *New England Journal of Medicine*, vol. 376, no. 11, pp. 1038–1046, 2017.