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ORIGINAL RESEARCH

Intrathecal transplantation of olfactory ensheathing cells by lumbar puncture for thoracic spinal cord injury in mice

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Objectives: To investigate the distribution and function of olfactory ensheathing cells (OECs) following lumbar puncture (LP) transplantation in mouse spinal cord injury (SCI).

Methods: OECs were transplanted by LP at level L3–5, 1 week after transected SCI at T8 vertebra. Mice were killed at 3, 21, and 56 days after LP transplantation, and the relative distribution of cells at T8 vertebra was quantitated. The injured spine was also tested by immunohistochemistry to assess neuron regeneration and scar bridging at 8 weeks posttransplantation. Motor functions of mice were evaluated during the observation period using the Basso Mouse Scale.

Results: OECs were examined and confirmed by studying cell morphology under phase contrast and immunostaining of NGFR p75. LP-transplanted OECs could be detected just 3 days after transplantation ($p75^+$ area: 0.16 mm^2) and accumulated to 0.31 and 0.30 mm^2 at 21 and 56 days postengraftment, respectively. The number of endogenous neurons, −400 to +400 μm, far from the epicenter, in OEC-transplanted mice was more than that in SCI mice without engraftment. SCI lesion of mice in the control group (164.3±3.97 μm) was much longer than that in OEC-grafted group (116.7±3.60 μm). Grafts of OECs induced significant functional improvement in mice that underwent T8 vertebral transection, just from 3 days after cell injection.

Conclusion: LP is a minimally invasive method for OEC transplantation to treat SCI. This is the first study to visualize the distribution and functions of LP-transplanted OECs in the intact and injured spinal cord.

Keywords: olfactory ensheathing cells, spinal cord injury, intrathecal transplantation, lumbar puncture, neuron regeneration, scar bridging

Introduction

Spinal cord injury (SCI) is a devastating disease that has significant human and societal costs.[1,2] In People's Republic of China, there are >400,000 people living with SCI, and it is estimated that 10,000 new cases occur every year, the majority of which result from motor vehicle incidents or sports injuries, violence, or falls from a height. Treatments for SCI include surgical stabilization of the spinal column, decompression surgery, administration of high-dose steroids, and rehabilitation.[3–5] Although substantial progress has been made in the survival of injured individuals, attempts to restore function have remained essentially ineffective.[6]

Stem cell transplantation provides hope for neurorestoration.[7,8] Olfactory ensheathing cells (OECs) are unique glial cells found in the olfactory systems that support olfactory neurogenesis and the retargeting across the peripheral nervous system/the central nervous system boundary in the olfactory system. These cells are able to stimulate tissue sparing and neuroprotection, enhance outgrowth of both intact and

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lesioned axons, activate angiogenesis, and remyelinate axons after a range of demyelinating insults.[9–11]

Currently, OEC transplantation post-SCI is commonly implemented by direct delivery into the injured parenchyma.[12] However, there are many disadvantages of this method, including additional trauma in intramedullary transplantation surgery and the loss of grafted cells in a hostile environment, but intrathecal transplantation by lumbar puncture (LP) and intravascular delivery are minimally invasive techniques. Considering the impenetrable blood–brain barrier, LP is a higher efficient access for cellular transplants.[13,14] Although numerous studies have characterized the neuroprotective effects of OECs in SCI, no studies have explored the distribution and function of OECs following LP transplantation into the injured mouse spinal cord.

We now report a remarkable capability of intrathecal transplanted OECs leading to functional improvement in the lesioned spinal cord of mice. These findings indicate that, despite the inhibitory milieu of the injured spine, OECs could migrate to the sites of SCI, resulting in the formation of new neuronal connection scar bridging that induces activity and locomotion recovery.

Materials and methods

Animals

All the animals were housed according to the guidelines of the Laboratory Animal Center, Soochow University. Also, the surgical procedures and postoperative care were conducted in accordance with the protocols approved by the Laboratory Animal Center, Soochow University.

Preparation of OECs from c57bl/6 mice

OECs were generated from the olfactory bulbs of newborn mice. Each culture was prepared from 10 to 12 dissected postnatal day (P) P1–P3 mouse olfactory bulbs digested with 0.125% trypsin enzyme (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at 37°C, before centrifugation and plating. Briefly, the caudal two-thirds of the olfactory bulbs was dissected free of meninges and white matter, minced with scalpel blades, and incubated for 15 min in 0.125% trypsin enzyme at 37°C in a 5% CO_2 incubator. The tissue was then gently dissociated mechanically and washed. The cell suspension was incubated for 12 h in Dulbecco's modified Eagle medium/F12 (Thermo Fisher Scientific) medium supplemented with 10% fetal bovine serum (Hyclone, South Logan, Utah, USA). Then, we changed the Petri dish and let the suspended cells grow in the Dulbecco's Modified Eagle Medium/F12 medium with 5–10 ng/mL basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO, USA). Next day, we changed the culture medium and obtained the OECs. The purity of OECs was evaluated by immunostaining of anti-p75 (Abcam, Cambridge, MA, USA).

Immunocytochemistry

OECs were fixed in cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.2) overnight, then washed three times with PBS (5 min) and incubated with primary antibodies (Abs) for 2 h at room temperature or overnight at 4°C. Anti-p75 (1:500) was diluted in PBS/0.02% NaN_3/3% bovine serum albumin/0.2% Triton X-100. After incubation with primary Ab, the cells were rinsed with PBS three times for 5 min each before secondary Ab application. Fluorescein isothiocyanate-conjugated rabbit antimouse Abs (Proteintech Group, Chicago, IL, USA) were diluted 1:150 in PBS/0.02% NaN_3/3% bovine serum albumin and applied to the cells for 1 h at room temperature in the dark. The cells were subsequently washed in PBS three times, and the cell nuclei were counterstained with Hoechst 33258 (1:100; Sigma-Aldrich).

Animal model of SCI

A total of 110 adult female c57bl/6 mice (20–25 g) were subjects of this study. The mice were anesthetized with a single intraperitoneal injection of 4% chloral hydrate (4 mg/kg). A sterile laminectomy was performed at the vertebral T8 level without disrupting the dura. The spinal cord was cut into two parts absolutely. After injury, the muscles and skin were closed in layers. The procedure resulted in hind limb paralysis in all the animals. Bicillin (60,000 U/kg, intramuscular route) was administered daily for 7 days after injury to prevent wound and bladder infections, with bladder expression performed twice daily.

Cell transplantation by LP

Cell transplantation was performed at the seventh day after SCI. Briefly, mice were anesthetized as described above and placed prone, and OECs were transplanted by LP. A small longitudinal incision was made over the L3–4 spinous processes, and the skin was retracted. A 50 μL microsyringe needle was advanced into the spinal canal at L3–4. Approximately 1×10^5 OECs diluted in 40 μL PBS were slowly injected into the intrathecal space, and 10 μL PBS was used to flush the needle. To prevent backflow of the injected contents, the LP needle remained in place for at least 2 min and then was slowly withdrawn, after which the skin was sutured with 3-0 nylon.

Immunohistochemistry

For watching OEC distribution, the mice were sacrificed at different time points (3, 21, and 56 days) after LP. For testing

the function of homing OECs, animals were killed at 56 days posttransplantation with an overdose of chloral hydrate (100 mg/kg, intraperitoneally) and perfused transcardially with PBS followed by phosphate-buffered, 4% paraformaldehyde (pH 7.4). The vertebral T8 spinal cords (injury site) were dissected, postfixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose over 1 day, and frozen in isopentane over dry ice. Thoracic segments were cryosectioned sagittally into 20 μm sections and stored at −80°C. Frozen sections were thawed on a slide warmer for 5 min, rehydrated in PBS, permeabilized for 1 h in 0.1% Triton-X 100, and incubated with 10% goat serum for 20 min to prevent nonspecific binding. The following primary Abs were used: rabbit anti-p75-GFP (1:500; Abcam), rabbit anti-NeuN-GFP (1:500; Abcam), and mouse anti-GFAP-Cy3 (1:800; Sigma-Aldrich).

Digital images were captured with an Axioplan microscope (Zeiss, Jena, Germany), an inverted fluorescence microscope (Carl Zeiss Meditec AG, Jena, Germany), and Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON, Canada), and were processed by using GraphPad prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Assessment of locomotor behavior

Using the Basso Mouse Scale (BMS) locomotor rating scale, all the mice were tested for locomotor function by two observers blinded to the treatment groups. BMS testing was performed weekly, from the day of SCI to 8 weeks posttransplantation.

Quantitative and statistical analysis

Neurons in ten sections of a spinal sample at different positions from the SCI epicenter (from 0 to 600 μm both rostral and caudal) were calculated. Lesion size was quantified by measuring the distance between bilateral ends of SCI scar.

All data are presented as the mean ± standard error of mean. Statistical analysis was performed with Student's *t*-test or analysis of variance followed by a Bonferroni–Dunn multiple comparisons test (GraphPad Prism 6 software, GraphPad Software, Inc.). Differences were considered statistically significant when $P<0.05$.

Results

Cell morphology

The morphology of primary OECs was described in our previous report.[15] Significant changes were observed in the cell morphology in culture. OECs displayed random orientation with various shapes after 24 h in culture. On day 4, with the elongation and spreading of OECs, the cells started to cluster and change to bipolar or tripolar shape, and spindle-shaped cell bodies were evident (Figure 1A, B). On day 7, cell protrusions became long and thin and formed a complex network (Figure 1C). Characteristic protein markers for OECs (NGFR p75) were studied by immunocytochemistry. Fluorescence microscopy showed that OECs were all marked by p75 positively before transplantation (Figure 1D).

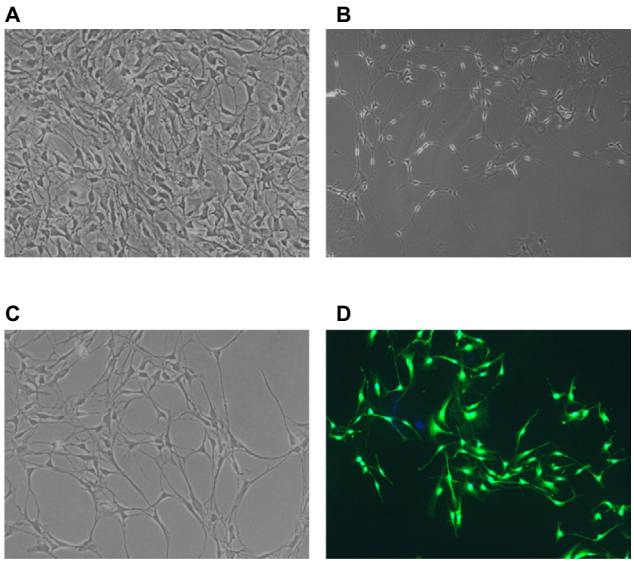

Figure 1 Phenotypes of olfactory ensheathing cells in culture before transplantation.
Notes: Cells were cultured in DMEM/F12 (**A**–**C**) phase contrast and expressed high levels of p75 (green) as shown with (**D**) immunocytochemistry. Magnification: ×200.
Abbreviation: DMEM, Dulbecco's Modified Eagle Medium.

Relative distribution of OECs following LP transplantation

The mouse spinal cord was transected by surgery blade at T8 vertebra. After a 7-day window phase, which is a tough time for transplanted cells to survive, OECs were grafted into intrathecal space by LP. Figure 2 illustrates the OECs were detected in the GFAP-devoid scars after a long homing journey. Also, the homing cells accumulated as time prolonged during the observation. Transplanted OECs could be detected just 3 days after transplantation ($p75^+$ area: 0.16 mm^2; Figure 2A) and increased to 0.31 and 0.30 mm^2 at 21 (Figure 2B) and 56 (Figure 2C) days post-engraftment, respectively. Meanwhile, we noticed that transplanted OECs establish the consummate compensation and the connection between the lacerated ends 56 days after transplantation.

OEC grafts by LP after T8 vertebral transection: endogenous neuron regeneration, scar bridging, and functional improvement

OECs have exceptional plasticity and can support neurogenesis and enhance axonal regeneration throughout life.

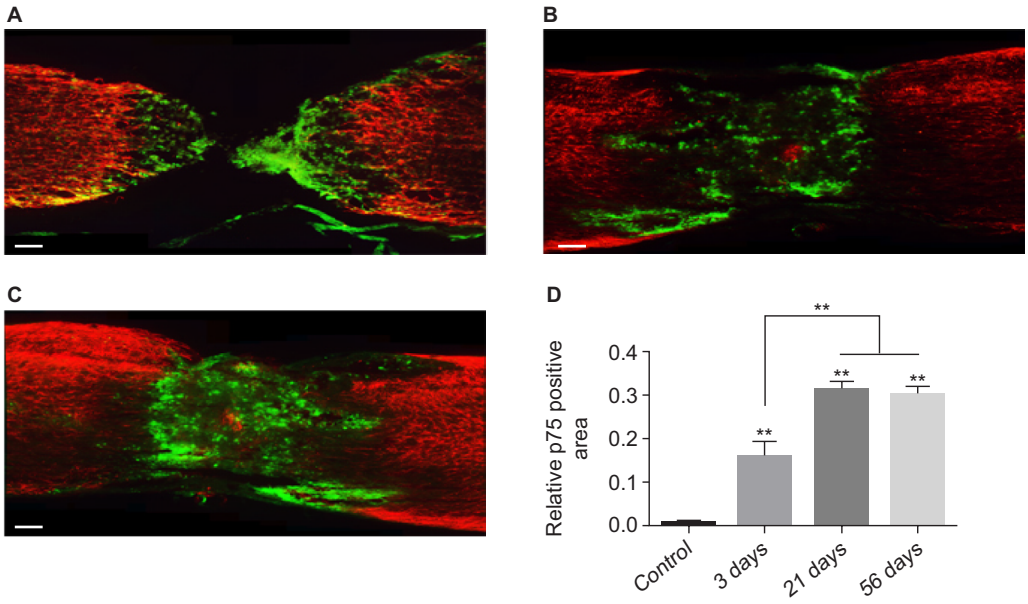

Figure 2 Immunostaining of OECs transplantation.
Notes: Immunohistochemistry showing olfactory ensheathing cells in transected SCI lesion sites (T8) at 3, 21, and 56 days after lumbar puncture transplantation: (**A**) 3 days, (**B**) 21 days, (**C**) 56 days. Sections were double-stained for GFAP (red) and p75 (green). The GFAP-devoid area represents the scar regions after SCI. Scale bar = 100 μm. (**D**) Relative p75 positive area in T8 vertebral section of different groups. **P<0.01.
Abbreviations: OECs, olfactory ensheathing cells; SCI, spinal cord injury.

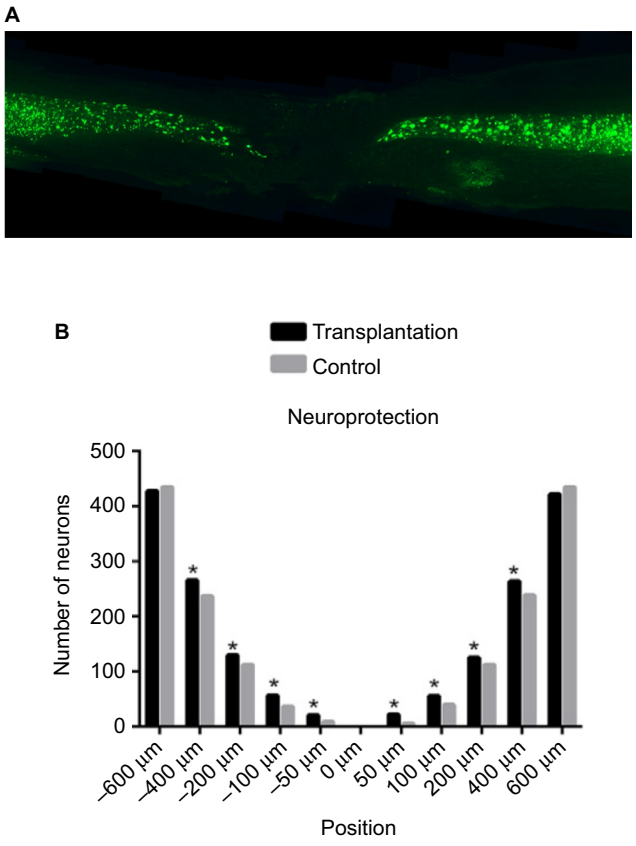

Figure 3 Number of endogenous neurons at different positions from the SCI epicenter.
Notes: (**A, B**) Immunohistochemistry was used to visualize neurons (anti-NeuN-GFP marked, green) in sagittally cryosectioned spine. Neurons were counted at different positions from the epicenter of SCI. Mice in the control group underwent only T8 vertebral transection without OEC injection. Position – to +: rostral–caudal direction. *P<0.05 compared with control.
Abbreviations: SCI, spinal cord injury; OECs, olfactory ensheathing cells.

To clarify the function for SCI of homing OEC grafts by LP, we tested endogenous neuron regeneration by immunohistochemistry. Neurons were stained by NeuN, its specific Ab (Figure 3A). Neurons in ten sections of a spine sample at different positions from the SCI epicenter (from 0 to 600 μm both rostral and caudal) were calculated. As is shown in Figure 3B, the number of endogenous neurons, from −400 to +400 μm, in OEC-transplanted mice is more than that in SCI mice without engraftment. However, it shows no difference exceeding 600 μm far from the epicenter both rostral and caudal.

We also noticed that scar bridging was more explicit in OEC-engrafted mice. Then, a statistical analysis was conducted to assess the distance between bilateral ends of SCI scar (Figure 4A, B; GFAP-avoid area). SCI lesion of mice in the control group (164.3±3.97 μm) was much longer than that in OEC-grafted group (116.7±3.60 μm). The result of statistical analysis are shown in Figure 4C. Surprisingly, we could even see a nearly full bridging at 56 days after OEC transplantation (Figure 4A).

As neuron regeneration and scar bridging occur, we also monitored locomotor recovery from 1 to 56 days posttransplantation by BMS score. Grafts of OECs induced significant functional improvement in mice that underwent T8 vertebral complete transection just 3 days after cell injection (Figure 5), with movement about all joints of the lower extremities.

To distinguish OECs and other cells in mice, we labeled OECs with Hoechst 33258 (Figure 6).

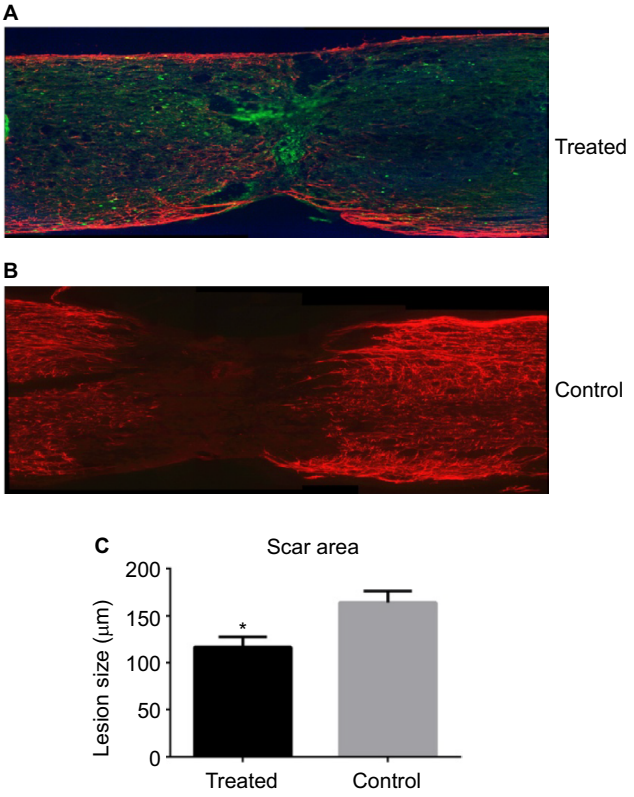

Figure 4 Scar bridging after intrathecal injection of OECs by immunohistochemistry.
Notes: (**A**, **B**) Representative images of scar healing in OEC-treated and control groups, respectively. Anti-GFAP-Cy3 antibody was used to reveal the SCI scar which is not stained red. Mice in control group were subjected to only T8 vertebral transection without OEC injection. (**C**) Distance between the rostral and the caudal cliff was calculated and analyzed. Data are shown as the mean ± SEM from at least three independent experiments (*P<0.05 vs control).
Abbreviation: OECs, olfactory ensheathing cells; SCI, spinal cord injury; SEM, standard error of mean.

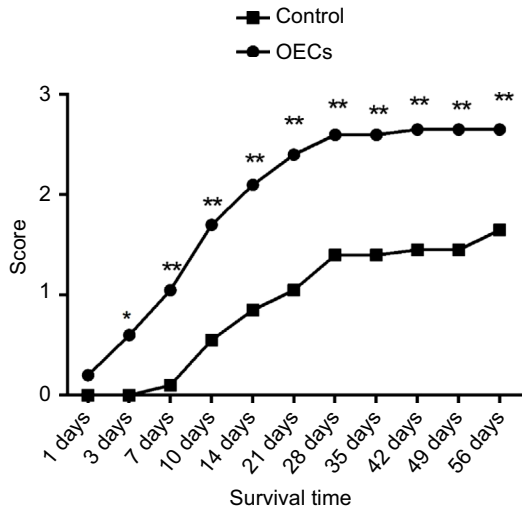

Figure 5 Significant functional improvement was found after OEC grafts were transplanted by lumbar puncture injection to sites of T8 complete transection (*P<0.05, **P<0.01).
Note: Mice locomotion was evaluated from 1 to 56 days posttransplantation using BMS.
Abbreviations: BMS, Basso Mouse Scale; OECs, olfactory ensheathing cells.

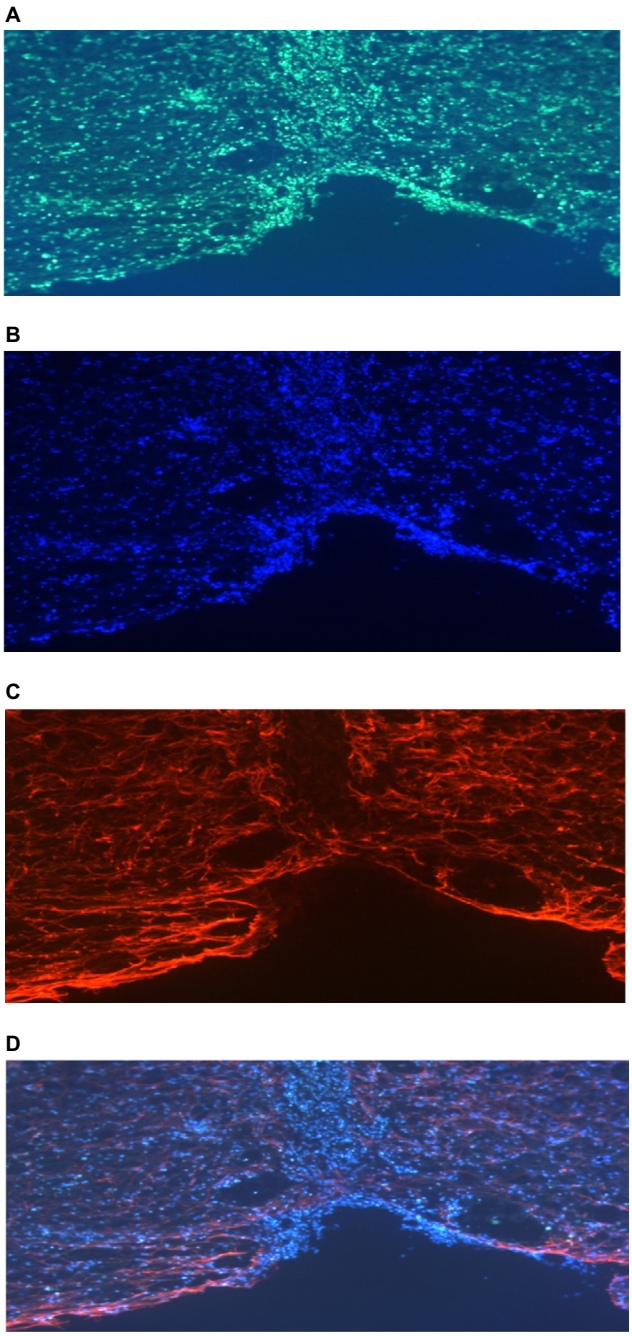

Figure 6 Immunostaining of OECs 7 days after transplantation.
Notes: (**A**) OECs stained with p75 (green). (**B**) OECs labeled with Hoechst 33258 (blue). (**C**) The spinal cord stained for GFAP (red), which shows the edge of the scar. (**D**) Merged figure of (**A**–**C**). All these indicate OECs have been transplanted successfully.
Abbreviation: OECs, olfactory ensheathing cells.

Discussion

In recent years, much attention has been paid to secondary cord injury because this appears to be susceptible to therapeutic intervention. Delivery of cells to an already compromised spinal cord is a major unresolved problem in SCI. Direct injection has been commonly used to transplant cells into injured spinal cord parenchyma.[16] Although this

is acceptable in animal experiments, it is hard to implement in clinical treatment of human SCI with a neurosurgical invasive manipulation. Another problem accompanies direct parenchymal injections, which is additional trauma to the spine in intramedullary transplantation.

To overcome these limitations, some investigators have introduced intravascular delivery of therapeutic cells.[17] However, intra-arterial delivery of cells for the treatment of spinal cord disorders is limited by the impenetrable blood–brain barrier and the multisegmental arterial supply of the spinal cord, which requires highly selective and technically challenging cannulation of the spinal arteries.[13]

LP provides ideal access for cellular transplants with higher efficiency and less host immune response, and is a minimally invasive procedure that can be performed at the bedside after injection of a local anesthetic.[18] In humans, LP is performed at the L3–4 level, far away from the cervical or thoracic spinal cord that most commonly suffers from SCI. This makes LP delivery of stem cells relatively safe and unlikely to worsen compromised patients as a direct result of the intervention. Currently, no studies have characterized the distribution and function of OECs following LP transplantation after SCI.

We found that OECs migrate into the SCI scars after a long journey and the homing cells start to accumulate just 3 days after transplantation.

Three days after cell transplantation (10 days postinjury), some early signs are found which indicate the ability for late restoration. Actually, in our study, we could find some positive changes in behavioral testing. We can speculate that OECs secreted some cell inducible factors, which can increase the survival ability of neurons. These changes lead to the survival of more active cells, and thus, we may find some exciting results in our study. Meanwhile, we noticed that transplanted OECs establish perfect compensation and connection between the lacerated terminal 56 days after transplantation (Figure 2). It is confirmed that LP is an ideal passage for OEC grafts to locate at the epicenter of SCI. Also, the number of homed cells is considerable as time goes by.

Cell transplantation paradigms have shown great promise in improving outcomes after SCIs; different cell types have been tested in various injury models.[19] OEC, as a hopeful candidate for treating SCI, has been found to reduce the volume of injured tissue, promote axonal regeneration, and recover some functions.[20] The proposed mechanisms of grafted OECs include their interaction with astrocytes, modulation of early inflammation after injury, remyelination and enhancement of axonal regeneration, and stimulation of angiogenesis.[21–23]

As SCI induces apoptosis or death of neurons and scar formation, we examined neuron regeneration and scar bridging to evaluate the therapeutic efficacy of LP-transplanted OECs.[24–26] Figure 3 shows that the number of endogenous neurons, from −400 to +400 μm, in OEC-transplanted mice is more than that in SCI mice without engraftment. Intrathecal transplanted OECs could promote neuron regeneration in this range. This could be a result of the OECs inducing the differentiation of stem cells into functional neurons and the modulation of early neuroinflammatory response.[27,28]

We also noticed that scar bridging was more explicit in OEC-engrafted mice. It is evident from Figure 4 that LP-transplanted OECs accelerate scar bridging. Surprisingly, we could even see a nearly full bridging at 56 days after OEC transplantation. The chemotactic factors, such as CCL2 and CCL3, can promote astrocytes to be active and accumulative in an injured region.[29] Activated astrocytes can form glial scars, which block axonal regeneration and repair of nerve cells.[30,31] OECs can significantly reduce the expression of CCL2 and CCL3, reducing the activation of astrocytes, which leads to scar repair.[24]

In addition, this study also showed that intrathecal grafted OECs resulted in substantial improvement in motor function by higher open-field BMS score. This is the "golden standard" to prove a therapy method is effective. Neuron regeneration and scar bridging could be partly responsible for this functional improvement in OEC-treated SCI mice.

Currently, accumulating evidence recommends the possible use of OECs in the treatment of brain or spinal cord disorders.[32,33] However, delivery methods of OECs should be reformed with higher efficiency and less side effects, rather than direct injection. Our study confirmed that LP-transplanted OECs respond to the inflammatory chemokines secreted and migrate toward the site of injury. The homing cells facilitate neuron regeneration and scar bridging after SCI and lead to locomotory improvement. Thus, future efforts should reveal the underlying mechanism by which the OECs locate at the sites of SCI. More intensive studies are required to further delineate whether this delivery access is suitable and safe enough to use in clinical treatment.

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Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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