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Original Article

Brg1 promotes osteogenic differentiation of mesenchymal stem cells by regulating Runx2-mediated Wnt and PI3K/AKT pathways

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Abstract: *Objective*: Brahma-related gene 1 (Brg1) has been reported to be related with osteoblast differentiation; however, exact mechanism is unclear. We aimed to investigate the functional role of Brg1 in osteoblast differentiation of bone marrow-derived mesenchymal stem cells (MSCs), as well as underlying mechanism. *Methods*: MSCs obtained from bone marrow of rats were transfected with pcDNA3.1-Brg1 or empty vector, and the transfection efficiency was confirmed. Thereafter, effects of Brg1 overexpression on osteogenesis markers Runt-related transcription factor 2 (Runx2), special protein 7 (Sp7), collagen I and osteopontin (OPN) were investigated, as well as cell viability, adhesion ability and migration ability of MSCs. In addition, the interaction between Brg1 and Runx2 was explored by co-immunoprecipitation (Co-IP). Moreover, after transfection with pcDNA3.1-Brg1, MSCs were incubated with or without the inhibitor of Runx2, CADD522, and then the expression of Wnt and PI3K/AKT pathways key proteins was determined. *Results*: Brg1 expression vector was successfully transfected into MSCs. Overexpression of Brg1 significantly upregulated expression of Runx2, Sp7, collagen I and OPN, cell viability and adhesion ability (all $P < 0.05$), but had no effect on migration ability. Additionally, Runx2 was able to coprecipitate with Brg1 and was regulated by Brg1. The expressions of Wnt and PI3K/AKT pathways key proteins were statistically increased by overexpression of Brg1 ($P < 0.05$); however, administration of CADD522 markedly reversed the effects ($P < 0.05$ or $P < 0.01$). *Conclusion*: Brg1 promotes osteogenic differentiation of MSCs, and the effects might be by regulating Runx2-mediated downstream Wnt and PI3K/AKT pathways.

Keywords: Brahma-related gene 1, osteoporosis, mesenchymal stem cells, osteogenic differentiation, Runt-related transcription factor 2, Wnt and PI3K/AKT pathways

Introduction

Osteoporosis is a skeletal chronic disease and a major cause of morbidity in the elderly, particular in women. It is characterized by reduced bone strength, low bone mass, enhanced skeletal fragility and increased fracture risk [1]. Osteoporosis has become a major public health problem in aging societies [2]. It has been estimated that approximately 200 million people worldwide suffer from the disease [3]. The prevalence of osteoporosis in older adults ranged from 13% to 21.2% [4, 5], with a prevalence rate of 15.7% in China [6]. However, the prevalence has been considered to be gradually grown due to the increasing age of the total population. Although tremendous advance has been made in recent years in understanding the pathogenesis of osteoporosis and in the investigation of novel treatments, osteoporosis is still a prevalent health problem.

Currently, there are two conservative treatments of osteoporosis, including physical exercise and pharmacological treatment. However, compliance is relatively low and pharmacological strategies can be rather expensive [3]. In recent years, transplantation of marrow mesenchymal stem cells (MSCs) has gained great attention in tissue repair, immune regulation, and cell therapy [7-10]. Differentiation of bone marrow-derived MSCs to other lineages, particularly osteoblasts, has been a hotspot in the treatment of osteoporosis [11]. Therefore, bet-

ter understanding the regulatory mechanism of differentiation of MSCs to osteoblasts is essential and significant for developing strategies to treat osteoporosis.

Brahma-related gene 1 (Brg1) is a critical component of the SWI/SNF chromatin-remodeling complex through a DNA-dependent ATPase activity, which has been reported to be a potential tumor suppressor gene in a variety of tumor cell lines [12]. It has been well demonstrated that Brg1 plays a significant role in regulation of cell proliferation [13], apoptosis [14], differentiation [15], migration and invasion [16]. Additionally, an increasing number of evidence has suggested that Brg1 is involved in modulation of cell growth arrest, apoptosis, and senescence of MSCs [17, 18]. However, the exact regulatory mechanism has yet to be fully elucidated.

Therefore, in the present study, we aimed to explore the regulatory mechanism of Brg1 in osteoblast differentiation of bone marrow-derived MSCs, along with underlying mechanism. Our study might provide a new insight into theoretical basis for treatment of osteoporosis.

Materials and methods

Isolation of MSCs

Adult male Sprague-Dawley (SD) rats weighing between 220 and 250 g (9 weeks old) were obtained from SLRC Laboratory Animal Company (Shanghai, China) and were maintained under specific pathogen-free (SPF) conditions. The animal experiment was performed according to the Principles of Laboratory Animal Care and approved by the Ethics Committee of our university. After 1 week of acclimation, the rats were killed under anesthesia, and femurs and tibias were removed. Rat bone marrow was collected from the rat femurs and tibias, maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, USA), filtered, and centrifuged. The collected cells were then cultured in Iscove's Modified Dulbecco's Medium (IMDM; Gibco) supplemented with 10% FBS (Sigma-Aldrich, St Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich) and 100 μg/ml streptomycin (Sigma-Aldrich). Cells were maintained in 5% CO_2 at 37%. Non-adherent cells were removed after 24 h and the medium was changed every 3 days. The residual attached cells were maintained at in 5% CO_2 at 37% by replacing the culture media every 3 days.

Plasmids transfection

A Brg1 expression vector (pcDNA3.1-Brg1) was constructed by sub-cloning the full-length wild-type Brg1 coding sequence into pcDNA3.1 (+), and confirmed by sequencing. The empty construct pcDNA3.1 was used as a control. Cell transfections were conducted by using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. Stable transfected cells expressing Brg1 were generated under G418 selection (Life Technologies, Burlington, ON, Canada).

Cell viability

After transfection with pcDNA3.1-Brg1 or empty vector, the cell viability was determined by a 3-(4, 5-dimethylthiazol-2-yl)-2 5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay according to standard methods. Briefly, MSCs were seeded in 96-well plates and transfected with pcDNA3.1-Brg1 or empty vector. Forty-eight hours later, 20 μL 5 mg/mL MTT (Gibco) were added to the plates and incubated at 37°C for 4 h. Thereafter, 100 μL dimethylsulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals. Absorbance at 590 nm was read by a microplate reader (Bio-Rad Benchmark, Hercules, CA, USA).

Adhesion assay

The cell adhesion assay was carried out as previously described [19]. Briefly, 96-well plates coated with 10 μg/ml fibronectin (FN) were incubated overnight at 4°C. Bovine serum albumin (0.2%; Sigma-Aldrich) was used to block non-specific binding sites. After 48 h of transfection with pcDNA3.1-Brg1 or empty vector, MSCs were resuspened in serum-free medium and allowed to adhere for 2 h at 37°C. Non-attached cells were removed and the adherent cells were fixed with paraformaldehyde and stained with crystal violet. The cells were then washed and quantified by determining the absorbance at 570 nm on a microplate reader (Bio-Rad).

Migration assay

Cell migration was tested by using a Transwell chamber with a pore size of 8 mm. In brief,

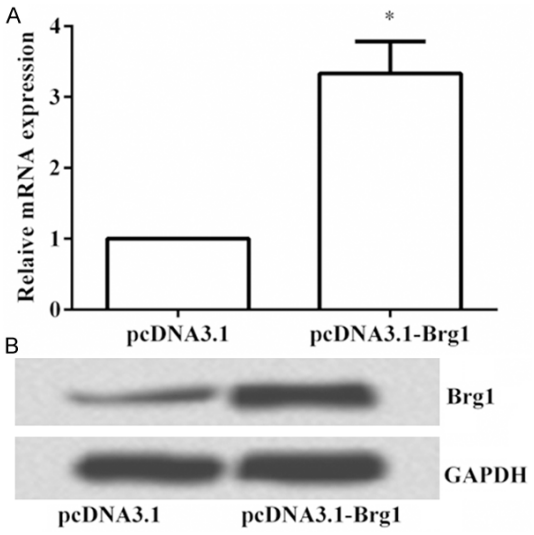

Figure 1. Transfection efficiency of Brg1. A: Relative mRNA expression levels of Brg1; B: Protein expression levels of Brg1. $^{*}P < 0.05$ compared to the control group.

MSCs were suspended in serum-free medium and plated onto the upper compartment of 24-well Transwell chamber. In addition, the lower compartment was added by complete medium. After 12 h of incubation at 37°C, these cells were fixed with methanol and non-migrated cells were removed from the upper compartment with a cotton swab. Migrated cells were stained with crystal violet and counted under a microplate reader (Bio-Rad).

Quantitative real time polymerase chain reaction (qRT-PCR)

After transfection with pcDNA3.1-Brg1 or empty vector, total RNA was isolated from MSCs using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. The RNA was then reverse-transcribed with the Multiscribe RT kit (Qiagen, Germany) following the manufacturer's procedure. Reverse transcribed RNA was subsequently amplified by PCR using Go Taq polymerase (Promega). The sequences of the primers were as follows, Brg1 forward primer: 5'CCTCTCTCAACGCTGTC CAACTG'3, reverse primer: 5'ATCTTGGCGAGGATGTGCTTGTCTT'3. GAPDH forward primer: 5'GCACCGTCAAGGCTGAGAAC'3, reverse primer: 5'TGGTG AAGACGCCAGTGGA'3.

Co-immunoprecipitation (Co-IP)

Co-IP was used to determine the interaction between Brg1 and Runt-related transcription factor 2 (Runx2) using an antibody-coupling gel to precipitate the bait protein (Brg1) and co-immunoprecipitate the interacting prey protein (Runx2). Anti-Brg1 antibody (ab4081; Abcam, Cambridge, UK) was coupled to an amine-reactive gel (ProFound co-immunoprecipitation kit, Pierce) overnight using slow agitation at room temperature. The eluted proteins were then subjected to a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Thereafter, membranes were blocked, probed with anti-Brg1 or anti-Runx2, and incubated with horseradish peroxidase anti-rabbit IgG using SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL).

Western blot

After transfection with pcDNA3.1-Brg1 or empty vector or treatment with the inhibitor of Runx2 (CADD522), total protein was extracted from MSCs using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors (Applied Science). The proteins were quantified using a BCA assay kit (Pierce, Rockford, IL). The protein samples were separated on 10-12% SDS-PAGE gels and transferred to PVDF membranes. Thereafter, the membranes were blocked with 5% skim milk in Tris Buffered Saline with Tween (TBST) for 1 h and then maintained in the following primary antibodies overnight at 4°C: anti-Brg1 antibody (ab4081; Abcam), anti-Runx2 antibody (ab23981; Abcam), anti-Wnt3a antibody (ab28472; Abcam), anti-β-Catenin antibody (ab16051; Abcam), anti-Wnt5a antibody (ab72583; Abcam), anti-phosphoinositide 3-kinase (PI3K) antibody (ab18-9403; Abcam), anti-AKT antibody (sc-5298, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-p-Ser473-AKT (sc-24500; Santa Cruz). GAPDH was used as a loading control. The membranes were then washed and incubated with secondary antibody marked by horseradish peroxidase for 2 h at room temperature. The protein bands were visualized with enhanced chemiluminescence (ECL; Pierce, Rochford, IL, USA) and was quantified using Image Lab™ Software (Bio-Rad).

Statistical analysis

Each experiment was repeated three times. The data are presented as the mean ± standard deviation (SD). Statistical analyses were

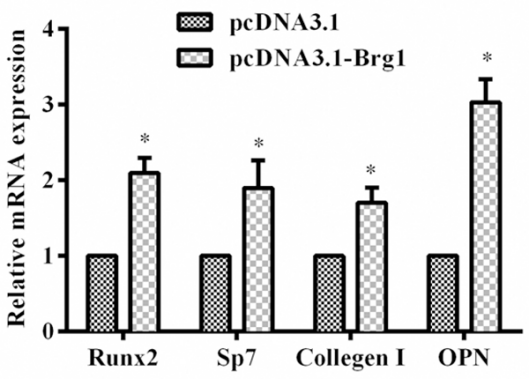

Figure 2. Effects of Brg1 overexpression on osteogenesis markers expressions. The results showed that the mRNA levels of Runx2, Sp7, collagen I and OPN were all statistically upregulated by overexpression of Brg1 compared to the control group. $^{*}P < 0.05$ compared to the control group.

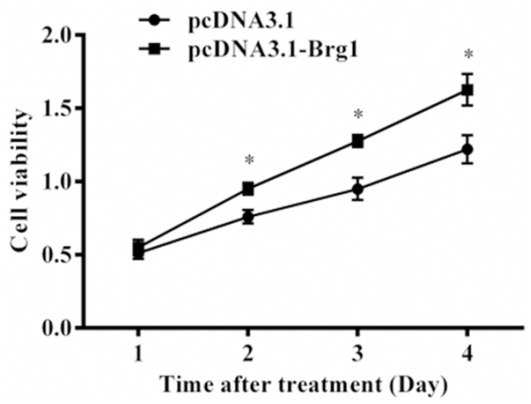

Figure 3. Effects of Brg1 overexpression on cell viability. The results showed that overexpression of Brg1 could dramatically increase the cell viability at 2, 3, and 4 days compared with the control group. $^{*}P < 0.05$ compared to the control group.

performed using Statistic Package for Social Science (SPSS; SPSS Inc., Chicago, IL, USA) version 19.0 statistical software. Statistical significance between groups was measured by paired t tests or one-way analysis of variance (ANOVA). The $P < 0.05$ was defined as statistically significant.

Results

Transfection efficiency of Brg1

To determine the influence on its expression of Brg1 after transfection with pcDNA3.1-Brg1 into MSCs, qRT-PCR and Western blot were performed to confirm the transfection efficiency of Brg1. As shown in **Figure 1A**, the mRNA expression levels of Brg1 were significantly increased by transfection with pcDNA3.1-Brg1 compared to the control group ($P < 0.05$). Similar results were observed in protein levels (**Figure 1B**). The results indicated that the transfection efficiency was high and could be used for further analysis.

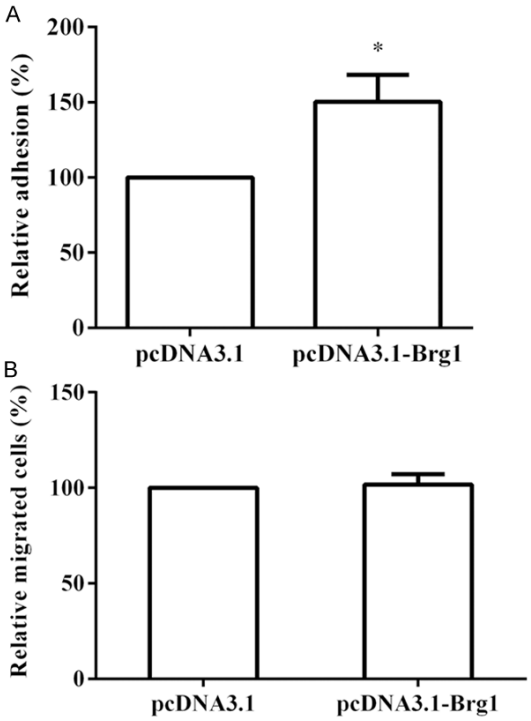

Figure 4. Effects of Brg1 overexpression on cell adhesion and migration. A: Effects of Brg1 overexpression on cell adhesion; B: Effects of Brg1 overexpression on cell migration. $^{*}P < 0.05$ compared to the control group.

Effects of Brg1 overexpression on osteogenesis markers expressions

After overexpression of Brg1 by transfection with pcDNA3.1-Brg1 into MSCs, we analyzed the effects of Brg1 overexpression on the mRNA expression of osteogenesis markers Runx2, special protein 7 (Sp7), collagen I and OPN. As shown in **Figure 2**, the results demonstrated that the mRNA levels of Runx2, Sp7, collagen I and OPN were all statistically upregulated by overexpression of Brg1 compared to the control group (all $P < 0.05$). The results implied that overexpression of Brg1 could promote osteogenesis of MSCs.

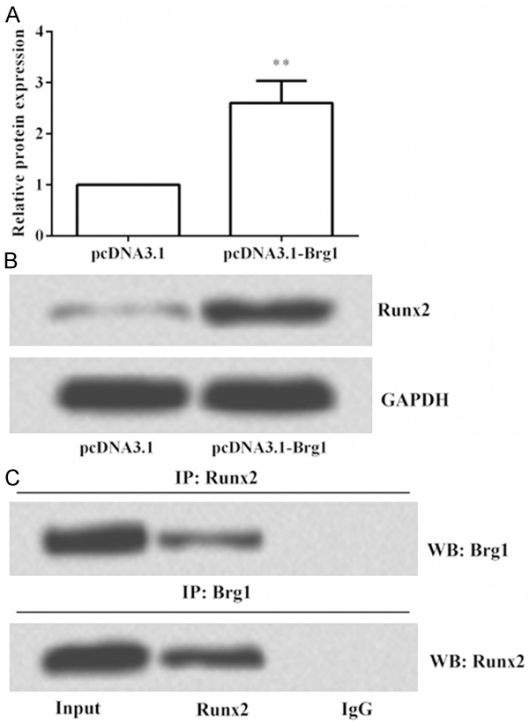

Figure 5. Effects of Brg1 overexpression on expression of Runx2 and the interaction between Brg1 and Runx2. A: Quantitative analysis of Brg1 overexpression on Runx2 protein expression; B: Representative Western blot picture; C: Interaction between Brg1 and Runx2. $^{**}P < 0.01$ compared to the control group.

Effects of Brg1 overexpression on cell viability

To investigate the effects of Brg1 overexpression on cell viability, MTT was performed after 1, 2, 3, and 4 days transfection with pcDNA3.1-Brg1 into MSCs. We found that overexpression of Brg1 could dramatically increase the cell viability at 2, 3, and 4 days compared with the control group (all $P < 0.05$) (**Figure 3**). However, no significant differences were observed at 1 d between the two groups. The results suggested that overexpression of Brg1 could promote the cell viability of MSCs.

Effects of Brg1 overexpression on cell adhesion and migration

Subsequently, the effects of Brg1 overexpression on cell adhesion and migration were determined. Our data revealed that the relative adhesion ability of MSCs was markedly raised by overexpression of Brg1 compared with the control group ($P < 0.05$) (**Figure 4A**). However, there was no significant difference in migration ability of MSCs between the two groups (**Figure 4B**). These results suggested that overexpression of Brg1 enhanced the adhesion ability of MSCs but had no effective response on migration ability of MSCs.

Effects of Brg1 overexpression on expression of Runx2 and the interaction between Brg1 and Runx2

RUNX2 is an important transcription factor associated with osteoblast differentiation. Therefore, we speculated that the effects of Brg1 on MSCs might be by regulating the expression of Runx2. To confirm the hypothesis, we measured the expression of Runx2 after overexpression of Brg1. As indicated in **Figure 5A** and **5B**, the results showed that the relative protein expression of Runx2 was significantly elevated by overexpression of Brg1 compared to the control group ($P < 0.01$). The Co-IP results showed that Runx2 was able to coprecipitate with Brg1 (**Figure 5C**). The results implied that Runx2 was directly bound to Brg1 and regulated by Brg1.

Effects of Brg1 overexpression on Wnt and PI3K/AKT pathways

To further demonstrate the underling associated signaling pathways with respect to the effects of Brg1 overexpression on MSCs, we determined the protein expression levels of Wnt3a, β-Catenin, Wnt5a, PI3K, and p/t-AKT after transfection with pcDNA3.1-Brg1 and/or treatment with the inhibitor of Runx2 (CADD522). Non-treated cells were considered as control group. As expected, the results revealed that the protein expression levels of Runx2 were significantly elevated by pcDNA3.1-Brg1 but decreased by administration of CADD522. Additionally, the protein levels of Wnt3a, β-Catenin, Wnt5a, PI3K, and p/t-AKT were all remarkably increased by overexpression of Brg1 compared to the control group (all $P < 0.05$) (**Figure 6A** and **6B**). However, these results were reversed by administration of CADD522. That means, after administration of CADD522, the protein levels were all obviously decreased even though overexpression of Brg1 ($P < 0.05$ or $P < 0.01$). These data suggested that Brg1 could regulate Runx2-mediated downstream Wnt and PI3K/AKT pathways.

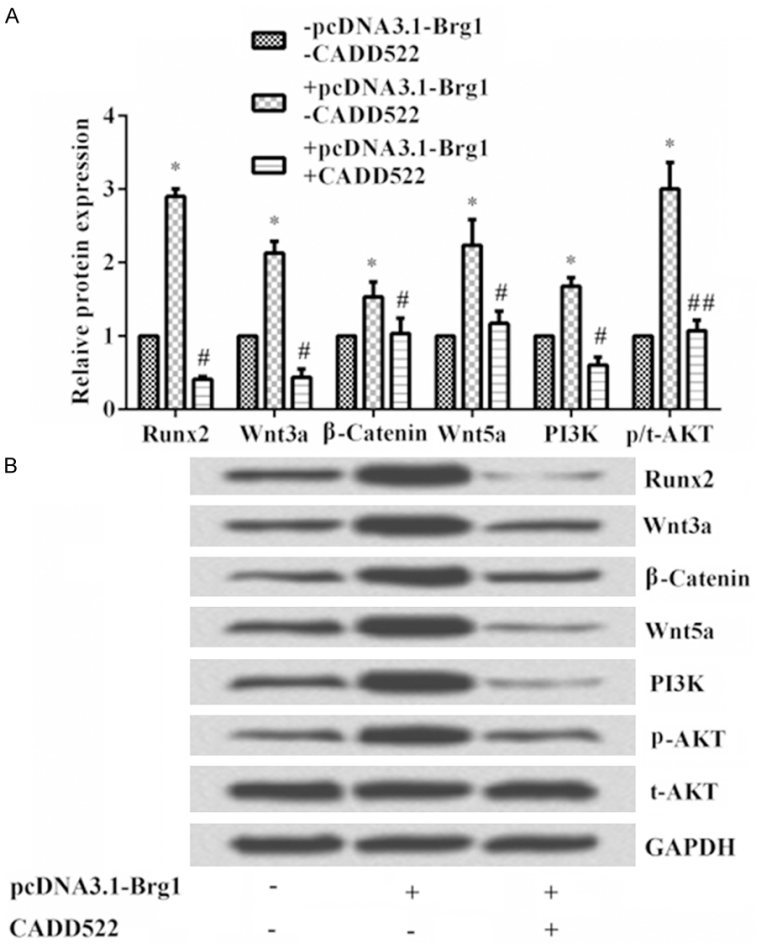

Figure 6. Effects of Brg1 overexpression on Wnt and PI3K/AKT pathways. A: Quantitative analysis of Brg1 overexpression on Wnt and PI3K/AKT pathways key proteins expression; B: Representative Western blot picture. $^{*}P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the pcDNA3.1-Brg1 group; $^{\#\#}P < 0.01$ compared to the pcDNA3.1-Brg1 group.

Discussion

In the present study, we investigated the functional role of Brg1 in osteogenic differentiation of MSCs, together with the potential underlying mechanism. Our data revealed that overexpression of Brg1 could increase the expression of osteogenesis markers. Overexpression of Brg1 also elevated the cell viability and adhesion ability of MSCs, but had no effect on migration ability. Moreover, we observed that Brg1 overexpression significantly raised the expression of Runx2, and Co-IP demonstrated that Runx2 was able to coprecipitate with Brg1. Furthermore, the results demonstrated that overexpression of Brg1 was capable of activating Runx2-mediated Wnt and PI3K/AKT pathways. Taken together, our results suggested that Brg1 promoted osteogenic differentiation of MSCs through activating Runx2-mediated Wnt and PI3K/AKT pathways.

Osteoporosis is a silent debilitating bone disorder that affects millions of people worldwide, primarily women. Current treatments for osteoporosis are mainly focus on bone-resorbing drugs, which has been reported to be associated with several side effects. The therapeutic use of stem cells as agents of repair human diseases including musculoskeletal disorders has garnered great interest recently due to the potential tissue regeneration [20-23]. It has been acknowledged that stem cell therapy for osteoporosis has the potential ability of decreasing the susceptibility of fractures and enhancement lost mineral density via upregulating the numbers or restoring the function of resident stem cells [21]. Exogenous introduction of MSCs are capable of recruiting endogenous stem cells to osteoporotic sites. MSCs could be derived from bone marrow [24], adipose tissue [25], muscle [26], synovium [27], periosteum [28], umbilical cord blood [29], or other tissues. However, how to control the stem cell fate, for example differentiation of MSCs, has been the main obstacle to achieve the desired outcomes. It has been reported that the differentiation of MSCs is controlled by both intrinsic and extrinsic regulations, such as epigenetic modifications and signaling pathway [30]. However, the exact regulatory mechanisms still remain incomplete. Over the last decades, the molecular analysis of the regulatory mechanisms of Brg1 on MSCs has raised great concerns in various fields of medicine [17, 18, 31]. Brg1 is a critical component of the evolutionarily conserved SWI-SNF chromatin remodeling complex. Depletion of Brg1 has been reported to affect the survival of totipotent cells and impair the ability of mouse embryonic stem cells (mESCs) differentiating into

ectoderm, mesoderm and endoderm layers [32, 33]. In addition, aberrant expression of Brg1 has been reported to enhance the senescence of MSCs, which contributes to transcriptional circuitry regulating the functions of stem cells [17].

In the present study, we focused on the functional role of Brg1 in osteogenic differentiation of MSCs. Our data also provided the evidence that Brg1 was a significant regulator in osteogenic differentiation of MSCs. After overexpression of Brg1, we determined the expression of Runx2, Sp7, collagen I and OPN. Runx2 and Sp7 (also called osterix) are two important osteoblast-determining transcription factors, which play an important role in regulation of osteoblast development and differentiation [34, 35]. Sp7 functions downstream of Runx2 during bone formation and mediates the expression of osteoblast markers, such as collagen I and OPN [35]. As shown in the results, we found that overexpression of Brg1 could upregulate the expression of Runx2, Sp7, collagen I and OPN, indicating a potential ability of Brg1 to differentiate into osteoblast. Moreover, the results demonstrated that overexpression of Brg1 significantly increased the cell viability and adhesion ability of MSCs but not migration ability, suggesting that Brg1 promotes differentiation and proliferation ability of MSCs. Our results were in line with previous studies [31]. It has been reported that expression of Brg1 depends on bone morphogenetic proteins (BMPs)-induced Runx2 expression [36]. Therefore, we speculated that there might be an interaction between Brg1 and Runx2. To confirm the results, we performed Co-IP and observed that Runx2 was directly bound to Brg1 and regulated by Brg1. This was the new evidence that Runx2 was regulated by Brg1. Subsequently, we further investigated the underlying singling pathways. It has been well acknowledged that several key signaling cascades are involved in MSCs differentiation including Wnt [37] and PI3K/AKT pathways [38]. Wnts are a family of secreted cysteine-rich glycoproteins that plays critical roles in stem cell maintenance, differentiation, and proliferation during embryonic development [39]. Previous study has confirmed that canonical Wnt signaling could increase cytoplasmic β-catenin stability and enhance β-catenin translocation into the nucleus [39]. β-catenin has been shown to recruit many cofactors including Brg1 and is involved human diseases [40]. PI3K/AKT signaling pathways presents several pro-survival abilities, such as cell proliferation, differentiation, survival, migration, invasion and metabolism. Our results showed that overexpression of Brg1 elevated the expression of Wnt3a, Wnt5a, β-catenin, PI3K, and AKT levels, indicating that Brg1 activated the both two signaling pathways. However, administration of the inhibitor of Runx2, CADD522, reversed all the protein levels, demonstrating that Runx2 medicated the two pathways. Taken together, Brg1 activated the both two signaling pathways by regulating the expression of Runx2.

In conclusion, our results suggest that Brg1 promotes osteogenic differentiation of MSCs, and the effects might be by regulating Runx2-mediated downstream Wnt and PI3K/AKT pathways. It reveals that the potential therapeutic targets for Brg1 in treatment of osteoporosis.

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Disclosure of conflict of interest

None.

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