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Shuanglongjiegu pill promoted bone marrow mesenchymal stem cell osteogenic differentiation by regulating the miR-217/RUNX2 axis to activate Wnt/β-catenin pathway

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Abstract

This study aimed to investigate the effects of Shuanglongjiegu pill (SLJGP) on the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) and explore its mechanism based on miR-217/RUNX2 axis. Results found that drug-containing serum of SLJGP promoted BMSCs viability with a dose-dependent effect. Under osteogenic differentiation conditions, SLJGP promoted the expression of ALP, OPN, BMP2, RUNX2, and the osteogenic differentiation ability of BMSCs. In addition, SLJGP significantly reduced miR-217 expression, and miR-217 directly targeted RUNX2. After treatment with miR-217 mimic, the promoting effects of SLJGP on proliferation and osteogenic differentiation of BMSCs were significantly inhibited. MiR-217 mimic co-treated with pcDNA-RUNX2 further confirmed that the miR-217/RUNX2 axis was involved in SLJGP to promote osteogenic differentiation of BMSCs. In addition, analysis of Wnt/β-catenin pathway protein expression showed that SLJGP activated the Wnt/β-catenin pathway through miR-217/RUNX2. In conclusion, SLJGP promoted osteogenic differentiation of BMSCs by regulating miR-217/RUNX2 axis and activating Wnt/β-catenin pathway.

Keywords Bone marrow mesenchymal stem cells, Traditional Chinese medicine, Shuanglongjiegu pill, Osteogenic differentiation, miR-217/RUNX2 axis, Wnt/β-catenin pathway

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Introduction

Osteoporosis is a progressive systemic bone disease characterized by low bone mass and degradation of bone tissue microstructure, which will lead to increased bone fragility, thus increasing the risk of fracture [1]. The treatment of osteoporosis mainly includes drug therapy and lifestyle interventions [2]. The drugs of modern medicine have certain curative effects, but there are also adverse reactions such as gastrointestinal reactions, hypercalcemia, fever, and kidney stones, and their long-term application is limited [3]. Traditional Chinese medicine (TCM) believes that osteoporosis belongs to the category of "bone impotence", and has been proven to have a unique advantage in preventing and treating osteoporosis [4]. Shuanglongjiegu pill (SLJGP) is an empirical formula for fracture treatment developed by Professor Zheng Huaixian, an expert in the orthopedics department of Chinese medicine. It includes fifteen Chinese medicines, including *Eupolyphaha steleophaga*, *Angelicae sinensis* radix, *Resina Draconis*, *Pheretima*, *Wine Dipsaci* radix, *Sappan lignum*, *Poria*, cooked *Rhubarb*, *Aucklandiae* radix, *Albizziae* cortex, *Fossilizid*, *Paeoniae* radix alba, *Achyranthis Bidentatae* radix, vinegar *olibanum*, and vinegar *myrrh*. SLJGP is believed to have the effect of promoting blood circulation and qi, dispersing blood stasis and clearing collateral-arteries, reducing swelling and relieving pain, strengthening muscles and bones, and can be used for osteoporosis [5, 6].

The etiology of osteoporosis is complex, but all of them are mainly manifested as the damage of bone remodeling balance due to decreased bone formation and increased bone resorption [7]. Promoting bone formation and inhibiting bone resorption to replace lost bone tissue is the key to the treatment of osteoporosis [8]. As the main source of osteoblasts, bone marrow mesenchymal stem cells (BMSCs) have weakened osteogenic differentiation ability and enhanced lipogenic differentiation ability, which is one of the important causes leading to osteoporosis [9]. By exploring the molecular mechanism of osteogenic differentiation of BMSCs, improving the osteogenic differentiation ability of BMSCs and increasing the number of osteoblasts is of great significance for alleviating osteoporosis [10]. At present, the effects and mechanism of SLJGP on the osteogenic differentiation of BMSCs remain unclear.

Runt-related transcription factor 2 (RUNX2) is an important transcription factor in bone development and plays an important role in the regulation of osteoblast differentiation, chondrocyte maturation, osteoclast differentiation, and extracellular matrix secretion [11]. The study of RUNX2 gene expression regulation and conduction pathway is of great significance for the treatment of bone metabolic diseases [12]. RUNX2 can be regulated by a variety of upstream factors, including miRNAs, and bioinformatics analysis showed that RUNX2 has a direct binding site with miR-217. MiR-217, a member of the miRNA group, is located on chromosome 2P16.1 and is involved in a variety of human diseases including angiogenesis, kidney disease, and tumors [13–15]. In addition, miR-217 plays different roles as a promoter or suppressor in osteogenic differentiation, which promotes osteogenic differentiation of BMSCs by targeting DKK1 in steroid-associated osteonecrosis [16], but inhibiting osteogenic differentiation of BMSCs in the development of bone loss by targeting RUNX2 [17]. The miR-217/RUNX2 axis plays an important role in the osteogenic differentiation of BMSCs. However, whether SLJGP regulates the osteogenic differentiation of BMSCs through the miR-217/RUNX2 axis needs further study.

In this study, rat BMSCs were used to observe the effects of SLJGP on the proliferation and osteogenic differentiation of BMSCs by serum pharmacology. And the role of miR-217/RUNX2 regulatory network in SLJGP's regulation of BMSCs osteogenic differentiation was further analyzed to explore the mechanism of SLJGP in the treatment of osteoporosis, and to provide a more substantial theoretical basis for clinical application.

Methods

Animals

Six specific-pathogen-free (SPF) Sprague Dawley (SD) rats, 3 months old, were purchased from Chengdu Dossy experimental animals Co., LTD. (SCXK (chuan) 2019-028). Rats were fed adaptively for 7 days and randomly divided into control group and SLJGP group. Rats in the SLJGP group was intragastric administration of 0.92 g/kg SLJGP based on the human-animal drug dose conversion Tables [18, 19] while the control group was intragastric administration of equal volume normal saline twice a day for consecutive 3 days. 2 h after the last administration, the rats were anesthetized by intraperitoneal injection of pentobarbital sodium, and blood was collected from the abdominal aorta under sterile conditions. After standing at room temperature, the serum was centrifuged, filtered, sterilized, and then inactivated at 56℃ for 30 min for the cell experiment [20, 21]. This experiment was approved by the Ethics Committee of Chengdu Sport University (CDSU Ethical Audit [2023] No. 8; Chengdu, China).

Preparation of rat BMSCs

BMSCs were extracted from the bone marrow cavities of the femur and tibia of rats. The rats were sacrificed by cervical dislocation, soaked in 75% ethanol, and the femur and tibia samples were separated under sterile conditions and immersed in sterile PBS solution containing penicillin-streptomycin (BL505A, Biosharp, China). The muscles and connective tissue of the femur and tibia were discarded, and the bone end was cut off to expose

the bone marrow cavity. Then, the bone marrow cavity was repeatedly rinsed with complete medium, the bone marrow fluid was collected, centrifuged, and the complete medium of rat BMSCs (CM-R131, Procell, China) were added and cultured in a constant temperature incubator of 37℃ and 5% CO_2. The passage started when the cells were fused to 80%.

Osteogenesis induction of BMSCs

The third generation of about 1×10^5 BMSCs at the logarithmic growth stage were inoculated in 12-well plates and incubated at 37℃ and 5% CO_2. Osteogenic differentiation was induced by rat BMSCs osteogenic induction differentiation medium (PD-008, Procell, China). The medium was changed every other day. After 21 days of induction, alizarin red staining was used for identification. In brief, the cells were washed twice with PBS and fixed with fixative for 20 min. Discard fixing solution, wash twice with PBS, and soak with alizarin red dye solution for 30 min. Discard the dye solution and wash twice with PBS until there is no excess dye solution. PBS was added to cover the cells and the percentage of calcification was calculated after microscope photography.

Cell experimental design

Firstly, the effects of different concentrations of drug-containing serum of SLJGP on the activity of BMSCs were analyzed, and the experiment was divided into control group, negative serum control group (NC), low concentration SLJGP group (L-SLJGP, 2.5% SLJGP-serum), medium concentration SLJGP group (M-SLJGP, 5% SLJGP-serum) and high concentration SLJGP group (H-SLJGP, 10% SLJGP-serum). The total serum content of the BMSCs culture accounted for 10% of the medium. After 48 h of different treatments, the proliferation activity of BMSCs was detected. Next, according to the results of cell proliferation activity detection, the cell experiments were divided into four groups: common medium (without osteogenic inducers) group (control), common medium+medium concentration SLJGP group (SLJGP), osteogenic induction group, and osteogenic induction+medium concentration SLJGP group, to analyze the effects of SLJGP on osteogenic differentiation of BMSCs. The third part of the experiment was cultured in the osteogenic differentiation induction medium, and was divided into control group, SLJGP group, NC mimic+SLJGP group, miR-217 mimic+SLJGP group, miR-217 mimic+SLJGP+pcDNA-NC group, and miR-217 mimic+SLJGP+pcDNA-RUNX2 group, to analyze the role of miR-217/RUNX2 axis in SLJGP regulation of BMSCs differentiation.

Cell transfection

NC mimic, miR-217 mimic, pcDNA-NC, and pcDNA-RUNX2 were provided by Ribobio (China) for transfection of BMSCs. The mimic was transfected by a RiboFect CP Transfection kit (C10511-05, Ribobio, China). First, dilute 5 μL 20 μM miRNA mimic with 120 μL 1×riboFect CP Buffer. 12 μL riboFect CP Reagent was added and mixed, incubated at room temperature for 15 min, and the transfection complex was obtained. Then, the riboFect CP mixture was added to 1863 μL cell medium and gently mixed. The prepared transfection reagent was added to the corresponding 6-well plate at the rate of 2 mL/well and cultured in an incubator at 37℃. For plasmid transfection, 125 μL Opti-MEM I medium (31985088, Gibco, USA) was added to two clean sterile centrifuge tubes, then 2.5 μg plasmid DNA was added to one tube, and 5 μL Lipo2000 transfection reagent (11668500, Gibco, USA) was added to the other tube. After mixing, stand at room temperature for 5 min, mix the two tubes, stand at room temperature for 5 min, and mix with the appropriate medium for use. The prepared transfection reagent was added to the corresponding 6-well plate at the rate of 2 mL/well and cultured in the incubator. 6 h after transfection, all cells were replaced with fresh complete medium, and the culture was continued for 42 h for follow-up experiments.

Cell proliferative activity

About 5×10^3 BMSCs suspension was inoculated into 96-well plates and incubated at 37℃ and 5% CO_2. According to the experimental protocol, the BMSCs were cultured in the medium with the corresponding concentration of serum for 48 h, or transfected for 48 h, and the supernatant was discarded. CCK-8 working solution (BS350B, Biosharp, China) was added at 37℃ and cultured at 5% CO_2 for 2 h. The light absorption values of each well were measured at the wavelength of 450 nm using an enzyme marker (ELx800, BioTek, USA).

ELISA detection

BMSCs were collected, washed and suspended with PBS, and broken by repeated freezing and thawing. The cell lysate was centrifuged at 1500×g for 10 min, and the supernatant was collected for ELISA detetion. The expression levels of alkaline phosphatase (ALP), osteopontin (OPN), bone morphogenetic protein 2 (BMP2), and runt-related transcription factor 2 (RUNX2) in BMSCs were detected using rat ALP ELISA kit (ZC-36805, ZCIBIO, China), rat OPN ELISA kit (ZC-36664, ZCIBIO, China), rat BMP2 ELISA kit (ZC-36653, ZCIBIO, China), rat RUNX2 ELISA kit (ZC-36954, ZCIBIO, China), respectively. The operation was conducted in strict accordance with the kit instructions. All samples were repeated three times.

Dual-luciferase reporter assay

The binding relationship between miR-217 and RUNX2 was predicted by the targetscan website (https://www.targetscan.org/vert_80/), and confirmed by dual-luciferase reporter assay. RUNX2 wild-type (RUNX2-WT) and mutant-type (RUNX2-Mut) sequences were constructed into the luciferase reporter gene pmirGLO vector and transfected into cells. After transfection, luciferase expression was detected using a dual luciferase reporting system (DD1205-01, Vazyme, China). By comparing the changes in luciferase after overexpression of miR-217, the inhibitory effect of miRNA on RUNX2 was reflected.

Immunofluorescent staining

After washing with PBS, BMSCs were immersed in 5% membrane breaker (0694, Biosharp, China) at room temperature for 10 min, and washed with PBS. Goat serum sealer (AR1009, Boster, China) was added and treated at room temperature for 20 min. Primary antibody β-catenin (1:100; 51067-2-AP, proteintech, USA) was incubated at 4℃ overnight. Then, the secondary antibody FITC labeled goat anti-rabbit (1:100; GB22303, Servicebio, China) was incubated at 37℃ for 30 min. DAPI working solution (ZLI-9557, ZSBIO, China) was incubated at room temperature for 10 min for nucleation. Then, the images were collected by digital scanning and browsing software (OlyVIA, OLYMPUS, Japan), Image-J analysis system (National Institutes of Health, USA) was used to measure the mean fluorescence intensity of each image [22].

Western blot analysis

Total protein was extracted from BMSCs by cell lysis buffer (P0013, Beyotime, China) containing protease inhibitor (BL612A, Biosharp, China) and phosphatase inhibitor (BL615A, Biosharp, China). The BCA protein concentration assay kit (P0009, Beyotime, China) was used to quantify the protein content. The normalized total protein was separated by SDS-PAGE, and the isolated protein was transferred to the Immobilon-PSQ PVDF membrane (ISEQ00010, Sigma-Aldrich, USA). After 5% defatted milk was treated for 2 h, β-actin (1:50000; AC026, abclonal, China), RUNX2 (1:1000; A2851, abclonal, China), Wnt1 (1:2000; AF5315, Affinity, China) and β-Catenin (1:2000; A11932, abclonal, China) antibodies were incubated overnight at 4℃. HRP goat anti-mouse IgG (H+L) (1:5000; AS003, abclonal, China) and goat anti-rabbit IgG (H+L) (1:5000; S0001, Affinity, China) antibodies were incubated at room temperature for 2 h. Visualization of proteins was performed using the hypersensitive ECL western HRP substrate (17046, zenbio, China) and fluorescence image analysis system (5200 Multi, Tanon, China).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from BMSCs using Molpure cell/tissue total RNA kit (19221ES50, YEASEN, China). Total RNA was reverted into cDNA using the PrimeScript RT reagent kit (RR047A, Takara, China), and PCR was performed by TB Green Premix Ex TaqII (RR820A, Takara, China) using QuantStudio real-time PCR System (ThermoFisher, USA). Primers used were as follows: β-actin: Forward primer: 5′-GGGAAATCGTGCGTGACATT-3′, Reversed primer: 5′-GCGGCAGTGGCCATCTC-3′; RUNX2: Forward primer: 5′-CGGCAAGATGAGCGACGTGAG-3′, Reversed primer: 5′-TGCTGCTGCTGCTGCTGTTG-3′. In addition, reverse transcription and PCR reactions were performed on miRNA by Bulge-Loop miRNA qRT-PCR primer (R10031.8, Ribobio, China) and Bulge-Loop miRNA qRT-PCR starter kit (R11067.3, Ribobio, China). The relative gene expression was calculated by $2^{-\triangle\triangle CT}$.

Statistical analysis

The experimental data were statistically analyzed by SPSS 28.0 software, and those conforming to normal distribution were expressed as mean±standard deviation. Comparison between two groups was performed by T-test, and comparison between multiple groups was performed by one-way analysis of variance. $P<0.05$ was considered statistically significant.

Results

SLJGP promoted osteogenic differentiation of BMSCs

After BMSCs were treated with normal serum from rats in the control group and low, medium, and high content of SLJGP serum from rats in the SLJGP group, the cell viability was analyzed by CCK-8. The results showed that compared with the control group, the NC group had no significant effect on the cell activity of BMSCs ($P>0.05$, Fig. 1A). And compared with the NC group, L-SLJGP, M-SLJGP, and H-SLJGP all significantly promoted the activity of BMSCs in a dose-dependent effect ($P<0.01$, Fig. 1A). Then, M-SLJGP was selected to analyze the effects of SLJGP on the osteogenic differentiation of BMSCs. Similarly, as shown in Fig. 1B, compared with the control group, SLJGP significantly promoted the activity of BMSCs in both the normal medium and osteogenic differentiation induction medium ($P<0.01$). Compared with the normal medium, the osteogenic induction medium also significantly promoted the activity of BMSCs ($P<0.01$, Fig. 1B). In addition, ELISA was used to detect the expressions of ALP (Fig. 1C), OPN (Fig. 1D), BMP2 (Fig. 1E), and RUNX2 (Fig. 1F), and the results showed that the expressions of ALP, OPN, BMP2, and RUNX2 were significantly increased in BMSCs after osteogenic induction ($P<0.01$). And compared with the

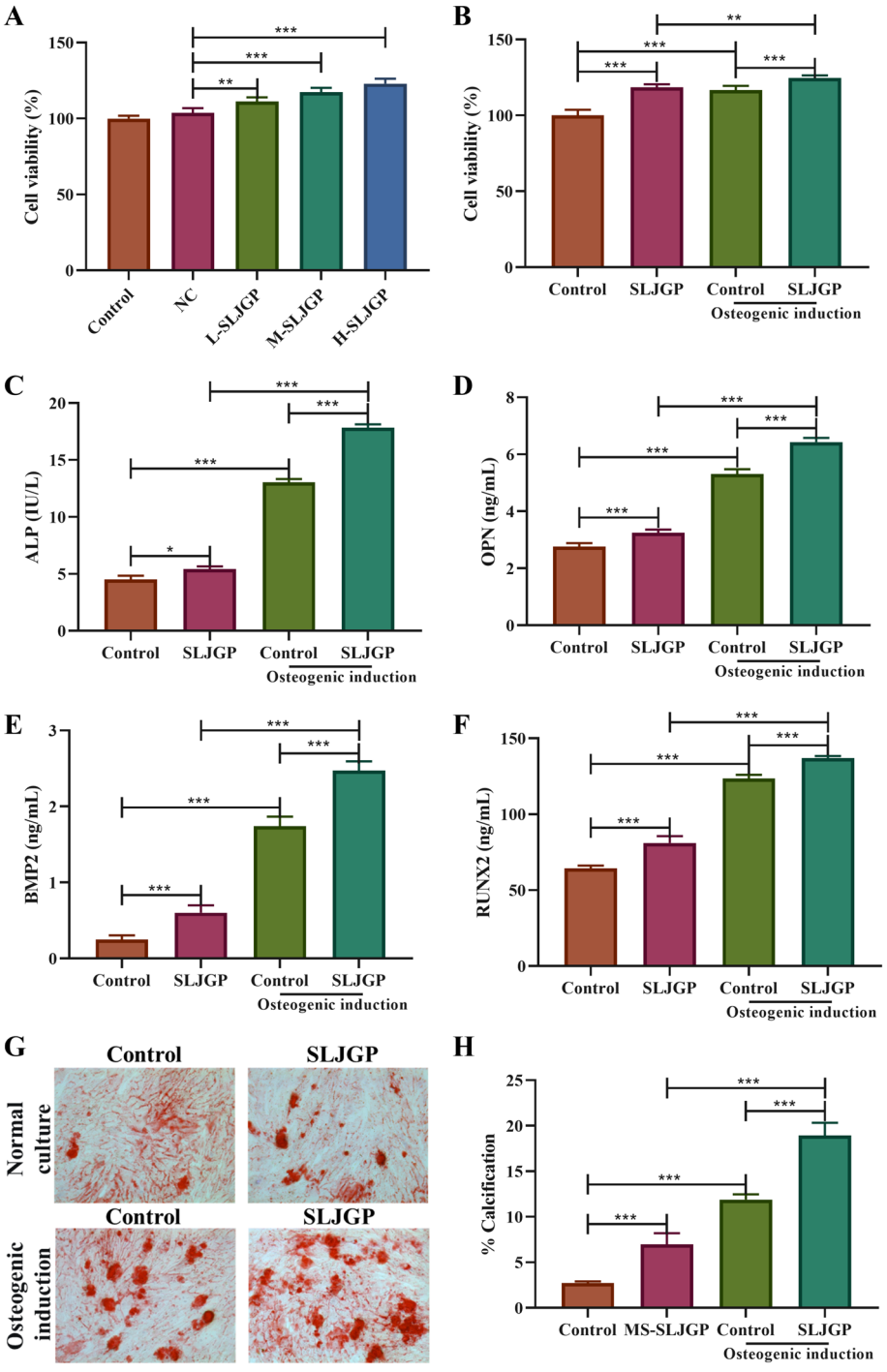

Fig. 1 SLJGP promoted osteogenic differentiation of BMSCs. **(A)** The effects of different doses of SLJGP on the viability of BMSCs were detected by CCK-8. **(B)** After osteogenic differentiation induction, the effects of SLJGP on cell activity were detected by CCK-8. The expression levels of osteogenic factors ALP **(C)**, OPN **(D)**, BMP2 **(E)**, and RUNX2 **(F)** were determined by ELISA. **(G)** Alizarin red staining was used to observe the formation of calcium nodules. **(H)** Histogram of calcification percentage. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NC: negative serum control group; SLJGP: drug-containing serum of SLJGP; ALP: alkaline phosphatase; OPN: osteopontin, BMP2: bone morphogenetic protein 2, RUNX2: runt-related transcription factor 2

control group, SLJGP significantly promoted the expression of ALP, OPN, BMP2, and RUNX2 in BMSCs in both normal medium and osteogenic induction medium ($P<0.01$, Fig. 1C-F). Similarly, alizarin red staining was used to analyze the calcification ratio in BMSCs, which also showed that SLJGP significantly promoted the calcification of BMSCs in both normal medium and osteogenic induction medium compared to the control group ($P<0.01$, Fig. 1G and H). These results all suggested that SLJGP could promote osteogenic differentiation of BMSCs.

SLJGP inhibited the expression of miR-217, which directly targeted RUNX2

A large number of studies have confirmed that miR-217 plays an important role in the osteogenic differentiation of BMSCs [16, 17]. In order to confirm whether miR-217 is involved in the regulation of SLJGP on BMSCs differentiation, RT-qPCR was used to detect the influence of SLJGP on the expression of miR-217 in BMSCs. The results showed that the expression of miR-217 in BMSCs was significantly decreased after osteogenic differentiation induction ($P<0.001$, Fig. 2A). And compared with the control group, SLJGP further reduced the expression of miR-217 in BMSCs induced by osteogenic differentiation ($P<0.001$, Fig. 2A). Further prediction of the downstream target genes of miR-217 showed that there was a direct binding site between miR-217 and the osteogenic differentiation-related gene RUNX2 (Fig. 2B). Dual-luciferase reporter assay was used to confirm the binding relationship between miR-217 and RUNX2, and the results showed that compared with the NC mimic, the miR-217 mimic had no significant effect on the luciferase activity of RUNX2-mut, while that of RUNX2-WT, miR-217 mimic could significantly reduce the luciferase activity, confirming the binding relationship between miR-217 and RUNX2. The above results suggested that SLJGP may promote osteogenic differentiation of BMSCs through the miR-217/RUNX2 axis.

SLJGP regulated miR-217/RUNX2 to promote osteogenic differentiation of BMSCs

To further elucidate the role of miR-217/RUNX2 axis in SLJGP promoting osteogenic differentiation of BMSCs, the miR-217 mimic and/or pcDNA-RUNX2 were used to transfected BMSCs. First, RT-qPCR was used to examine the expressions of miR-217 and RUNX2 in BMSCs of different treatment groups. Consistent with previous results, compared with the control group, SLJGP could significantly reduce the expression of miR-217 ($P<0.001$, Fig. 3A) and promote the expression of RUNX2 ($P<0.001$, Fig. 3B). Compared with the NC mimic+SLJGP group, miR-217 mimic+SLJGP could significantly increase the expression of miR-217 and

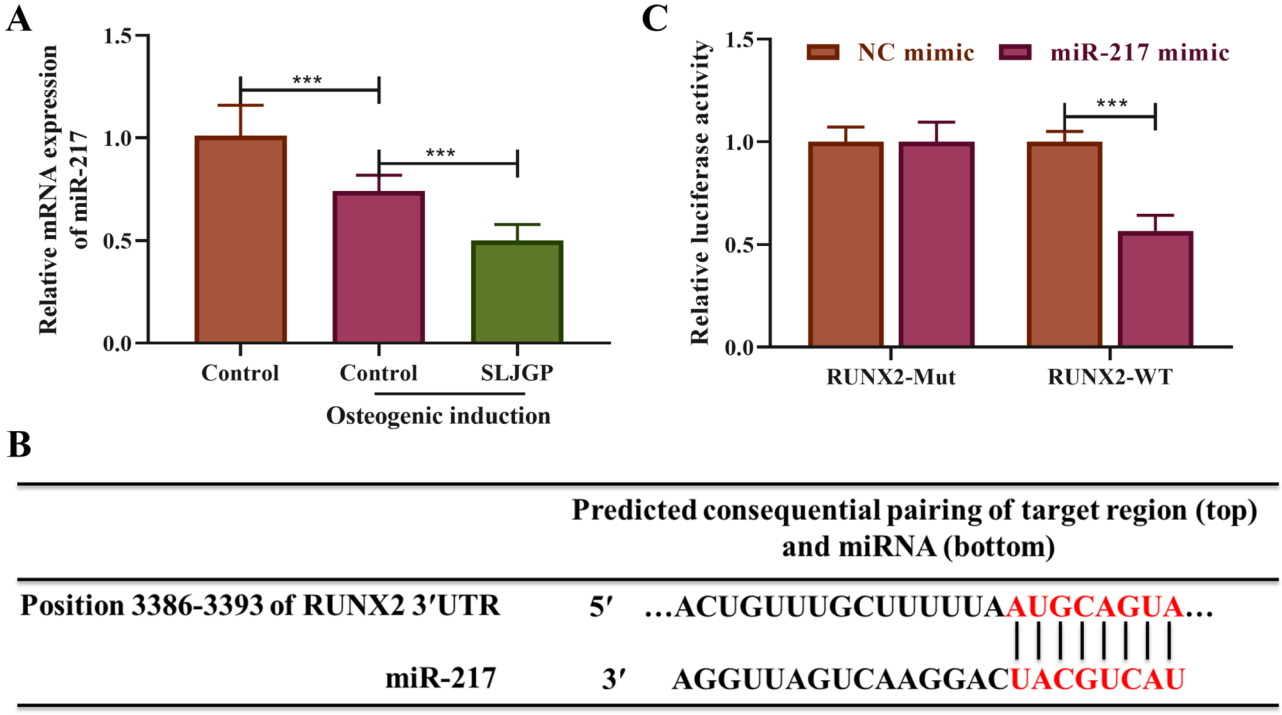

Fig. 2 SLJGP inhibited the expression of miR-217, which directly targeted RUNX2. **(A)** The expression of miR-217 in SLJGP-treated BMSCs was detected by RT-qPCR. **(B)** The binding relationship between miR-217 and RUNX2 was predicted by the targetscan website. **(C)** Dual-luciferase report assay verified the binding relationship between miR-217 and RUNX2. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. SLJGP: drug-containing serum of SLJGP

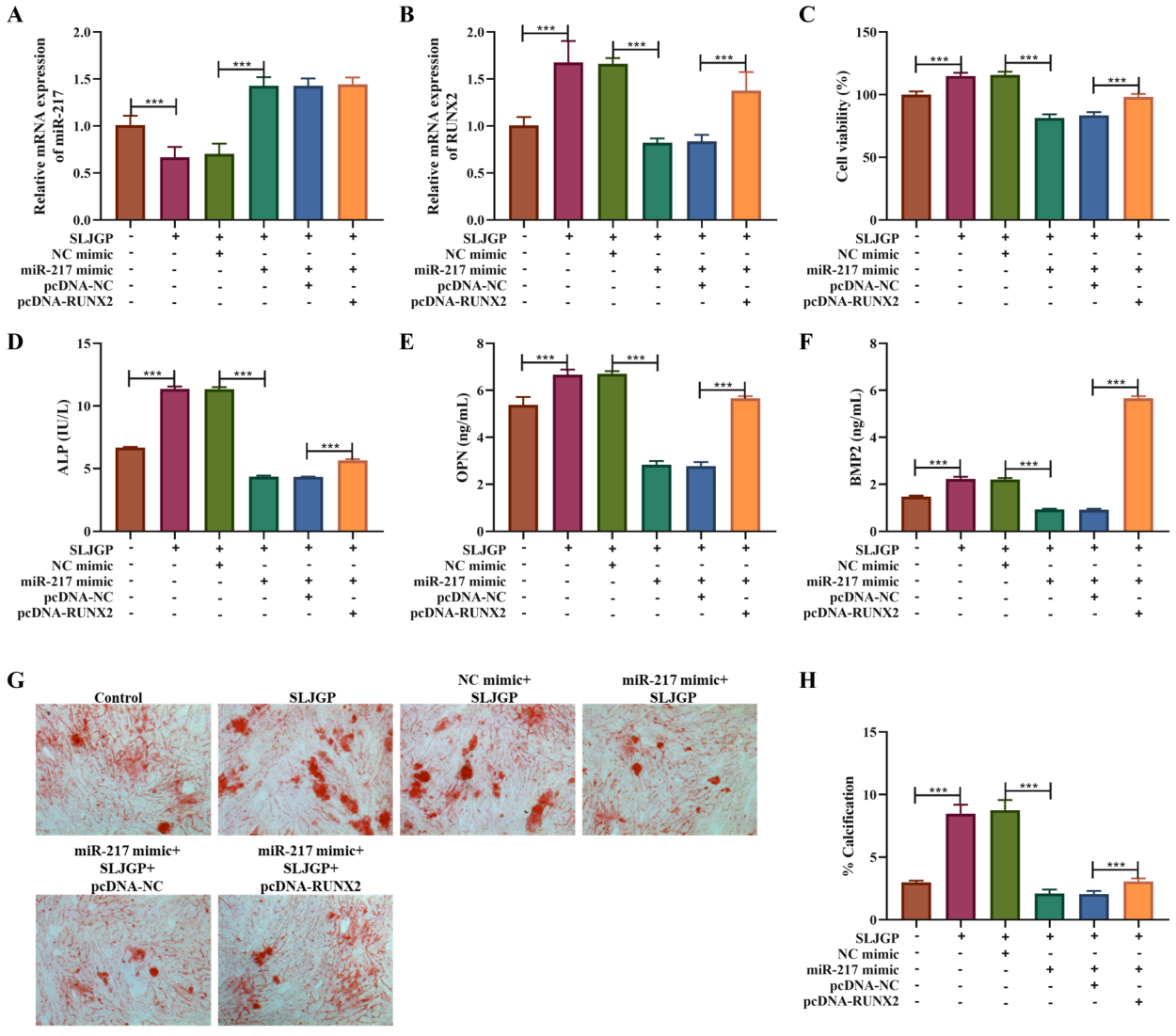

Fig. 3 SLJGP regulated miR-217/RUNX2 to promote osteogenic differentiation of BMSCs. **(A)** The expression level of miR-217 was detected by RT-qPCR. **(B)** The expression level of RUNX2 was detected by RT-qPCR. **(C)** CCK-8 was used to detect the cell activity in each group. **(D-F)** ELISA was used to detect the expression levels of ALP, OPN, and BMP2. **(G)** Alizarin red staining was used to observe the formation of calcium nodules. **(H)** Histogram of calcification percentage. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. SLJGP: drug-containing serum of SLJGP; ALP: alkaline phosphatase; OPN: osteopontin, BMP2: bone morphogenetic protein 2, RUNX2: runt-related transcription factor 2

reduce the expression of RUNX2, and inhibit the effect of SLJGP ($P<0.001$, Fig. 3A and B). Compared with the miR-217 mimic+SLJGP+pcDNA-NC group, pcDNA-RUNX2 transfection could significantly increase RUNX2 expression ($P<0.001$, Fig. 3B), but had no significant effect on the expression of miR-217 ($P>0.05$, Fig. 3A). Similarly, compared with the control group, SLJGP significantly promoted the activity of BMSCs ($P<0.001$, Fig. 3C). And compared with the NC mimic+SLJGP group, miR-217 mimic+SLJGP significantly inhibited the promoting effects of SLJGP on the activity of BMSCs ($P<0.001$, Fig. 3C). Compared with the miR-217 mimic+SLJGP+pcDNA-NC group, pcDNA-RUNX2 transfection significantly increased the activity of BMSCs and reversed the inhibitory effect of miR-217 mimic on SLJGP ($P<0.001$, Fig. 3C). The expressions of ALP (Fig. 3D), OPN (Fig. 3E), and BMP2 (Fig. 3F) in BMSCs were further analyzed, and the results showed that miR-217 mimic+SLJGP group significantly inhibited the promoting effects of SLJGP on the expressions of ALP, OPN, and BMP2 ($P<0.001$). Compared with the miR-217 mimic+SLJGP+pcDNA-NC group, pcDNA-RUNX2 transfection significantly reversed the inhibitory effect of miR-217 mimic on SLJGP ($P<0.001$, Fig. 3D-F). Alizarin red staining results also showed that SLJGP significantly promoted the calcification of BMSCs, and the

effect could be reversed by miR-217 mimic ($P<0.001$, Fig. 3G and H). Similarly, compared with the miR-217 mimic+SLJGP+pcDNA-NC group, calcification of miR-217 mimic+SLJGP+pcDNA-RUNX2 group was significantly increased ($P<0.001$, Fig. 3G and H), suggesting that pcDNA-RUNX2 transfection could reverse the inhibitory effect of miR-217 mimic on SLJGP. All the results confirmed that the miR-217/RUNX2 axis was involved in the promotion of SLJGP on the osteogenic differentiation of BMSCs.

SLJGP regulated the miR-217/RUNX2 axis to activate the Wnt/β-catenin pathway in BMSCs

The Wnt/β-catenin pathway has been confirmed to play an important role in the osteogenic differentiation of BMSCs, and miR-217 was involved in the regulation of the Wnt/β-catenin pathway. Therefore, the protein expressions in the Wnt/β-catenin pathway were detected, and the results showed that compared with the control group, SLJGP significantly promoted the expression of Wnt1, β-catenin, and RUNX2 ($P<0.001$, Fig. 4A-D). And compared with the NC mimic+SLJGP group, miR-217 mimic+SLJGP significantly inhibited the promoting effects of SLJGP on the expressions of Wnt1, β-catenin, and RUNX2 in BMSCs ($P<0.001$, Fig. 4A-D). Compared with the miR-217 mimic+SLJGP+pcDNA-NC group, pcDNA-RUNX2 transfection significantly increased the expressions of Wnt1, β-catenin, and RUNX2 in BMSCs and reversed the inhibitory effect of miR-217 mimic on SLJGP ($P<0.001$, Fig. 4A-D). The results of immunofluorescence staining were consistent with those of WB (Fig. 4E and F). These results suggested that SLJGP may promote the Wnt/β-catenin pathway in BMSCs by regulating the miR-217/RUNX2 axis.

Discussion

Bone turnover imbalance is one of the basic pathological mechanisms of osteoporosis, and is directly related to the imbalance of osteogenic and lipogenic differentiation ability of BMSCs [23]. BMSCs are stem cells derived from the mesoderm with multidirectional differentiation potential and are the most abundant in bone marrow tissues [24]. They have the ability to differentiate into adipocytes, osteoblasts, and chondroblasts, providing an important source of cells for bone repair [25]. A large number of studies have shown that BMSCs are closely related to osteoporosis, and their weakened osteogenic differentiation ability or enhanced lipogenic differentiation ability will lead to the occurrence of osteoporosis [26]. The osteogenic differentiation ability of BMSCs was demonstrated to be weakened in osteoporosis patients [27]. Enhancing the osteogenic differentiation ability and promoting the proliferation of BMSCs is one of the important ways to prevent and treat osteoporosis [10, 28, 29]. This study found that SLJGP, a Traditional Chinese medicine formula, could significantly promote the

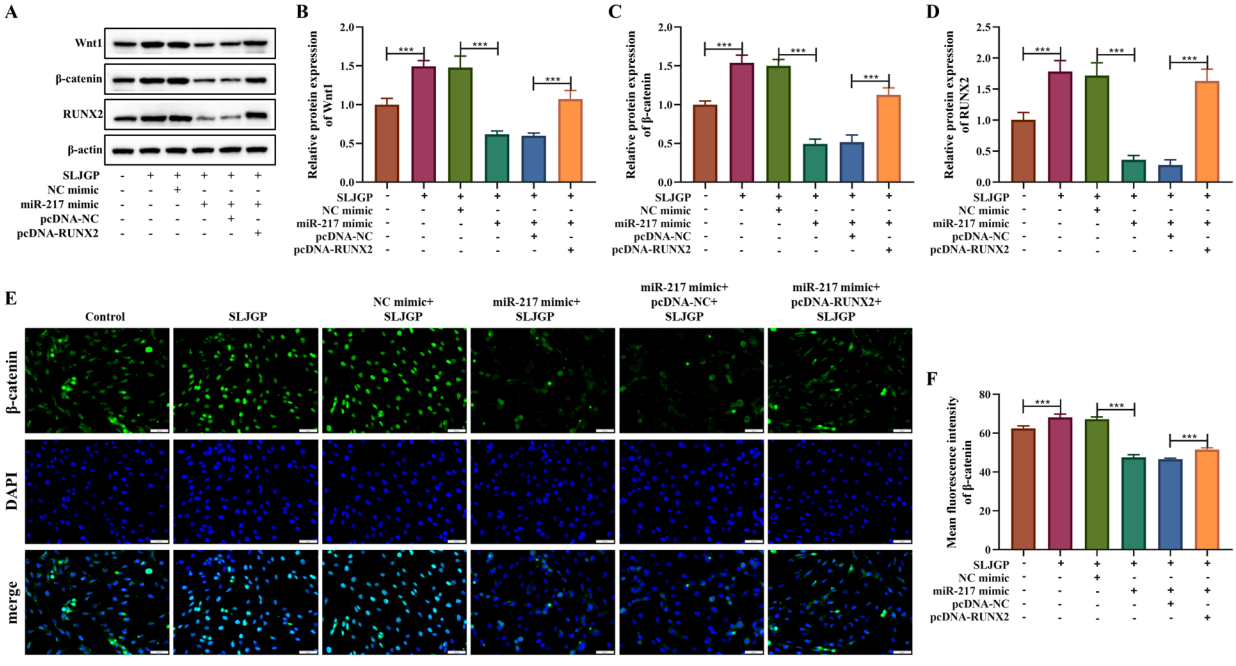

Fig. 4 SLJGP regulated the miR-217/RUNX2 axis to activate the Wnt/β-catenin pathway in BMSCs. **(A)** The expressions of Wnt1, β-catenin, and RUNX2 were detected by WB. **(B)** Relative protein expression levels of Wnt1. **(C)** Relative protein expression levels of β-catenin. **(D)** Relative protein expression levels of RUNX2. **(E)** Immunofluorescence detection of β-catenin. **(F)** Mean fluorescence intensity of β-catenin. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. SLJGP: drug-containing serum of SLJGP; RUNX2: runt-related transcription factor 2

proliferation and osteogenic differentiation of BMSCs, and regulate the expression of miR-217 in BMSCs, which was confirmed to directly target RUNX2. Further studies confirmed that SLJGP promoted osteogenic differentiation of BMSCs by activating the Wnt/β-catenin pathway through the miR-217/RUNX2 axis.

In recent years, Chinese medicine and its metabolites have been playing an increasing role in human health [30–32]. SLJGP is a classic formula for the treatment of fracture, among which *Fossilizid* and *Pheretima* are sovereign medicinal, *Fossilizid* sweet astringent, that can soothe the mind and panic, astringent solid astringent, *Pheretima* salty cold, that can relax sinews and activate collaterals, clearing heat and clearing collaterals. Vinegar *olibanum* and vinegar *myrrh* as minister medicinal, can dispel wind and move qi, disperse blood stasis and detumescence, promote blood circulation and relieve pain. *Eupolyphaha steleophaga*, *Angelicae sinensis* radix, *Resina Draconis*, *Wine Dipsaci* radix, *Poria*, cooked *Rhubarb*, *Aucklandiae* radix, *Albizziae* cortex, *Paeoniae* radix alba, *Achyranthis Bidentatae* radix, vinegar *olibanum*, and vinegar *myrrh* are assistant medicinal, which can tonify blood and qi, warm the meridians and collaterals, strengthen tendons and bones. The combination of all kinds of medicines can activate blood and move qi, disperse blood stasis and collaterals, reduce swelling and relieve pain, and strengthen muscles and bones. Modern pharmacological studies have also shown that *Angelicae sinensis* radix is the most common herb in the treatment of osteoporosis [4] and could promote osteogenic differentiation of MSCs [33]. *Achyranthis Bidentatae* radix could treat osteoporosis by regulating bone metabolism and cell proliferation [34]. *Dipsaci* Radix expressed its therapeutic effect on osteoporosis by regulating immune system-related pathways [35]. *Poria Cocos* could improve bone loss and inhibit osteoclast formation in vitro in ovariectomized mice [36]. *Myrrh* inhibited osteoporosis by inhibiting osteoclast generation [37]. This study showed that different concentrations of SLJGP all promoted the proliferation of BMSCs, and SLJGP could promote the expression of ALP, OPN, BMP2, and RUNX2 in BMSCs.

ALP is a homologous dimer glycoprotein synthesized by osteoblasts in the early stage of osteogenic differentiation, which can degrade phosphatase, assist mineralization, and eliminate the inhibitory effect of osteocalcin on bone salts [38]. Its activity can reflect the degree of cell differentiation and is often used as a specific marker of the degree of differentiation in the early stage of osteogenic differentiation. OPN is a phosphorylated vulcanized glycoprotein, which can effectively adsorb hydroxyapatite, guide mineralization and promote bone formation, and is a marker of osteoblast differentiation [39]. BMP2, a member of the B-superfamily of transforming growth factors, has the ability to induce the directed differentiation and proliferation of MSCs into chondroblasts and osteoblasts, and participate in bone and cartilage growth and reconstruction process, and then accelerate bone defect repair [40]. RUNX2 is a transcription factor that plays a decisive role in the differentiation of osteoblasts and affects the osteoblastic differentiation of MSCs [41]. The promotion effects of SLJGP on the expression of ALP, OPN, BMP2, and RUNX2 demonstrated that SLJGP could promote the osteogenic differentiation of BMSCs.

BMSCs differentiate into osteoblasts, which are the main cells that play the function of bone formation, synthesize and secrete extracellular matrix components, and are responsible for the synthesis and mineralization of bone matrix [42]. RUNX2 is the primary determinant of osteogenic differentiation of BMSCs and the promoter of major bone matrix gene expression in the early stage of osteoblast differentiation [43, 44]. RUNX2 can be regulated by a variety of upstream factors, which influence osteogenic development, bone repair and regeneration, and bone remodeling through RUNX2 regulation [45]. Small interfering RNAs (siRNAs) that act on gene regulation can participate in bone repair processes and serve as molecular targets for the study of bone-related diseases, including tendon structural proteins [46], inflammatory processes in rheumatoid arthritis [47], and metabolic processes in osteoporosis [48]. In addition, miRNAs, as a class of evolutionarily conserved endogenous noncoding RNAs, regulate the differentiation, proliferation and apoptosis of bone cells by binding to specific target genes in different signaling pathways and promoting or inhibiting their expression [49, 50]. It has been found that miRNA, a single-stranded small molecule, regulates bone formation and the proliferation and differentiation of bone cells by inhibiting the expression of RUNX2 [51, 52]. Upstream miR-217 has been shown to regulate RUNX2 and affect the osteogenic differentiation of rat BMSCs [17]. This study found that miR-217 expression decreased after osteogenic differentiation of BMSCs and further decreased after SLJGP treatment, suggesting that miR-217 was involved in the osteogenic differentiation of BMSCs and SLJGP had a significant regulatory effect on it. A large number of studies have confirmed that miR-217, as an upstream regulatory gene, can directly target RUNX2, the master regulator of osteogenesis [53, 54]. This study also confirmed that miR-217 has a direct binding relationship with RUNX2. Further studies confirmed that miR-217/RUNX2 axis was involved in the promotion of SLJGP on the osteogenic differentiation of BMSCs.

In addition, SLJGP was demonstrated to promote the expression of Wnt/β-catenin pathway proteins. The Wnt/β-catenin pathway has been identified as a potential target for osteoporosis treatment [29, 55]. Chemicals, mechanical stimuli, and extracellular Wnt proteins

can activate the Wnt/β-catenin pathway and promote bone formation [56]. In this study, the promoting effects of SLJGP on the Wnt/β-catenin pathway changed after interference with the miR-217/RUNX2 axis, suggesting that SLJGP regulated the Wnt/β-catenin pathway through the miR-217/RUNX2 axis.

In conclusion, SLJGP could significantly promote the proliferation of BMSCs, the expression of ALP, OPN, BMP2, and RUNX2, and the osteogenic differentiation of BMSCs. In addition, SLJGP reduced miR-217 expression in BMSCs, while miR-217 directly targeted RUNX2. Use of miR-217 mimic inhibited the promoting effects of SLJGP on proliferation and osteogenic differentiation of BMSCs. The co-transfection of miR-217 mimic with pcDNA-RUNX2 reversed the inhibition effect of miR-217 mimic on SLJGP. Further studies showed that SLJGP could promote the activation of the Wnt/β-catenin pathway, and miR-217/RUNX2 was also involved. This study confirmed that SLJGP promoted osteogenic differentiation of BMSCs by activating the Wnt/β-catenin pathway through the miR-217/RUNX2 axis, and clarified the possible mechanism of SLJGP in the treatment of osteoporosis.

Of course, there are some limitations in this study. For example, the regulatory mechanisms of miRNAs and signaling pathways are complex and diverse, involving the interaction of multiple levels and links. This study only focuses on the miR-217/RUNX2 axis, and can only clarify the role of miR-217/RUNX2 network. The promoting role of SLJGP is a comprehensive effect of multiple factors and pathways, other important regulatory factors and pathways may be overlooked. Similarly, the miR-217/RUNX2 axis and its relationship with SLJGP can be analyzed with more experiments. While our research is currently focused on the miR-217/RUNX2 axis, we are also aware of the complexity of this process and plan to further explore other possible mechanisms in future work. In addition, the loss of bone and increase of adipose in osteoporosis is related to the imbalance of osteoblastation-lipid differentiation of BMSCs. In the pathological process of osteoporosis, the abnormal differentiation of BMSCs towards lipogenesis is an important promoting factor, which not only reduces the number of BMSCs available for osteogenesis, but also further affects the bone microenvironment and accelerates bone loss by secreting factors specific to fat cells. Another limitation of this study is that it only analyzed the effect of SLJGP on the osteogenic differentiation of BMSCs. Further analysis and evaluation of the effect of SLJGP on the lipogenic process of BMSCs, as well as other related cellular and molecular mechanisms in subsequent further studies, will be of great significance for the comprehensive evaluation of its potential to prevent and treat osteoporosis.

Author contributions
You-li Tan, Shao-hua Ju, and Qiang Wang designed the research; You-li Tan and Shao-hua Ju performed the research; Qiang Wang, Yu-shi Hu, Zhang-meng Xu and Ji-hai Gao analyzed the data; You-li Tan, Ming-jian Wang and Ya-lan Kang wrote the paper. All authors agreed to publish the final version.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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