**NEURAL REGENERATION RESEARCH**
Volume 7, Issue 34, December 2012

www.nrronline.org

*doi:10.3969/j.issn.1673-5374.2012.34.006 [http://www.crter.org/nrr-2012-qkquanwen.html]*
*Zhou J, Tian GP, Wang JG, Luo XG, Zhang SY, Li JP, Li L, Xu B, Zhu F, Wang X, Jia CH, Zhao WJ, Zhao DY, Xu AH. Neural cell injury microenvironment induces neural differentiation of human umbilical cord mesenchymal stem cells. Neural Regen Res. 2012;7(34): 2689-2697.*

# Neural cell injury microenvironment induces neural differentiation of human umbilical cord mesenchymal stem cells☆

**Jin Zhou[1], Guoping Tian[1], Jinge Wang[1], Xiaoguang Luo[2], Siyang Zhang[3], Jianping Li[4], Li Li[1], Bing Xu[1], Feng Zhu[1], Xia Wang[1], Chunhong Jia[1], Weijin Zhao[1], Danyang Zhao[1], Aihua Xu[1]**

1 *Department of Neurology, First People's Hospital of Shenyang, Shenyang 110041, Liaoning Province, China*
2 *First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China*
3 *College of Basic Medical Sciences, China Medical University, Shenyang 110001, Liaoning Province, China*
4 *Liaoning Provincial Blood Center, Shenyang 110044, Liaoning Province, China*

## Abstract

This study aimed to investigate the neural differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs) under the induction of injured neural cells. After *in vitro* isolation and culture, passage 5 hUCMSCs were used for experimentation. hUCMSCs were co-cultured with normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate in a Transwell co-culture system. Western blot analysis and flow cytometry results showed that choline acetyltransferase and microtubule-associated protein 2, a specific marker for neural cells, were expressed in hUCMSCs under various culture conditions, and highest expression was observed in the hUCMSCs co-cultured with injured PC12 cells. Choline acetyltransferase and microtubule-associated protein 2 were not expressed in hUCMSCs cultured alone (no treatment). Cell Counting Kit-8 assay results showed that hUCMSCs under co-culture conditions promoted the proliferation of injured PC12 cells. These findings suggest that the microenvironment during neural tissue injury can effectively induce neural cell differentiation of hUCMSCs. These differentiated hUCMSCs likely accelerate the repair of injured neural cells.

## Key Words

stem cell; umbilical cord mesenchymal stem cell; co-culture; induction; differentiation; neural cell; microtubule-associated protein 2; injured cell; Transwell; neural regeneration; regeneration

## Research Highlights

(1) We investigated the neural cell differentiation of human umbilical cord mesenchymal stem cells under induction by injured neural cells.
(2) Human umbilical cord mesenchymal stem cells were co-cultured with normal or injured PC12 cells, PC12 cell supernatant or PC12 cell lysate in a Transwell co-culture system.
(3) *In vitro* cultured human umbilical cord mesenchymal stem cells differentiated into neural cells under the various induction methods, as demonstrated by the positive expression of choline acetyltransferase and microtubule-associated protein 2, a specific marker for neural cells.
(4) Under co-culture, human umbilical cord mesenchymal stem cells more effectively promoted the proliferation of injured PC12 cells.

Jin Zhou☆, M.D., Chief physician, Department of Neurology, First People's Hospital of Shenyang, Shenyang 110041, Liaoning Province, China

Corresponding author: Jin Zhou, Department of Neurology, First People's Hospital of Shenyang, Shenyang 110041, Liaoning Province, China
zhoujinhaoyun@yahoo.com.cn

Received: 2012-09-11
Accepted: 2012-11-23
(N20120808002/H)

## INTRODUCTION

Stem cells are a cell population capable of long-term self-renewal, with strong proliferation and multilineage differentiation potential[1-5]. They are also one of the most studied cell types in the field of cell transplantation.

Compared with bone marrow stromal stem cells (BMSCs)[6], transplantation of human umbilical cord mesenchymal stem cells (hUCMSCs) is associated with a lower rate of immunological rejection, and these cells have stronger proliferative capacity[7-10]. UCMSCs have been used in cell transplantation for the treatment of various nervous system diseases because of their strong self-renewal and multilineage differentiation potential[11-17]. These cells have the potential to differentiate into various cell types, and are ideal for the repair and regeneration of organ tissues because they can be obtained in large quantity and exhibit strong proliferation potential. In addition, they exhibit low immunogenicity and have a distinct immune regulatory function. Furthermore, these cells will not cause immunological rejection in autologous transplantation and they can reduce immunological reactions in allogeneic transplantation[16, 18]. There is no need for anesthesia and there is no pain or adverse reaction during harvesting of UCMSCs. An increasing amount of research has focused on whether umbilical cord mesenchymal stem cells can substitute for bone marrow stromal stem cells as a source rich in stem cells for use in cell transplantation[19-20].

There is strong evidence that, when co-cultured with some types of cells, stem cells can be induced to differentiate into specific cell types. For example, *in vitro* co-culture of hUCMSCs and umbilical vein endothelial cells can improve angiogenesis by vascular endothelial cells and osteogenic mineralization of BMSCs[21]. When co-cultured with LO2 human hepatocytes, hUCMSCs can be induced to differentiate into hepatocyte-like cells[22]. A co-culture of olfactory ensheathing cells with neural stem cells can promote the proliferation and neuronal differentiation of neural stem cells[23]. When co-cultured with Schwann cells, some BMSCs can be induced to differentiate into nerve tissue cells and express a Schwann cell surface marker[24]. It has also been reported that stem cells co-cultured with injured cells can differentiate into certain cell types. Zhang *et al* [25] found that when co-cultured *in vitro* with adriamycin-damaged myocardial cells, to simulate the microenvironment of injured myocardial cells, BMSCs can be successfully differentiated into myocardial-like cells. Based on these previous studies, we hypothesized that when co-cultured with injured neural cells, hUCMSCs can be induced to differentiate into neuron-like cells.

In this study, we co-cultured hUCMSCs and $A\beta_{1-40}$-injured PC12 cells in a Transwell co-culture system and examined the expression of neuronal markers by the differentiating hUCMSCs using flow cytometry and western blot analysis.

## RESULTS

### hUCMSC morphology

At 24 hours after seeding, a small number of adherent cells exhibited a multipolar or round appearance. After 5–7 days of culture, cells proliferated rapidly and gradually formed a single layer of cell clusters. After 2–3 weeks of culture, the majority of cells exhibited a uniform spindle shape and were arranged similar to fibroblasts (Figure 1). Passage 5 cells were used for experiments.

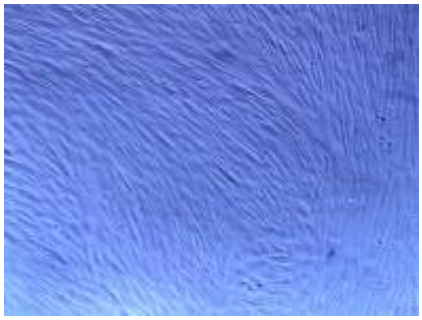

Figure 1 Morphology of primary human umbilical cord mesenchymal stem cells cultured for 3 weeks (inverted microscope, × 200).

Cells exhibit uniform long spindle-shaped appearance and are arranged like fibroblasts.

### hUCMSC identification

To confirm that the isolated and purified hUCMSCs were the non-differentiated mesenchymal stem cells required for the experiments, we selected six surface markers for mesenchymal stem cells: cell surface receptor molecule CD44, adhesion molecule CD29, hematopoietic stem/progenitor cell marker CD34, hematopoietic cell marker CD45, neural cell adhesion molecule CD56, and tumor stem cell marker CD133. The expression of these markers was examined by flow cytometry. CD44 and CD29 were highly expressed on the hUCMSCs, while CD34, CD45, CD56 and CD133 were barely detected on these cells (Figure 2).

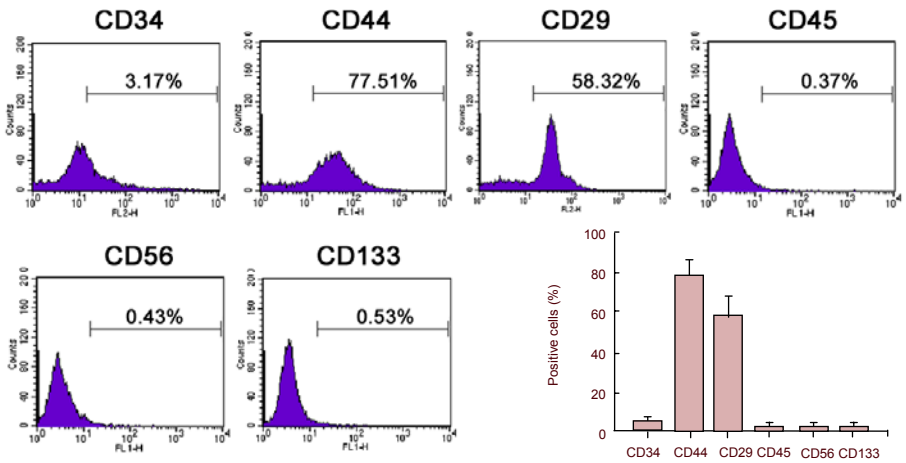


Figure 2 Expression of human umbilical cord mesenchymal stem cell (hUCMSC) surface markers.

CD44 and CD29 are highly expressed in hUCMSCs, and CD34, CD45, CD56 and CD133 are minimally expressed on these cells.

### Expression of neural cell-specific markers on hUCMSCs co-cultured with injured PC12 cells

Neuron-like differentiation of hUCMSCs was induced with normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate. After induced differentiation of hUCMSCs, the expression of microtubule-associated protein 2 (MAP2), a specific marker for neural cells, was examined using flow cytometry. Compared with hUCMSCs cultured alone, MAP2 expression appeared in hUCMSCs co-cultured with normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate. The proportion of MAP2-positive cells among hUCMSCs co-cultured with $A\beta_{1-40}$-injured PC12 cells was 17.1 ± 2.1%, which was significantly higher than that among hUCMSCs co-cultured with normal PC12 cells, PC12 cell supernatant or PC12 cell lysate ($P < 0.05$, Figure 3).

### Effects of hUCMSCs on the proliferation of co-cultured injured PC12 cells

A previous study examined the differentiation of stem cells and their ability to promote the proliferation of cells surrounding the site of injury during tissue injury repair[26]. In this study, Cell Counting Kit-8 assay was used to investigate the effects of hUCMSCs on the proliferation of co-cultured injured PC12 cells. Compared with hUCMSCs cultured alone, normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant and PC12 cell lysate promoted the proliferation of injured PC12 cells to different degrees. At the end of co-culture (3 days of culture), hUCMSCs had the strongest ability to promote the proliferation of injured PC12 cells ($P < 0.05$; Figure 4). These findings suggest that hUCMSCs co-cultured with injured PC12 cells exhibit the strongest effects on tissue injury repair.

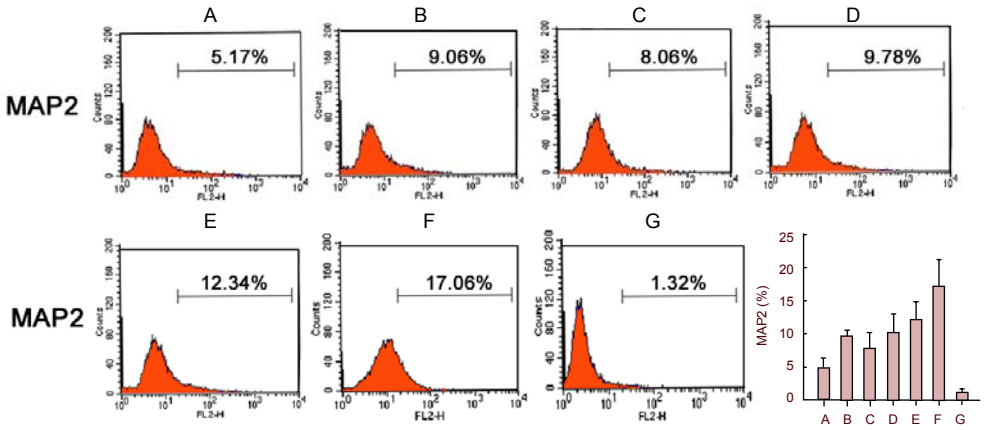


Figure 3 Proportion of microtubule-associated protein 2 (MAP2)-positive cells among human umbilical cord mesenchymal stem cells (hUCMSCs) co-cultured with normal (A) or $A\beta_{1-40}$-injured (B) PC12 cell supernatant, normal (C) or $A\beta_{1-40}$-injured (D) PC12 cell lysate, normal (E) or $A\beta_{1-40}$-injured (F) PC12 cells, or hUCMSCs alone (G).

Normal and $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant and PC12 cell lysate induced differentiation to varying degrees. hUCMSCs co-cultured with $A\beta_{1-40}$-injured PC12 cells had the strongest effects on neuronal differentiation of hUCMSCs ($P < 0.05$).

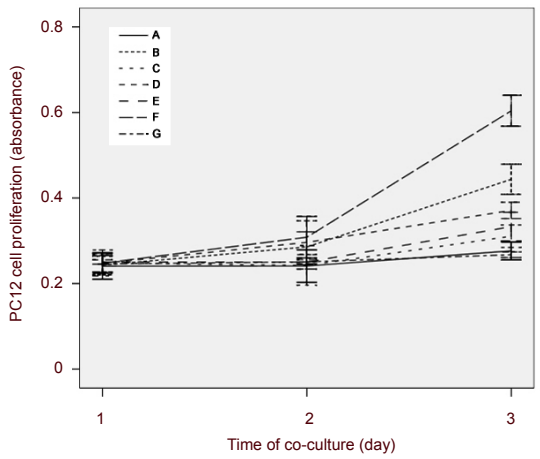


Figure 4 Effects of human umbilical cord mesenchymal stem cells (hUCMSCs) co-cultured with normal (A) or $A\beta_{1-40}$-injured (B) PC12 cell supernatant, normal (C) or $A\beta_{1-40}$-injured (D) PC12 cell lysate, normal (E) or $A\beta_{1-40}$-injured (F) PC12 cells, or hUCMSCs cultured alone (G) on the proliferation of injured PC12 cells.

hUCMSCs co-cultured with injured PC12 cells most strongly promoted the proliferation of injured PC12 cells ($P < 0.05$). Measurement data are expressed as mean ± SD. Each experiment was run in triplicate.

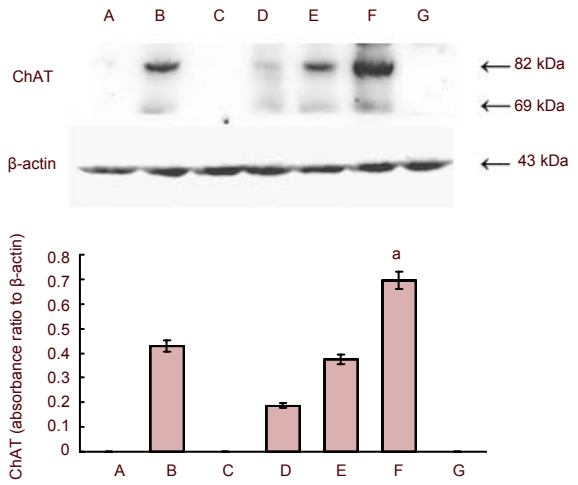


Figure 5 Choline acetyltransferase (ChAT) expression in human umbilical cord mesenchymal stem cells (hUCMSCs) co-cultured with normal (A) or $A\beta_{1-40}$-injured (B) PC12 cell supernatant, normal (C) or $A\beta_{1-40}$-injured (D) PC12 cell lysate, normal (E) or $A\beta_{1-40}$-injured (F) PC12 cells, and in hUCMSCs cultured alone (G).

ChAT expression was strongest in hUCMSCs co-cultured with injured PC12 cells. $^aP < 0.01$, *vs*. the other six groups (one-way analysis of variance). Measurement data were expressed as mean ± SD. Each experiment was run in triplicate.

### Choline acetyltransferase (ChAT) expression in hUCMSCs co-cultured with injured PC12 cells

To investigate whether hUCMSCs can synthesize ChAT, similar to neural cells, we measured expression of the protein in hUCMSCs co-cultured with normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate. ChAT was not expressed in hUCMSCs cultured alone, while the enzyme was up-regulated to different degrees in hUCMSCs co-cultured with normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate (Figure 5). ChAT expression was highest in the hUCMSCs co-cultured with injured PC12 cells ($P < 0.05$, Figure 5). These findings suggest that the induced hUCMSCs can synthesize ChAT, similar to neural cells, and that hUCMSCs co-cultured with injured PC12 cells exhibit the strongest capacity to produce the enzyme.

## DISCUSSION

The pathological changes in Alzheimer's disease (AD) include a greatly decreased number of neurons and a significant reduction in ChAT and acetylcholine levels[27-35]. ChAT is a key enzyme for the synthesis of the important neurotransmitter acetylcholine in neural cells[36-42]. A great decrease in ChAT and acetylcholine levels is the main pathological change in neurodegenerative diseases, such as AD[43-44].

In recent years, various types of stem cells have been used for the treatment of AD, and great advances have been made[45-47]. Babaei *et al* [45] reported that learning and memory abilities were significantly improved 10 months after injection of bone marrow mesenchymal stem cells into the hippocampus of AD rats. Zhang *et al* [46] reported that hUCMSCs expressed the neuron-specific marker MAP2 and extended neurite-like structures after stimulation with neural cell induction medium. Furthermore, these cells expressed ChAT after addition of hippocampal cholinergic neurostimulating peptide or hippocampal extract. In the clinic, intrathecal injection of hUCMSCs can improve clinical symptoms in patients with spinocerebellar ataxia and/or cerebellar atrophy, and this strategy is safe and effective[47]. Induced pluripotent stem cells derived from dermal fibroblasts of AD patients have been proven to be capable of differentiating into cholinergic neurons[48]. Park *et al* [49] reported that neural stem cells over-expressing ChAT restore cognition in a rat model of cognitive dysfunction. Kitiyanant *et al* [50] reported that neural cells co-cultured with human neural progenitor cells secreting neurotrophic factors were protected from Aβ-induced cell death, and these neurotrophic factors can increase intracellular ChAT expression. Therefore, in this study, ChAT expression level was used as an index for evaluating the functional recovery of neural cells.

Results from this study showed that ChAT expression was increased in hUCMSCs co-cultured with normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate, and the highest expression level was found in the hUCMSCs co-cultured with injured PC12 cells. These findings suggest that when co-cultured with injured cells, hUCMSCs can better differentiate into neural cells.

Compared with other cell types, UCMSCs show great clinical application potential because of ease of isolation, no injury to donors, stronger capacity to proliferate and differentiate, low immunogenicity, and low incidence of graft *versus* host disease. Local transplantation of hUCMSCs into ischemic skin flap can increase vascular endothelial growth factor and basic fibroblast growth factor expression, increase capillary density, and effectively promote skin flap survival. This method can be applied for the treatment of large skin defects or deep skin injury[51]. Use of hUCMSCs in the treatment of optic neuritis can greatly improve symptoms, decrease focus volume and reduce adverse reactions in humans[52]. Transfusion of hUCMSCs for treatment of decompensated cirrhosis can effectively reduce ascites and improve liver function[53]. Xue *et al* [54] performed an intravenous transfusion of hUCMSCs to treat bone nonunion complicated by nerve injury, and they observed absence of fracture stumps and an increase in nervous reflex and muscular strength. These findings suggest that hUCMSCs can be used for repair of various tissue injuries.

There is evidence that hUCMSCs can differentiate into Schwann cell-like cells in the presence of glial growth factors and can secrete various neurotrophic factors that promote axon formation when co-cultured with neurons[55]. When co-cultured with nucleus pulposus cells, hUCMSCs can differentiate into nucleus pulposus-like cells that express the specific marker for nucleus pulposus cells[56]. Therefore, co-culture is an effective means of inducing the differentiation of mesenchymal stem cells.

In this study, we investigated the effects of co-culture with normal and/or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate on the induced differentiation of hUCMSCs. Co-culture with injured cells had the greatest effect and induced hUCMSCs to differentiate into neuron-like cells expressing the neuron-specific marker MAP2. These findings suggest that the microenvironment following nervous tissue injury can effectively induce the directed differentiation of hUCMSCs. Thus, local transfusion of hUCMSCs into injured tissue may be an effective strategy for repair of nervous tissue injury. The differentiated hUCMSCs can promote the proliferation of injured cells. This suggests that injured cells and hUCMSCs may interact with each other to promote the repair of injured tissue. However, the underlying molecular mechanisms are unknown and require further investigation.

In summary, the results of this study indicate that during induced differentiation, hUCMSCs display certain morphological and biochemical characteristics of neural cells. These results provide experimental support for the *in vivo* transplantation of hUCMSCs for the treatment of nervous system injury and disease.

## MATERIALS AND METHODS

### Design

A controlled, observational, *in vitro* study.

### Time and setting

This study was performed at the Experimental Center, China Medical University, between January 2009 and December 2010.

### Materials

Umbilical cord tissue from healthy normal full-term delivery babies was provided by the First People's Hospital of Shenyang, China. According to the *Administrative Regulations on Medical Institution* issued by the State Council of the People's Republic of China, mothers or their relatives provided informed consent[57].

### Methods

#### *hUCMSC preparation*

Under sterile conditions, umbilical cord tissue was harvested. Following thorough washes with Hanks' balanced salt solution, umbilical vein, umbilical artery and umbilical cord adventitia were removed and chopped into 1 mm × 1 mm × 1 mm pieces. Then, these tissue blocks were transferred into a mixture of collagenase II (1 g/L; Nanjing KeyGen Biotechnology Co., Ltd., Nanjing, China) and DMEM for digestion. Digestion solution containing cells was diluted in 30 volumes of DMEM and centrifuged at 1 500 r/min for 10 minutes, and the supernatant was discarded. Cells were thoroughly resuspended with DMEM/F12 culture medium (Invitrogen, Carlsbad, CA, USA) containing 15% fetal bovine serum, plated into a culture flask at a density of $1 \times 10^6$/mL and cultured at 37°C in an incubator

containing 5% $CO_2$. On day 3, after cell adherence, half of the culture medium was refreshed, non-adherent cells were discarded, and culture medium was changed once every 3 days thereafter. When cells reached 90% confluency, they were digested with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid and passaged at 1:3. Passage 5 cells were used for co-culture experiments.

### *Analysis of hUCMSC immunophenotype by flow cytometry*

Passage 5 hUCMSCs were digested with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid and then prepared into a single-cell suspension at a density of $1 \times 10^6$/mL. After addition of phycoerythrin-labeled mouse anti-human CD34, CD44, CD29, CD45 or CD56 monoclonal antibodies (BD Bioscience Pharmingen Inc., San Diego, CA, USA), the cell suspension was incubated at room temperature for 30 minutes and then centrifuged for 10 minutes at 1 000 r/min. After one PBS wash, the cell suspension was centrifuged at 1 000 r/min for 10 minutes. After cell resuspension with PBS, cell immunophenotype was analyzed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). This experiment was performed in triplicate. The proportion of MAP2-positive cells within the total cell count was calculated using CellQuest software (Becton Dickinson).

### *PC12 cell culture and passage*

PC12 cells (Cell Research Institute, Chinese Academy of Sciences) were used as a substitute for neural cells and were grown in PC12 cell culture medium (containing 10% horse serum and 5% fetal bovine serum 1640). PC12 cells grew as a suspension. After 7–8 passages, cells adhered to the flask and were used for experimentation. PC12 cells were resuspended in PC12 cell culture medium, and a 300 μL aliquot of $1 \times 10^7$ cells/mL was transferred into the wells of a 24-well plate[58-59].

### *Experimental procedures and grouping*

Preparation of injured PC12 cell supernatant: PC12 cells were incubated with 10 μg/mL $A\beta_{1-40}$ (Sigma, St. Louis, MO, USA) overnight[60-63]. After removal of the supernatant, cells were cultured with 1640 culture medium for 24 hours, and then the supernatant was collected as injured PC12 cell-conditioned culture medium. Simultaneously, normal PC12 cell-conditioned culture medium was collected using the same method.

Preparation of injured PC12 cell lysate: Under sterile conditions, PC12 cells were collected during preparation of injured PC12 cell supernatant, pre-cooled with PBS, centrifuged, and washed with sterile PBS three times. After adding a sufficient amount of sterile PBS, cell clumps were dispersed to prepare a single-cell suspension. Cells at a density of $1 \times 10^4$ cells/mL were sonicated in an ice bath (350 W; working time: 2 seconds; interval: 10 seconds; 40 times). After decontamination, cells were stored at –80 °C for later use.

Preparation of transwells: 16 wells were selected from the 24-well plate and filled with 400 μL DMEM. hUCMSCs at a density of $1 \times 10^4$ cells/well were cultured in the transwells (aperture: 0.4 μm; Corning, Steuben County, NY, USA)[64].

Preparation of PC12 cells: PC12 cells were digested and plated in the 24-well plate at a density of $1 \times 10^4$ cells/well.

hUCMSCs co-cultured with normal or $A\beta_{1-40}$-injured PC12 cells, PC12 cell supernatant or PC12 cell lysate: Normal or injured PC12 cells were incubated in the Transwell upper chamber for 24 hours and then transferred into the lower chamber for another 24 hours of culture.

The seven groups used in this study were as follows: group A: normal PC12 cell supernatant + hUCMSCs; group B: injured PC12 cell supernatant + hUCMSCs; group C: normal PC12 cell lysate + hUCMSCs; group D: injured PC12 cell lysate + hUCMSCs; group E: normal PC12 cells + hUCMSCs; group F: injured PC12 cells + hUCMSCs; group G: hUCMSCs cultured alone.

### *Western blot analysis*

hUCMSCs were washed twice with ice-cold PBS and lysed on ice. The lysing solution was composed of 20 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, 50 mM NaCl, 50 mM NaF, 1 mM $Na_3VO_4$, 1% Triton X-100 and 1 mM phenylmethyl sulfonylfluoride. At 4°C, hUCMSCs were centrifuged at 22 000 × *g* for 30 minutes. The supernatant was collected for determination of protein concentration using the Coomassie brilliant blue protein assay[46]. 50 μg of total protein was separated on 12% SDS-PAGE gels and then transferred onto a PVDF membrane. The membrane was blocked with 5% defatted milk at room temperature for 2 hours, incubated with mouse anti-human ChAT and β-actin monoclonal antibodies (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, and then with goat anti-mouse IgG secondary antibody (1:200; Beijing Zhongshan) at 37°C for 2 hours. Bands were revealed with an enhanced chemiluminescence agent (KeyGen

Biotechnology Co., Ltd.). This experiment was performed in triplicate. The absorbance of each protein band was determined using Image J software (National Institutes of Health, Rockville, MD, USA). The absorbance ratio of target protein to β-actin was calculated as the relative expression of target protein.

### *Expression of neural cell-specific protein MAP2 in hUCMSCs assessed with flow cytometry*

hUCMSCs were digested with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid, and then prepared into a single-cell suspension at a density of $1 \times 10^6$/mL. After addition of Cy5-labeled rabbit anti-MAP2 polyclonal antibody (BioLegend, San Diego, CA, USA), hUCMSCs were incubated for 1 hour at room temperature in the dark, centrifuged at 1 000 r/min for 10 minutes, washed three times with PBS, and centrifuged again at 1 000 r/min for 10 minutes. Finally, cells were resuspended, and MAP2 expression in hUCMSCs was detected using a FACScalibur flow cytometer (Becton Dickinson). This experiment was performed in triplicate. The proportion of MAP2-positive cells within the total cell count was calculated using CellQuest software (Becton Dickinson).

### *Effects of induced hUCMSCs on the proliferation of injured PC12 cells*

The proliferation of injured PC12 cells was determined using 24-well plates. The initial cell density was adjusted to $2.5 \times 10^3$ cells/well for hUCMSCs and $1 \times 10^5$ cells/well for $A\beta_{1\text{-}40}$-injured PC12 cells. Cell proliferation after 48 hours in culture (5% $CO_2$, 37 °C) was determined using the Cell Counting Kit-8 assay (Dojindo, Japan). Briefly, 10 μL of Cell Counting Kit-8 reagent was added to each well. After incubation at 37°C for 2 hours, the absorbance of each well was measured at 450 nm with an ELISA reader (Thermo Fisher Scientific, Boston, MA, USA). This experiment was performed in triplicate.

### *Statistical analysis*

All measurement data were statistically processed using SPSS 13.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used for comparison between groups. Results were expressed as mean ± SD. A level of $P < 0.05$ was considered statistically significant.

**Author contributions:** Jin Zhou and Guoping Tian designed the experiments, analyzed the data and wrote the manuscript. Guoping Tian, Jinge Wang, and Xiaoguang Luo checked and revised the manuscript. Siyang Zhang, Jianping Li, Li Li, Bing Xu, Feng Zhu, Xia Wang, Chunhong Jia, Weijin Zhao, Danyang Zhao, Aihua Xu performed the experiments. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** Animal-related procedures were reviewed and approved by the Animal Care and Use Committee of China Medical University.

**Author statements:** The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

## REFERENCES

[1] Buzanska L, Machaj EK, Zablocka B, et al. Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci. 2002;115:2131-2138.

[2] Jeong JA, Gang EJ, Hong SH, et al. Rapid neural differentiation of human cord blood-derived mesenchymal stem cells. Neuroreport. 2004;15:1731-1734.

[3] Sanchez-Ramos JR. Neural cells derived from adult bone marrow and umbilical cord blood. J Neurosci Res. 2002; 69:880-893.

[4] Jang YK, Park JJ, Lee MC, et al. Retinoic acid-mediated induction of neurons and glial cells from human umbilical cord-derived hematopoietic stem cells. J Neurosci Res. 2004;75:573-584.

[5] Jiang CC. Effects of co-engraftment of Schwann cells with neural stem cells into rats with Parkinson disease. Chin Med J. 2006;119:1030-1033.

[6] Kingham PJ, Kalbermatten DF, Mahay D, et al. Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Exp Neurol. 2007;207:267-274.

[7] Wu KH, Wu HP, Chan CK, et al. The role of mesenchymal stem cells in hematopoietic stem cell transplantation: from bench to bedsides. Cell Transplant. in press.

[8] Forraz N, McGuckin CP. The umbilical cord: a rich and ethical stem cell source to advance regenerative medicine. Cell Prolif. 2011;44 Suppl 1:60-69.

[9] Fan CG, Zhang QJ, Zhou JR. Therapeutic potentials of mesenchymal stem cells derived from human umbilical cord. Stem Cell Rev. 2011;7:195-207.

[10] Kang PB, Lidov HG, White AJ, et al. Inefficient dystrophin expression after cord blood transplantation in Duchenne muscular dystrophy. Muscle Nerve. 2010;41:746-750.

[11] Burra P, Arcidiacono D, Bizzaro D, et al. Systemic administration of a novel human umbilical cord mesenchymal stem cells population accelerates the resolution of acute liver injury. BMC Gastroenterol. 2012;12:88.

[12] Zhang S, Liu XZ, Liu ZL, et al. Stem cells modified by brain-derived neurotrophic factor to promote stem cells differentiation into neurons and enhance neuromotor function after brain injury. Chin J Traumatol. 2009;12: 195-199.

[13] Kadam S, Govindasamy V, Bhonde R. Generation of functional islets from human umbilical cord and placenta derived mesenchymal stem cells. Methods Mol Biol. 2012; 879:291-313.
[14] Pelosi E, Castelli G, Testa U. Human umbilical cord is a unique and safe source of various types of stem cells suitable for treatment of hematological diseases and for regenerative medicine. Blood Cells Mol Dis. 2012;49(1): 20-28.
[15] Yang S, Huang S, Feng C, et al. Umbilical cord-derived mesenchymal stem cells: strategies, challenges, and potential for cutaneous regeneration. Front Med. 2012; 6(1):41-47.
[16] Lee JY, Lee JH. Peripheral nerve regeneration with transplantation of umbilical cord mesenchymal stem cell and schwann cell in the rat sciatic nerve defect. Korea: Seoul National University. 2008.
[17] Cho SR, Yang MS, Yim SH, et al. Neurally induced umbilical cord blood cells modestly repair injured spinal cords. Neuroreport. 2008;19:1259-1263.
[18] Cho SR, Yang MS, Yim SH, et al. Neurally induced umbilical cord blood cells modestly repair injured spinal cords. Neuroreport. 2008;19:1259-1263.
[19] Lu LL, Liu YJ. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. Haematologica, 2006;91:1017-1026.
[20] 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.
[21] Liu CX, Wu HH, Ma HY, et al. Experimental study on the co-culture of human bone marrow mesenchymal stromal cells and human umbilical vein endothelial cells in vitro. Zuzhi Gongcheng yu Chongjian Waike Zazhi. 2011;7: 65-69.
[22] Han CP, Liu JY, Gao L, et al. Human umbilical cord blood mesenchymal stem cells differentiate into hepatocypte-like cells: feasibility of co-culture with human hepatocyte line LO2 in vitro. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2010;14:48-52.
[23] Wang XJ, Sheng BY, Huang ZY, et al. Proliferation and differentiation from neural stem cells into neurons following coculture with olfactory ensheathing cells. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2010;14:7501-7504.
[24] Lin WW, Chen X, Li Y, et al. Inducing effect of Schwann cells on differentiation of bone marrow stromal stem cells into nerve tissue cells. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2009;13:7865-7870.
[25] Zhang W, Zhang RQ, Jia GL, et al. Differentiation of bone marrow mesenchymal stem cells co-cultured with cardiomyocytes damaged by adyiamycin in vitro. Disi Junyi Daxue Xuebao. 2004;25:1101-1105.
[26] Park SI, Lim JY, Jeong CH, et al. Human umbilical cord blood-derived mesenchymal stem cell therapy promotes functional recovery of contused rat spinal cord through enhancement of endogenous cell proliferation and oligogenesis. J Biomed Biotechnol. 2012;2012:362473.
[27] Lou G, Zhang Q, Xiao F, et al. Intranasal administration of TAT-haFGF(14-154) attenuates disease progression in a mouse model of Alzheimer's disease. Neuroscience. 2012; 223:225-237.
[28] Perry EK, Johnson M, Ekonomou A, et al. Neurogenic abnormalities in Alzheimer's disease differ between stages of neurogenesis and are partly related to cholinergic pathology. Neurobiol Dis. 2012;47:155-162.
[29] Borlongan CV. Recent preclinical evidence advancing cell therapy for Alzheimer's disease. Exp Neurol. 2012;237: 142-146.
[30] Ray B, Bailey JA, Simon JR, et al. High-affinity choline uptake (HACU) and choline acetyltransferase (ChAT) activity in neuronal cultures for mechanistic and drug discovery studies. Curr Protoc Neurosci. 2012;Chapter 7:Unit 7.23.
[31] Cai J, Zhong YW, Li F, et al. Anti-amyloid beta single-chain Fv ameliorates behavioral impairment in Alzheimer’s disease mice via adeno-associated virus delivery. Neural Regen Res. 2011;6(2):96-100.
[32] Zhan Y, Ma DH, Zhang Y. Effects of cotransplantated Schwann cells and neural stem cells in a rat model of Alzheimer's disease. Neural Regen Res. 2011;6(4): 245-251.
[33] Yu M, Qin L, Wang Z, et al. Effects of MCI-186 on intracellular calcium ion concentration and membrane fluidity of hippocampal neurons in a rat model of Alzheimer’s disease. Neural Regen Res. 2011;6(29): 2245-2250.
[34] Tan Y, Deng YL, Qing H. Calcium channel blockers and Alzheimer’s disease. Neural Regen Res. 2012;7(2): 137-140.
[35] Zhang P, Zhao GY, Kang XJ, et al. Effects of lateral ventricular transplantation of bone marrow-derived mesenchymal stem cells modified with brain-derived neurotrophic factor gene on cognition in a rat model of Alzheimer’s disease. Neural Regen Res. 2012;7(4): 245-250.
[36] Bailey JA, Lahiri DK. Chromatographic separation of reaction products from the choline acetyltransferase and carnitine acetyltransferase assay: differential ChAT and CrAT activity in brain extracts from Alzheimer’s disease versus controls. J Neurochem. 2012;122:672-680.
[37] Nunes-Tavares N, Santos LE, Stutz B, et al. Inhibition of choline acetyltransferase as a mechanism for cholinergic dysfunction induced by amyloid-β peptide oligomers. J Biol Chem. 2012;287:19377-19385.
[38] Yu J, Guo KH, Li DP, et al. Tolerance of nestin+ cholinergic neurons in the basal forebrain against colchicine-induced cytotoxicity. Neural Regen Res. 2011; 6(24):1860-1864.
[39] Yang ZJ, Yan HW, Zhang GM, et al. Estrogen intervention in microvascular morphology and choline acetyltransferase expression in rat hippocampal neurons in chronic cerebral ischemia. Neural Regen Res. 2011; 6(17):1285-1290.

[40] Zhao CL, Chen Y, Zhang CL, et al. Impairment of cognitive function and reduced hippocampal cholinergic activity in a rat model of chronic intermittent hypoxia. Neural Regen Res. 2011;6(17):1301-1305.
[41] Yu LL, Ding YD, Spencer A, et al. Dorsal root ganglia progenitors differentiate to gamma-aminobutyric acid- and choline acetyltransferase-positive neurons. Neural Regen Res. 2012;7(7):485-491.
[42] Liu B, Deng CY, Zhang YQ, Zhang JX. Wnt3a expression during the differentiation of adipose-derived stem cells into cholinergic neurons. Neural Regen Res. 2012;7(19): 1463-1468.
[43] Park D, Joo SS, Kim TK, et al. Human neural stem cells overexpressing choline acetyltransferase restore cognitive function of kainic acid-induced learning and memory deficit animals. Cell Transplant. 2012;21: 365-371.
[44] Mengel-From J, Christensen K, Thinggaard M, et al. Genetic variants in the choline acetyltransferase (ChAT) gene are modestly associated with normal cognitive function in the elderly. Genes Brain Behav. 2011;10: 876-882.
[45] Babaei P, Soltani Tehrani B, et al. Transplanted bone marrow mesenchymal stem cells improve memory in rat models of Alzheimer's disease. Stem Cells Int. 2012;2012: 369417.
[46] Zhang L, Tan X, Dong C, et al. In vitro differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs), derived from Wharton's jelly, into choline acetyltransferase (ChAT)-positive cells. Int J Dev Neurosci. in press.
[47] Dongmei H, Jing L, Mei X, et al. Clinical analysis of the treatment of spinocerebellar ataxia and multiple system atrophy-cerebellar type with umbilical cord mesenchymal stromal cells. Cytotherapy. 2011;13:913-917.
[48] Ooi L, Sidhu K, Poljak A, et al. Induced pluripotent stem cells as tools for disease modelling and drug discovery in Alzheimer's disease. J Neural Transm. in press.
[49] Park D, Lee HJ, Joo SS, et al. Human neural stem cells over-expressing choline acetyltransferase restore cognition in rat model of cognitive dysfunction. Exp Neurol. 2012;234:521-526.
[50] Kitiyanant N, Kitiyanant Y, Svendsen CN, et al. BDNF-, IGF-1- and GDNF-secreting human neural progenitor cells rescue amyloid β-induced toxicity in cultured rat septal neurons. Neurochem Res. 2012;37:143-152.
[51] Leng X, Zhang Q, Zhai X, et al. Local transplant of human umbilical cord matrix stem cells improves skin flap survival in a mouse model. Tohoku J Exp Med. 2012;227: 191-197.
[52] Lu Z, Ye D, Qian L, et al. Human umbilical cord mesenchymal stem cell therapy on neuromyelitis optica. Curr Neurovasc Res. in press.
[53] Zhang Z, Lin H, Shi M, et al. Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients. J Gastroenterol Hepatol. 2012;27 Suppl 2:112-120.
[54] Xue G, He M, Zhao J, et al. Intravenous umbilical cord mesenchymal stem cell infusion for the treatment of combined malnutrition nonunion of the humerus and radial nerve injury. Regen Med. 2011;6:733-741.
[55] Peng J, Wang Y, Zhang L, et al. Human umbilical cord Wharton's jelly-derived mesenchymal stem cells differentiate into a Schwann-cell phenotype and promote neurite outgrowth in vitro. Brain Res Bull. 2011;84: 235-243.
[56] Ruan D, Zhang Y, Wang D, et al. Differentiation of human Wharton's jelly cells toward nucleus pulposus-like cells after coculture with nucleus pulposus cells in vitro. Tissue Eng Part A. 2012;18:167-175.
[57] State Council of the People's Republic of China. Administrative Regulations on Medical Institution. 1994-09-01.
[58] Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A. 1976;73:2424-2428.
[59] Zigmod MJ, Stricker EM. Animal models of Parkinsonism using selective neurotoxins: clinical and basic implications. J Int Rev Neurobiol. 1989;31:1-99.
[60] Pike CJ, Burdick D, Walencewicz AJ, et al. Neurodegeneration induced by beta-amyloid peptide in vitro: the role of peptide assembly state. J Neurosci. 1993;13:1676.
[61] Harkany T, Abrahám I, Kónya C, et al. Mechanisms of beta-amyloid neurotoxicity: perspectives of pharmacotherapy. Rev Neurosci. 2000;11:329-382.
[62] Harkany T, Abrahám I, Timmerman W, et al. Beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis. Eur J Neurosci. 2000;12:2735-2745.
[63] Lorenzo A, Yuan M, Zhang Z, et al. Amyloid beta interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. Nat Neurosci. 2000; 3:460-464.
[64] Bossolaso P, Cova L, Calzarossa C, et al. Neuro-glial differentiation of human bone marrow stem cells in vitro. J Exp Neurol. 2005;193:312-325.

(Edited by Zhu XJ, Yang XY/Song LP)