Functional recovery in acute traumatic spinal cord injury after transplantation of human umbilical cord mesenchymal stem cells

Sheng-Li Hu, MD; Hai-Shui Luo, MD; Jiang-Tao Li, MM; Yong-Zhi Xia, MD; Lan Li, MM; Li-Jun Zhang, MD; Hui Meng, MD; Gao-Yu Cui, MD; Zhi Chen, MD; Nan Wu, MD; Jiang-Kai Lin, MD; Gang Zhu, MD; Hua Feng, MD

Objective: **Spinal cord injury results in loss of neurons, degeneration of axons, formation of glial scar, and severe functional impairment. Human umbilical cord mesenchymal stem cells can be induced to form neural cells *in vitro*. Thus, these cells have a potential therapeutic role for treating spinal cord injury.**

Design and Setting: **Rats were randomly divided into three groups: sham operation group, control group, and human umbilical cord mesenchymal stem cell group. All groups were subjected to spinal cord injury by weight drop device except for sham group.**

Subjects: **Thirty-six female Sprague-Dawley rats.**

Interventions: **The control group received Dulbecco's modified essential media/nutrient mixture F-12 injections, whereas the human umbilical cord mesenchymal stem cell group undertook cells transplantation at the dorsal spinal cord 2 mm rostrally and 2 mm caudally to the injury site at 24 hrs after spinal cord injury.**

Measurements: **Rats from each group were examined for neurologic function and contents of brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and neurotrophin-3. Survival, migration, and differentiation of human umbilical cord mesenchymal stem cells, regeneration of axons, and formation of glial scar were also explored by using immunohistochemistry and immunofluorescence.**

Main Results: **Recovery of hindlimb locomotor function was significantly enhanced in the human umbilical cord mesenchymal stem cells grafted animals at 5 wks after transplantation. This recovery was accompanied by increased length of neurofilament-positive fibers and increased numbers of growth cone-like structures around the lesion site. Transplanted human umbilical cord-mesenchymal stem cells survived, migrated over short distances, and produced large amounts of glial cell line-derived neurotrophic factor and neurotrophin-3 in the host spinal cord. There were fewer reactive astrocytes in both the rostral and caudal stumps of the spinal cord in the human umbilical cord-mesenchymal stem cell group than in the control group.**

Conclusions: **Treatment with human umbilical cord mesenchymal stem cells can facilitate functional recovery after traumatic spinal cord injury and may prove to be a useful therapeutic strategy to repair the injured spinal cord. (Crit Care Med 2010; 38:2181–2189)**

Key Words: functional recovery; mesenchymal stem cells; spinal cord injury; transplantation; umbilical cord

Spinal cord injury (SCI) always results in severe functional impairment and leads to high cost for the family and society. A variety of methods including medicine, surgery, rehabilitation, and gene therapy have been used to treat SCI. Although these treatments may provide benefits, many patients still cannot obtain substantial functional recovery (1, 2). Thus, the search for effective therapeutic methods for SCI is of major public health importance.

In recent years, stem cell treatment for SCI has been extensively investigated and may be a viable therapeutic strategy. Previous research found that embryonic stem cells (3) and adult stem cells, including bone marrow-mesenchymal stem cells (BM-MSCs; 4) and neural stem cells (5), were able to promote locomotor recovery after SCI. However, the application of embryonic stem cells is limited in a clinical setting because of ethical issues arising from the destruction of human embryo when the inner mass is removed (6, 7). BM-MSCs also have their own limitations due to the disadvantage of low frequencies and limited proliferation/differentiation capacity with age (8, 9). Furthermore, neural stem cells decrease in neurogenesis and undergo replicative senescence over time (10).

Obtained studies have found that stem cells could be obtained from embryo-derived tissues, such as amnion (11), amniotic fluid (12), umbilical cord blood (13), and umbilical cord, itself (14, 15). These tissues are discarded after birth, so isolation of these stem cells is noninvasive and safe for both mothers and children. In 2003, Mitchell et al (16) firstly identified MSCs from human umbilical cord-Wharton's jelly. It was reported that human umbilical cord (hUC)-MSCs have greater *ex vivo* expansion capabilities, faster proliferation, and lower immunogenicity than BM-MSCs (17, 18). Furthermore, they can be induced to differentiate *in vitro* into bone, cartilage, adipose (19, 20), skeletal muscle cells (21), cardiomyocytes (22, 23), endothelium (24), and especially neural cells (16, 25–27). Therefore, the cells posses a potential therapeutic role for treating patients with neurodegenerative diseases and central nervous system injuries.

For these reasons, the purpose of this study is to explore the potential efficacy of hUC-MSCs transplanted into the spinal cord to promote axon regeneration and functional recovery after SCI by using a contusion model in rats. We provide the

From the Department of Neurosurgery, Southwest Hospital, Third Military Medical University, Chong-Qing, China.

This work was supported, in part, by grants from the National Natural Science Foundation of China, Peking, China (0330220, 30572167, 30872735), and from the Natural Science Foundation of Chong-Qing, Chong-Qing, China (2008BA5002).

The authors have not disclosed any potential conflicts of interest.

For information regarding this article, E-mail: fenghua8888@yahoo.com.cn

DOI: 10.1097/CCM.0b013e3181f17c0e

first demonstration that hUC-MSCs obtained from Wharton's jelly can survive, migrate, and produce large amounts of glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3) in the rat spinal cord without immunologic suppression therapy, which may help spinal cord repair.

MATERIALS AND METHODS

Cell Culture

All parts of this study, especially the isolation of the human umbilical cord (hUC), were performed according to the Declaration of Helsinki. Ethical approval was obtained from the Third Military Medical University (Chong-Qing, China), and written informed consent was obtained from donors of UC. The isolation and culture of hUC-MSCs was carried out according to the methods previously described (28). In brief, each hUC was collected from full-term Caesarian section births and processed within 3–6 hrs. Umbilical arteries and veins were removed, and the remaining tissue was transferred to a sterile container in Dulbecco's modified essential media/nutrient mixture F-12 (DMEM/F12; Hyclone, Fisher Scientific, Logan, UT) and diced into small fragments. The explants were transferred to 50-mL culture flasks containing the DMEM/F12 along with 10% fetal bovine serum (FBS; PAA, Linz, Austria). They were left undisturbed for 4–6 days to allow migration of cells from the explants, at which point the media was replaced. Cultures were maintained at 37°C in an incubator containing 5% CO2. They were re-fed and passaged as necessary.

Animal Preparation and Experimental Protocol

The animal study protocol used in this research was approved by the ethics committee for animal experimentation and was conducted according to the Guidelines for Animal Experimentation of our institute. Thirty-six female Sprague-Dawley rats (from Experimental Animal Center of Third Military Medical University) weighing between 230 and 270 g were used. Rats were allowed free access to food and water before and after the surgical procedure. They were randomly divided into three groups: sham operation group (n = 12), control group (n = 12), and hUC-MSC group (n = 12). All groups were subjected to SCI by weight drop device based on the technique described by Wamil et al (29) except for the sham group. The control group received DMEM/F12 injections, whereas the hUC-MSC group undertook cells suspension treatments. Rats from each group were examined for neurologic function and contents of brain-derived neurotrophic factor (BDNF), GDNF, and NT-3. Survival, migration, and differentiation of hUC-MSCs, regeneration of axons, and formation of glial scar were also explored with immunohistochemistry and immunofluorescence.

Surgical Procedures

Animals were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). A 4-cm-long skin incision was made in the midline and a laminectomy was carried out at the thoracic 9–11 level to expose the thoracic 9–11 spinal segment, leaving the dura intact. An area of the thoracic 10 segment in the spinal cord was contused by delivery of a 12.5$g \times$ cm force, produced by dropping a 10-g weight a distance of 1.25 cm through a stainless steel guide tube onto a 2.3-mm diameter steel cylinder. The sham operation rats were treated identically except for the contusion. After impact, the skin and muscles incision was closed by using sutures.

Transplantation of Superparamagnetic Iron Oxide-Labeled hUC-MSCs

One day after SCI, rats were anesthetized with pentobarbital (40 mg/kg, intraperitoneal). The spinal cord was reopened at the injury area. To prepare the cell suspension, passage 5 hUC-MSCs were collected and resuspended in the appropriate volume of DMEM/F12 to produce the final concentration ($4 \times 10^4/\mu l$). Cell viability was assessed by trypan blue. The cells were then loaded into a 5-μl microsyringe mounted on a stereotactic apparatus for transplantation. Cells were injected into the dorsal spinal cord 2 mm rostrally and 2 mm caudally to the injury site at a depth of 1.2 mm and 0.75 mm laterally from midline at a rate of 1 μl/min. At each site, 2.5 μl of cell suspension was injected. The needle was left in position for a further 2 min before being slowly withdrawn. A total volume of 10 μl of cell suspension was injected (total 4×10^5 cells). After operation, incision was closed by using sutures, and animals were allowed to recover. Control group rats received 10-μl DMEM/F12 injections.

Basso, Beattie, and Bresnahan Open Field Locomotion Score

The Locomotor Rating Scale was used to assess locomotor recovery in an open field (30). Before testing, bladders were expressed, because spontaneous bladder contraction often accompanies hind-limb activity. For examination, the rats were placed individually in an open field with a nonslippery surface. The 22-point (0–21) Basso, Beattie, and Bresnahan (BBB) scale was used to assess hindlimb locomotor recovery including joint movements, stepping ability, coordination, and trunk stability. A score of 21 indicates unimpaired locomotion as observed in uninjured rats. The duration of each session was 5 min per rat. BBB tests were performed at 1 day after injury, and after transplantation of hUC-MSCs, the rats were tested at 1 wk, 3 wks, 5 wks, 7 wks, and 8 wks.

In Vitro Immunocytochemistry

For *in vitro* immunocytochemistry, passage 4/5 hUC-MSCs were collected and plated on glass slides placed in 24-well plate. The cells were grown in a culture medium containing DMEM/F12 plus 10% fetal bovine serum (FBS). Then, the cultures were grown for 2–3 days, at which time they were fixed with 4% paraformaldehyde (PFA) and washed three times with PBS. The cultures were blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 1 hr at room temperature and exposed to the following primary antibodies: rabbit anti-nestin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-GDNF (1:50; Bo-Ao-Sen, Peking, China), rabbit anti-BDNF (1:50; Santa Cruz Biotechnology), and rabbit anti-NT-3 (1:100; Santa Cruz Biotechnology). The cultures were incubated with the primary antibody for 1.5 ht at room temperature or overnight at 4°C. Cultures were washed three times with PBS, treated with fluorescent fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:300; Millipore, Billerica, MA) for 1 hr, washed three times with PBS, counterstained the nuclei with 4′, 6-diamidino-2-phenyl-indole, and coverslipped. The images were taken by using a Zeiss (Thornwood, NY) 510META laser confocal microscope.

Assessment of hUC-MSCs Migration and Differentiation

For the assessment of the possible differentiation of hUC-MSCs into subpopulations of neurons, astrocytes, or oligodendrocytes, we applied double staining for human nuclei (hNu) antigen (31) and β-tubulin III, glial fibrillary acidic protein (GFAP), and platelet-derived growth factor-α receptor, respectively.

Animals were killed with an overdose of pentobarbital and perfused transcardially with 4% PFA in 0.1 M PBS, pH 7.4. The spinal cords were subsequently postfixed in the perfusing solution for 24 hrs at 4°C. Then, the tissues were cryoprotected in 30% sucrose in PBS for 24 hrs at 4°C. A 1.5 cm length of the spinal cord centered at the injury site was separated and embedded in tissue embedding medium on dry ice. Cryostat sections (20 μm) were cut and mounted onto poly-L-lysine-coated slides and stored at −70°C. For immunostaining, the frozen slides were air dried at room tem-

perature for 30 min and washed three times with PBS. Then they were blocked by using 5% normal goat serum and 0.3% Triton X-100 in PBS for 1 hr at room temperature, and the primary antibody was applied for 1.5 hrs at room temperature or overnight at 4°C.

The following primary antibodies were used: rabbit anti-platelet-derived growth factor-α receptor (1:50; Santa Cruz Biotechnology) for oligodendrocyte progenitor cells, rabbit anti-GFAP (1:50; Zhong-Shan, Peking, China) for astrocytes, and rabbit anti-β-tubulin III (1:50; Sigma-Aldrich, St. Louis, MO) for neurons. The slides were washed in PBS three times and incubated with fluorescent fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:300; Millipore, Billerica, MA) for 1 hr. In double staining for human nuclei, the slides were treated with mouse anti-hNu (to label cells of human origin; 1:30; Millipore, Billerica, MA) first and then incubated with Cy3-conjugated goat anti-mouse secondary antibody (1:400; Bi-Yun-Tian, Peking, China). The slides were washed three times with PBS, counterstained the nuclei with 4′, 6-diamidino-2-phenylindole, and coverslipped. The images were taken by using a Zeiss (Thornwood, NY) 510META laser confocal microscope.

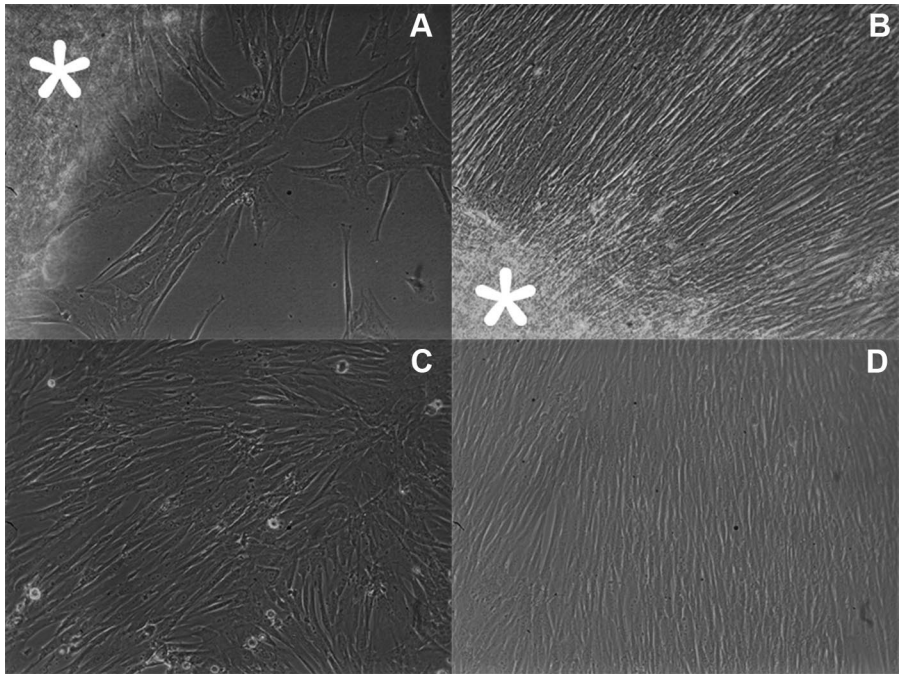

Figure 1. Characteristics of human umbilical cord mesenchymal stem cells. *A* and *B*, The primary isolates migrate from the Wharton's jelly tissue (asterisk) and demonstrate a fibroblast-like or spindle-shaped morphology in culture (original magnification, ×100). *C* and *D*, After passaged, human umbilical cord mesenchymal stem cells still grow as adherent cells with a spindle morphology (original magnification, ×100).

Immunohistochemistry on Tissue Sections

Spinal cord sections were treated with a blocking solution for 1 hr in order to prevent nonspecific antibody-antigen binding. Immunohistochemistry was performed by using primary antibodies against neurofilament-200 (NF-200; 1:100; Bo-Ao-Sen) for axons, GFAP (1:50; Zhong-Shan, Beijing, China) for astrocytes, and growth associated protein-43 (GAP-43; 1:100; Bo-Ao-Sen) for growth cone, as well as biotin-conjugated goat anti-rabbit secondary antibodies (working dilution; Zhong-Shan), followed by avidin-horseradish peroxidase complex (SP KIT, Zhong-Shan) and 3,3′-diaminobenzidine (DAB KIT, Zhong-Shan). Tissue sections were dehydrated and coverslipped. Pathologic changes and quantification of the nerve fibers and astrocytes in the injured site of the spinal cord were observed under an optical microscope and analyzed by software ImagePro Plus 5.1 (Media Cybernetics, Inc., Silver Spring, MD).

Enzyme-Linked Immunosorbent Assay (ELISA) of Neurotrophin Expression in the Injured Spinal Cord

A total of six animals received contusive SCI as described above. One day later, they were then randomly divided into two groups, which received hUC-MSCs or DMEM/F12 in-

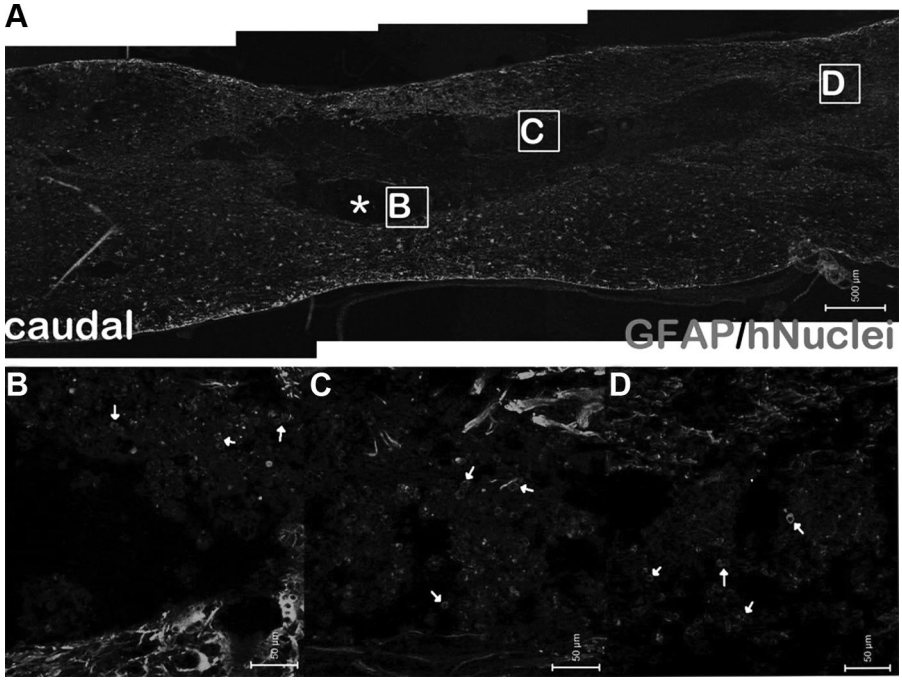

Figure 2. Human umbilical cord mesenchymal stem cells survive and engraft within the injured spinal cord. Immunofluorescence for human nuclei (*hNuclei*) antigen and glial fibrillary acidic protein (*GFAP*) reveals extensive human cells survival and engraftment within the injured rat spinal cord. *A–D*, By 8 wks, many human cells have migrated away from the implantation site for approximately 5 mm along the rostrocaudal axis into the rim of the injured spinal cord and even at the epicenter. *B*, Injury site (*asterisk*); *D*, implantation site; *arrow*, Human umbilical cord mesenchymal stem cells bar. Bar, 500 μm (*A*); bar, 50 μm (*B–D*).

jections, respectively, at four sites as described above. Three weeks after transplantation, animals were anesthetized with pentobarbital (40 mg/kg) and transcardially perfused with 250 mL of ice-cold normal saline, and 1.3 cm of spinal cord centered at injured site was used for protein isolation. Spinal cords were homogenized with buffer consisting of 50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and protease inhibitor (Bi-Yun-Tian) and centrifuged for 15 min at 1500 × *g* at 4°C to remove cell debris. Expression of GDNF, BDNF, and NT-3 was examined by commercially available sandwich technique ELISA kit (R&D Systems, Minneapolis, MN) following the recommended protocol of the manufacturer. Oxidized horseradish peroxidase enzymatic products in the microplate wells were read at 450 nm absorbance using a Bio-Rad Model 550 plate reader (Hercules, CA). Human recombinant GDNF, BDNF, and NT-3 (R&D Systems) was used to generate a standard curve. Neurotrophin levels were then normalized against total protein.

Statistical Analysis

All data are reported as $\bar{\chi} \pm s$. Statistical analysis of changes in relative length of NF were performed by using two-tailed unpaired *t* test. Changes in optical density (OD) of GFAP staining and contents of GDNF, BDNF, and NT-3 were compared by using one-way ANOVA. BBB scores of the three rat groups were analyzed by repeated measure ANOVA. A *p* value of ≤ 0.05 was considered significant.

RESULTS

Culture of MSCs in hUC

The hUC-MSCs were successfully isolated from all of the 10 umbilical cords and examined by phase contrast imaging. The primary isolates demonstrated a fibroblast-like or spindle-shaped morphology in confluent layers in culture (Fig. 1, *A* and *B*). We isolated 5×10^5 cells per centimeter of cord length. After passaged, hUC-MSCs grew as adherent cells with a spindle morphology that was evident as early as passage 1 (Fig. 1*C*). At passage 9, the populations were morphologically similar to the parent cells (Fig. 1*D*).

hUC-MSCs Survive and Engraft within the Injured Spinal Cord

Anti-hNu antigen immunostaining was used to trace the survival and migration pattern of the hUC-MSCs. Rats receiving hUC-MSCs grafts were euthanized at 4 and 8 wks posttransplantation. Immunofluorescence for hNu antigen revealed extensive human cells survival and engraftment within the injured rat spinal cord. A series of sections showed that large numbers of hUC-MSCs survived at least for 8 wks after transplantation. At 4 wks posttransplantation, numerous human cells had migrated out from the injection site. By 8 wks, many human cells

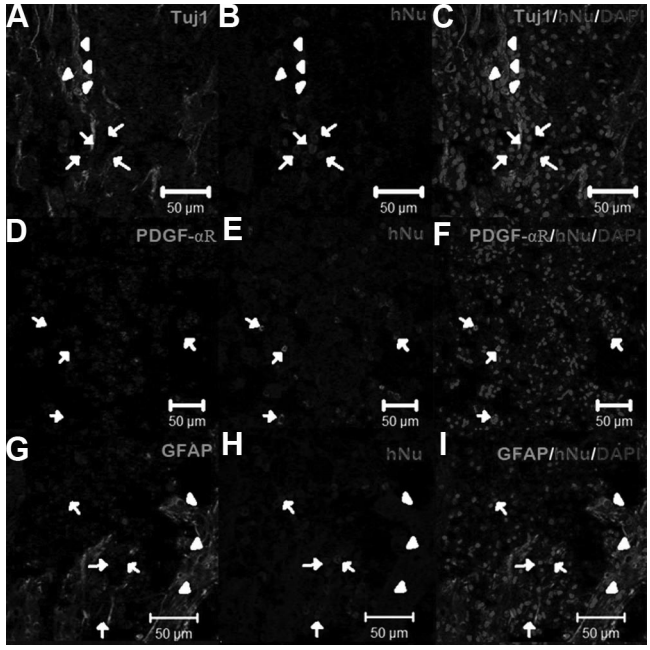

Figure 3. Human umbilical cord-mesenchymal stem cells (hUC-MSCs) remain undifferentiated in the host spinal cord. *A–C*, Double-staining of anti-human nuclei antigen (*hNu*) and anti–tubulin III (*Tuj1*) reveals that most hUC-MSCs do not differentiate into neurons. *D–F*, Double-staining of anti-hNu antigen and antiplatelet-derived growth factor-receptor (*PDGF-R*) shows that the majority of hUC-MSCs have not differentiated into oligodendrocytes. *G–I*, Staining with anti-hNu antigen and anti-glial fibrillary acidic protein (*GFAP*) indicates that most hUC-MSCs are GFAP-negative cells, suggesting these cells do not differentiate into astrocytes. (*Arrow*, hUC-MSCs; *arrowhead*, host cells; bar, 50 μm).

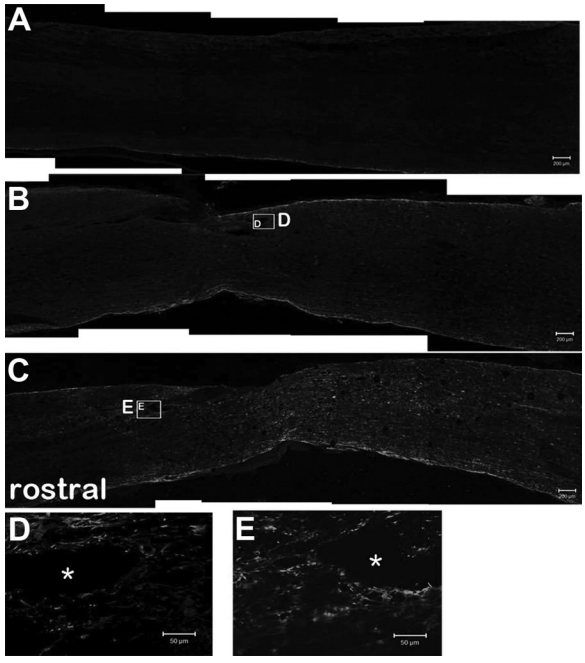

Figure 4. Immunofluorescence for glial fibrillary acidic protein reveals that human umbilical cord-mesenchymal stem cells inhibit activation of astrocytes. *A*, Sham group. Immunofluorescence shows increased expression of glial fibrillary acidic protein in spinal tissues near the injury site in the control group (*C* and *E*) compared with the human umbilical cord-mesenchymal stem cell group (*B* and *D*). Bar, 200 μm, *A–C*; bar, 50 μm, *D* and *E*; injury site (*asterisk*).

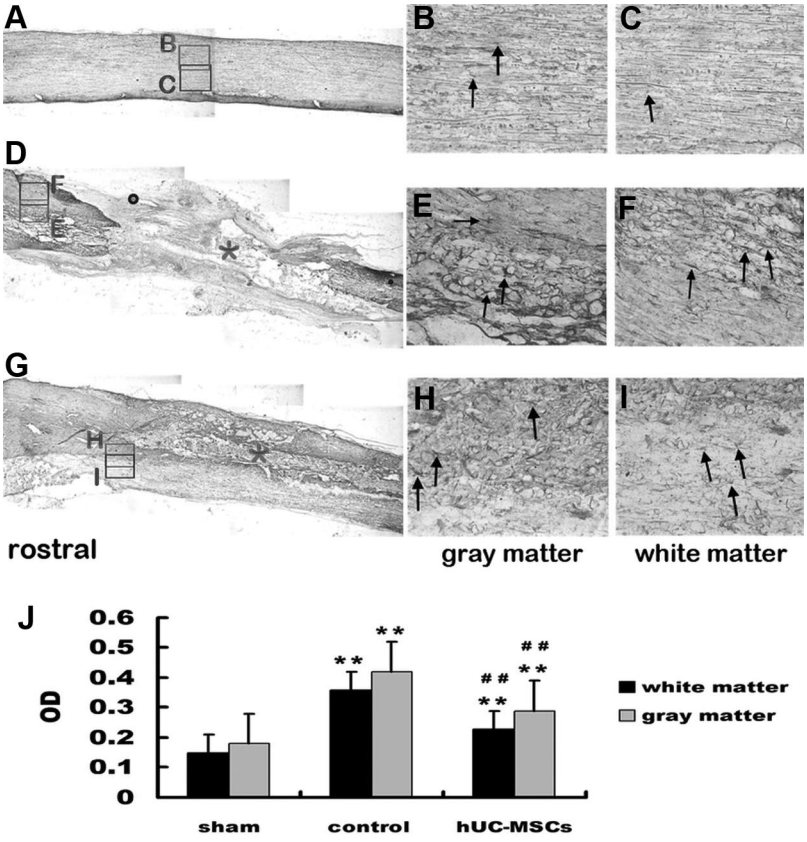

Figure 5. Immunohistochemistry for glial fibrillary acidic protein reveals that human umbilical cord-mesenchymal stem cells (*hUC-MSCs*) inhibit formation of glial scar in injured spinal cord. *A–C*, Sham group. Astrocytes appear to be permissive and do not form a prominent glial limitans in the hUC-MSCs group (*G–I*), which is different from the formation of dense scar barrier in the control group (*D–F*). Quantitative analysis shows that the stains of glial fibrillary acidic protein in the hUC-MSCs group decreased significantly in the white and gray matters near the injury site compared with the control group (*J*). *Arrow*, astrocytes; *A*, *D*, and *G*, original magnification ×40 ; *B*, *C*, *E*, *F*, *H*, and *I*, original magnification, ×250; injury site (*asterisk*); $^{**}p < .01$ compared with the sham group; $^{\#\#}p < .01$ compared with the control group.

had migrated away from the implantation site for approximately 5 mm along the rostrocaudal axis. hNu-immunopositive cells were found in both gray and white matter. In sagittal sections, migration into the rim of spared tissue in the contused cord, even at the epicenter, was frequently observed (Fig. 2, *A–D*).

hUC-MSCs Remain Undifferentiated in the Host Spinal Cord

Double-staining of anti-hNu antigen and anti-β-tubulin III revealed that most hUC-MSCs did not differentiate into neurons (Fig. 3, *A–C*). Double-staining of anti-hNu antigen and anti-platelet-derived growth factor-α receptor showed that the majority of hUC-MSCs had not differentiated into oligodendrocytes (Fig. 3, *D–F*). Staining with anti-hNu antigen and anti-GFAP indicated that most hUC-MSCs were GFAP-negative cells, suggesting these cells did not differentiate into astrocytes (Fig. 3, *G–I*).

hUC-MSCs Inhibit Formation of Glial Scar

Anti-GFAP immunostaining was performed to assess the distribution of astrocytes and formation of glial scar at 8 wks after contusion. Immunofluorescence showed increased expression of GFAP in spinal tissues near the injury site in the control group compared with the hUC-MSCs group (Fig. 4, *A–E*). These astrocytes were packed tightly together as a scar barrier (Fig. 5, *D–F*). However, in the hUC-MSCs group, the astrocytes appeared to be permissive and did not form a prominent glial limitans to completely block regenerative axons (Fig. 5, *G–I*). Our quantitative analysis showed that the stains of GFAP in the hUC-MSCs group decreased significantly in the white and gray matters near the injury site compared with the control group (Fig. 5*J*).

hUC-MSCs Enhance Neuroregeneration and Provide Neuroprotection

Eight weeks after contusion, the neurofilament-positive fibers near the lesion site were scarce in the control group (Fig. 6, *A* and *B*). Quantification was performed in spinal sections obtained from the site. The average relative length of neurofilament-positive axons in the control group was 53.6 ± 3.4. In the hUC-MSCs group, the neurofilament-positive fibers near the lesion site were prominently labeled (Fig. 6, *C* and *D*). The average relative length was 89.2 ± 11.3, which was statistically greater than that of the control group (Fig. 6*E*). In the hUC-MSCs group, numerous GAP-43-positive cells and growth cone-like structures were observed near the injury site, whereas they were scarce in the control group (Fig. 7, *A–F*).

hUC-MSCs Express Neurotrophin in Rat Spinal Cord

In vitro immunocytochemisty showed that most hUC-MSCs were GDNF- and NT-3-positive (Fig. 7, *G* and *H*), whereas they did not express nestin and BDNF (data not shown). We also used an ELISA assay to examine whether hUC-MSCs could stably secret GDNF, NT-3, and BDNF into the injured spinal cord after transplantation. As shown in Figure 7*I*, expression of GDNF and NT-3 in animals that received grafts of hUC-MSCs (33.38 ± 5.18 pg/mg and 18.76 ± 2.39 pg/mg) was significantly higher compared with ones that received grafts of DMEM/F12 (23.09 ± 2.39 pg/mg and 12.20 ± 1.20 pg/mg) at 3 wk posttransplantation. But there is no statistical significance in the expression of BDNF between the hUC-MSCs group (43.60 ± 10.02 pg/mg) and the control group (41.19 ± 9.34 pg/mg) (Fig. 7*I*).

hUC-MSCs Promote Locomotor Recovery

There is no evident locomotor dysfunction in the sham group. BBB scores were improved over time in the hUC-MSCs group and control group. Scores were significantly higher at week 3, 5, 7, and 8 than at week 1 after injury. More importantly, BBB scores in hUC-MSCs-

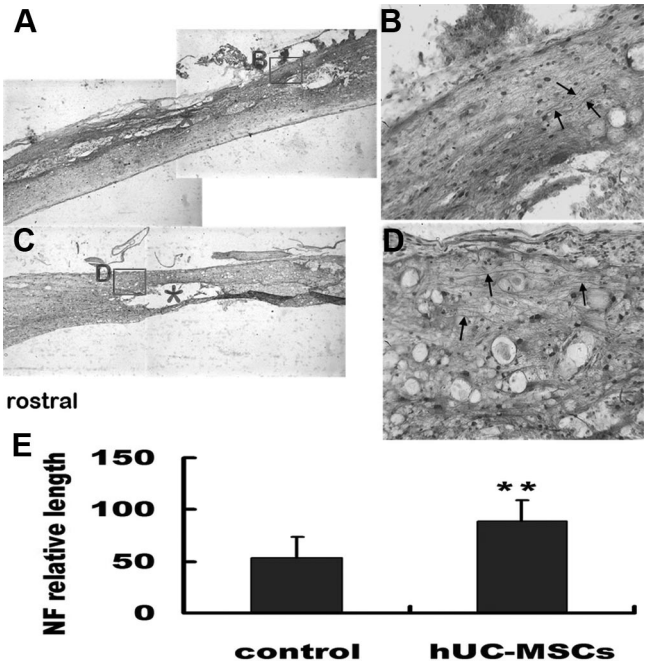

Figure 6. Human umbilical cord mesenchymal stem cells (*hUC-MSCs*) provide neuroprotection. The neurofilament (*NF*)-200-positive fibers near the lesion site are scarce in the control group (*A* and *B*) whereas in the hUC-MSCs group, the NF-positive fibers are prominently labeled (*C* and *D*). Quantitative analysis shows that the average relative length of NF-positive axons in the hUC-MSCs group is statistically greater than that in the control group (*E*). *Arrow*, NF200-positive fibers; Original magnification, ×40, *A* and *C*; original magnification, ×250, *B* and *D*; injury site (*asterisk*); $^{**}p < .01$ compared with the control group.

grafted animals were 13.3, 14.8, and 15.8 at 5, 7, and 8 wks after graft, respectively, which were significantly higher than those of the DMEM/F12-grafted group (12.4, 13.3, and 14.0). BBB scores between the two groups were not significantly different from week 1 to week 3 after injury (Fig. 7*J*).

DISCUSSION

Wharton's jelly of umbilical cord is the way through which totipotent cells, such as primordial germ cells and hematopoetic stem cells, migrate from the yolk sac to target tissues in the embryo and fetus during embryogenesis (32). Therefore, it is a rich source of primitive cell types and may be a convenient source of stem cells because it is always discarded after birth. In 2003, Mitchell et al firstly identified MSCs from human umbilical cord-Wharton's jelly (16). Further research find that the hUC-MSCs can be isolated from umbilical cord blood (33, 34), perivacular regions (20, 35), and umbilical vein subendothelium (36–38) of the umbilical cord. hUC-MSCs express matrix markers (CD90) and MSCs markers (CD73, CD105) but do not express hematopoietic lineage markers (CD34, CD45, CD14) and HLA-DR, which is similar to MSCs derived from bone marrow, umbilical cord blood, and placenta (21, 22, 37). More important, OCT-4 is also expressed by hUC-MSCs (39–41), which is marker of undifferentiated state and not expressed by BM-MSCs. Expression of OCT by hUC-MSCs indicates that these cells may be an intermediate hierarchy of stem cells between embryonic stem cells and adult stem cells. We also successfully isolate hUC-MSCs from Wharton's jelly of umbilical cord and find that they express similar surface markers to other sources of MSCs (28). In the present study, approximately 5×10^6 hUC-MSCs are collected from a 10 cm-umbilical cord. hUC-MSCs may be superior to BM-MSCs and embryonic stem cells for translational medicine because they have characteristics of easily availability, great expansion capabilities, and fast proliferation (17, 42, 43).

Recent studies have shown that hUC-MSCs can be induced *in vitro* to form neurons, glial cells (16, 25, 26), and especially dopaminergic neurons (27). In addition, emerging data have demonstrated that transplantation of hUC-MSCs can promote neuroprotection or locomotion recovery in experimental models of cerebral global ischemia (40, 44), Parkinson's disease (27, 39), and retinal degenerative diseases (45). Thus, we hypothesize that the hUC-MSCs may be an ideal cell source for treating SCI.

It is always considered that stem cells transplantation for SCI could affect histologic and/or functional outcome in a number of ways including differentiating into neural cells such as neurons or oligodendrocytes to replace damaged and diseased cells and promoting host neuronal or oligodendroglial survival and regeneration by providing cytokines or neurotrophins (46, 47). In our study, recovery of hindlimb locomotor function is significantly enhanced in the hUC-MSCs grafted animals compared with the control group rats. This recovery is accompanied by increased length of neurofilament-positive fibers and increased numbers of growth cone-like structures around the lesion site. These results suggest that hUC-MSCs survival in the host plays an important role in the maintenance of improved performance. We also find the transplanted human cells survive and engraft within the injured rat spinal cord, and at 8 wks, most of them have migrated for about 5 mm distance along the rostrocaudal axis into the rim of the injured spinal cord, and even at the epicenter. However, the majority of the implanted hUC-MSCs do not differentiate into neurons, astrocytes, and oligodendrocytes. Therefore, the mechanism underlying the functional recovery after transplantation of hUC-MSCs is not the differentiation of these cells into neuronal or glial cells. In addition, our results show that most hUC-MSCs express GDNF and NT-3 and stably secrete GDNF and NT-3 into the injured spinal cord after transplantation. Then, taken together, these data suggest that cytokines or growth factors released by implanted undifferentiated hUC-MSCs could be the mechanisms for sustained locomotor recovery. Similar conclusions have been reported by Yang et al (39) and Weiss et al (48).

Past studies have suggested that the mechanisms by which hUC-MSCs promote functional recovery involved: 1) differentiation into neural cells (44); 2) production of cytokines or neurotrophins (39, 45, 48); 3) inhibition of the host immune response and inflammation (39, 48); 4) improvement of vascularization (44); 5) suppression of glial scar formation (48), and 6) activation of endogenous neural stem cells (49). Our results show that the production of GDNF

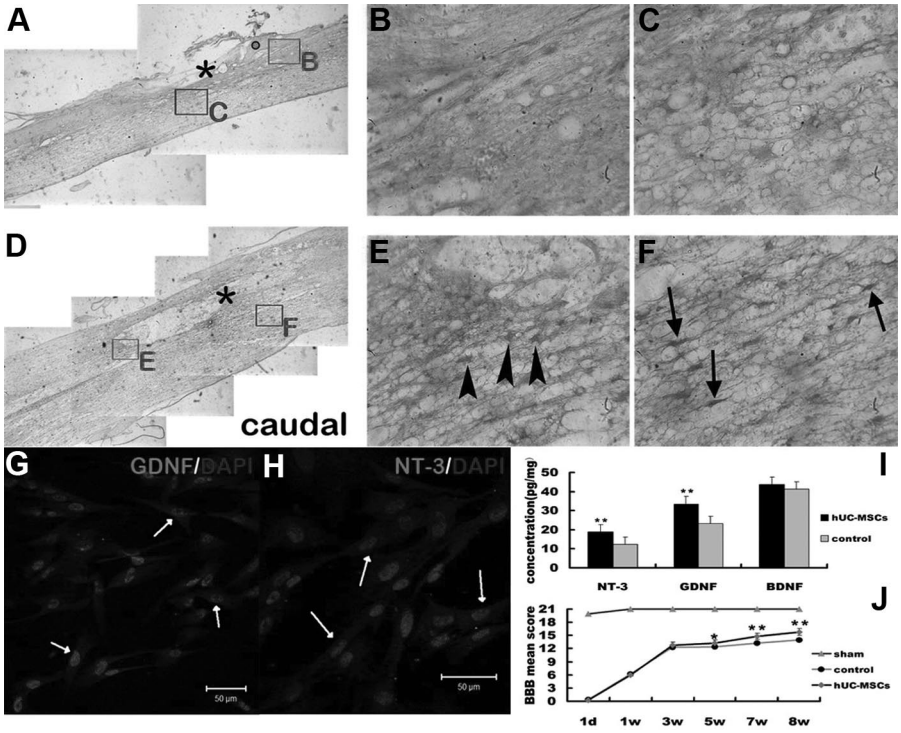

Figure 7. Human umbilical cord mesenchymal stem cells (hUC-MSCs) enhance neuroregeneration, produce neurotrophins, and facilitate functional recovery. In the hUC-MSCs group (*D–F*), numerous growth-associated protein-43-positive cells and growth cone-like structures are observed near the injury site, whereas they are scarce in the control group (*A–C*). *In vitro* immunocytochemisty shows that most hUC-MSCs express glial cell line-derived neurotrophic factor (*GDNF*, *G*) and neurotrophin-3 (*NT-3*, *H*). Enzyme-linked immunosorbent assay shows expression of GDNF and NT-3 in animals that received grafts of hUC-MSCs is significantly higher compared with ones that received grafts of Dulbecco's modified essential media/nutrient mixture F-12. But there is no statistical significance in the expression of brain-derived neurotrophic factor between the hUC-MSCs group and the control group (*I*). Basso, Beattie, and Bresnahan (*BBB*) scale scores in hUC-MSCs-grafted animals at 5, 7, and 8 wks (*w*) after graft are significantly higher than those of the Dulbecco's modified essential media/nutrient mixture F-12-grafted group (*J*). *A* and *D,* injury site (*asterisk*); *E*, arrowheads, growth-associated protein-43 positive cells; *F,* arrow, growth cone-like structures; *G,* arrow, GDNF-positive cells; *H,* arrow, NT-3-positive cells. *A* and *D*, Original magnification, ×40; *B*, *C*, *E*, and *F*, original magnification, ×250; bar, 50 μm, *G* and *H*; **p* .05; ***p* .01, compared with the control group. *DAPI*, 4, 6-diamidino-2-phenyl-indole.

and NT-3 by hUC-MSCs and inhibition of glial scar formation could be the underlying mechanism.

GDNF is one of the most potent trophic factors for motor neuron survival, connectivity, and axonal growth. Rooney et al (50) found that GDNF genetically modified MSCs survived posttransplantation and possessed therapeutic benefits without differentiating into neural cells.

NT-3 is one kind of neutrophin that regulates neuronal development and axonal regeneration (51). Administration of NT-3 can promote the growth of corticospinal axons and improve functional deficits after chronic scar resection (52). Transplantation of NT-3-expressing glial-restricted precursor cells can also facilitate functional recovery after traumatic SCI (53).

After SCI, the astrocytes response begins immediately after injury and evolves over time and finally forms glial scar, which is not only a physical barrier but also an inhibitory environment for axonal regeneration and remyelination (54). The main inhibitory molecules produced by reactive astrocytes is chondroitin sulfate proteoglycans (55). These molecules have been shown to inhibit axonal regeneration, and degradation of them by chondroitinases has been demonstrated to improve functional recovery after SCI (56). Transplanted stem cells can secrete extracellular matrix and cytokines that help provide counterbalance to the inhibitory effects of the glial scar to promote cell migration and axonal regeneration. Our studies show that astrocytes appear to be permissive and do not form a prominent glial limitans to completely block regenerative axons in the hUC-MSCs group, which is different from the formation of dense scar barrier in the control group.

This study demonstrates that hUC-MSCs can survive and migrate in the rats spinal cord after transplantation without immunologic suppression therapy and produce large amounts of GDNF and NT-3, which inhibits the formation of glial scar, enhances neuroregeneration, and facilitates functional recovery after traumatic SCI. Although functional recovery is significantly enhanced in the hUC-MSCs-grafted animals compared with control group. The improvement is small. Therefore, further research is needed to be explored in future work. Firstly, we may increase the amounts of implanted cells and study the dose-dependent effects of hUC-MSCs transplantation on SCI. Secondly, we may take some measures to promote hUC-MSCs to differentiate into neural cells *in vivo*.

CONCLUSION

Our study provide evidence that transplantation of hUC-MSCs from Wharton's jelly of the umbilical cord can be considered as a potential therapeutic strategy for the treatment of SCI. Although our transplantation strategies are successful in the acute phase of SCI, we still need to overcome the inhibitory obstacles presented by chronic SCI that interfere with cell transplantation.

ACKNOWLEDGMENTS

The authors thank Jia-Si Bai, PhD (Department of Central Laboratory, Southwest Hospital, Third Military Medical University) for his technical assistances.

REFERENCES

1. Lim PAC, Tow AM: Recovery and regeneration after spinal cord injury: A review and summary of recent literature. *Ann Acad Med Singapore* 2007; 36:49–57
2. Willerth SM, Sakiyama-Elbert SE: Cell therapy for spinal cord regeneration. *Adv Drug Deliv Rev* 2008; 60:263–276
3. Keirstead HS, Nistor G, Bernal G, et al: Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci* 2005; 25:4694–4705
4. Hofstetter CP, Schwarz EJ, Hess D, et al: Marrow stromal cells from guiding strands in the injured spinal cord and promote recovery. *Proc Natl Acad Sci U S A* 2002; 99: 2199–2204
5. Karimi-Abdolrezaee S, Eftekharpour E, Wang J, et al: Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recov-

ery after spinal cord injury. *J Neurosci* 2006; 26:3377–3389
6. Civin CI: Commitment to biomedical research: Clearing unnecessary impediments to progress. *Stem Cells* 2002; 20:482–484
7. Freed CR: Will embryonic stem cells be a useful source of dopamine neurons for transplant into patients with Parkinson's disease? *Proc Natl Acad Sci U S A* 2002; 99:1755–1757
8. Rao MS, Mattson MP: Stem cells and aging: Expanding the possibilities. *Mech Ageing Dev* 2001; 122:713–734
9. Romanov YA, Svintsitskaya VA, Smirnov VN: Searching for alternative sources of postnatal human mesenchymal stem cells: Candidate MSC-like cells from umbilical cord. *Stem Cells* 2003; 21:105–110
10. Wright LS, Prowse KR, Wallace K, et al: Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro. *Exp Cell Res* 2006; 312: 2107–2120
11. Marcus AJ, Coyne TM, Rauch J, et al: Isolation, characterization, and differentiation of stem cells derived from the rat amniotic membrane. *Differentiation* 2008; 76: 130–144
12. De Coppi P, Bartsch G Jr., Siddiqui MM, et al: Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 2007; 25:100–106
13. Lee OK, Kuo TK, Chen WM, et al: Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004; 103: 1669–1675
14. Lu LL, Liu YJ, Yang SG, et al: Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica* 2006; 91:1017–1026
15. Secco M, Zucconi E, Vieira NM, et al: Multipotent stem cells from umbilical cord: Cord is richer than blood! *Stem Cells* 2008; 26: 146–150
16. Mitchell KE, Weiss ML, Mitchell BM, et al: Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells* 2003; 21:50–60
17. Troyer DL, Weiss ML: Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells* 2008; 26:591–599
18. Weiss ML, Anderson C, Medicetty S, et al: Immune properties of human umbilical cord Wharton's jelly-derived cells. *Stem Cells* 2008; 26:2865–2874
19. Karahuseyinoglu S, Cinar O, Kilic E, et al: Biology of the stem cells in human umbilical cord stroma: In situ and in vitro surveys. *Stem Cells* 2007; 25:319–331
20. Sarugaser R, Lickorish D, Baksh D, et al: Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors. *Stem Cells* 2005; 23:220–229
21. Conconi MT, Burra P, Di Liddo R, et al: CD105(+) cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential. *Int J Mol Med* 2006; 18:1089–1096
22. Wang HS, Hung SC, Peng ST, et al: Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* 2004; 22:1330–1337
23. Chang SA, Lee EJ, Kang HJ, et al: Impact of myocardial infarct proteins and oscillating pressure on the differentiation of mesenchymal stem cells: Effect of acute myocardial infarction on stem cell differentiation. *Stem Cells* 2008; 26:1901–1912
24. Wu KH, Zhou B, Lu SH, et al: In vitro and in vivo differentiation of human umbilical cord derived stem cells into endothelial cells. *J Cell Biochem* 2007; 100:608–616
25. Fu YS, Shih YT, Cheng YC, et al: Transformation of human umbilical mesenchymal cells into neurons in vitro. *J Biomed Sci* 2004; 11:652–660
26. Ma L, Feng XY, Cui BL, et al: Human umbilical cord Wharton's Jelly-derived mesenchymal stem cells differentiation into nerve-like cells. *Chin Med J (Engl)* 2005; 118:1987–1993
27. Fu YS, Cheng YC, Lin MY, et al: Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: Potential therapeutic application for parkinsonism. *Stem Cells* 2006; 24: 115–124
28. Hu SL, Zhang JQ, Hu X, et al: In vitro labeling of human umbilical cord mesenchymal stem cells with superparamagnetic iron oxide nanoparticles. *J Cell Biochem* 2009; 108: 529–535
29. Wamil AW, Wamil BD, Hellerqvist CG: CM101-mediated recovery of walking ability in adult mice paralyzed by spinal cord injury. *Proc Natl Acad Sci U S A* 1998; 95:13188–13193
30. Basso DM, Beattie MS, Bresnahan JC: Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. *Exp Neurol* 1996; 139:244–256
31. Zhang SC, Wernig M, Duncan ID, et al: In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 2001; 19:1129–1133
32. Kadner A, Zund G, Maurus C, et al: Human umbilical cord cells for cardiovascular tissue engineering: A comparative study. *Eur J Cardiothorac Surg* 2004; 25:635–641
33. Jang YK, Jung DH, Jung MH, et al: Mesenchymal stem cells feeder layer from human umbilical cord blood for ex vivo expanded growth and proliferation of hematopoietic progenitor cells. *Ann Hematol* 2006; 85: 212–225
34. Gao L, Chen X, Zhang X, et al: Human umbilical cord blood-derived stromal cell, a new resource of feeder layer to expand human umbilical cord blood CD34+ cells in vitro. *Blood Cells Mol Dis* 2006; 36:322–328
35. Baksh D, Yao R, Tuan RS: Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells* 2007; 25:1384–1392
36. Kim JW, Kim SY, Park SY, et al: Mesenchymal progenitor cells in the human umbilical cord. *Ann Hematol* 2004; 83:733–738
37. Panepucci RA, Siufi JL, Silva WA Jr, et al: Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. *Stem Cells* 2004; 22: 1263–1278
38. Kaltz N, Funari A, Hippauf S, et al: In vivo osteoprogenitor potency of human stromal cells from different tissues does not correlate with expression of POU5F1 or its pseudogenes. *Stem Cells* 2008; 26:2419–2424
39. Weiss ML, Medicetty S, Bledsoe AR, et al: Human umbilical cord matrix stem cells: Preliminary characterization and effect of transplantation in a rodent model of Parkinson′s disease. *Stem Cells* 2006; 24:781–792
40. Jomura S, Uy M, Mitchell K, et al: Potential treatment of cerebral global ischemia with Oct-4$^+$ umbilical cord matrix cells. *Stem Cells* 2007; 25:98–106
41. Zhang ZY, Teoh SH, Chong MS, et al: Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. *Stem Cells* 2009; 27:126–137
42. Cao FJ, Feng SQ: Human umbilical cord mesenchymal stem cells and the treatment of spinal cord injury. *Chin Med J (Engl)* 2009; 122:225–231
43. Bailey MM, Wang L, Bode CJ, et al: A comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. *Tissue Eng* 2007; 13:2003–2010
44. Ding DC, Shyu WC, Chiang MF, et al: Enhancement of neuroplasticity through upregulation of beta1-integrin in human umbilical cord-derived stromal cell implanted stroke model. *Neurobiol Dis* 2007; 27:339–353
45. Lund RD, Wang S, Lu B, et al: Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. *Stem Cells* 2007; 25:602–611
46. Coutts M, Keirstead HS: Stem cells for the treatment of spinal cord injury. *Exp Neurol* 2008; 209:368–377
47. Cummings BJ, Uchida N, Tamaki SJ, et al: Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci U S A* 2005; 102:14069–14074
48. Yang CC, Shih YH, Ko MH, et al: Transplantation of human umbilical esenchymal stem cells from Wharton's Jelly after complete transection of the rat spinal cord. *PLoS ONE* 2008; 3:e3336
49. Koh SH, Kim KS, Choi MR, et al: Implantation of human umbilical cord-derived mesenchymal stem cells as a neuroprotective therapy for ischemic stroke in rats. *Brain Res* 2008; 1229:233–248
50. Rooney GE, McMahon SS, Ritter T, et al: Neurotrophic factor-expressing mesenchymal stem cells survive transplantation into

the contused spinal cord without differentiating into neural cells. *Tissue Eng Part A* 2009; 15:3049–3059
51. Xu XM, Guenard V, Kleitman N, et al: A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. *Exp Neurol* 1995; 134:261–272
52. Tuszynski MH, Grill R, Jones LL, et al: NT-3 gene delivery elicits growth of chronically injured corticospinal axons and modestly improves functional deficits after chronic scar resection. *Exp Neurol* 2003; 181:47–56
53. Cao Q, Xu XM, Devries WH, et al: Functional recovery in traumatic spinal cord injury after transplantation of multineurotrophin-expressing glial-restricted precursor cells. *J Neurosci* 2005; 25:6947–6957
54. Fitch MT, Silver J: CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp Neurol* 2008; 209:294–301
55. Fok-Seang J, Smith-Thomas LC, Meiners S, et al: An analysis of astrocytic cell lines with different abilities to promote axon growth. *Brain Res* 1995; 689:207–223
56. Bradbury EJ, Moon LD, Popat RJ, et al: Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 2002; 416:636–640