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Umbilical cord-derived mesenchymal stem cells retain immunomodulatory and anti-oxidative activities after neural induction[★]

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Abstract

The immunomodulatory and anti-oxidative activities of differentiated mesenchymal stem cells contribute to their therapeutic efficacy in cell-replacement therapy. Mesenchymal stem cells were isolated from human umbilical cord and induced to differentiate with basic fibroblast growth factor, nerve growth factor, epidermal growth factor, brain-derived neurotrophic factor and forskolin. The mesenchymal stem cells became rounded with long processes and expressed the neural markers, Tuj1, neurofilament 200, microtubule-associated protein-2 and neuron-specific enolase. Nestin expression was significantly reduced after neural induction. The expression of immunoregulatory and anti-oxidative genes was largely unchanged prior to and after neural induction in mesenchymal stem cells. There was no significant difference in the effects of control and induced mesenchymal stem cells on lymphocyte proliferation in co-culture experiments. However, the expression of human leukocyte antigen-G decreased significantly in induced neuron-like cells. These results suggest that growth factor-based methods enable the differentiation of mesenchymal stem cell toward immature neuronal-like cells, which retain their immunomodulatory and anti-oxidative activities.

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Key Words

umbilical cord; mesenchymal stem cell; immunomodulation; oxidative stress; neural induction; neural regeneration

Research Highlights

(1) Umbilical cord-derived mesenchymal stem cells can differentiate into neuron-like cells following induction with basic fibroblast growth factor, nerve growth factor, epidermal growth factor, brain-derived neurotrophic factor and forskolin.

(2) Real-time quantitative reverse transcription-PCR and co-culture of induced mesenchymal stem cells with lymphocytes verified that neural induction with growth factors did not greatly influence the immunomodulatory and anti-oxidative activities of mesenchymal stem cells.

Abbreviation

UC-MSCs, umbilical cord-derived mesenchymal stem cells

INTRODUCTION

Mesenchymal stem cells (MSCs) are a highly promising source of adult stem cells for cell-based therapeutics because of their substantial multilineage differentiation capacity and secretory activities^[1-4]. Allogeneic umbilical cord and autologous bone marrow may be ideal practical sources of MSCs because of their accessibility and lack of ethical concerns. In addition to their differentiation into osteoblasts, chondroblasts and adipocytes^[5], MSCs also have the capacity to differentiate into neuron-like cells^[6-8]. Therapeutic benefit has been shown in cerebral ischemia^[9-10] and trauma^[11] following administration of MSCs. *In vitro* neurally induced human MSCs have been shown to influence injured brain tissue repair *in vivo*^[12].

Recent studies have suggested that the immunomodulatory activity of MSCs is of crucial importance to cell therapy using MSCs^[13]. There is *in vitro* evidence that MSCs can directly modulate the function of T-cells. Moreover, they inhibit the maturation and migration of various antigen-presenting cells, suppress B-cell activation, induce suppressor T-cell formation, and alter the expression of several receptors necessary for antigen capture and processing^[14-15]. This immunosuppressive activity of MSCs may play an important role in the repair of nervous system injuries. In addition, the anti-oxidative effects of MSCs can improve the survival of injured neuronal cells. Expression of the heme-oxygenase-1 protein within MSCs decreased cytotoxicity and inhibited apoptosis induced by oxidative stresses^[16].

Immunomodulatory and anti-oxidative activities are key properties of MSCs. However, whether neurally-differentiated MSCs retain these properties is unclear. In the present study, we isolated MSCs from umbilical cord and analyzed the immunomodulatory and anti-oxidative properties of these umbilical cord-derived MSCs (UC-MSCs) before and after neural induction at the cellular and molecular levels.

RESULTS

Biological characteristics of UC-MSCs

Adherent cells with a fibroblastic morphology were observed as early as 48 hours after establishing explant cultures of umbilical cord tissue^[17]. The cells formed a monolayer of homogeneous bipolar spindle-like cells with a whirlpool like morphology within 2 weeks (Figure 1A).

Surface antigens expressed by the cultured cells at passage 5 were detected by fluorescence-activated cell sorting. The results showed that the cells expressed CD29, CD44, CD73, CD90, CD105 and CD106, but did not express CD34 and CD45, consistent with the phenotype of MSCs (Figure 1B).

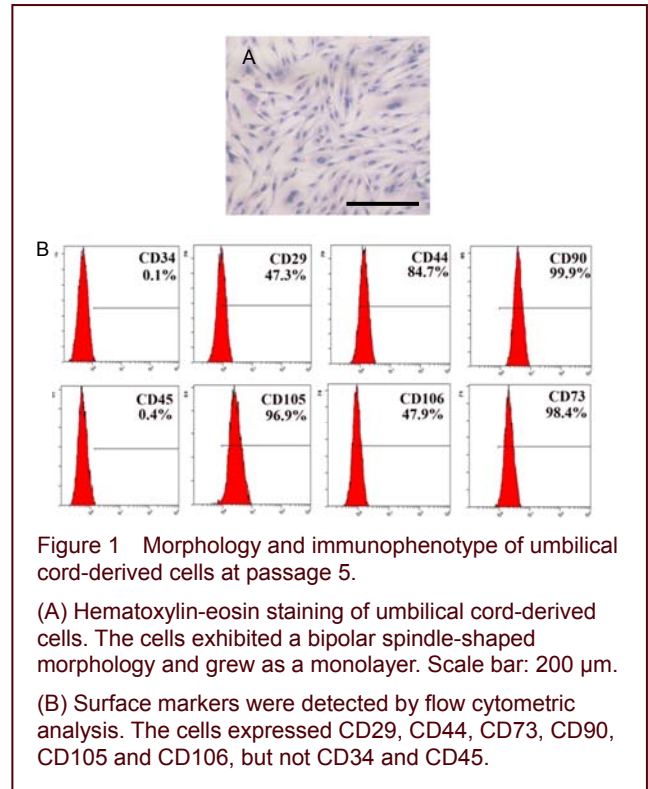


Figure 1 Morphology and immunophenotype of umbilical cord-derived cells at passage 5.

(A) Hematoxylin-eosin staining of umbilical cord-derived cells. The cells exhibited a bipolar spindle-shaped morphology and grew as a monolayer. Scale bar: 200 μ m.

(B) Surface markers were detected by flow cytometric analysis. The cells expressed CD29, CD44, CD73, CD90, CD105 and CD106, but not CD34 and CD45.

Osteogenic and adipogenic differentiation capacities of MSCs

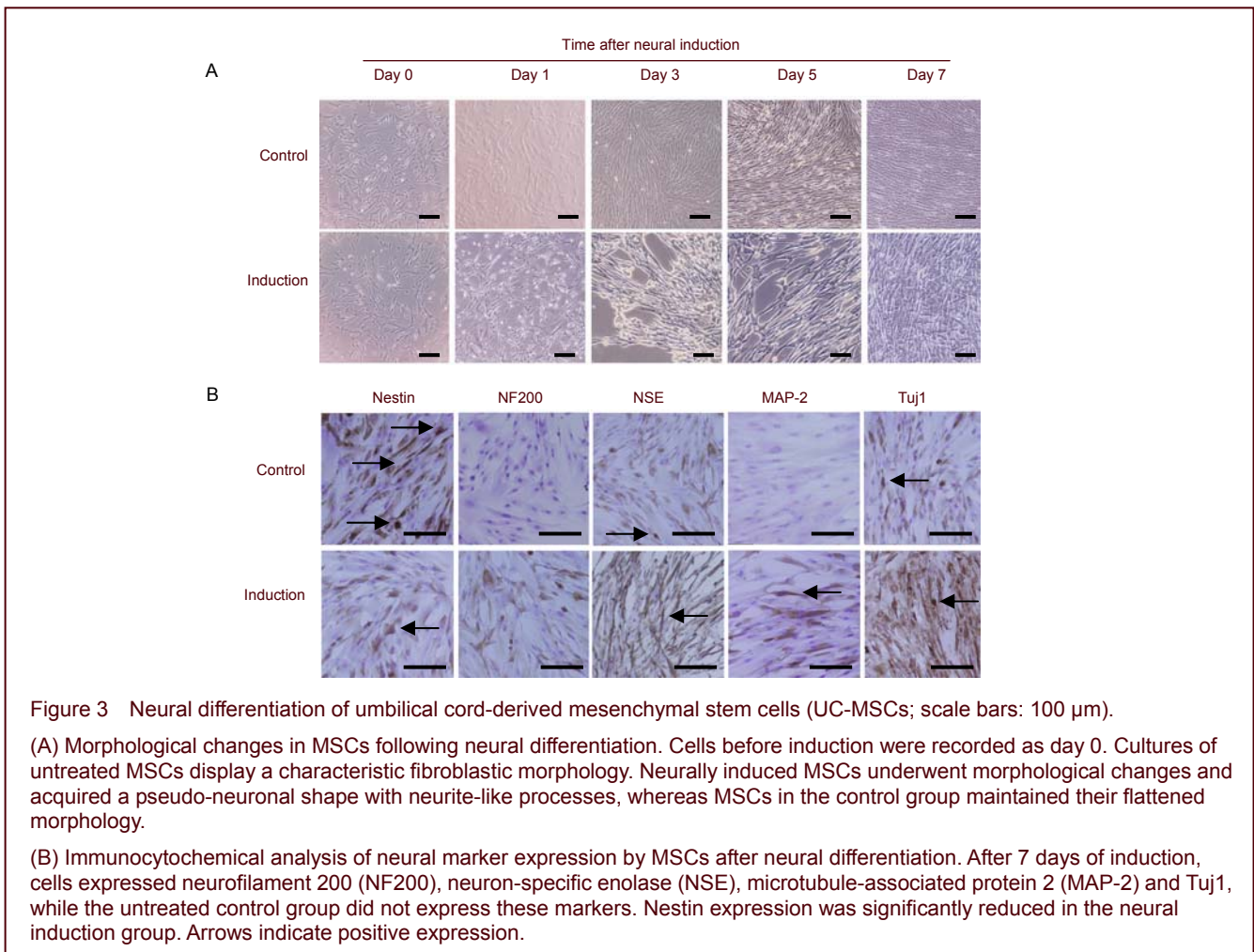
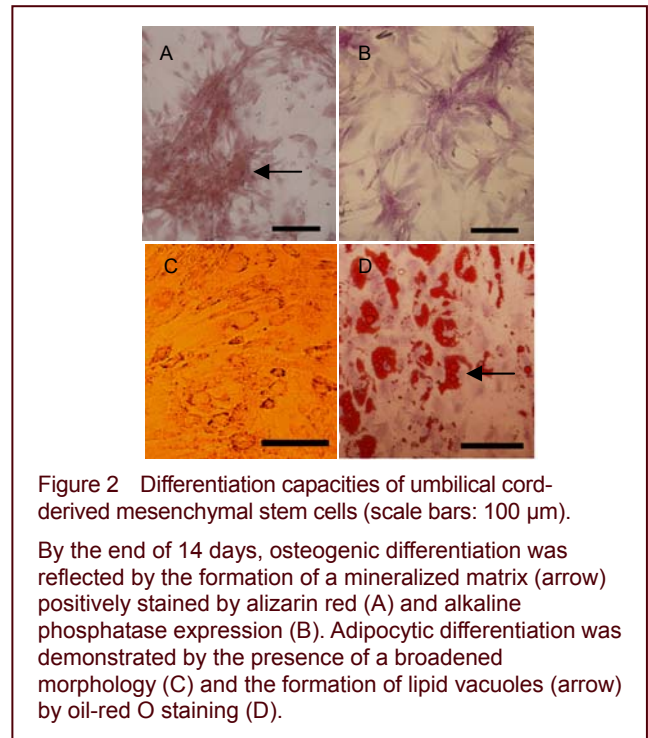
The differentiation capacity of the MSCs was assessed using passage 4 cells derived from umbilical cord. When induced to differentiate under osteogenic conditions, the MSCs increasingly congregated with increasing time of induction and formed a mineralized matrix, as confirmed by alizarin red staining (Figure 2A). Most MSC-like cells became positive for alkaline phosphatase by the end of 14 days (Figure 2B). No mineralized matrix was observed in the cells kept in regular growth medium. The spindle-shaped MSCs flattened and broadened after 1 week of adipogenic induction (Figure 2C). Small oil droplets gradually appeared in the cytoplasm. By the end of the second week, almost all of the cells contained numerous oil red O-positive lipid droplets (Figure 2D). The cells maintained in regular growth medium did not stain with oil red O.

Induced differentiation of UC-MSCs into neuron-like cells

When MSCs were exposed to neural induction medium, they rapidly underwent dramatic morphological changes.

Within a few hours, the majority of the cells had rounded up and extended long dendritic cellular processes. MSCs in the control group maintained their flattened morphology. The morphology of the induced cells was almost indistinguishable from control cells after 6 days of continuous induction because the cells became confluent (Figure 3A). In addition to the morphological evidence, we therefore compared the expression of neural specific markers in MSCs, *i.e.*, nestin, Tuj1 (neuronal class III beta-tubulin), neurofilament 200, microtubule-associated protein 2, and neuron-specific enolase, by immunocytochemistry prior to and after neural induction. As shown in Figure 3B, MSCs expressed neurofilament 200, neuron-specific enolase, microtubule-associated protein 2 and Tuj1 after 7 days of induction. Cells in the untreated control group did not show any immunoreactivity for neurofilament 200, neuron-specific enolase, microtubule-associated protein 2 and Tuj1. Nestin expression was significantly reduced in cells cultured in neural induction medium. The reduction of nestin expression in the induced cells reflected their differentiation^[18]. Overall, the results suggest that

UC-MSCs can be induced to differentiate into neuron-like cells *in vitro*.



Neural induction does not change the immunomodulatory activity of MSCs

The immunosuppressive effects of MSCs in response to neural induction were analyzed *in vitro* using a lymphocyte co-culture assay. Approximately 1.9×10^4 MSCs and 4×10^4 peripheral blood mononuclear cells were seeded into each well of a 96-well plate. Our results showed that MSCs both prior to and after neural induction inhibited phytohemagglutinin-stimulated peripheral blood mononuclear cell proliferation. Although the inhibitory activity of uninduced MSCs was higher than neurally induced MSCs, there was no significant difference ($P = 0.209$; Figure 4A).

Expression of immunoregulatory genes by MSCs prior to and after neural induction

Real-time PCR quantification of eleven immunoregulatory-related genes was performed.

Upregulation of four genes indoleamine

2,3-dioxygenase-1 (IDO-1), cyclooxygenase-2, hepatocyte growth factor, and inducible nitric oxide synthase) and downregulation of six genes (human leukocyte antigen-G, interleukin-10, tumor growth factor- β 1, matrix metalloproteinase-2, matrix metalloproteinase-9, and heme-oxygenase-1) were confirmed in neurally induced MSCs, but no significant difference was observed compared with the control group ($P > 0.05$; Figure 4B). It is also worth noting that human leukocyte antigen-G was significantly downregulated in induced MSCs ($P = 0.034$; Figure 4B).

The expression of heme-oxygenase-1 protein was chosen as an indicator of anti-oxidative activity^[19]. On western blot analysis, there were no significant differences between the heme-oxygenase-1 expression levels before and after neural induction (Figure 4C). The ratios of heme-oxygenase-1 to GAPDH in the untreated group and the neurally induced group were 1.18 and 1.07, respectively.

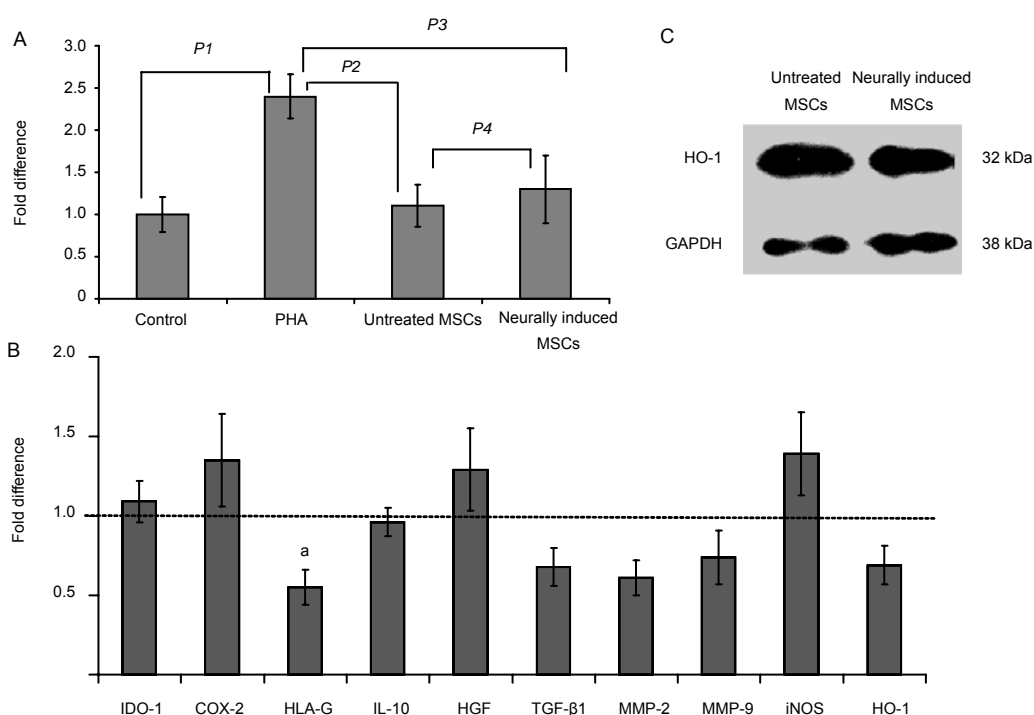


Figure 4 Influence of neural induction on the immunomodulatory and anti-oxidative activities of mesenchymal stem cells (MSCs).

(A) Neural induction does not affect the cells' capacity to suppress allogeneic lymphocyte proliferation. The proliferation of lymphocytes in the MSC and induced groups was significantly lower than in the phytohemagglutinin (PHA) stimulated group (P values: $P_1 = 0.003$ 1; $P_2 = 0.007$ 8; $P_3 = 0.012$ 1), while there was no significant difference between the MSCs and induced cells (P values: $P_4 = 0.209$). Quantitative data are expressed as mean \pm SD.

(B) Real-time PCR quantification of immunoregulatory-related genes. The untreated control group values are set at fold change = 1. Only the expression of human leukocyte antigen-G (HLA-G) was reduced significantly ($^aP = 0.034$, vs. control group by unpaired Student's t -test). Quantitative data are expressed as mean \pm SD.

(C) Western blotting showed no significant difference in heme-oxygenase-1 (HO-1) expression before and after neural induction. GAPDH was used as the internal control.

IDO-1: Indoleamine 2,3-dioxygenase-1; COX-2: cyclooxygenase-2; IL-10: interleukin-10; HGF: hepatocyte growth factor; TGF- β 1: tumor growth factor- β 1; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; iNOS: inducible nitric oxide synthase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

DISCUSSION

Cell-based therapy for nerve repair has achieved promising functional recovery. MSCs constitute an interesting cellular source for promoting neural regeneration in patients with neurodegenerative diseases because MSCs have the capacity to differentiate into cells with a neuronal phenotype *in vivo*^[20-21] or *in vitro* under specific culture conditions^[7, 22-23]. However, the purity and yield of cells differentiated from stem cells must be sufficient to exert meaningful therapeutic effects. Therefore, pre-induction differentiation *in vitro* prior to transplantation is essential. The earliest neuronal differentiation protocols were based on the use of a mixture of chemical compounds (beta-mercaptoethanol, dimethyl sulfoxide and butylated hydroxyanisole) that induced MSCs to rapidly acquire a neuronal-like morphology characterized by the expression of specific neuronal markers^[8, 24-25]. However, many subsequent studies demonstrated that the morphological and molecular changes in MSCs were probably because of a stress response, rather than real differentiation into neuronal cells. Despite the neuronal-like morphology and neural protein expression, induced MSCs did not have basic neuronal functional properties^[26].

The latest methods for inducing the differentiation of MSCs into neuron-like cells *in vitro* include the use of cytokines^[27-29] and signal pathway inhibitors^[30]. These combinations are more moderate but equally effective for inducing differentiation compared with chemical methods. Thus, in the present study, we also attempted to induce the neuronal differentiation of MSCs using a cytokine combination that included nerve growth factor, epidermal growth factor, basic fibroblast growth factor and brain-derived neurotrophic factor together with forskolin. Our observations were in accordance with previously published results^[31-33]. Morphological changes in MSCs were accompanied by protein expression changes: a decrease in the immature stem cell marker, nestin, together with an increase in the neuronal immature marker, Tuj1, and thereafter of the more mature neuronal markers, microtubule-associated protein 2, neurofilament 200 and neuron-specific enolase. Moreover, a decrease in nestin expression, which is consistent with ongoing maturation, was also observed when MSCs were differentiated according to the protocol of Woodbury *et al*^[8]. Although microtubule-associated protein 2 is not specific to neuronal cells, the increased expression of this gene might be involved in neurite initiation^[34]. However, because transfusion therapy does not require terminal

differentiation, we did not analyze electrophysiological and other functional indicators.

The consequences of physical injuries and neurodegenerative diseases in the central nervous system are severe because of acute inflammation/immune response, trophic factor withdrawal, oxidative stress, excitotoxicity, hypoxia or anoikis. To exploit the immunosuppressive and neuroprotective activities of MSCs, we further hope that MSC-derived neural cells may retain the secretory and anti-oxidative activities of MSCs. Our neural induction protocol led to changes in the expression of immune-related genes such as IDO-1, cyclooxygenase-2, hepatocyte growth factor, inducible nitric oxide synthase, interleukin-10, tumor growth factor- β 1, matrix metalloproteinase-2, matrix metalloproteinase-9 and heme-oxygenase-1, but without statistical significance. IDO-1 and cyclooxygenase-2 participate in the synthesis of prostaglandin E₂ in MSCs. Prostaglandin E₂ is the major soluble factor responsible for the *in vitro* inhibition of the allogeneic lymphocyte reaction^[35]. IDO-1 is an enzyme that catabolizes tryptophan, an essential amino acid, and plays a critical role in immunosuppression mediated by MSCs of various tissue origins^[36]. In accordance with the role of this secretory protein, we showed similar results for other well-known immunosuppressive factors, *i.e.*, hepatocyte growth factor, interleukin-10 and tumor growth factor- β 1^[37-39]. Matrix metalloproteinase-2 and matrix metalloproteinase-9 secreted by MSCs modulate the immune system by reducing the surface expression of CD25 on responding T-cells^[40]. More importantly, we showed that inducible nitric oxide synthase and heme-oxygenase-1, which play important roles in both immunoregulation and anti-oxidative processes^[41-42], did not change significantly after neural induction. Altogether, these phenomena are in accordance with the results of the lymphocyte proliferation assays. There was no significant difference between the activities of MSCs and induced cells in lymphocyte co-cultures.

Although the immunosuppressive activity of neurally induced MSCs was retained, human leukocyte antigen-G was significantly downregulated in MSCs and the cells lost their non-immunogenic properties. It is widely regarded that human leukocyte antigen-G expressed on placenta and umbilical cord inhibits immune responses to foreign antigens *via* these actions on immune cells^[43].

In conclusion, neural induction does not change the immunosuppressive and anti-oxidative activities of MSCs. The suppression of host innate inflammatory responses

may be a key factor for improving MSC survival and engraftment^[29]. Neuronal-primed human UC-MSCs may be clinically useful for treating Parkinson's disease, spinal cord injuries and optic nerve injuries. However, their immune privilege may gradually diminish upon differentiation *in vivo*. Hence, the question of how to maintain the immunoprivileged status of the cells has attracted particular attention.

In summary, growth factor-based methods allowed MSC differentiation toward immature neuronal-like cells. The induced neuron-like cells retained their immunomodulatory properties *in vitro*, but their immunoprivileged status was gradually lost following neural differentiation.

MATERIALS AND METHODS

Design

A comparative observation at the cellular and molecular levels.

Time and setting

The experiments were performed at the Cryomedicine Laboratory of Qilu Hospital, Shandong University, China from October 2011 to May 2012.

Materials

Umbilical cords ($n = 10$; clinically normal pregnancies) were obtained from Qilu Hospital of Shandong University after normal full-term deliveries. Informed consent was obtained from the mothers. Tissue collection for research was approved by the Ethics Committee of Qilu Hospital.

Methods

Isolation and culture of MSCs

Umbilical cords were excised and washed in 0.1 M PBS (pH 7.4) to remove excess blood. The cords were dissected and blood vessels removed. The remaining tissue was cut into small pieces (1–2 cm²) and placed in plates containing low-glucose Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco-BRL). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. The medium was changed every 3–4 days. After 7 to 12 days in culture, adherent cells were observed growing out from the individual tissue explants. The adherent fibroblast-like cells became confluent after 2–3 weeks in culture. They were then trypsinized using 0.25% trypsin (Gibco-BRL)

and passaged at 1×10^4 cells/cm² in the medium described above. After three or more passages, the cells were analyzed^[44].

Cell surface antigen phenotyping by flow cytometry

Passages 15–17 cells were collected and treated with 0.25% trypsin. The cells were individually stained with fluorescein isothiocyanate or phycoerythrin-conjugated anti-marker monoclonal antibodies in 100 µL PBS for 15 minutes at room temperature or for 30 minutes at 4°C, as recommended by the manufacturer. The antibodies used were specific for the human antigens, CD29, CD34, CD44, CD45, CD73, CD90, CD105 and CD106 (10 µL for 1×10^6 cells; SeroTec, Raleigh, NC, USA). Cells were analyzed on a flow cytometry system (Cytometer 1.0, Cytomics™FC500, Beckman Coulter, Brea, CA, USA). Positive cells were counted and the signals for the corresponding immunoglobulin isotypes were compared^[45].

Determination of the differentiation capacities of the cultured cells

To investigate the differentiation potential of the fibroblast-like cells, passage 4 cells were cultured under conditions appropriate for inducing their differentiation into each lineage. The cell density was 2×10^4 cells per cm², and the medium was replaced every 3–4 days for the differentiation assays. The osteogenic differentiation medium consisted of low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.1 µM dexamethasone, 50 mM β-glycerol phosphate and 0.2 mM ascorbic acid (Sigma, St. Louis, MO, USA). The adipogenic differentiation medium consisted of high-glucose Dulbecco's modified Eagle's medium supplemented with 0.25 mM 3-isobutyl-1-methylxanthine, 0.1 µM dexamethasone, 0.1 mM indomethacin (Sigma), 6.25 µg/mL insulin (Peptotech EC Ltd., London, UK) and 10% fetal bovine serum (Gibco-BRL). Cells kept in the regular growth medium served as controls. For evaluation of mineralized matrix formation, cells were fixed with 4% formaldehyde and stained with 1% alizarin red S (Sigma) solution in water for 10 minutes. Positive staining was indicated by brown staining of the cells. For alkaline phosphatase staining, cells were incubated with 300–400 µL (enough to coat the well) nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate chromogen solution (Jiancheng Bioengineering, Nanjing, China) for 8–10 minutes at room temperature. Positive staining was indicated by distinct purple staining of the cell surface. For oil red O staining, cells were fixed with 4% formaldehyde, stained with oil red O

(Sigma) for 10 minutes, and then counterstained with Mayer hematoxylin (Sigma) for 1 minute. The formation of neutral lipid vacuoles was visualized by red staining^[45].

Neuronal differentiation of MSCs

To initiate neural differentiation, subconfluent MSCs at passages 4–7 were treated with neural induction medium consisting of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 2% B27 (Invitrogen, Carlsbad, CA, USA), 5 μ M forskolin (Sigma), 125 μ M 3-isobutyl-1-methylxanthine (Sigma), 10 μ M β -mercaptoethanol and the following cytokines: 10 ng/mL basic fibroblast growth factor, 50 ng/mL nerve growth factor, 10 ng/mL epidermal growth factor, 10 ng/mL brain-derived neurotrophic factor, and 10 ng/mL fibroblast growth factor-2 (all from Peprotec, Rocky Hill, NJ, USA). The cells were incubated for 7 days before fixation or harvesting^[46-48].

Immunocytochemical analysis of the expression of neuron-specific markers after neural induction

Immunocytochemical staining was performed in cells grown on glass coverslips. After washing in PBS, cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 minutes and rinsed again with PBS. The cells were permeabilized with 0.1% Tween-PBS for 5 minutes and treated with 3% bovine serum albumin/0.1% Tween/PBS for 30 minutes. Thereafter, they were incubated overnight at 4°C with a primary monoclonal antibody (mouse anti-human nestin, Abcam, 1:250; rabbit anti-human Tuj1, Abcam, 1:200; mouse anti-human neurofilament 200, Sigma, 1:50; rabbit anti-human microtubule-associated protein 2, Sigma, 1:250; rabbit anti-human neuron-specific enolase, Abcam, 1:500) in 1% bovine serum albumin/PBS. Cells were then incubated with goat anti-mouse or mouse anti-rabbit secondary antibodies (1:2 000; Zhongshan Golden Bridge, Beijing, China) at room temperature for 1.5 hours. The 3,3'-diaminobenzidine peroxidase substrate system (Sigma) was used. Images were captured with an Olympus MagnaFire S99800 digital camera mounted on an IX71 Olympus inverted microscope (Olympus, Tokyo, Japan).

Peripheral blood mononuclear cell proliferation assay after neural induction

Neurally induced MSCs and the untreated control MSCs must adapt to co-culture medium (RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin) by graded reduction of the proportion of

Dulbecco's modified Eagle's medium. Subsequently, MSCs were plated in triplicate in 96-well plates in 100 μ L of co-culture medium at 100% confluence. Allogeneic peripheral blood mononuclear cells were isolated from peripheral blood by Ficoll/Hypaque gradient centrifugation, resuspended at 4×10^5 /mL, and added to wells (4×10^4 cells/well in 100 μ L of medium) containing or lacking MSCs in the presence of 10 μ g/mL phytohemagglutinin (Sigma). After 48 hours, 100 μ L of cells from each well were transferred to new 96-well plates containing 10 μ L of Cell Counting Kit-8 reagent (Dojindo, Kumamoto, Japan). The absorbance at 450 nm was measured with a model 450 microplate reader (Bio-Rad Labs, Richmond, CA, USA). All experiments were performed in triplicate and were repeated at least twice^[49].

Real-time quantitative reverse transcription-PCR analysis of immunomodulatory gene mRNA expression after neural induction

Total RNA was purified from each sample of UC-MSCs using Trizol (Invitrogen) as per the manufacturer's protocol. Measures were taken to guarantee that equal amounts of RNA from each subject were used. The expression levels of immunomodulatory genes were analyzed by quantitative reverse transcription-PCR. Total RNA was reverse transcribed to cDNA with the Omniscript cDNA Synthesis Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Briefly, approximately 0.2 μ g of total RNA was reverse transcribed in a 20 μ L reaction mixture containing the following components: 1 \times RT buffer, deoxynucleotide triphosphate mix (5 mM each), RNase inhibitor (10 U/ μ L RNase Out, Invitrogen), and 4 U Omniscript RT. The samples were incubated at 37°C for 60 minutes. The resulting cDNA was stored at -80°C prior to real-time PCR.

All reagents and primers were obtained from Bioasi Co., Ltd., Shanghai, China. Beta-actin was validated as an internal control. The expression of each gene relative to β -actin was determined using the $2^{-\Delta\text{CT}}$ method, where $\Delta\text{CT} = (\text{CT}_{\text{target gene}} - \text{CT}_{\beta\text{-actin}})$. Real-time PCR conditions were as follows: 95°C for 4 minutes, 94°C for 15 seconds, and 60°C for 1 minute, for a total of 40 cycles. Quantitative RT-PCR was performed using an ABI 7500 PCR system (Applied Biosystems, Foster City, CA, USA) and the SYBR green I dye (Toyobo, Osaka, Japan). Successful amplification was defined by the presence of a single dissociation peak on the thermal melting curve. Data were analyzed with Sequence Detection Software 1.4 (Applied Biosystems). Results are given as the fold change relative to untreated control group cDNA, while the control group values are set at fold change = 1.

Reported data are representative of at least three independent experiments. The primers used are listed below.

Primer	Sequence (5' -3')	Size (bp)
Interleukin-10	Forward: GCC TTG TCT GAG ATG ATC CAG TT	85
	Reverse: TCA CAT GCG CCT TGA TGT CT	
Hepatocyte growth factor	Forward: CCC TGA AGG ATC AGA TCT GGT T	81
	Reverse: AGG TAA ATC AAT CGT ACT AAC AAA ATC ATC	
Indoleamine 2,3-dioxygenase-1	Forward: GCC CTT CAA GTG TTT CAC CAA	90
	Reverse: GCC TTT CCA GCC AGA CAAATA T	
Tumor growth factor- β 1	Forward: GGG AAA TTG AGG GCT TTC G	83
	Reverse: GAA CCC GTT GAT GTC CAC TTG	
Matrix metalloproteinase-2	Forward: GGC TGG AGC ACT CCC AAG A	82
	Reverse: ATC CTG GGA CAG ACG GAA GTT	
Matrix metalloproteinase-9	Forward: CGC TGG GCT TAG ATC ATT CC	107
	Reverse: GTG CCG GAT GCC ATT CAC	
Inducible nitric oxide synthase	Forward: GGT GGA AGC GGT AAC AAA GG	81
	Reverse: TGC TTG GTG GCG AAG ATG A	
Cyclooxygenase-2	Forward: GGT CTG GTG CCT GGT CTG AT	80
	Reverse: TCC TGT TTA AGC ACA TCG CAT ACT	
Heme-oxygenase-1	Forward: AGG GAA GCC CCC ACT CAA C	81
	Reverse: ACT GTC GCC ACC AGA AAG CT	
Human leukocyte antigen-G	Forward: GGA GCC CCT CAT GCT GAG A	80
	Reverse: GGA CAA CCA GGC CAG CAA	
β -actin	Forward: GGA CAT CCG CAA AGA CCT GTA	80
	Reverse: GCA TCC TGT CGG CAA TGC	

Western blot analysis of heme-oxygenase-1 expression in MSCs after neural induction

For heme-oxygenase-1 western blot analysis, after separation by 7.5% SDS-PAGE, proteins (70 μ g) were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 hour in 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with the mouse anti-heme-oxygenase-1 antibody (7.25 μ g/mL; Abcam, Cambridge, UK) and mouse anti-GAPDH antibody (1:1 000; Abcam) overnight at 4°C. After washing in Tris-buffered saline containing 0.05% Tween

20, membranes were incubated for 1 hour at room temperature with peroxidase-labeled goat anti-mouse secondary antibodies (1:2 000; Zhongshan Golden Bridge). The immunoreactive bands were visualized using a SuperEnhanced chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China) and subsequent exposure of the blots to Biomax L film (Kodak). Image analysis software, GIS 1D Ver 4.0 (Tanon Science & Technology Co., Ltd., Shanghai, China), was used to determine band intensities. GAPDH was used as the internal control.

Statistical analysis

Data were analyzed using SPSS software version 14.0 (SPSS Inc, Chicago, IL, USA). Quantitative data were expressed as mean \pm SD. Statistical analysis was performed using unpaired Student's *t*-test for comparisons between two groups, and by one-way analysis of variance followed by Bonferroni test for comparisons among at least three groups. A value of *P* < 0.05 was considered statistically significant.

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Author contributions: Jianjun Li designed the experiments and wrote the manuscript. Dong Li provided research funding and finalized the manuscript. Jianjun Li, Xiuli Ju and Qing Shi carried out the laboratory experiments and interpreted the results. Dakun Wang analyzed the data. Fengcai Wei defined the research theme and co-worked on data analysis. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: All experimental protocols were approved by the Ethics Committee of Qilu Hospital, Shandong University, China.

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/funding source disputations.

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