

# Effects of ginsenoside Rg1 on proliferation and directed differentiation of human umbilical cord mesenchymal stem cells into neural stem cells

Li Xiao<sup>a,b</sup>, Maoyuan Wang<sup>a,b</sup>, Kang Zou<sup>c</sup>, Zuoyong Li<sup>d</sup> and Jun Luo<sup>e</sup>

**Objective** Human umbilical cord mesenchymal stem cells (hUCMSCs) can be transformed into neural stem cells (NSCs) and still maintain immunomodulatory and antioxidant effects. Transplantation of NSCs induced by hUCMSCs would be a promising therapeutic strategy for the treatment of neurological diseases. Ginsenoside Rg1 has neuroprotective effects and influences cell proliferation and differentiation. In this study, we further evaluated the effects of ginsenoside Rg1 on the proliferation and directional differentiation of hUCMSCs into NSCs.

**Methods** The CCK-8 assay was used to determine the optimal dose of ginsenoside Rg1 with respect to hUCMSC proliferation and differentiation. NSCs were authenticated using immunofluorescence staining and flow cytometry and were quantified in each group. RT-PCR was used to screen the signaling pathway by which ginsenoside Rg1 promoted the differentiation of hUCMSCs into NSCs.

**Results** The optimal dose of Rg1 to promote hUCMSC proliferation and differentiation to NSCs was 10  $\mu\text{mol/l}$ . Flow cytometry and immunofluorescence showed that induced NSCs expressed nestin and sex-determining region Y-box 2, with higher expression levels in the Rg1 group than that in the negative control group. RT-PCR

showed that Rg1 downregulates the expression of genes involved in Wnt/ $\beta$ -catenin and Notch signaling pathways in the induction process.

**Conclusion** Ginsenoside Rg1 not only promotes the proliferation and viability of hUCMSCs in the process of differentiation into NSCs but also improves the differentiation efficiency. This study provides a basis for the development of hUCMSC-derived NSCs for the treatment of nervous system diseases and for analyses of underlying biological mechanisms. *NeuroReport* 33: 413–421 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.

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**Keywords:** differentiation, ginsenoside Rg1, human umbilical cord mesenchymal stem cells, neural stem cells, proliferation

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## Introduction

The incidences of cerebral apoplexy, neurological degeneration, and neurological diseases caused by trauma are increasing year by year; however, the clinical treatments are insufficient [1]. The regeneration of nerve cells and neural stem cell (NSC) transplantation provide new strategies for the treatment of neurological diseases [2]. In particular, human NSCs derived from embryonic tissues and the brains of aborted fetuses are promising candidate cells for the treatment of neurodegenerative diseases and other central nervous system diseases. However, immune rejection, ethical issues, and cost limit the use of allogeneic NSCs [3].

Human umbilical cord mesenchymal stem cells (hUCMSCs) have various advantages over stem cells from other

sources for practical applications, including the wide range of sources, lack of ethical controversy, efficient amplification, and very low immunogenicity [4]. Previous studies have shown that hUCMSCs can differentiate into NSCs [5]. Furthermore, hUCMSCs maintain immunomodulatory and antioxidant activities after induction into nerve cells [6].

Various agents, including transretinoic acid, Centella asiatica, and cyclic adenosine monophosphate-elevating agents, can promote the differentiation of hUCMSCs into NSCs. However, these agents are cytotoxic; they have been shown to reduce cell proliferation [7] or promote apoptosis [8].

Ginsenoside Rg1 is the main active component of ginseng. It has neuroprotective effects [9] and promotes cell proliferation [10] and differentiation [11]. Therefore, in this study, we evaluated, for the first time, whether ginsenoside Rg1 promotes the induction of hUCMSCs into NSCs.

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## Materials and methods

### Isolation and culture of human umbilical cord mesenchymal stem cells

Human umbilical cords ( $n = 6$ ) were obtained from women who had a healthy full-term pregnancy and who underwent a cesarean delivery. Women had no infectious diseases or genetic history. The fetuses had no congenital diseases. Each umbilical cord unit was handled in a sterile biosafety cabinet (Airtech, Suzhou, China) and cut into approximately 5 cm sections. The pieces were washed two or three times with sterile PBS (SolarBio, Beijing, China) to remove the blood. Subsequently, veins and arteries were removed to avoid contamination by endothelial cells. Using sterile scissors and tweezers, Wharton's jelly was cut into 1-mm pieces and spread evenly in a 175-cm<sup>2</sup> sterile plastic culture bottle (Thermo, Waltham, Massachusetts, USA). Then, 12-ml of mesenchymal stem cell (MSC) proliferation medium [Alpha-MEM (REF. No. 41061-029; Gibco, Waltham, Massachusetts, USA) supplemented with 10% volume/volume (v/v) fetal bovine serum (FBS) (Cat. No. FNA500; ExCell Bio, Shanghai, China) and 1% v/v Penicillin–Streptomycin Solution (Cat. No. BL505A; Biosharp, Beijing, China)] was added to the cell and tissue culture flasks. The fragments were then cultured in an incubator (Thermo) at 37 °C with 5% CO<sub>2</sub>/95% air and 95% humidity until hUCMSCs began to migrate out of the umbilical cord pieces, forming well-defined colonies. Then, umbilical cord fragments were removed from the flasks, and cells were passaged.

For passaging, cells were dissociated using 0.25% Trypsin-EDTA (Gibco, REF. No. 25200-056). Cells were counted and seeded at a density of 10 000 cells/cm<sup>2</sup> in MSC proliferation medium and maintained in a CO<sub>2</sub> incubator (as described above) until reaching 80–90% confluence.

### Multilineage differentiation of human umbilical cord mesenchymal stem cells

The osteogenic, adipogenic, and chondrogenic differentiation capacities of isolated hUCMSCs were assessed using cells at the third passage (P3). For osteogenic and adipogenic differentiation, cells were plated at 20 000 cells/cm<sup>2</sup> in six-well cell culture clusters (Corning, Inc., Corning, New York, USA) and cultured with MSC proliferation medium (as described above) until reaching 70–100% confluence. The medium was then removed; osteogenic (HUXUC-90021; Cyagen, Santa Clara, California, USA) or adipogenic differentiation medium (HUXUC-90031; Cyagen) was added; and cells were cultured for 2–4 weeks. The cells were then stained with Alizarin Red and Oil Red O. For chondrogenic differentiation, 4 × 10<sup>5</sup> cells were transferred to a new 15-ml centrifuge tube and washed with semicomplete chondrogenic differentiation medium (BGsciences, Guangzhou, China) three times. Then, 0.5 ml of complete chondrogenic differentiation medium was added and centrifuged (150 × *g*, 5 min). The

cap of the centrifuge tube was unscrewed (to facilitate gas exchange), and the tube was placed vertically in the incubator. The medium was changed every 2–3 days. After 20–30 days of continuous induction, the cartilage globules were fixed with formalin and sectioned with paraffin embedding, and then stained with Alcian blue.

### Flow cytometry

Cells at passage 5 were collected and treated with 0.25% Trypsin-EDTA. The cells were individually stained with fluorescein isothiocyanate or phycoerythrin-conjugated antimarker monoclonal antibodies in 50 µl of PBS for 30 min at 4 °C according to the recommendations of the Biologend manufacturer. The antibodies were specific for the human antigens CD34, CD44, CD45, CD73, CD90, CD105, and HLA-DR (5 µl for 1 × 10<sup>6</sup> cells; Biologend, San Diego, California, USA). Positive cells were counted, and the signal intensities for the corresponding immunoglobulin isotypes were compared. MSC-derived neurospheres (days 0, 3, 7, and 14 postinduction) were dissociated into a single-cell suspension with Accutase Cell Dissociation Reagent (Gibco, REF. No. 25200-056) and subjected to the same monochromatic flow cytometry analysis. The antibodies were specific for the human antigens nestin and sex-determining region Y-box 2 (SOX2) (5 µl for 1 × 10<sup>6</sup> cells; Biologend). Cells in the blank group (day 0), control group (hUCMSC-derived NSCs induced with NSC culture medium), and Rg1 group [hUCMSC-derived NSCs induced with NSC culture medium supplemented with 10 µmol/l ginsenoside Rg1 (Macklin, Shanghai, China)] were analyzed using a flow cytometry instrument and CellQuest Pro (BD FACSCalibur Flow Cytometer; BD Biosciences, Franklin Lakes, New Jersey, USA). Unstained control was performed for each group to compare with stained samples.

### Generation of neurospheres from human umbilical cord mesenchymal stem cells

hUCMSCs at passage 5 were dissociated with 0.25% trypsin-EDTA, washed twice with PBS, and plated on T25 plastic tissue culture flasks (Corning) at 4 × 10<sup>5</sup> cells/ml in NSC culture medium containing neurobasal medium with 20-ng/ml epidermal growth factor (EGF; PeproTech, Rocky Hill, New Jersey, USA), 20-ng/ml basic fibroblast growth factor (bFGF; PeproTech), 1% GlutaMAX (Gibco, REF: 35050-061), 2% B27 supplement (Gibco, REF: 17504-044), and 1% v/v Penicillin–Streptomycin Solution [5,12]. Additionally, 10-µmol/l ginsenoside Rg1 was added to the cell culture medium of the Rg1 group. When the cell spheres turned black (about every 2–3 days) under the light microscope, they were too large, and cells in the middle could not obtain sufficient nutrition. In this case, cell spheres were washed with PBS, dissociated with Accutase Cell Dissociation Reagent for 5 min at 37 °C, and gently pipetted using 200-µl pipette tips. After cleaning and centrifugation at

350 × g, cells were added to culture flasks at 4 × 10<sup>5</sup> cells/ml in new NSC culture medium.

#### Secondary human umbilical cord mesenchymal stem cells-derived neurosphere formation assay

hUCMSC-derived NSCs were digested with Accutase Cell Dissociation Reagent for 5 min at 37 °C and gently pipetted using 200- $\mu$ l pipette tips to ensure that a single-cell suspension was obtained under a microscope. The cells were then cultured in new NSC culture medium. One thousand cells were then added to each well of a 96-well culture plate (Corning), and positive neurosphere formation was scored after 5 days.

#### Cell-counting kit-8 assays

MSC proliferation medium containing 0-, 0.1-, 1-, 10-, and 100- $\mu$ mol/l ginsenoside Rg1 was prepared. hUCMSCs at passage 5 were cultured in 96-well plates at a concentration of 3000 cells per well for 12, 24, 36, 48, 60, and 72 h in different media. The medium was replaced after 48 h. The effects of various concentrations of ginsenoside Rg1 on cell growth were examined by the Cell-Counting Kit-8 (CCK-8) assay (Glpbio, Montclair, California, USA). The optical density (OD) of each sample was measured at a wavelength of 450 nm, with a reference wavelength of 630 nm (Cmax Plus, Molecular Devices, Sunnyvale, California, USA).

hUCMSCs at passage 5 were treated with various concentrations of NSC culture medium (0-, 0.1-, 1-, 10-, and 100- $\mu$ mol/l ginsenoside Rg1). Then, 10 000 cells were added to each well of the 96-well plate. OD values were measured by the same method on days 1, 2, 3, 4, 5, and 6. Ginsenoside Rg1 at 10  $\mu$ mol/l was used in subsequent analyses.

#### Immunofluorescence staining

On days 2 and 6, after hUCMSC-derived NSC formation, neurosphere-like structures were plated (5–10 neurospheres per well) on tissue culture-treated 24-well plate cell slides and cultured in NSC culture medium for 24 h. On the following day, NSC markers were detected by immunofluorescence staining, as described later. On day 7, after hUCMSC-derived NSC formation, the neurosphere-like structures were dissociated with Accutase Cell Dissociation Reagent for 5 min at 37 °C and gently pipetted using 200- $\mu$ l pipette tips to ensure that a single-cell suspension was obtained under a microscope. The cells were then plated on tissue culture-treated 24-well plate cell slide and cultured in NSC culture medium supplemented with 10% FBS. Ginsenoside Rg1 (10  $\mu$ mol/l) was added to the cell culture medium of the Rg1 group. Ten days after induction, neurogenic differentiation was assayed by immunofluorescence staining to detect microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) protein expression.

Cells were washed with PBS and fixed with 4% paraformaldehyde and 0.1% Triton X-100 (Bioss, Beijing, China). Then, cells were incubated for 12 h overnight at 4 °C with the following antibodies: rabbit mAb (mAb) to nestin (Abcam, Cambridge, UK; 1/200 dilution), rabbit mAb to SOX2 (Bioss; 1/100 dilution), rabbit mAb to GFAP (Bioss; 1/100 dilution), and rabbit mAb to MAP2 (Bioss; 1/100 dilution). Secondary CoraLite488-goat anti-Rabbit IgG (Proteintech, Rosemont, Illinois, USA; 1/200 dilution) was incubated for 1 h at 18–25 °C in the dark. Cell nuclei were counterstained with DAPI (Bioss). After labeling, the cells were covered with Antifade Mounting Medium (Bioss). Slides were immediately examined under a fluorescence microscope (Nexcope, Ningbo, China). The mean fluorescence intensity for nestin and SOX2 in the control group and Rg1 group was evaluated using Image J (NIH, Bethesda, Maryland, USA). Each experiment was repeated three times independently, and three images were taken randomly each time.

#### Real-time quantitative PCR

Cells from the control group and the Rg1 group were collected 3 days after induction. RNA was isolated from each group using TRIzol Reagent (Invitrogen, Carlsbad, California, USA). cDNA was transcribed following the manufacturer's instructions (Eppendorf, Hamburg, Germany). Primers for real-time PCR are provided in Table 1. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. The normalized relative expression levels were obtained using the 2<sup>- $\Delta\Delta$ CT</sup> method.

#### Statistical analysis

All values are expressed as means  $\pm$  SD. Two groups were compared by the *t*-test for two independent samples, and more than two groups were analyzed by one-way analysis of variance followed by either a Dunnett or a Tukey post hoc test. All statistical tests were two-sided, and *P* < 0.05 was considered statistically significant. All analyses were performed using SPSS 23 (IBM, Chicago, Illinois, USA).

## Results

### Characterization of human umbilical cord mesenchymal stem cells

At days 5–7, the primary cells grew out of the umbilical cord tissue and formed whirlpool-like colonies at about 2 weeks. The cells were passaged every 3 days. Passage 3 cells could be obtained at about 6 weeks, and passage 5 cells were obtained at about 8 weeks (Fig. 1a). In our experiment, hUCMSCs could be successfully induced into osteogenic, adipogenic, and chondrogenic cells (Fig. 1b).

Cells at passage 5 were evaluated by flow cytometry. These cells expressed CD44, CD73, CD90, and CD105 (all >95%) and did not express CD34, CD45, and HLA-DR (all <2%), consistent with the phenotype of MSCs (Fig. 1c).

**Table 1 Primer used for real-time PCR**

Primer name	Primer sequence (5'-3')	Product size (bp)
GSK3 $\beta$		
Forward	GTTAGCAGAGACAAGGACGGCA	183
Reverse	GCAATACITTTCTTGATGGCGAC	
$\beta$ -catenin		
Forward	GCCAAGTGGGTGGTATAGAGG	192
Reverse	GGGATGGTGGGTGTAAGAGC	
Notch1		
Forward	GGACGGCGTGAACACCTACAA	100
Reverse	GCAGGCATTTGGCATCAGC	
Hes1		
Forward	ATTCTGGAATGACAGTGAAGCAC	168
Reverse	CACCTCGGTATTAACGCCCTC	
JAK2		
Forward	GCTAACAGTTGGCATGGGC	182
Reverse	TCCTGAAGAATGTCCCTTTGGC	
STAT3		
Forward	GAGAAGGACATCAGCGGTAAG	138
Reverse	CAGTGAGACACCAGGATATTG	
PI3K		
Forward	TACACTGTCCTGTGCTGGCTACT	293
Reverse	GAGATCCCATGCGCTCGTA	
Akt		
Forward	GCTCAGCCCACCCCTCAAG	112
Reverse	GCTGTCATCTTGGTCAGGTGGT	
SHH		
Forward	CAGCGGAAGGTATGAAGGGAA	150
Reverse	GCGTTCAACTGTCTTACACCTC	
PTCH1		
Forward	TGTGGGTGGAAGTTGGAGGAC	164
Reverse	TGCCGAGTCCAGGTGTTGTAG	
GAPDH		
Forward	GGAAGCTTGTCAATGAAATC	168
Reverse	TGATGACCCTTTGGCTCCC	

bp, base pair; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; JAK2, Janus kinase 2; PI3K, phosphatidylinositol 3-kinase; PTCH1, patched1; SHH, Sonic Hedgehog; STAT3, signal transducer and activator of transcription.

### Generation and identification of neural stem cells from human umbilical cord mesenchymal stem cells

hUCMSCs were induced under serum-free suspension culture using induction medium for NSCs (including neurobasal medium, 20 ng/ml EGF, 20 ng/ml bFGF, 1% GlutaMAX, 2% B27 supplement, and 1% v/v Penicillin–Streptomycin Solution). In the Rg1 group, ginsenoside Rg1 was added to the induction medium at 10  $\mu$ mol/l. The hUCMSCs in both the control group and the RG1 group formed neurosphere-like structures within 6 h.

To confirm that the hUCMSC-derived neurospheres were NSCs rather than aggregates of hUCMSCs, self-renewal and pluripotency were evaluated. hUCMSC-derived nerve globules were isolated as single cells and cultured continuously in new complete culture medium. The neurosphere-like cells at passage 2 were observed. Additionally, after further culture in a 96-well plate with clonal densities, secondary neurospheres formed within 5 days. Neurospheres in the Rg1 group were larger and more compact than those in the control group, as shown in Fig. 1d. Nerve globules at passage 2 were isolated as single cells and cultured in induction medium supplemented with 10% FBS. After 10 days, immunofluorescence staining showed the positive expression of MAP2,

a neuronal marker, and GFAP, a glial cell marker (Fig. 1e and f).

The main markers for the identification of NSCs are nestin and SOX2. Both cell immunofluorescence and flow cytometry indicated that these proteins were expressed in hUCMSC-derived neurosphere cells. However, the expression of nestin in hUCMSCs before induction was negative, as shown in Figs 2 and 3.

### Effects of various concentrations of ginsenoside Rg1 on human umbilical cord mesenchymal stem cell growth

hUCMSCs were cultured in MSC medium with various concentrations of ginsenoside Rg1 (0, 0.1, 1, 10, and 100  $\mu$ mol/l), and the medium was replaced after 48 h. The OD value for each group was determined by a CCK-8 assay every 12 h. At 48 and 72 h, the OD values for all ginsenoside Rg1 groups were higher than those for the control group (0  $\mu$ mol/l) ( $P < 0.001$ ), as shown in Fig. 4a.

### Effects of various concentrations of ginsenoside Rg1 on human umbilical cord mesenchymal stem cell-derived neurosphere growth

hUCMSCs were treated with NSC induction medium containing 0-, 0.1-, 1-, 10-, and 100- $\mu$ mol/l ginsenoside Rg1. On day 2, the OD value was highest for the group treated with 10- $\mu$ mol/l ginsenoside Rg1, and on days 4, 5, and 6, the OD values for this group were still higher than those of the control group ( $P < 0.001$ ), as shown in Fig. 4b. Accordingly, a ginsenoside Rg1 concentration of 10  $\mu$ mol/l was selected for subsequent experiments.

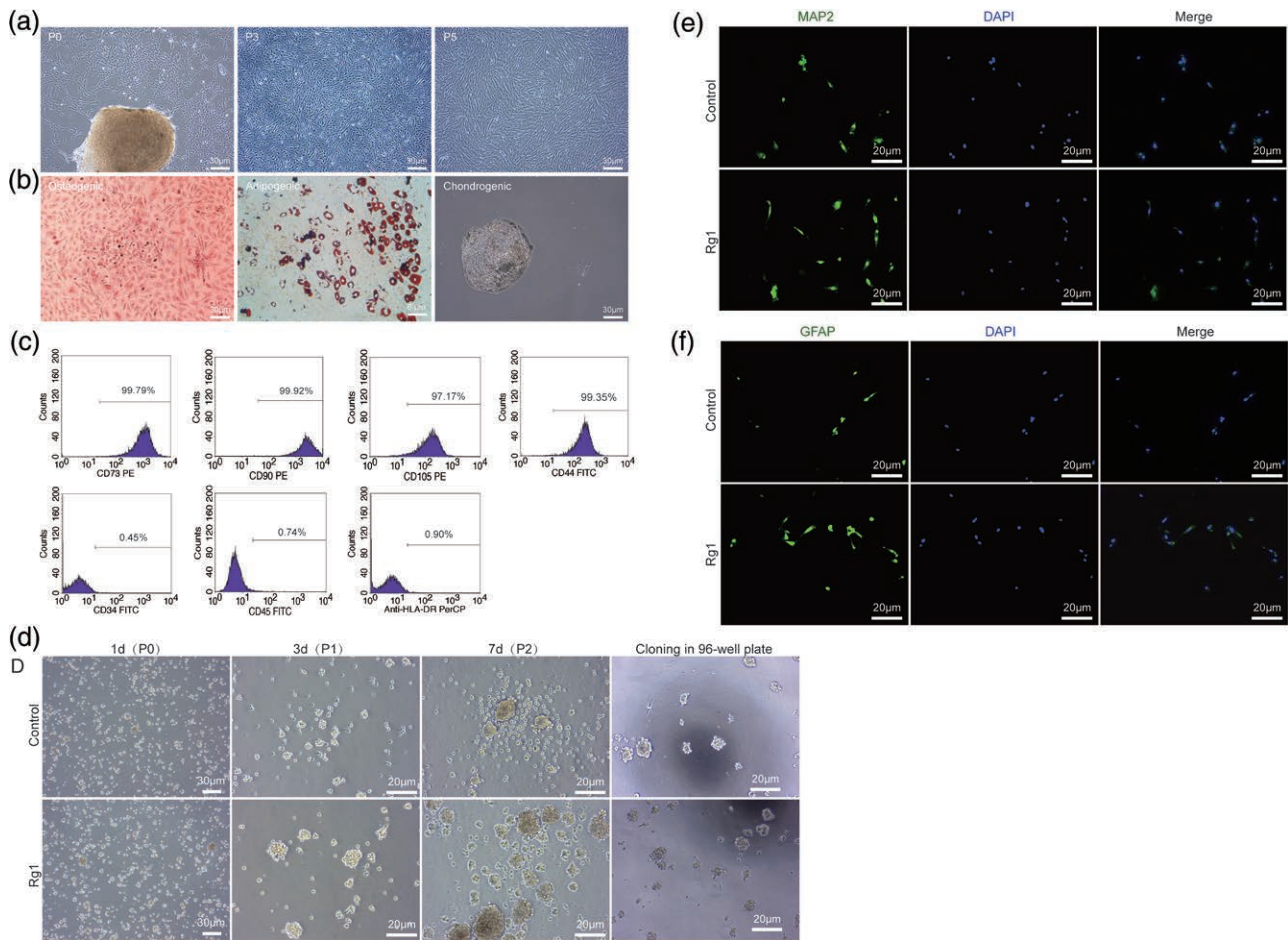
### Ginsenoside Rg1 upregulated nestin and sex-determining region Y-box 2 in neural stem cells induced from human umbilical cord mesenchymal stem cells

Flow cytometry was performed to evaluate hUCMSCs before induction (day 0) and dissociated neurosphere cells in the RG1 group (10  $\mu$ mol/l Rg1) and the control group (0- $\mu$ mol/l Rg1) at 3, 7, and 14 days after induction. Before induction, hUCMSCs did not express nestin. On day 3, after induction, nestin was expressed in both the negative group and the Rg1 group, with significantly higher levels in the Rg1 group than that in the negative control group ( $P < 0.001$ ). On day 7, the positive rates of nestin in both groups were higher than those on day 3, and the positive rate of nestin in the Rg1 group was higher than that in the control group ( $P < 0.001$ ). After 14 days of induction, the expression levels of nestin in the two groups were lower than those on day 7; however, the expression of nestin in the Rg1 group was still higher than that in the control group ( $P < 0.001$ ), as shown in Fig. 2a and c.

During the induction of hUCMSCs into NSCs, the proportions of SOX2-positive cells in both the negative group and the RG1 group on day 7 were higher than those on



Fig. 1



Identification of hUCMSCs and NSC derived from hUCMSCs. (a) Morphology of hUCMSCs (40 $\times$  magnification). (b) hUCMSCs differentiated into osteocytes (40 $\times$  magnification), adipocytes (200 $\times$  magnification), and chondrocytes (40 $\times$  magnification). (c) The expression levels of CD73, CD90, CD105, and CD44 of hUCMSCs were more than 95%, and the expression levels of CD34, CD45, and anti-HLA-DR were less than 2%. An open profile represents an isotype control for background fluorescence. The percentage figure indicates the percentage of positive cells in the total number of cells. (d) Morphology of neurosphere (100 $\times$  magnification). (e and f) The neurosphere derived from hUCMSCs could continue to differentiate into neurons and glial cells. FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; P0, passage 0; P3, passage 3; P5, passage 5; PE, phycoerythrin.

day 3 ( $P < 0.001$ ). In addition, the proportions of SOX2-positive cells in the Rg1 group were higher than those in the negative group on both days 3 and 7 ( $P < 0.001$ ), as shown in Fig. 2b and d.

#### Immunofluorescence analysis of the effect of ginsenoside Rg1 on nestin and sex-determining region Y-box 2 expression in neural stem cells induced from human umbilical cord mesenchymal stem cells

Immunofluorescence staining showed that uninduced hUCMSCs (day 0) did not express nestin, whereas induced cells (control group and Rg1 group) were positive for nestin (Fig. 3a). As determined using ImageJ, the mean fluorescence intensity was higher on day 7 than on day 3 in both the control group and the Rg1 group. Notably, the mean fluorescence intensity was significantly

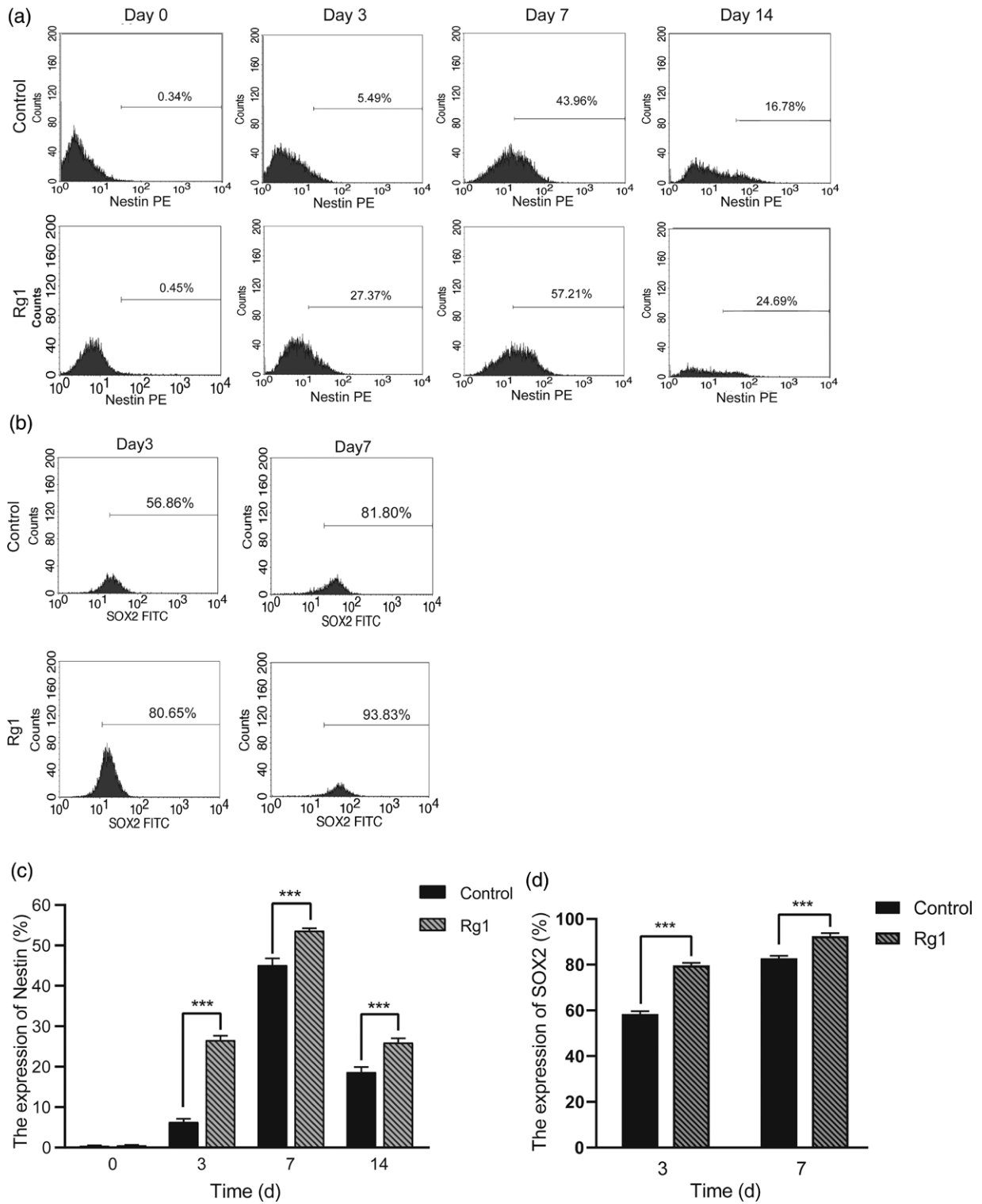
higher in the Rg1 group than that in the control group on days 3 and 7 ( $P < 0.05$ ) (Fig. 3c).

Immunofluorescence staining also showed that SOX2 was expressed in both the negative control group and the Rg1 group after induction (Fig. 3b). Similar to the results for nestin, the expression of SOX2 on day 7 was higher than that on day 3, and the mean fluorescence intensity was higher in the Rg1 group than that in the control group (Fig. 3d).

#### Real-time quantitative PCR

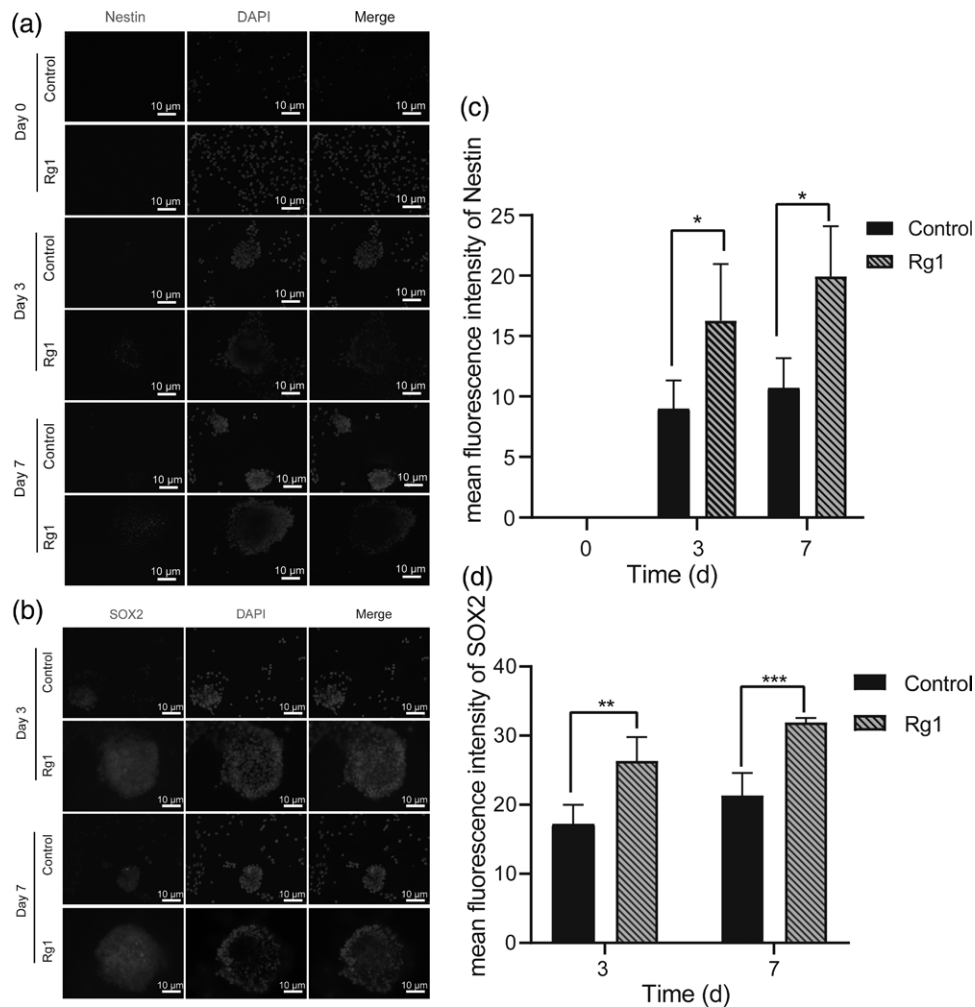
Gene expression levels in the Rg1 group and control group were evaluated by real-time quantitative PCR on day 3 after induction. The expression levels of Wnt/ $\beta$ -catenin and Notch signaling pathway genes (*GSK3 $\beta$* ,  $\beta$ -catenin, *Notch1*, and *Hes1*) were lower in the

Fig. 2



Ginsenoside Rg1 upregulated the expression of nestin and SOX2 proteins determined by flow cytometry. (a and b): Expression of nestin (a) and SOX2 (b) in cells before induction (day 0) and in induced groups with or without Rg1 as measured by flow cytometry. An open profile represents an isotype control for background fluorescence. The percentage figure indicates the percentage of positive cells in the total number of cells. (c and d): Quantitative analysis of nestin+ cells (c) and SOX2+ cells (d). Significant differences were determined by *t*-tests for two independent samples; \*\*\**P* < 0.01. FITC, fluorescein isothiocyanate; PE, phycoerythrin; SOX2, sex determining region Y-box 2.

Fig. 3



Ginsenoside Rg1 upregulated the expression of nestin and SOX2 proteins determined by immunofluorescence. (a and b): Immunofluorescence microscopic images of nestin (a) and SOX2 (b). DAPI represents nuclear staining (200 $\times$  magnification). DAPI represents nuclear staining (200 $\times$  magnification). (c and d): Quantitative analysis of nestin (c) and SOX2 (d) expression by mean fluorescence intensity of neurosphere. Significant differences were determined by *t*-tests for two independent samples; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . SOX2, sex determining region Y-box 2.

Rg1 group than that in the control group (all  $P < 0.01$ ) (Fig. 5).

In this study, all experiments were independently repeated three times.

## Discussion

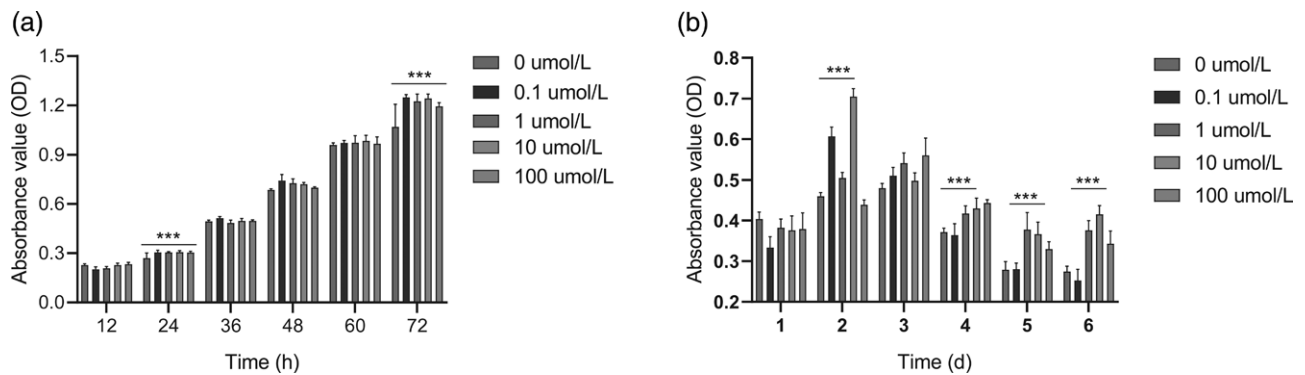
Ginsenoside Rg1 (molecular formula:  $C_{42}H_{72}O_{14}$ ) is a natural active ingredient extracted from the root or stem of ginseng. Rg1 exerts beneficial health effects by various mechanisms. For example, Rg1 can be used to treat diabetes, mastitis, and liver and kidney diseases by the inhibition of inflammation [13] and can protect the brain, liver, and heart by antioxidant activity [14]. In addition, Rg1 inhibits cell apoptosis. Previous studies have shown that ginsenoside Rg1 can effectively regulate the proliferation, differentiation, apoptosis, and senescence of MSCs

in different microenvironments in vivo and in vitro [15]. In NSCs, Rg1 can promote proliferation and directed differentiation [10]. In this study, we investigated whether Rg1 could effectively promote hUCMSC proliferation and differentiation into NSCs.

hUCMSCs were successfully cultured from tissue blocks in Wharton's jelly of human umbilical cords. These cells showed plastic-adherent growth and had self-renewal ability. In addition, they had multipotency with osteogenic, adipogenic, and chondrogenic potentials. Based on the positive expression of CD44, CD73, CD90, and CD105 and negative expression of CD34, CD45, and HLA-DR, the cells met the criteria for human MSCs proposed by the International Society for Cell Therapy in 2006 [16].

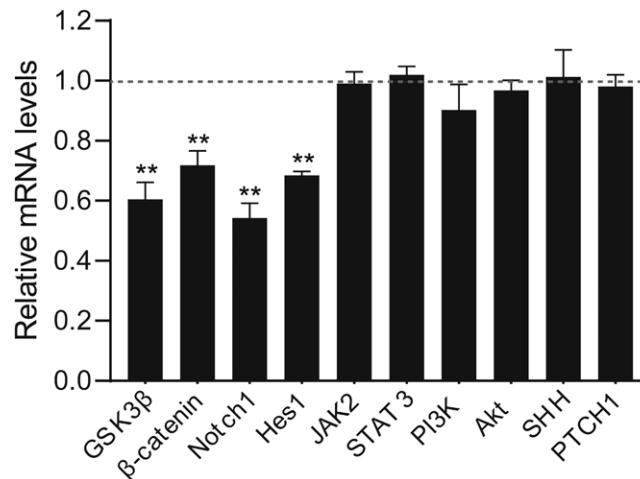
hUCMSCs were induced to form neurosphere-like structures in NSC induction medium. We verified that these

Fig. 4



Effects of various concentrations of ginsenoside Rg1 on hUCMSCs and hUCMSC-derived neurosphere growth determined by CCK-8 assay. (a) Rg1 promotes the proliferation of hUCMSCs. (a) 10  $\mu\text{mol/L}$  Rg1 promoted the activity of hUCMSC-derived neurosphere. Data are presented as means  $\pm$  SD. Significant differences were determined by one-way analysis of variance; \*\*\* $P < 0.01$ .

Fig. 5



Ginsenoside Rg1 downregulates genes related to Wnt/ $\beta$ -catenin and Notch signaling pathways to promote hUCMSCs induced into NSCs. Red dotted line represents the relative expression level in the negative control group after normalization to GAPDH (value = 1.0). Bars represent means  $\pm$  SD. Significant differences were determined by *t*-tests for two independent samples; \*\* $P < 0.01$ . GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; JAK2, Janus kinase 2; PI3K, phosphatidylinositol 3-kinase; PTCH1, patched 1; SHH, Sonic Hedgehog; STAT3, signal transducer and activator of transcription 3.

cells were NSCs and not simple aggregates of hUCMSCs by secondary hUCMSC-derived neurosphere formation assay, nestin and SOX2 detection, and differentiation into neurons and glial cells. These results are consistent with those of previous study [5]. Notably, these results not only proved that ginsenoside Rg1 did not impede the induction of hUCMSCs into NSCs but also prompted the neurospheres to grow larger and more compact than the control group.

The results of CCK-8 showed that Rg1 was not only non-toxic to hUCMSCs but also promoted cell proliferation. In addition, Rg1 increased the activity of NSCs induced by hUCMSCs. The optimal concentration of ginsenoside

Rg1 in promoting the proliferation and differentiation of hUCMSCs into NSCs was 10  $\mu\text{mol/L}$ .

Nestin is an intermediate filament protein that was first detected in NSCs [17]. It contributes to the basic functions of stem cells, including self-renewal, proliferation, differentiation, and migration [18]. Nestin knockout results in embryonic death and the loss of the self-renewal ability of NSCs [19]. In addition to NSCs, nestin is expressed in a variety of tissues, including the bone, marrow, immune system, muscle, lung, gastrointestinal tract, kidney, and urinary bladder [18]. Therefore, an additional marker is necessary to identify NSCs. SOX2, a persistent marker for multipotential NSCs [20], is



essential for the maintenance of embryonic stem cells and NSCs and for the self-renewal or pluripotency of undifferentiated embryonic stem cells [21]. A SOX2 deficiency leads to neurodegeneration and premature aging in the brains of adult mice [22]. Both SOX2 and nestin are characteristic markers of NSCs [23]. In our study, the expression levels of nestin and SOX2 in the Rg1 group were higher than those in the control group in both flow cytometry and immunofluorescence experiments. These results suggested that ginsenoside Rg1 could promote the directed differentiation of hUCMSCs into NSCs.

Both the Wnt and Notch signaling pathways are involved in the regulation of stem cell self-renewal and differentiation [24]. Wnt, Notch, FGF, Hedgehog, and bone morphogenetic protein signaling networks are involved in the maintenance of tissue homeostasis by regulating the proliferation or differentiation of stem cells and progenitor cells [25]. In our study, we found that Rg1 could promote the differentiation of MSCs into NSCs by downregulating *GSK3 $\beta$* ,  $\beta$ -catenin, *Notch1*, and *Hes1* in the Wnt/ $\beta$ -catenin and Notch signaling pathways.

This study had some limitations. Analyses of the mechanism underlying the effects of Rg1 are still in the preliminary stage, and animal experiments have not been carried out. In the future, we will continue to characterize the effects of Rg1.

## Conclusion

In conclusion, ginsenoside Rg1 can promote the proliferation of hUCMSCs and increase cell viability during the differentiation of hUCMSCs to NSCs. In addition, quantitative analyses of nestin and SOX2 protein expression indicated that ginsenoside Rg1 can significantly increase the differentiation of hUCMSCs into NSCs. The promotion of differentiation by Rg1 may be mediated by the downregulation of genes in the Wnt/ $\beta$ -catenin and Notch signaling pathways. These findings provide a novel therapeutic strategy that may be applicable to stroke, neurodegenerative disease, and neurological diseases caused by trauma.

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Ethics statement: all procedures were conducted in accordance with the ethical guidelines of the First Affiliated Hospital of Gannan Medical University. The research scheme was approved by the Medical Ethics Committee of the First Affiliated Hospital of Gannan Medical University (approval number LLSC-2021030201) and was conducted according to the ethical standards of the 2013 Declaration of Helsinki. Prior to the study, all subjects provided informed consent.

## Conflicts of interest

There are no conflicts of interest.

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