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RESEARCH ARTICLE

Salidroside induces rat mesenchymal stem cells to differentiate into dopaminergic neurons

Hong-Bin Zhao[1]*, Hui Ma[1], Xiao-Qin Ha[2], Ping Zheng[2], Xiao-Yun Li[1], Ming Zhang[1], Ju-Zi Dong[2] and Yin-Shu Yang[1]

1 Institute of Orthopedics, General Hospital of Lanzhou Military Command of the PLA, Lanzhou, Gansu 730050, China
2 Department of Clinical Laboratory, General Hospital of Lanzhou Military Command of the PLA, Lanzhou, Gansu 730050, China

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterised by the loss of substantia nigra dopaminergic neurons that leads to a reduction in striatal dopamine (DA) levels. Replacing lost cells by transplanting dopaminergic neurons has potential value to repair the damaged brain. Salidroside (SD), a phenylpropanoid glycoside isolated from plant *Rhodiola rosea*, is neuroprotective. We examined whether salidroside can induce mesenchymal stem cells (MSCs) to differentiate into neuron-like cells, and convert MSCs into dopamine neurons that can be applied in clinical use. Salidroside induced rMSCs to adopt a neuronal morphology, upregulated the expression of neuronal marker molecules, such as gamma neuronal enolase 2 (*Eno2/NSE*), microtubule-associated protein 2 (*Map2*), and beta 3 class III tubulin (*Tubb3/β-tubulin III*). It also increased expression of brain-derived neurotrophic factor (*BDNF*), neurotrophin-3 (*NT-3*) and nerve growth factor (*NGF*) mRNAs, and promoted the secretion of these growth factors. The expression of dopamine neurons markers, such as dopamine-beta-hydroxy (*DBH*), dopa decarboxylase (*DDC*) and tyrosine hydroxylase (*TH*), was significantly upregulated after treatment with salidroside for 1–12 days. DA steadily increased after treatment with salidroside for 1–6 days. Thus salidroside can induce rMSCs to differentiate into dopaminergic neurons.

Keywords: dopaminergic neuron; mesenchymal stem cells; salidroside

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterised by the loss of substantia nigra dopaminergic neurons that leads to a reduction in striatal dopamine (DA) levels. Replacing lost cells by transplanting dopaminergic neurons has the potential value to repair the damaged brain (Ben et al., 2004). Many kinds of cells, such as primary mesencephalic cultures (Erceg et al., 2008), embryonic stem cells (Montzka et al., 2009), and sympatho-adrenal cells (Wu et al., 1998), have the potential to differentiate into dopaminergic neurons. However, these cells face at least two major challenges: on the one hand, because they are from normal tissues, it is very difficult to obtain them, but easy to damage the function of their normal host tissues. On the other hand, the socio-ethical problems of embryonic stem cells limit their clinical application. To address these issues, bone marrow-derived mesenchymal stem cells (MSCs) have been used for neuron-like differentiation. MSCs have the potential to differentiate into several types of cells, including osteogenic, chondrogenic, adipogenic and neuronal lineages in response to stimulation by multiple environmental factors (Jiang et al., 2002; Pereira et al., 1995), they thus possess tremendous clinical potential for use in regenerative medicine. So far, serum deprivation and/or treatment with cyclic AMP (cAMP) analogs, retinoic acid (RA), bone morphogenetic proteins (BMPs) (Tio et al., 2010), nerve growth factor (NGF) (Brederlau et al., 2002), brain-derived neurotrophic factor (BDNF) (Shi et al., 2012), glial cell-derived neurotrophic factor (GDNF) and other factors (Trzaska et al., 2009) have been used for neuron-like differentiation of several mouse and human cells. Despite their effectiveness as inducers to differentiate into dopaminergic neurons, a new type of inducer that can rapidly and reliably produce desired quantities of dopaminergic neurons with a long-term survival rate is urgently needed.

Kirilow Rhodiola Root and Rhizome is a traditional Chinese medicinal herb with multiple pharmacological

*Corresponding author: e-mail: zhao6703@aliyun.com

effects, such as anti-hypoxia, anti-oxidant and neuroprotective effects (Choe et al., 2012; Xu and Li, 2012). Salidroside, the active constituent, has multiple biological effects (Zhang et al., 2011). An important role for salidroside is to protect the central nerve, and thus it is reasonable to hypothesise that salidroside can induce MSCs to differentiate into neuron-like cells, perhaps also into dopaminergic neurons.

To test this hypothesis, we have investigated the effects of salidroside on the induction of rat MSCs (rMSCs) to differentiate into dopaminergic neurons in comparison to RA. Salidroside effectively induces dopaminergic neuron differentiation of rMSCs by upregulating the expression of BDNF, neurotrophin-3 (NT-3), and NGF.

Materials and methods

Isolation and culture of rMSCs

Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at the General Hospital of Lanzhou Military Command of the PLA. Bone marrow-derived MSCs were isolated and harvested from bone marrow of the tibias of 2- to 3-month-old male Sprague–Dawley rats by inserting a 21-ga needle into the shaft of the bone and flushing it with 30 mL Dulbecco's modified Eagle/F12 medium (DMEM/F-12; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Grand Island, NY). After centrifugation and resuspension, isolated cells were grown in a culture flask and cultured under a routine condition (37°C, air plus 5% CO_2) for 48 h. Non-adherent cells were removed by changing the medium and the resulting monolayer of cells was trypsinised. Aliquots were cultured further, or frozen and stored. The MSCs were used for the following experiments when they were at the third passage.

Flow-cytometric determination of cell-surface antigen profiles

One millilitre of CD106-PE, CD90-FITC, CD45-PE or CD34-FITC rat-specific antibodies (BD Pharmingen, San Diego, CA) was added to the bottom of tubes, after which 100 μL (6×10^6 cells/mL) of a single-cell suspension of cultured rMSCs was added. The mixture was incubated for 30 min at 4°C in the dark and washed. Positive cells were detected by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ). Rat IgG1-FITC and IgG1-PE (BD Pharmingen) were used as isotype controls.

Cell morphology

Cells were seeded at 1×10^4 cells/mL in 24-well plates containing DMEM/F-12 and 10% FBS for 24 h, and then induced by 100 μg/mL salidroside (Purity ≥ 99.8%, Chinese Institute for Drug Assay, Beijing, China) for 24–72 h. They were fixed with 4% paraformaldehyde and incubated at 4°C overnight with rabbit monoclonal anti-β-tubulin antibody (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After three washes with PBS, the cells were incubated at 37°C for 1 h with secondary antibodies conjugated to fluorescein isothiocyanate (FITC, Abcam, Cambridge, MA). The cell number in each experimental condition was determined by counting the cells in four different fields on each coverslip in at least three independent experiments. The images were taken with an Olympus fluorescence microscope BX61 (Olympus, Tokyo, Japan).

Immunocytochemistry

rMSCs were seeded at 1×10^4 cells/well on 24-well plates with poly-L-lysine-coated coverslips, and treated with 100 μg/mL of salidroside for varying times. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min, rinsed thrice with phosphate-buffered saline (PBS), and blocked for 1 h in PBS containing 0.1% Triton X-100 and 2% goat serum. They were labelled with mouse monoclonal anti-neuron-specific enolase (NSE) antibody (1:1,000, Abcam), mouse monoclonal anti-microtubule associated protein-2 (MAP2) antibody (1:1,000, Abcam), mouse monoclonal anti-beta 3 class III tubulin (Tubb3/β-tubulin III) antibody (1:500, Abcam), goat polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:500, Abcam), mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (1:1,000, Santa Cruz Biotechnology), mouse monoclonal anti-dopamine-beta-hydroxy (DBH) antibody (1:1,000), or mouse monoclonal anti-dopa decarboxylase (DDC) antibody (1:1,000). After three washes with PBS, cells were incubated at 37°C for 30 min with secondary antibodies conjugated to FITC or Cy3 (1:1,000, Abcam). Cell nuclei were stained with DAPI. Images were taken with a fluorescence microscope (BX61; Olympus) under 20× objective. To determine the percentage of specific cell types in a particular condition, the total number of cells and the number of cells with a specific immunoreactivity were counted in 10–12 randomly selected fields of two or three different coverslips. Each experiment was repeated at least three times.

Real-time PCR analysis

Total RNA was extracted by using Trizol reagent (Invitrogen, Carisbad, CA) according to the manufacturer's instructions. cDNA was generated from 2 μg total RNA reacting with M-MLV reverse transcriptase (Applied TaKaRa Company, Dalian, China) and an oligo (dT) 18 primer. Real-time PCR amplification was done in 25 μL containing 600 ng cDNA, 20 μM each primer (Table 1), and 12.5 μL of the Power SYBR

Table 1 Real-Time PCR primer sequences.

mRNA (GenBank accession number)	Sequence
NSE (NM_013509.2)	
Sense	TCTGAACGTCTGGCGAAGTACAA
Antisense	CAGAGTGACTATGGCAGGTCAGG
MAP2 (NM_008632.2)	
Sense	AGTTTGGCTGAAGGTAGCTGAA
Antisense	GTCCGCTCTCTTCATTCATTC
β-tubulinIII (NM_013613.1)	
Sense	GCGATGAGCACGGCATAGAC
Antisense	GAAGGCACCACGCTGAAGGT
BDNF (NM_012513.3)	
Sense	ATCCACTGAGCAAAGCCGAAC
Antisense	CAGCCTTCATGCAACCGAAGTA
NT-3 (NM_031073.2)	
Sense	CATGTCGACGTCCCTGGAAATAG
Antisense	GGATGCCACGGAGATAAGCAA
NGF (NM_001277055.1)	
Sense	TCACTGGATCACGACTCCCAAC
Antisense	CAACTGGCACTCGGCATCA
TH (NM_012740.3)	
Sense	AGCTGTGCAGCCCTACCAAGA
Antisense	GTGTGTACGGGTCAAACTTCACAGA
GAPDH (NM_008084.2)	
Sense	TGTGTCCGTCGTGGATCTGA
Antisense	TTGCTGTTGAAGTCGCAGGAG

Green PCR Master Mix (Applied TaKaRa). The conditions were set as an initial denaturation of 10 min at 95°C, and 30 cycles of 95°C for 30 s and 60°C for 31 s. The fluorescent signals were normalised to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the threshold cycle (C_t) was set within the exponential phase of the PCR. The relative expression level between treatments was calculated using the following equation: relative gene expression = $2^{-}(DC_t \text{ sample} - DC_t \text{ control})$ (Livak and Schmittgen, 2001). The results were presented as the fold change in gene expression in the differentiated cells compared to that in the undifferentiated cells.

ELISA analysis

rMSCs were treated with 100 μg/mL of salidroside or 10 μg/mL of RA for 1–12 days. ELISA was used on cell supernatants to quantify BDNF, NT-3, NGF, and Dopamine (DA) (ELISA Ready-SET-GO, eBioscience, CA; Cat. Nos. 88-7346 and 88-7106, respectively). The absorbance at 450 nm (less 690 nm background) was read using a Microplate Reader (Bio-RAD Model 3550-UV, GMI, Inc., Ramsey, MN, USA).

Western blot analysis

Cultured rMSCs were rinsed with PBS and lysed for 30 min on ice in RIPA-B buffer (0.5% Nonidet P-40, 20 mM Tris (pH 8.0), 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM DTT, and 50 μg/mL phenylmethanesulfonylfluoride (PMSF)). The lysate was centrifuged at 14,000*g* for 30 min at 4°C, and supernatant containing 20 μg protein was run on 12% SDS–PAGE followed by Western blot analysis. The blots were blocked in TBS (PBS containing 5% BSA and 0.05% Tween-20). The membrane was incubated at 4°C overnight with the appropriate primary antibodies (anti-NSE mouse monoclonal antibody (1:1,000, Abcam), anti-β-tubulin III rabbit monoclonal antibody (1:2,000, Abcam) and anti-TH mouse monoclonal antibody (1:1,000, Santa Cruz Biotechnology). After incubating with primary antibodies, the blots were washed extensively in TBS. Thereafter, the samples were treated with a peroxidase-conjugated anti-rabbit and/or anti-mouse secondary antibody (Santa Cruz Biotechnology). The proteins were subsequently detected and visualised by electrochemiluminescence (ECL, Millipore Corporation, Billerica, USA). The results of two-dimensional gel electrophoresis were quantitated using the Alpha Imager 2000 (Alpha Innotech, San Leandro, CA) and Image-Pro Plus Version 6.0 (Media Cybernetics, Inc., MD, USA). The average area and band intensity from three to five independent blots were used for each data point. Actin levels were used to correct for loading in each sample, and fold changes were calculated.

Statistical analyses

Statistical analyses used SPSS for Windows version 17.0 (SPSS, Inc., Chicago, IL). The data are expressed as the mean ± SD unless otherwise indicated. Data were analysed by the Student's *t*-test for two group comparisons and $P < 0.05$ was considered significant.

Results

Salidroside induces rMSCs to adopt a neuronal morphology

rMSCs surface antigen profiles determined by staining with rat-specific monoclonal antibodies followed by flow cytometry revealed that rMSCs were strongly positive for typical MSCs markers CD106 and CD90, whereas they were negative for the hematopoietic markers CD45 and CD34 (Figure 1A). Thus, the cells retained the MSC phenotype. Cell morphology after 100 μg/mL of salidroside was examined along with staining for beta-tubulin (green). After 24 h of salidroside treatment, 33 ± 4.6% MSCs had phenotypes ranging from simple bipolar cells to large branched multipolar cells (Figure 1E: 24 h). The number of branched multipolar cells reached 64 ± 4.3% and 71.5 ± 2.7% by 48 and 72 h treatments, respectively (Figures 1F: 48 h and 1G: 72 h). Spindle-shaped cells predominated, but a few branched multipolar were seen in untreated MSCs (Figures 1B–1D).

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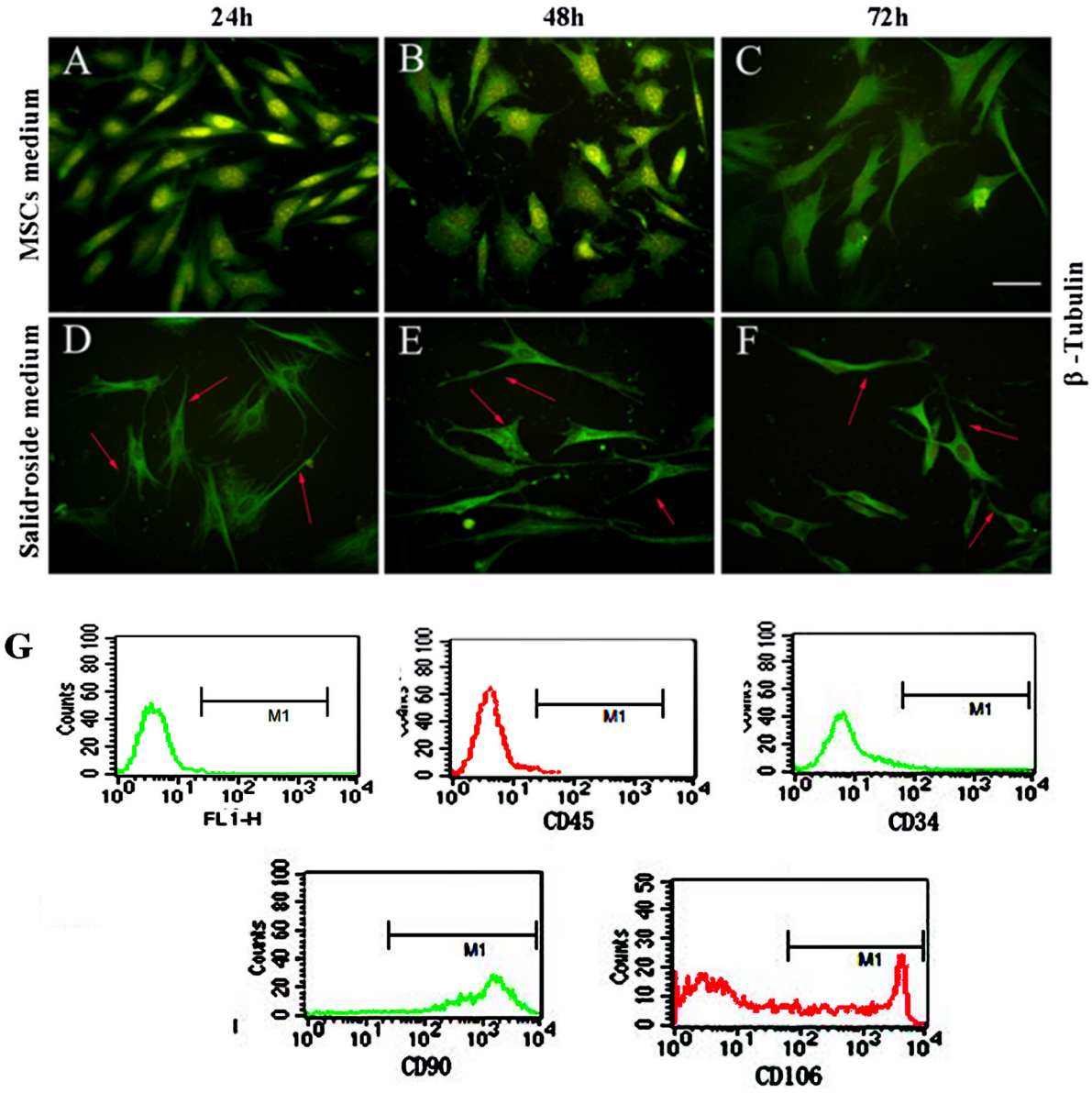

Figure 1 Salidroside induces rMSCs to adopt a neuronal morphology in vitro. MSCs were isolated from the tibias of rat and cultured in D/F12 medium, and subcultured for 24 h (A), 48 h (B) and 72 h (C). Neuronal morphology of rMSCs treated with 100 μg/mL salidroside for 24 h (D), 48 h (E), and 72 h (F). rMSC surface antigen profiles determined by staining with rat-specific monoclonal antibodies followed by flow cytometry (G). The images were taken by fluorescence microscopy (Olympus, U-TV1 X) under 40× objective. The images shown are a representative of three independent experiments.

Salidroside induces rMSCs to differentiate into neuron-like cells

To determine whether salidroside can induce rMSCs to differentiate into neuron-like cells in vitro, selected neuronal markers were assessed by immunostaining assay after MSCs were treated with salidroside (100 μg/mL) for 24–72 h (Figure 2A). With the time of induction increased the percentage of cells expressing NSE, MAP2, and β-tubulin III significantly increased from 24 to 72 h. These neural markers were expressed in a time-dependent manner after treatment with salidroside for 24–72 h (Figure 2B), whereas GFAP expression in cells treated with salidroside was lower at 24–72 h (Figure 2C).

To investigate the effects of salidroside treatment on rMSCs differentiation, Real time-PCR and Western blot analysis were used to measure expression of NSE, MAP2, and β-tubulin III after salidroside treatment for 24 h. Treatment with 100 μg/mL salidroside increased the expression of *NSE*, *MAP2*, and *β-tubulin III* mRNAs (Figure 2D) and NSE and

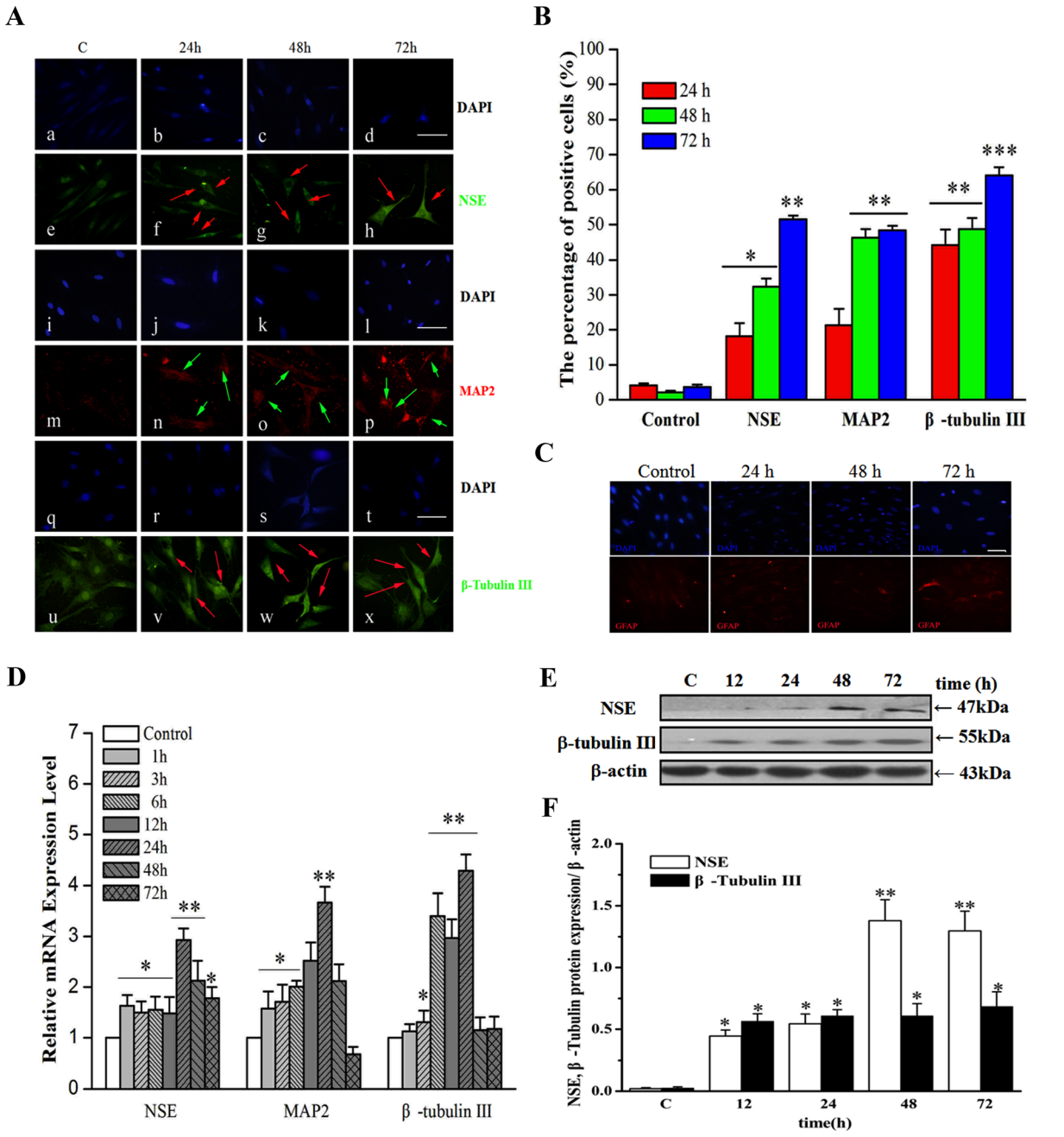

Figure 2 **Salidroside induces rMSCs to differentiate into neuronal-like cells in vitro.** (A and B) rMSCs were incubated in conditioned medium with 100 μg/mL salidroside for 24–72 h. Immunocytochemical detection of the markers of neuronal cells. The images shown are a representative of three independent experiments. Arrows: rMSCs expressing the neuronal markers NSE (green), MAP2 (red), β-tubulin III (green) and (C) GFAP (red), Nuclei were stained with DAPI (blue). Scale bar: 20 μm. (D) Real time-PCR analysis of the expression of *NSE*, *MAP2* and *β-tubulin III* mRNAs in rMSCs treated with 100 μg/mL salidroside (SD) for 24 h. GAPDH was used as an internal control. Data are shown as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. (E and F) Western blot analysis of the expression of *NSE* and *β-tubulin III* proteins in rMSCs treated with 100 μg/mL salidroside (SD) for 24 h. The blot shown is a representative of three independent experiments. The results of two-dimensional gel electrophoresis were quantitated using the Alpha Imager 2000 and Image-Pro Plus Version 6.0. The average area and band intensity from three to five independent blots were used for each data point. Actin levels were used to correct for loading in each sample, and fold changes were calculated. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

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β-tubulin III proteins (Figure 2E). *NSE*, *MAP2* and *β-tubulin III* reached their mRNA peaks at 24 h (Figure 2C). Both NSE and β-tubulin *III* reached their highest protein levels after 48 h (Figure 2F).

Salidroside upregulates the expression of BDNF, NT-3 and NGF mRNAs and promotes their secretion in rMSCs

To determine whether salidroside can induce rMSCs to express and secrete BDNF, NGF, and NT-3, Real-time PCR and ELISA analysis were used. At 100 μg/mL salidroside or 10 μg/mL RA for 1–12 days, expression of *BDNF*, *NT-3* and *NGF* mRNAs significantly increased in with salidroside for 1–6 days, but had decreased by day 12, with a similar pattern being seen in RA-treated rMSCs (Figures 3A–3C). ELISA analysis showed that BDNF levels were significantly increased in cells treated with salidroside for 1–12 days in comparison with untreated controls (Figure 3D), NT-3 and NGF increased for 1–3 days in comparison with untreated (Figures 3E–3F). The levels of BDNF, NT-3 and NGF increased in cells treated with salidroside for 1–3 days in comparison with RA treated, but there were no differences between RA and salidroside treated for 6 and 12 days (Figures 3D–3F).

Salidroside induces rMSCs to differentiate into dopaminergic neurons

To characterise the property of salidroside-induced rMSCs-differentiated neurons, the expression of DBH, DDC, and TH, the markers of dopaminergic neurons, were analysed by immunostaining assay, which showed that 100 μg/mL salidroside or 10 μg/mL RA for 1–12 days significantly increased the percentage of DBH^+ or DDC^+ cells compare to the control (Figures 4A–D). Expression of TH was also increased in salidroside (Figure 5C) compared with cells treated with RA (Figure 5B) and untreated control (Figure 5A). *TH* mRNA expression was increased after 1–6 days of treatment (Figure 5D), which was confirmed by Western blotting, compared with control or RA-treated cells (Figures 5F and G). ELISA was used to measure DA in MSCs that were treated with salidroside in comparison with the cells treated with RA or control for 1–12 days. DA was readily increased after treatment with salidroside for 1–3 days, but dropped off by 12 days (Figure 5E). These results suggest that increased DA is consistent with that of TH expression after treatment with salidroside for 1–3 days.

Discussion

The ability of MSCs to differentiate in vitro along a neural lineage allows potential therapeutic applications for the treatment of neurological diseases and CNS trauma. MSCs have the potential to trans-differentiation into neuronal phenotypes in vitro (Woodbury et al., 2000; Abouelfetouh et al., 2004). Our data clearly demonstrate that rMSCs transdifferentiate into neuronal phenotype in vitro. Clearly, after 72 h of induction, a neuronal-like phenotype accounted for >64% of the total population, which agrees with the expressions of neuronal markers, salidroside induced rMSCs to increase the percentage of NSE^+, $MAP2^+$ and β-tubulin III^+ cells (Figure 2A), and the upregulation of the expression of *NSE*, *MAP2*, and *β-tubulin III* mRNAs (Figures 2B and C), and NSE and β-tubulin III proteins (Figures 2D and E) for 24–72 h. The data indicate that salidroside induced rMSCs to differentiate into neuron-like cells in vitro.

To date, many ectogenic factors, such as cAMP analogs, RA, BMPs (Tio et al., 2010), NGF (Brederlau et al., 2002), BDNF (Shi et al., 2012), GDNF and other factors (Trzaska et al., 2009) have been used for neuron-like differentiation. The innate capacity of MSCs to influence neural cell growth, survival and differentiation, is to express the endogenic neurotrophic factors. Crigler et al. (2006) demonstrated that specific subpopulations of hMSCs expressed BDNF and β-NGF but not neurotrophin-3 and -4. They used a co-culture assay to demonstrate that BDNF expression levels correlated with the ability of MSC subclones to induce survival and neurite outgrowth in the SH-SY5Y neuroblastoma cell line. The effects were only partially inhibited by a neutralizing anti-BDNF antibody, indicating that other factors secreted by the MSCs also had neuroregulatory effects. Neurite extension MSCs secrete neurotrophic factors involving NGF and BDNF, and these neurotrophic factors can upregulate TH gene expression in PC12 cells and neural stem cells (Jin et al., 2008). In this study, salidroside induced rMSCs to upregulate the expression of *BDNF*, *NT-3* and *NGF* mRNAs and the secretion of these growth factors (Figure 3), especially, salidroside increase *NT-3* expression, these endogenic factors play a vital role in regulating MSCs differentiate into dopamine neurons in the presence of salidroside.

DBH and DDC are key synthetases of dopamine, which are responsible for the conversion of dopa to dopamine (Smeyne and Jackson-Lewis, 2005). TH is the rate-limiting enzyme in the synthesis of catecholamine and is widely used as a marker of dopaminergic cells (O'Byrne et al., 2000). In this study, to determine the efficacy of differentiation, cells were tested with the related markers of dopamine neurons. Immunostaining confirmed that salidroside increases the percentage of DBH^+, DDC^+ or TH^+ cells in the MSCs treated with salidroside in comparison with cells treated with RA (Figures 4 and 5A–C), Expression of *TH* mRNA and protein were upregulated after treatment with salidroside (Figures 5D, 5F and 5G). We also have demonstrated that the level of dopamine (DA) in MSCs increased in the presence with salidroside. DA release indicates that in vitro-generated DA neurons induced with salidroside are functional.

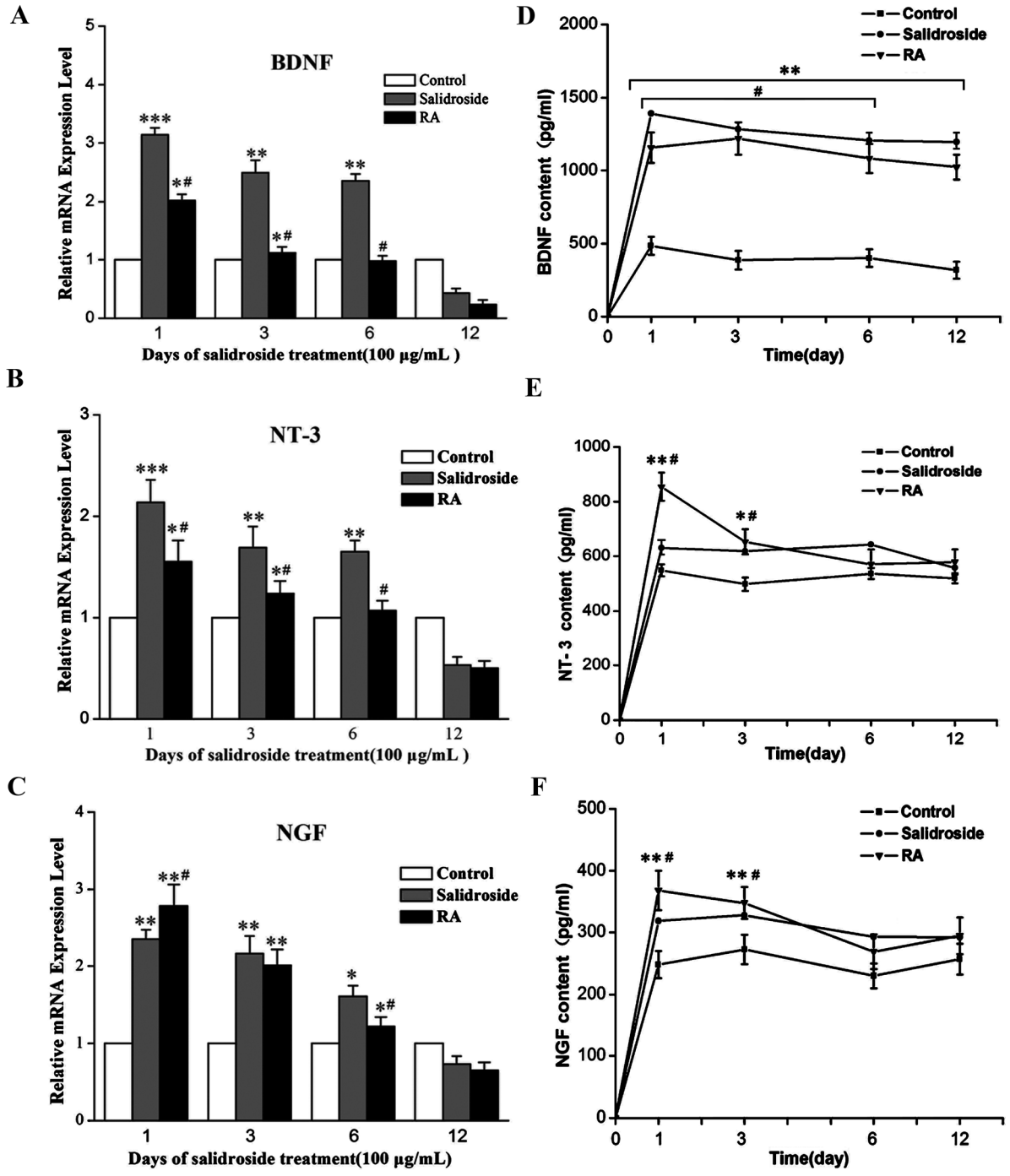

Figure 3 Salidroside upregulates the expression of BDNF, NT-3 and NGF mRNAs and promotes their secretion in rMSCs. (A–C) Real-time PCR analysis of the expression of *BDNF*, *NT-3* and *NGF* mRNAs in rMSCs treated with 100 μg/mL salidroside or 10 μg/mL retinoic acid (RA) for 1–12 days. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.01$ versus salidroside-treated group. (D–F) ELISA analysis of the secretion of BDNF, NT-3 and NGF in rMSCs treated with 100 μg/mL salidroside or 10 μg/mL retinoic acid (RA) for 1–12 h. Data are shown as mean ± SEM of three independent experiments. Different asterisk above each column in the bar graph indicate significant differences. Statistical analysis was done by the Student's *t*-test for two group comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#}P < 0.01$ versus RA-treated group.

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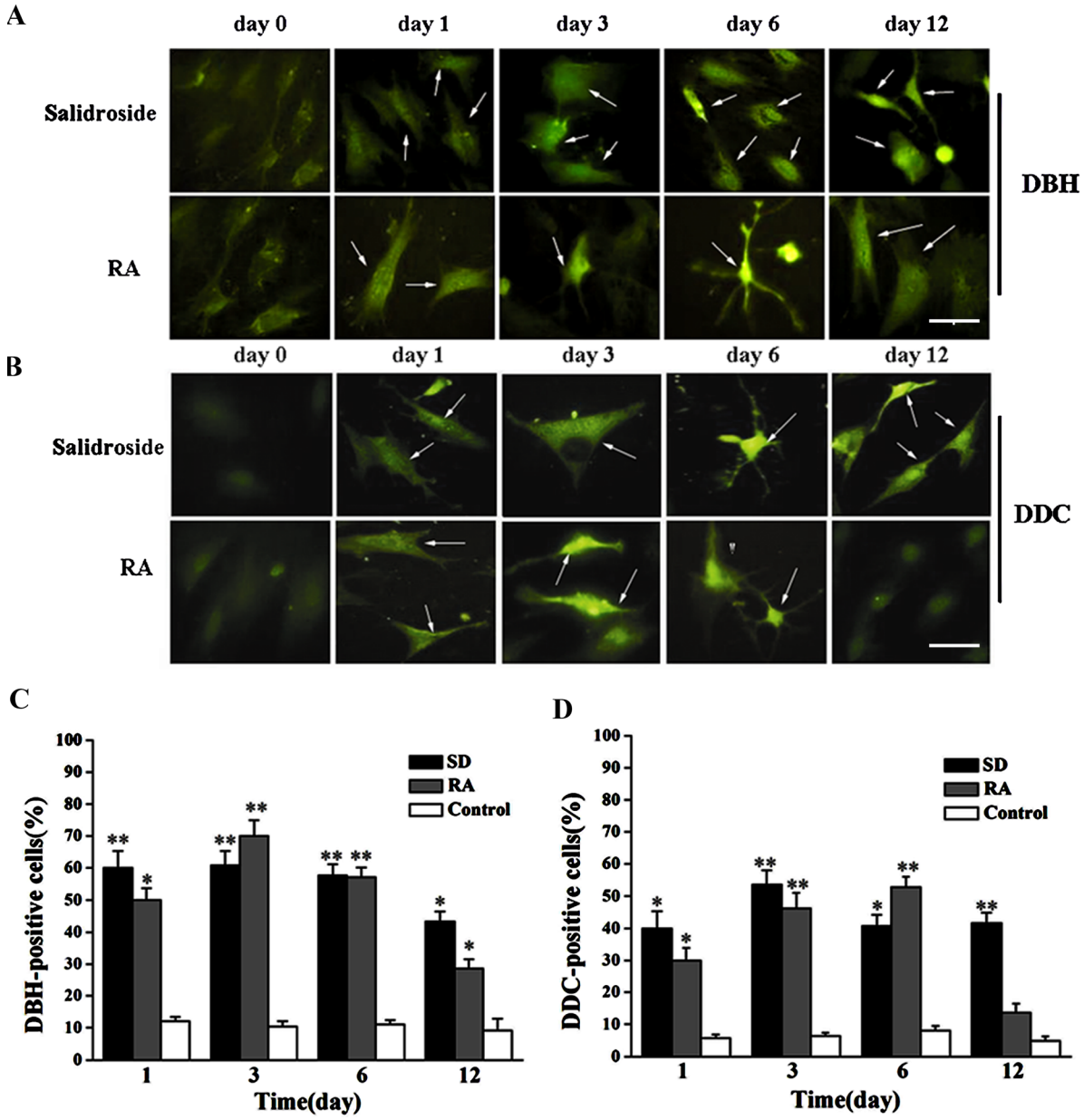

Figure 4 Salidroside increases the percentage of DBH $^{+}$ or DDC^{+} cells in rMSCs. rMSCs were treated with 100 μg/mL salidroside or 10 μg/mL retinoic acid (RA) for 1–12 days, followed by immunocytochemical analyses (A) DBH, (B) DDC. The images shown are a representative of three independent experiments. Scale bar: 50 μm. To determine the percentage of DBH (C) and DDC (D) positive cells in a particular condition, the total number of cells and the number of cells with a specific immunoreactivity were counted in 10–12 randomly selected fields of 2–3 different coverslips. Each experiment was repeated at least three times. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. Different asterisk above each column in the bar graph indicate significant differences. Statistical analysis was done by the Student's *t*-test for two group comparisons.

Salidroside not only induced MSCs to differentiate into dopaminergic neuron, but increased DA release. This is supported by the fact that salidroside enhanced secretion of BDNF, NT-3 and NGF may be responsible for MSCs expression of DBH, DDC and TH, which converted MSCs into dopaminergic neurons. It is interesting that the expression of *BDNF*, *NT-3*, *NGF*, DBH, DDC, TH and DA was clearly decreased after treatment with salidroside or RA for 12 days. There may be several reasons: (i) in our study, the longest time that salidroside induced MSCs is 12 days, but these growth factor or the markers of dopamine change over a longer time, which is unclear. (ii) Change of the growth factor or the markers of dopamine in MSCs after treatment with salidroside is different during the induction period;

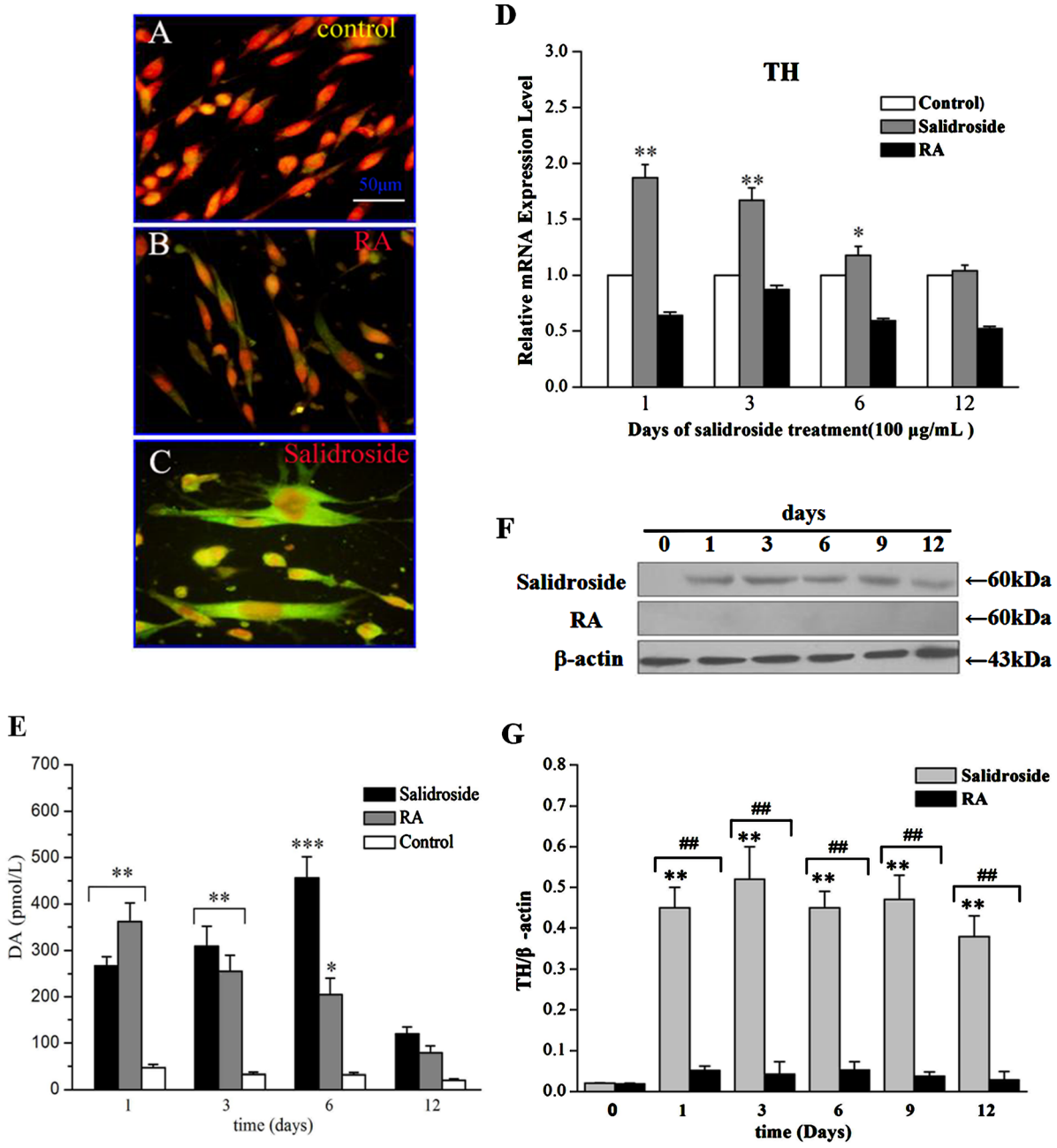

Figure 5 Salidroside increases the percentage of TH^+ cells and upregulates the expression of *TH* mRNA and protein in rMSCs. (A–C) rMSCs were treated with 100 μg/mL salidroside or 10 μg/mL retinoic acid (RA) for 3 days, followed by immunocytochemical analyses TH. The images shown are a representative of three independent experiments. Scale bar: 50 μm. (D) rMSCs were harvested for RNA preparations at 1–12 days after induction. Samples were analysed by Real Time-PCR to examine the expressions of *TH* mRNA. The data represent the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. (E) ELISA analysis of the secretion of DA in rMSCs treated with 100 μg/mL salidroside or 10 μg/mL retinoic acid (RA) for 1–12 days. Data are shown as mean ± SEM. Of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. (F and G) The expression of *TH* protein in rMSCs treated with 100 μg/mL salidroside or 10 μg/mL retinoic acid (RA) for 1–12 days was determined by Western blot. Actin levels were used to correct for loading in each sample, and fold changes were calculated. Data are shown as mean ± SEM. Of three independent experiments. $^{**}P < 0.01$ versus control, $^{\#\#}P < 0.01$ versus RA-treated.

increase was rapid in early differentiation, whereas these effects were slow in the later period. (iii) The inducing effect of salidroside may be important in early differentiated stage of MSCs, which conduce to dopaminergic neurons differentiation for early treatment of Parkinson's disease with MSCs in clinic. These reason may give impetus to future studies involving dopaminergic neurons of MSCs treated with salidroside.

This report provides a novel tactic for the application of active constituents of traditional Chinese medicinal herbs to the induction of MSCs differentiation towards dopaminergic neurons, which could have potential in clinical treatment.

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Author contribution

All authors participated in the experimental design, interpretation of the findings and manuscript review. Binhong Zhao, Hui Ma, Ming Zhang, Pin Zheng, Juzi Dong and Yinshu Yang conducted the experiments and analysed the data; Binhong Zhao wrote the manuscript; Xiaoqin Ha and Xiaoyun Li contributed constructive suggestions to the writing of the manuscript.

Conflict of interest

No competing financial interests exist.

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