

Original Article

Apoptosis of mesenchymal stem cells is regulated by *Rspo1* via the Wnt/ β -catenin signaling pathway

Xiao-Xia Cheng^a, Qiao-Yan Yang^{a,b}, Yong-Li Qi^{a,c}, Zhi-Zhen Liu^a, Dan Liu^a, Sheng He^b, Li-Hong Yang^{d,*}, Jun Xie^{a,**}

^a Department of Biochemistry and Molecular Biology, Shanxi Medical University, Taiyuan, Shanxi 030001, China

^b The First Affiliated Hospital of Shanxi Medical University, Taiyuan, Shanxi 030001, China

^c Huaihe Hospital of Henan University, Kaifeng, Henan 475000, China

^d Department of Pathology, Shanxi Medical University, Taiyuan, Shanxi 030001, China

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Abstract

Objective: The aim of this study was to investigate the effect and possible mechanism of action of roof plate-specific spondin1 (Rspo1) in the apoptosis of rat bone marrow mesenchymal stem cells (BMSCs).

Methods: Osteogenic and adipogenic differentiation of BMSCs was identified by Alizarin Red and Oil Red O staining, respectively. BMSC surface markers (cluster of differentiation 29 [CD29], CD90, and CD45) were detected using flow cytometry. BMSCs were transfected with an adenoviral vector encoding *Rspo1* (BMSCs-Rspo1 group). The expression levels of *Rspo1* gene and Rspo1 protein in the BMSCs-Rspo1 group and the two control groups (untransfected BMSCs group and BMSCs-green fluorescent protein [GFP] group) were analyzed and compared by quantitative polymerase chain reaction and Western blot. The occurrence of apoptosis in the three groups was detected by flow cytometry and acridine orange-ethidium bromide (AO-EB) double dyeing. The activity of the Wnt/ β -catenin signaling pathway was evaluated by measuring the expression levels of the key proteins of the pathway (β -catenin, c-Jun N-terminal kinase [JNK], and phospho-JNK).

Results: Osteogenic and adipogenic differentiation was confirmed in cultured BMSCs by the positive expression of CD29 and CD90 and the negative expression of CD45. Significantly increased expression levels of Rspo1 protein in the BMSCs-Rspo1 group compared to those in the BMSCs (0.60 ± 0.05 vs. 0.13 ± 0.02 ; $t=95.007$, $P=0.001$) and BMSCs-GFP groups (0.60 ± 0.05 vs. 0.10 ± 0.02 ; $t=104.842$, $P=0.001$) were observed. The apoptotic rate was significantly lower in the BMSCs-Rspo1 group compared with those in the BMSCs group ($[24.06 \pm 2.37]\%$ vs. $[40.87 \pm 2.82]\%$; $t = 49.872$, $P = 0.002$) and the BMSCs-GFP group ($[24.06 \pm 2.37]\%$ vs. $[42.34 \pm 0.26]\%$; $t = 62.358$, $P = 0.001$). In addition, compared to the BMSCs group, the protein expression levels of β -catenin (2.67 ± 0.19 vs. 1.14 ± 0.14 ; $t = -9.217$, $P = 0.000$) and JNK (1.87 ± 0.17 vs. 0.61 ± 0.07 ; $t = -22.289$, $P = 0.000$) were increased in the BMSCs-Rspo1 group. Compared to the BMSCs-GFP group, the protein expression levels of β -catenin (2.67 ± 0.19 vs. 1.44 ± 0.14 ; $t = -5.692$, $P = 0.000$) and JNK (1.87 ± 0.17 vs. 0.53 ± 0.06 ; $t = -10.589$, $P = 0.000$) were also upregulated in

* Corresponding author. 56 Xinjian South Road, Taiyuan, Shanxi 030001, China. Fax: +86 351 3985024.

** Corresponding author. 56 Xinjian South Road, Taiyuan, Shanxi 030001, China. Fax: +86 351 3985024.

E-mail addresses: ylh5577@yahoo.com (L.-H. Yang), junxie@sxmu.edu.cn (J. Xie).

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the BMSCs-Rspo1 group. Moreover, the protein expression levels of phospho-JNK were increased in the BMSCs-Rspo1 group compared to those in the BMSCs group (1.89 ± 0.10 vs. 0.63 ± 0.09 ; $t = -8.975$, $P = 0.001$) and the BMSCs-GFP group (1.89 ± 0.10 vs. 0.69 ± 0.08 ; $t = -9.483$, $P = 0.001$).

Conclusion: The Wnt/ β -catenin pathway could play a vital role in the Rspo1-mediated inhibition of apoptosis in BMSCs.

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Keywords: Rspo1; Bone marrow mesenchymal stem cells; Apoptosis; Wnt/ β -catenin signaling pathway

Introduction

Bone marrow mesenchymal stem cells (BMSCs) have been successfully used in cell transplantation therapy and are considered safe and effective seed cells in the field of regenerative medicine.¹ However, despite positive results from preclinical studies, data from phase I/II clinical trials are inconsistent and the improvement of organ function has been found to be quite limited. The major issues that BMSC therapy faces include inefficient cell delivery to the site of injury, low cell retention, and ineffectiveness of the stem cells in tissue regeneration.^{2–6} Moreover, these studies showed that genetic modification significantly improved the regenerative capacity of transplanted stem cells,⁶ and that genetic strategies may play a key role in improving the survival and differentiation of mesenchymal stem cells.^{7–11} Therefore, it is essential to find a gene or a set of genes that can improve the effect of BMSCs in the treatment of diseases. Recently, Zhao et al.¹² found that BMSCs overexpressing midkine could reduce the apoptosis rate in H9C2 cells (fetal rat cardiac cell line) and therefore improve cell survival. Compared with unmodified BMSCs, the improvement in heart function of rats with myocardial infarction was more obvious when treated with BMSCs overexpressing midkine. Moreover, mircoRNA-383, which enhances the expression of glial-derived neurotrophic factor, could improve the therapeutic effect of BMSCs on spinal cord injury.^{13–15} BMSCs overexpressing bone morphogenetic protein 2 (BMP-2) could also improve the biological function of the gastrocnemius tendon transplanted in the intramedullary cavity and promote tibia healing.¹⁶

Roof plate-specific spondin1 (Rspo1) is a member of the Rspo family that has a molecular weight of 35 kDa and is associated with activation of the Wnt signaling pathway.^{17,18} This family regulates the growth and development of animals, including the formation of blood vessels, muscles, and bones, as well as the development of limbs and the reproductive,

digestive, and respiratory systems.¹⁹ Rspo1 can bind to leucine-rich repeat-containing G protein-coupled receptors (LGRs) 4–6 and synergistically induce the phosphorylation of low-density lipoprotein receptor-related protein 6 (LRP6) with soluble Wnt3a, as well as promote the cytoplasmic stability and accumulation of β -catenin in the nucleus. The conformational changes of these proteins play an important role in cell proliferation, differentiation, and maintenance of stem cell function.²⁰ A recent study reported that Rspo1 could promote the osteogenesis of BMSCs by activating the Wnt/ β -catenin signaling pathway and rescue bone loss.²¹

It was recently reported that aspirin induced morphological apoptosis in rat tendon stem cells via the mitochondrial/caspase-3 pathway and induced cellular apoptosis in the Achilles tendon. Importantly, the Wnt/ β -catenin pathway played a vital role in aspirin-induced apoptosis by regulating mitochondrial/caspase-3 function.²² Wang et al.²³ found that inhibition of the Wnt/ β -catenin signaling pathway improved the therapeutic effect on transcatheter arterial chemoembolization by suppressing migration and invasion while promoting the apoptosis of transplanted hepatocellular carcinoma cells in rats. Moreover, Rspo1 activated the Wnt/ β -catenin signaling pathway, which is involved in the development, proliferation, and differentiation of stem cells, as well as the repair of tissue damage.^{17,18} Therefore, we first determined whether Rspo1 could indeed suppress BMSC apoptosis. Then, we analyzed the role of the Wnt/ β -catenin pathway in the inhibition of Rspo1 on the apoptosis of BMSCs.

Here, to test the possible effect and mechanism of action of Rspo1 in the apoptosis of rat BMSCs, we transfected BMSCs with an adenovirus carrying the *Rspo1* gene and measured the apoptosis rate and survival of BMSCs. The expression levels of β -catenin and c-Jun N-terminal kinase (JNK), which are key proteins in the Wnt/ β -catenin signaling pathway, were further explored for their roles in apoptosis.

Materials and methods

Animals

Healthy male Sprague–Dawley (SD) rats that weighed from 60 to 80 g were used to isolate the BMSCs. All rats were obtained from Animal Research Center of Shanxi Medical University. The experiments were performed in adherence to the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” (Publication No. 85-23, revised 1996) and were approved by Shanxi Medical University Committee on Animal Care (Approval No. 2018026).

Isolation and culture of BMSCs

SD rats were sacrificed by dislocation of cervical vertebra. The hind limbs to the femoral heads were cut and soaked in 75% alcohol for 5 min, and the femur and tibia were soaked in phosphate-buffered saline (PBS) solution. The bone marrow tissue was exposed and the bone marrow suspension was collected by flushing the marrow cavity with the medium. The collected bone marrow suspension was cultured for 24 h in 5% CO₂ at 37°C and washed with PBS to remove non-adherent cells. The morphology of the primary and passage BMSCs was monitored using an inverted microscope during the experiment.

Identification of BMSCs

The BMSC surface markers were identified by flow cytometry. Passage 3 (P3) cells were suspended in medium and the cell density was adjusted to 1×10^5 cells/ml. CD29-allophycocyanin (APC), CD90-fluorescein isothiocyanate (FITC), and CD45-phycoerythrin (PE) antibodies were added to the suspension and incubated at room temperature for 30 min for flow cytometry.

Osteogenic and adipogenic differentiation of BMSCs

The P3 cells with a degree of 90% fusion were obtained and inoculated in a 6-well plate at 1×10^5 cells/ml. The cells were induced with osteogenic and adipogenic differentiation media, and then stained with Alizarin Red and Oil Red O, respectively.

Transfection of BMSCs

Primary BMSCs (4×10^5 cells/well) were seeded in 6-well plates in complete culture medium. To construct

the adenovirus (ADV) encoding *Rspo1* plasmid (ADV-*Rspo1* vectors), the complementary DNA (cDNA) encoding rat *Rspo1* was synthesized and cloned into the restriction endonuclease sites of the ADV, a mammalian expression vector containing green fluorescent protein (GFP) and puromycin resistance genes (Shenggong, Shanghai, China). Twenty-four hours after seeding, BMSCs were infected with recombinant ADV (ADV-*Rspo1* vectors) and ADV control vectors. The recombinant ADV encoding GFP (BMSCs-GFP) was used as control. The cells were cultured for 72 h, and the transfection efficiency was determined using fluorescence microscopy and flow cytometry.

Apoptosis assay

The apoptosis rate of BMSCs was measured by Annexin V-APC/7-aminoactinomycin D (7-AAD) Apoptosis Kit (KeyGEN BioTECH, Nanjing, China). Briefly, the transfected BMSCs were collected by trypsin digestion without ethylenediaminetetraacetic acid (EDTA) and then washed with PBS by centrifugation at 2000 r/min for 5 min. To the cell suspension, 5 µl of Annexin V-APC and 5 µl of 7-AAD dye solution were added at room temperature and protected from the light for 5–15 mins. The cells were counted through flow cytometry within 1 h. Detection of apoptosis by AO-EB double staining (Solarbio, Beijing, China) was also performed. The cells were cultured in the 96-well plate; 72 h after transfection, the residual medium and the non-adherent cells were removed by washing with PBS and adding fresh PBS to the cells. A volume (20 µl) of working solution per millilitre of PBS was added (according to the dosage, mixing AO solution and EB solution into the working volume at a 1:1 ratio). After incubation for 2–5 min at room temperature, BMSCs were observed using a fluorescence microscope (Nikon, Tokyo, Japan).

Western blot analysis

The immunoblot was performed as previously described.²⁴ BMSCs were collected and total protein was extracted using radio-immunoprecipitation assay and phenylmethanesulfonyl fluoride (Thermo Fisher SCIENTIFIC, Waltham, MA, USA). Then, 25 µl of the protein sample was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked for 1 h, and incubated with a primary antibody (anti-Rspo1, 1:1000; anti-Bax, 1:1000; anti-Caspase-3, 1:2000; anti-Cleaved Caspase-

3, 1:1000; anti- β -catenin, 1:5000; anti-JNK, 1:1000; anti-phospho-JNK, 1:2000; Abcam, Cambridge, UK) at 4°C overnight. Then, the PVDF membrane was washed and incubated with the corresponding secondary antibody for 1 h at room temperature. Finally, the proteins were detected using an enhanced chemiluminescent reaction.

Detection of gene expression by quantitative polymerase chain reaction (qPCR)

Total RNA was isolated using the RNAiso Plus (Takara, Tokyo, Japan) and was converted to cDNA using the High Capacity cDNA RT Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNAs from three replicates for each treatment were pooled into a custom SYBR Array 48-Well FAST Plate. The fold changes of gene expression relative to β -actin (an endogenous control) were determined according to the $2^{-\Delta\Delta C_t}$ method. The PCR primers were as follows:

β -actin-F: 5'-GCTCTCTTCCAGCCTTCCTT-3';
 β -actin-R: 5'-AGGTCTTTACGGATGTCAACG-3';
Rspo1-F: 5'-TGTGAAATGAGCGAGTGGTC-3';
Rspo1-R: 5'-GAGCAGTTGGTTTGGTCTCC-3';
 β -catenin-F: 5'-GCAGTGAAGAATGCACACGA-3';
 β -catenin-R: 5'-CAAGCAAAGTCAGCACCACT-3';
JNK-F: 5'-TGGAGTCATAAGAGGGCAGC-3';
JNK-R: 5'-ACTGCTGTCTGTATCCGAGG-3'.

Statistical analysis

Numerical data with normal distribution were reported as the mean $\pm$ standard deviation (relative expression of genes [*Rspo1*, β -catenin and JNK] and proteins [*Rspo1*, Bax, Caspase-3, Cleaved Caspase-3, β -catenin, JNK and phospho-JNK] and the apoptotic rates of BMSCs). Statistical analysis was performed using Student's *t*-test for the comparison of two groups for multiple comparisons. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Characterization of BMSCs

The passage 0 (P0) cells were small with protrusions from the edges and were varied in shape—polygonal,

long fusiform, and irregular shapes. The P3 cells had an enlarged volume, had a long fusiform shape, were uniform in size, and were neatly arranged in a consistent direction (Fig. 1A, B). To identify the differentiation potential of BMSCs, orange-red round lipid droplets in the cytoplasm of Oil Red O-stained cells were observed after adipogenic differentiation for 20 days (Fig. 1E, F); after 21 days of osteogenic differentiation, the surface of Alizarin Red-stained cells had reddish brown calcium deposits that formed round calcium nodules (Fig. 1C, D). Moreover, BMSC surface markers were detected by flow cytometry. The results showed that BMSCs were positive for CD29 ($97.10 \pm 0.76\%$) and CD90 ($95.83 \pm 0.76\%$), and were negative for the hematopoietic stem cell surface marker CD45 ($3.93 \pm 0.60\%$) (Fig. 1G–I).

Rspo1 was successfully expressed in transfected BMSCs

The efficiency of viral transfection was determined by measuring the GFP signal using a fluorescence microscope. As shown in Fig. 2A–F, clear GFP signals were detected in the BMSCs-GFP and BMSCs-*Rspo1* groups, but not in the BMSCs group, demonstrating that ADV was successfully transfected into the cells. The transfection efficiency was further measured by flow cytometry. As shown in Fig. 2G–I, the ADV transfection efficiency in the BMSCs-*Rspo1* group was similar to that in the BMSCs-GFP group ($98.60 \pm 0.56\%$ vs. $98.43 \pm 0.50\%$; $t = 0.335$, $P = 0.754$), but was significantly higher than that in the BMSCs group ($98.60 \pm 0.56\%$ vs. $0.33 \pm 0.15\%$; $t = 58.973$, $P = 0.002$). The expression of *Rspo1* gene and *Rspo1* protein was confirmed by qPCR and Western blot. The qPCR data indicated that the expression level of *Rspo1* was higher in the BMSCs-*Rspo1* group than in the BMSCs group (5.05 ± 0.74 vs. 1.00 ± 0.00 ; $t = -13.490$, $P = 0.000$) and the BMSCs-GFP group (5.05 ± 0.74 vs. 0.96 ± 0.25 ; $t = -12.923$, $P = 0.000$) (Fig. 3A). The BMSCs-*Rspo1* group also exhibited higher levels of *Rspo1* protein than the BMSCs group (0.60 ± 0.05 vs. 0.13 ± 0.02 ; $t = 95.007$, $P = 0.001$) and the BMSCs-GFP group (0.60 ± 0.05 vs. 0.10 ± 0.02 ; $t = 104.842$, $P = 0.001$) (Fig. 3B, C). The results of qPCR and Western blot indicated that *Rspo1* was successfully expressed in transfected BMSCs.

Rspo1 could inhibit the apoptosis of BMSCs

To test whether overexpression of *Rspo1* could prevent the apoptosis of BMSCs, we compared the

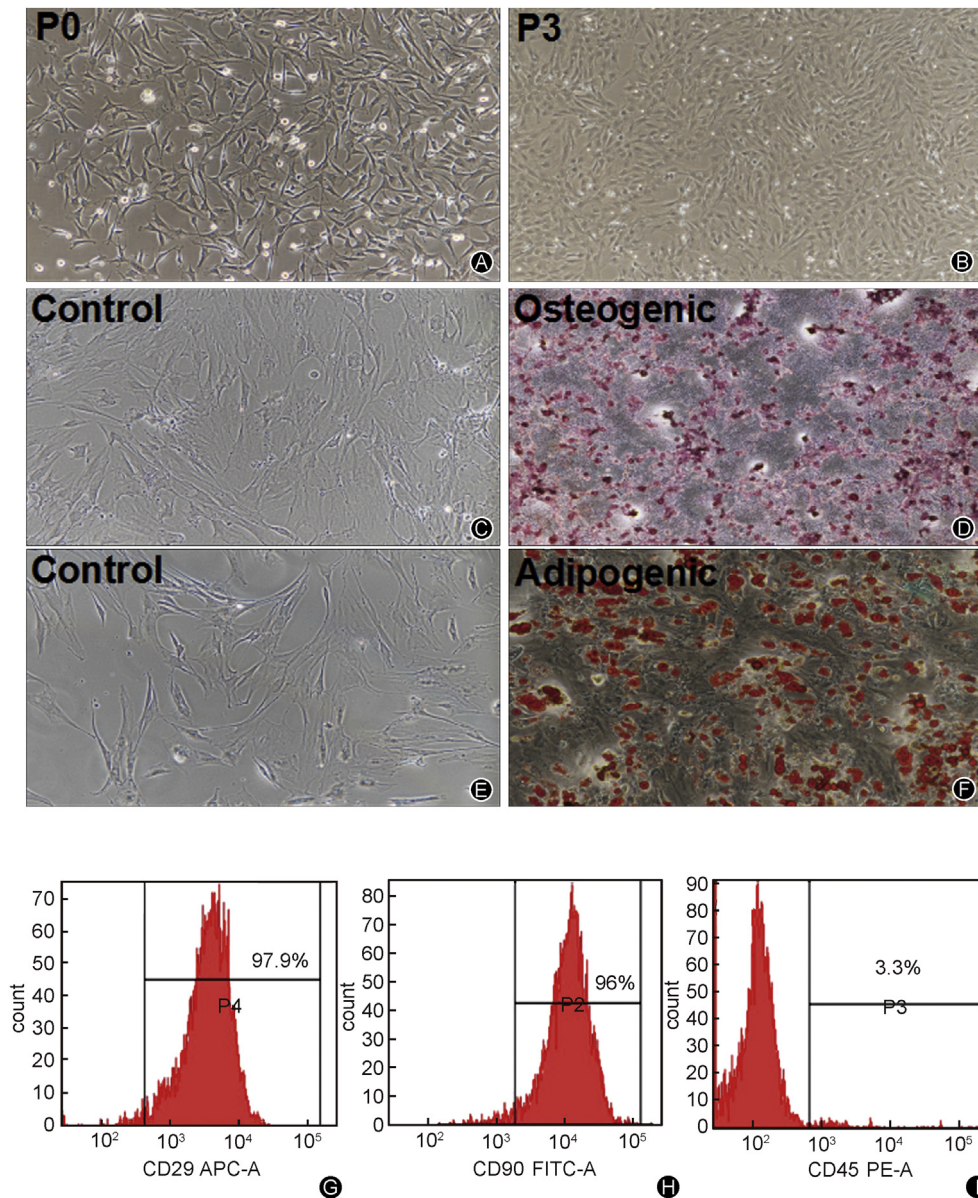


Fig. 1. Characterization of BMSCs. (A) represented P0 cells and (B) represented P3 cells (original magnification $\times 100$); (C) was a control for osteogenic differentiation and (D) was osteogenic differentiation (Alizarin Red staining, original magnification $\times 100$); (E) was an adipogenic differentiation control and (F) was adipogenic differentiation (Oil Red O staining, original magnification $\times 100$); and identification of BMSC surface markers CD29 (G), CD90 (H), and CD45 (I); $n = 3/\text{group}$. P0: passage 0 cells; P3: passage 3 cells; BMSCs: bone mesenchymal stem cells; CD: cluster of differentiation; APC: allophycocyanin; FITC: fluorescein isothiocyanate; PE: phycoerythrin.

number of apoptotic cells across groups. The apoptotic rate was significantly lower in the BMSCs-Rspo1 group compared with those in the BMSCs group ($[24.06 \pm 2.37]\%$ vs. $[40.87 \pm 2.82]\%$; $t = 49.872$, $P = 0.002$) and the BMSCs-GFP group ($[24.06 \pm 2.37]\%$ vs. $[42.34 \pm 0.26]\%$; $t = 62.358$, $P = 0.001$) (Fig. 4A–C). As shown in Fig. 4D–G, the results of AO-EB double dyeing displayed that the condensed or beaded apoptotic cells, which were

green and orange-red, were significantly less in the BMSCs-Rspo1 group than those in the BMSCs group ($[25.02 \pm 3.58]\%$ vs. $[43.63 \pm 2.16]\%$; $t = 44.906$, $P = 0.003$) and the BMSCs-GFP group ($[25.02 \pm 3.58]\%$ vs. $[44.56 \pm 2.04]\%$; $t = 67.676$, $P = 0.001$). Furthermore, it was more intuitive to verify that Rspo1 could inhibit the apoptosis of BMSCs. As expected, Western blot analysis demonstrated a significant decrease in Bax protein level in the BMSCs-

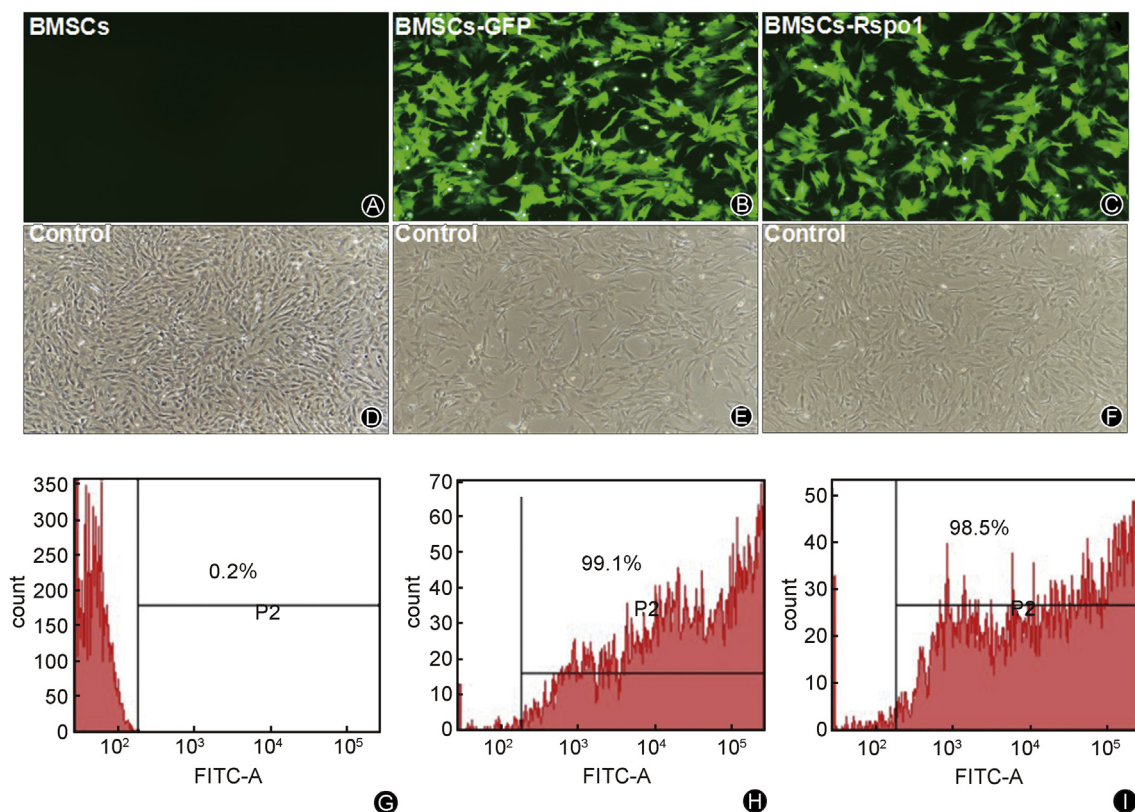


Fig. 2. Rspo1 was successfully expressed in transfected BMSCs. GFP was detected by fluorescence microscopy (original magnification $\times 40$). (A) and (D) were the fluorescence results and white light of the BMSCs group, respectively. (B) and (E) were the fluorescence results and white light of the BMSCs-GFP group, respectively. (C) and (F) were the fluorescence results and white light of the BMSCs-Rspo1 group, respectively. And flow cytometry detection results of apoptotic rates were shown in (G) for BMSCs, (H) for BMSCs-GFP and (I) for BMSCs-Rspo1. Rspo1: roof plate-specific spondin1; BMSCs: bone mesenchymal stem cells; GFP: green fluorescent protein; BMSCs-Rspo1: BMSCs overexpressing Rspo1; FITC: fluorescein isothiocyanate.

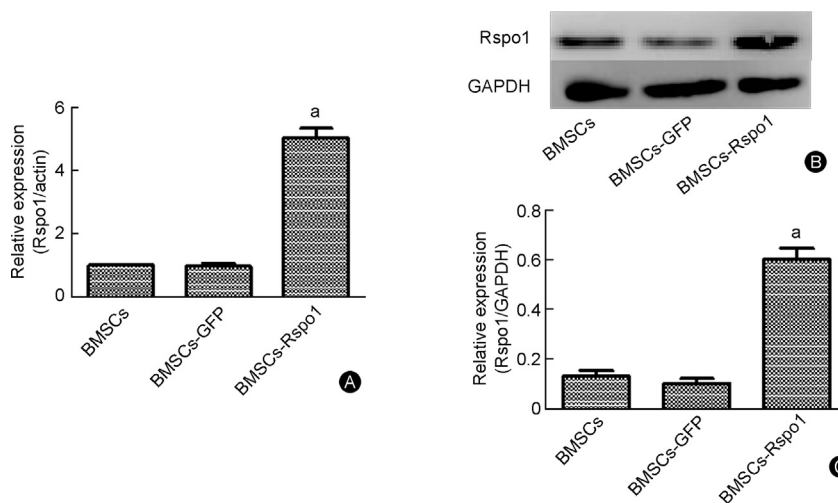


Fig. 3. Rspo1 was successfully expressed in transfected BMSCs. (A) Quantitative polymerase chain reaction analysis of Rspo1 mRNA; (B) Western blot band of Rspo1. (C) Histogram of Western blot of Rspo1 protein level in three groups. ^a $P < 0.01$ vs. BMSCs group and BMSCs-GFP group, $n = 3$ /group. Rspo1: roof plate-specific spondin1; BMSCs: bone mesenchymal stem cells; BMSCs-GFP: bone mesenchymal stem cells-green fluorescent protein; BMSCs-Rspo1: BMSCs overexpressing Rspo1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; mRNA: messenger RNA.

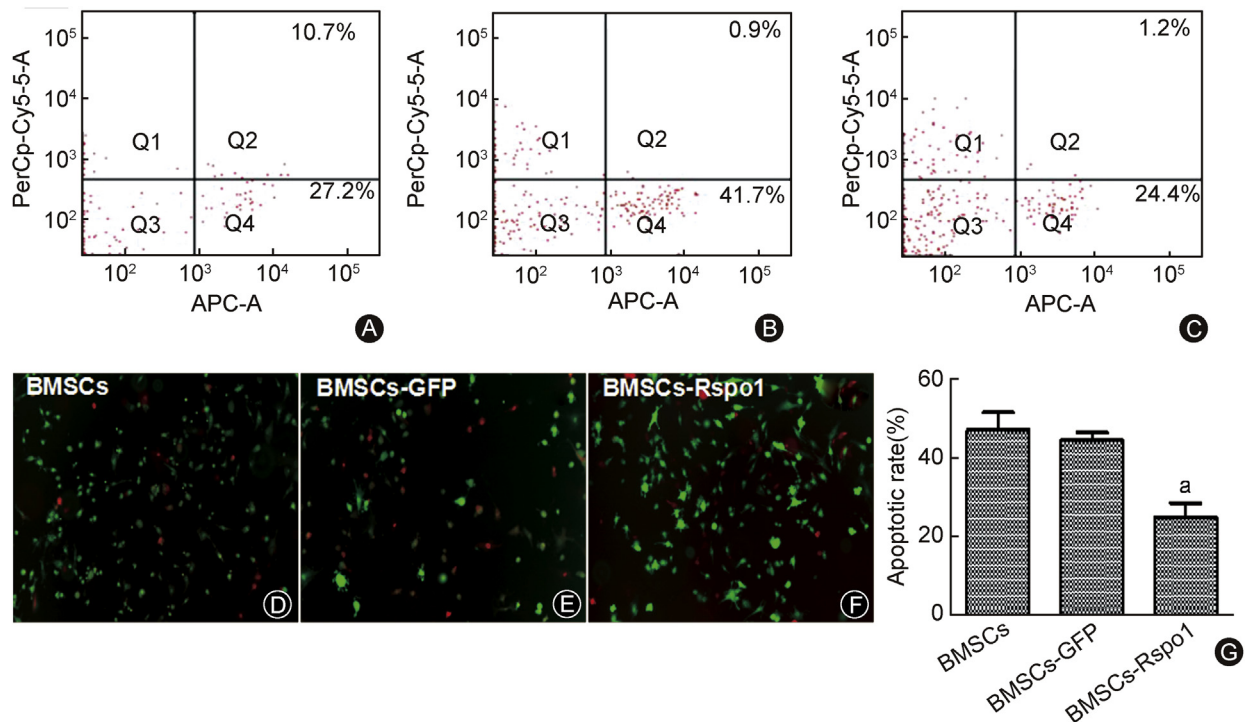


Fig. 4. Rspo1 could inhibit apoptosis in BMSCs. Flow cytometry analysis of cell apoptosis in all groups ([A] the apoptosis of BMSCs; [B] the apoptosis of BMSCs-GFP; [C] the apoptosis of BMSCs-Rspo1); the apoptosis of cells detected by AO-EB double staining ([D] the apoptosis of BMSCs; [E] the apoptosis of BMSCs-GFP; [F] the apoptosis of BMSCs-Rspo1) (original magnification $\times 40$); (G) histogram of apoptotic rates detected by AO-EB double staining. ^a $P < 0.01$ vs. BMSCs group and BMSCs-GFP group, $n = 3$ /group. Rspo1: roof plate-specific spondin1; BMSCs: bone mesenchymal stem cells; BMSCs-GFP: bone mesenchymal stem cells-green fluorescent protein; BMSCs-Rspo1: BMSCs over-expressing Rspo1; APC: allophycocyanin; AO-EB: acridine orange-ethidium bromide.

Rspo1 group compared with that in the BMSCs group (0.06 ± 0.03 vs. 0.61 ± 0.04 ; $t = 100.445$, $P = 0.001$) and the BMSCs-GFP group (0.06 ± 0.03 vs. 0.58 ± 0.04 ; $t = 108.736$, $P = 0.000$). The activity of Caspase-3 significantly decreased in the BMSCs-Rspo1 group compared with those in the BMSCs group (0.13 ± 0.05 vs. 1.56 ± 0.09 ; $t = 188.778$, $P = 0.000$) and the BMSCs-GFP group (0.13 ± 0.05 vs. 1.44 ± 0.05 ; $t = 354.715$, $P = 0.000$). Additionally, cleaved Caspase-3 was significantly decreased in the BMSCs-Rspo1 group compared with those in the BMSCs group (0.45 ± 0.05 vs. 0.97 ± 0.15 ; $t = 59.220$, $P = 0.003$) and the BMSCs-GFP group (0.45 ± 0.05 vs. 0.94 ± 0.12 ; $t = 73.664$, $P = 0.002$) (Fig. 5A–D). The results of Western blot indicated that Rspo1 could inhibit the apoptosis of BMSCs in protein levels.

The Wnt/ β -catenin pathway could play a vital role in the inhibition of Rspo1 on the apoptosis of BMSCs

As Rspo1 protein was recognized as an agonist of Wnt/ β -catenin signaling, we evaluated the signaling

level in the ADV-Rspo1-infected BMSCs during apoptosis. As expected, the expression levels of Wnt target genes (*CTNNB1* encoding β -catenin and *JNK*) were significantly increased ($P < 0.01$), which indicated the promoted activity of Wnt/ β -catenin signaling due to Rspo1 during apoptosis of BMSCs (Fig. 6). Consistent with the qPCR results, compared to the BMSCs group, the protein expression levels of β -catenin (2.67 ± 0.19 vs. 1.14 ± 0.14 ; $t = -9.217$, $P = 0.000$) and JNK (1.87 ± 0.17 vs. 0.61 ± 0.07 ; $t = -22.289$, $P = 0.000$) were increased in the BMSCs-Rspo1 group. Compared to the BMSCs-GFP group, the protein expression levels of β -catenin (2.67 ± 0.19 vs. 1.44 ± 0.14 ; $t = -5.692$, $P = 0.000$) and JNK (1.87 ± 0.17 vs. 0.53 ± 0.06 ; $t = -10.589$, $P = 0.000$) were also upregulated in the BMSCs-Rspo1 group (Fig. 7A–C). Moreover, the protein expression levels of phospho-JNK were increased in the BMSCs-Rspo1 group compared to those in the BMSCs group (1.89 ± 0.10 vs. 0.63 ± 0.09 ; $t = -8.975$, $P = 0.001$) and the BMSCs-GFP group (1.89 ± 0.10 vs. 0.69 ± 0.08 ; $t = -9.483$, $P = 0.001$) (Fig. 7A, D). This suggests that inhibition of the

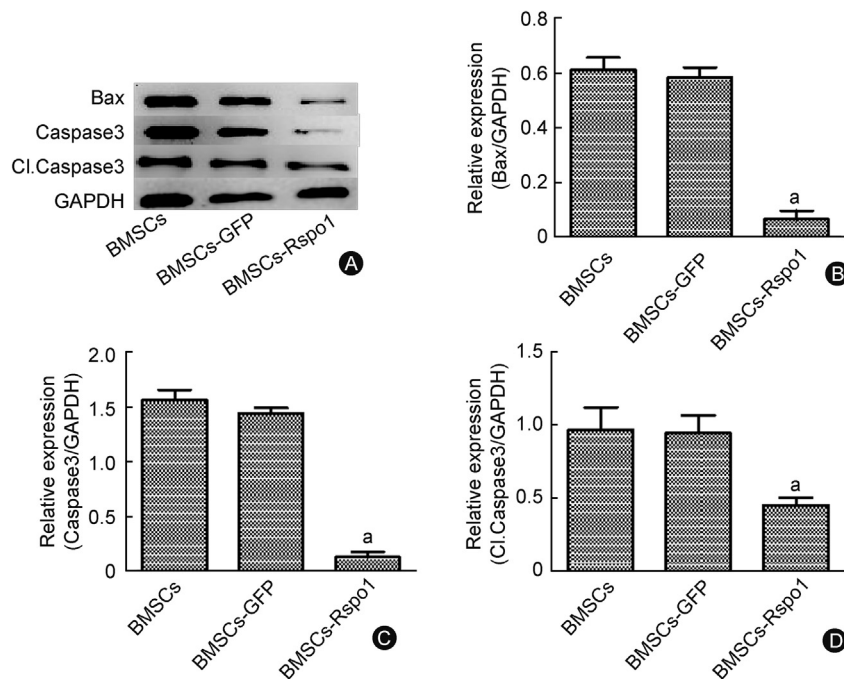


Fig. 5. Rspo1 could inhibit the apoptosis of BMSCs in protein levels. (A) Western blot band of Bax, Caspase3 and Cl. Caspase3 proteins. Western blot analysis confirmed that Rspo1 overexpression decreased the expression of Bax (B: Histogram of Western blot of Bax protein levels), Caspase3 (C: Histogram of Western blot of Caspase3 protein levels), and Cl. Caspase3 (D: Histogram of Western blot of Cl. Caspase3 protein levels) in BMSCs-Rspo1 group. ^a $P < 0.01$ vs. BMSCs group and BMSCs-GFP group, $n = 3$ /group. Rspo1: roof plate-specific spondin1; BMSCs: bone mesenchymal stem cells; BMSCs-GFP: bone mesenchymal stem cells-green fluorescent protein; BMSCs-Rspo1: BMSCs overexpressing Rspo1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Cl. Caspase3: cleaved-Caspase3.

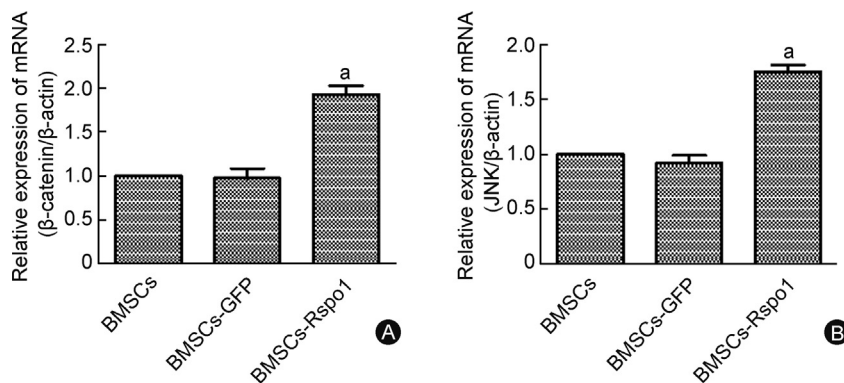


Fig. 6. The promoted activity of the Wnt/β-catenin signaling due to Rspo1 during apoptosis of BMSCs. Quantitative polymerase chain reaction analysis of β-catenin (A) and JNK (B) mRNAs; ^a $P < 0.01$ vs. BMSCs group and BMSCs-GFP group, $n = 3$ /group. mRNA: messenger RNA; Rspo1: roof plate-specific spondin1; BMSCs: bone mesenchymal stem cells; BMSCs-GFP: bone mesenchymal stem cells-green fluorescent protein; BMSCs-Rspo1: BMSCs overexpressing Rspo1; JNK: c-Jun N-terminal kinase.

apoptosis of BMSCs mediated by Rspo1 may be through activation of the Wnt/β-catenin pathway.

Discussion

BMSCs are capable of self-renewal and multi-differentiation. Moreover, there are no ethical problems regarding their use unlike with embryonic stem

cells, and there is no controversy about their gene stability unlike with induced pluripotent stem cells (iPSCs). In fact, depending on the environment, BMSCs can differentiate into a variety of cell types, such as cardiomyocytes, osteoblasts, endothelial cells, neurons, and fat cells *in vitro* and *in vivo*. Because of the homing ability of BMSCs, they can be used as tools for carrying various genes. For example, Zhang et al.²⁵

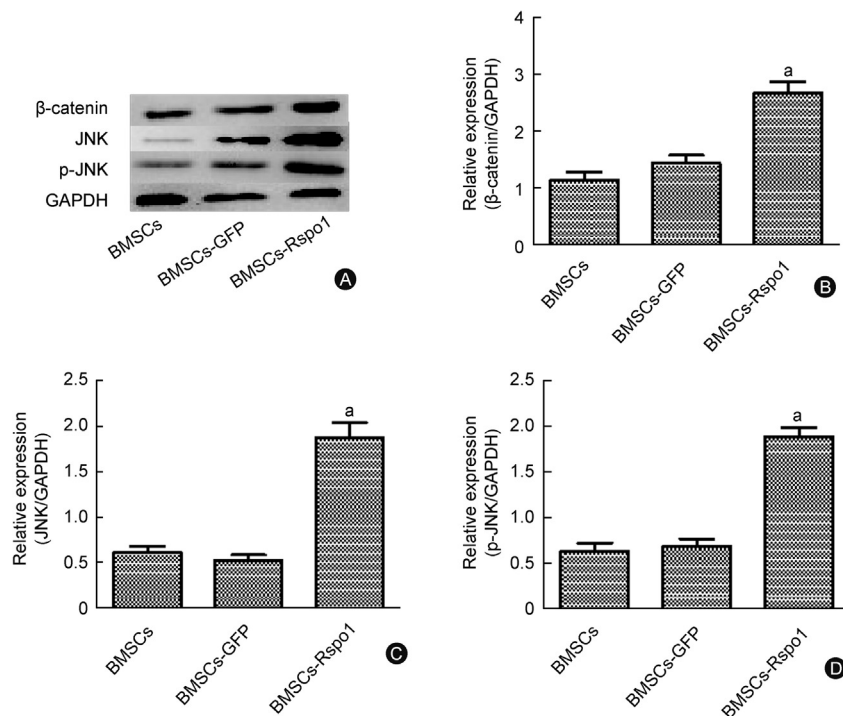


Fig. 7. The promoted activity of the Wnt/ β -catenin signaling due to Rspo1 during apoptosis of BMSCs at the protein levels. (A) Western blot band of β -catenin, JNK and p-JNK expression. (B) Histogram of Western blot of β -catenin protein levels; (C) histogram of Western blot of JNK protein levels; (D) histogram of Western blot of p-JNK protein levels. ^a $P < 0.01$ vs. BMSCs group and BMSCs-GFP group, $n = 3$ /group. Rspo1: roof plate-specific spondin1; JNK: c-Jun N-terminal kinase; p-JNK: phosphorylated JNK; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; BMSCs: bone mesenchymal stem cells; BMSCs-GFP: bone mesenchymal stem cells-green fluorescent protein; BMSCs-Rspo1: BMSCs over-expressing Rspo1.

used BMSCs as a vector for a tumor-targeting suicide gene to treat pulmonary metastases. Moreover, Ryu et al.²⁶ constructed a BMSCs-interferon β (IFN- β) gene complex to treat diseases.

Adenovirus (ADV) can be used as a gene vector because the ADV genome infects cells and integrates into the host chromosomes.²⁷ One of the significant challenges in using ADV as a gene vector is that virus particles can induce inflammatory reactions and cause damage to host cells.²⁸ In the present study, we transfected ADV with GFP and there was no significant difference in the expression of GFP between the BMSCs-GFP and BMSCs-Rspo1 groups. Since the spectral characteristics of GFP were similar to those of FITC, we used FITC channels to detect GFP expression in flow cytometry experiments. Using ADV as a gene vector, *Rspo1* was successfully expressed in transfected BMSCs. Our data showed that there was no significant difference in the expression of Rspo1 and in the apoptosis rate between the BMSCs group and the BMSCs-GFP group after ADV transfection. Similarly, ADV transfection did not affect the expression of apoptosis-related proteins Caspase-3, Bax, and cleaved

Caspase-3. Thus, our results revealed that the expression of the ADV genome in BMSCs did not interfere with the expression and function of Rspo1 in BMSCs.

Rspo1 activates β -catenin through a mechanism similar to that by the ligand in the classical pathway and enhances the biological activity of the Wnt/ β -catenin pathway.¹⁸ Several reports have shown that Rspo1 is involved in regulating cell proliferation and differentiation, as well as in the development of embryonic bone, blood vessels, muscles, and fingernails; it is also reported to have an effect on the development of the embryo digestive, respiratory, and reproductive systems, as well as limb formation. In addition, it plays an important role in the occurrence of many diseases.^{7–11} For example, after acute injury, Rspo1 was reported to be necessary for myogenic precursor cell differentiation at the appropriate time; at the same time, classical Wnt/ β -catenin signaling was activated during myogenic differentiation.²⁹ It was recently reported that the knockout of Wnt5a could inhibit the proliferation and promote the apoptosis of keratinocytes by suppressing Wnt/ β -catenin or Wnt5a/ Ca^{2+} signaling.³⁰ Okumura et al.³¹ have shown that Rspo1

regulated the proliferation and apoptosis of corneal endothelial cells by activating the Wnt/ β -catenin signaling pathway, effectively maintaining the function of corneal endothelial cells. Consistent with previous findings, our results showed that Rspo1 had a similar effect. The apoptosis rate of BMSCs-Rspo1 was significantly lower than those of the BMSCs and BMSCs-GFP groups, but no statistical difference was observed between the BMSCs and BMSCs-GFP groups. Moreover, the activity of Caspase-3, the expression levels of cleaved Caspase-3 protein, and the expression levels of Bax protein were significantly decreased in the BMSCs-Rspo1 group. This indicates that Rspo1 inhibited the apoptosis of BMSCs. In addition, the expression levels of *CTNBI* and *JNK* genes, as well as β -catenin, JNK, and phospho-JNK proteins in the BMSCs-Rspo1 group were significantly higher than those in the BMSCs and BMSCs-GFP groups. However, no statistical difference was observed between the BMSCs and BMSCs-GFP groups. Thus, to our knowledge, our results are the first to show that the Wnt/ β -catenin pathway may play a vital role in the inhibition of apoptosis of BMSCs and the consequent improved survival of BMSCs mediated by Rspo1.

Conflicts of interest

None.

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