Genistein induces adipogenic differentiation in human bone marrow mesenchymal stem cells and suppresses their osteogenic potential by upregulating PPARγ

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Abstract. Genistein is a soy isoflavone that exists in the form of an aglycone. It is the primary active component in soy isoflavone and has a number of biological activities (anti-inflammatory and anti-oxidative). However, the specific effect of genistein on human bone marrow mesenchymal stem cells (BMSCs) remains unclear. In the present study, the mechanism underlying the effect of genistein on the suppression of BMSC adipogenic differentiation and the enhancement of osteogenic potential was investigated using an MTT assay. It was observed that genistein significantly increased BMSC cell proliferation in a time- and dose-dependent manner (P<0.01). In addition, reverse transcription-quantitative polymerase chain reaction revealed that genistein significantly inhibited the expression of runt-related transcription factor 2 (Runx2), type I collagen (Col I) and osteocalcin (OC; P<0.01). Furthermore, 20 μm genistein significantly inhibited the activity of alkaline phosphatase (ALP) and increased the activity of triglycerides (TGs) increased (P<0.01) as determined by an enzyme-linked immunosorbent assay. Finally, western blotting revealed that BMSC pretreatment with 20 μm genistein significantly increased peroxisome proliferator-activated receptor γ (PPARγ) protein expression (P<0.01). This suggests that the downregulation of PPARγ may significantly reduce the effect of genistein on cell proliferation, suppress the expression of Runx2, Col I and OC mRNA, and reduce ALP and promote TG activity in BMSCs. Thus, the results of the present study conclude that genistein induces adipogenic differentiation in human BMSCs and suppresses their osteogenic potential by upregulating the expression of PPARγ. In conclusion, genistein may be a promising candidate drug for treatment against osteogenesis.

Introduction

Bone marrow mesenchymal stem cells (BMSCs) are fibrocyte-like stem cells that exist alongside hematopoietic stem cells within the marrow cavity. BMSCs are highly self-renewable with multipotential differentiation (1); they may develop into osteoblasts, chondrocytes and adipose cells through directional differentiation, and at present it is understood that all osteoblasts are derived from BMSCs (2).

Peroxisome proliferator-activated receptor γ (PPARγ) is a PPAR subtype that contributes towards the regulation of cell differentiation, proliferation and apoptosis (3,4). To date, it is understood that the PPARγ subtype is the primary regulator of fat differentiation, which serves a key regulatory role in the direction of BMSC differentiation (5). In the marrow cavity, osteoblasts and adipocytes are derived from BMSCs, and it is understood that there is an association between their expression levels (6). Previous studies demonstrate that PPARγ-mediated adipogenic differentiation of BMSCs directly affects the differentiation of osteoblasts (7,8). Osteoclasts are derived from hematopoietic stem cells in bone marrow, and are responsible for increased bone resorption and osteoporosis; this is demonstrated through an increase of fat in bone marrow cavities that occurs in every type of osteoporosis (9,10).

Soy isoflavone is a class 1 secondary metabolite that is formed during the growth of soybeans. In total, 12 types of natural isoflavones exist in soybeans, including daidzin, daidzein, genistin, genistein, glycitin and glycitein (11). Of these, genistein possesses the highest level of activity (12). Genistein possesses a number of bioactivities (13); it is an effective antioxidant, a protein tyrosine activating enzyme inhibitor and a phytoestrogen (14). In recent years, increasing evidence indicates that genistein may aid in the prevention and treatment of breast cancer, prostatic cancer, post-menopause syndrome, osteoporosis and angiocardiopathy (15,16). In the present study, the mechanisms underlying the effect of genistein on the suppression of human BMSC adipogenic differentiation

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and the enhancement of BMSC osteogenic potential were investigated.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and genistein (Fig. 1; ≥98%, high-performance liquid chromatography) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were provided by Sigma-Aldrich (St. Louis, MO, USA).

BMSC culture and identification. The use of animals in the present study was approved by the Animal Care and Use Committee of the Chinese People's Liberation Army General Hospital (Beijing, China). A total of 20 male and female Sprague-Dawley rats (age, 2-4 weeks; 100±10 g; male = 24, female = 24; purchased from Charles River Laboratories, Wilmington, MA, USA) were used to isolate marrow-derived BMSCs and were housed in an animal quarter (humidity, 60-70%; temperature, 23±1˚C; 12-h light-dark cycle) with *ad libitum* access to food and water. Rats were sacrificed by cervical dislocation and BMSCs were isolated according to a previously described method (17). Briefly, bone marrow was flushed from the femur and tibia with saline, and placed into 25 cm^2 flasks with DMEM supplemented with 10% FBS, 100 units penicillin and 100 μg-ml streptomycin (both purchased from Sigma-Aldrich), and was incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for 1 day. Following the incubation period, nonadherent cells were removed and adherent cells were washed with phosphate-buffered saline (PBS; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Next, adherent cells were incubated with DMEM for 2 h at 37˚C and washed with PBS once they reached 80-90% confluence. The cells were detached using 0.25% trypsin (Nanjing Sunshine Biotechnology, Co., Ltd., Nanjing, China) and the remaining cells were incubated in new 25 cm^2 flasks.

BMSCs were treated with genistein (0, 5, 10 and 20 μm) for 1, 2 or 3 days or GW9662 (1 mM; Invitrogen; Thermo Fisher Scientific, Inc.), a PPARγ inhibitor. BMSCs were then fixed using 5% pre-cooled paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd.) for 10-15 min at 4˚C, and cultured with hematoxylin and eosin staining (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min. Next, BMSCs were washed using tap and distilled water for 5-10 min. Stained BMSCs were dehydrated with 95% ethanol for 1-2 min and xylene (Shangbeijia Biological Technology Co., Ltd.) was applied for 5-10 min until transparent. BMSCs were analyzed using a microscope (TE2000; Nikon Corporation, Tokyo, Japan).

Grouping and cell proliferation assay. BMSCs were seeded in 96-well plates and incubated with different concentrations of genistein (0, 5, 10 and 20 μm) for 1, 2 and 3 days or GW9662 (1 mM). In the MTT cell proliferation assay, BMSCs were incubated with 20 μl MTT for 4 h at 37˚C in a humidified atmosphere containing 5% CO_2. Once the medium was removed, 150 μl dimethyl sulfoxide was added for 10 min at room temperature. The optical density was read at 570 nm (Epoch Microplate Spectrophotometer; BioTek Instruments, Inc., Winooski, VT, USA).
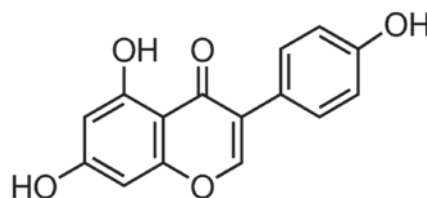

Figure 1. Chemical structure of genistein.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of runt-related transcription factor 2 (Runx2), collagen type I (Col I) and osteocalcin (OC). Following treatment with genistein, total RNA (1 μg) was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and BMSC cDNA (1 μg) was transcribed using RT-PCR Quick Master Mix (Toyoba Co., Ltd., Dalian, China) according to the manufacturer's protocol. qPCR was performed using LightCycler480 SYBR Green I Master (Roche Diagnostics, Indianapolis, IN, USA) at 94˚C for 45 sec, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 45 sec and 72˚C for 30 sec, and then 4˚C for 10 min. The primer sequences are listed in Table I. Samples were quantified using the $2^{-\Delta\Delta Cq}$ method (18).

Enzyme-linked immunosorbent assay of alkaline phosphatase (ALP) and triglyceride (TG). Following the treatment of BMSCs with genistein or GW9662, the activity of ALP and TG in cells was detected using an ALP and TG determination kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. The optical density was read using a microplate reader (LabSystems Miltiskan MS Plate Reader; Thermo Fisher Scientific, Inc.) at 405 nm.

Western blotting for PPARγ. Following the application of genistein or GW9662 to BMSCs, equal quantities of protein were analyzed using a BCA protein assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Protein was separated using 10% sodium dodecyl sulfate polyacrylamide (Sinopharm Chemical Reagent Co., Ltd.) gel electrophoresis (110 V; 45 min) and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membrane was blocked in 5% nonfat milk-PBS-Tween 20 solution (Shanghai Macklin Biochemical Co., Ltd.) for 1 h at room temperature, followed by separate incubation with polyclonal antibodies specific for PPAR (dilution, 1:1,000; goat anti-mouse; sc-1985; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and β-actin (dilution, 1:2,000; goat anti-mouse; sc-1616; Santa Cruz) at 4˚C overnight. The membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG; dilution, 1:5,000; sc-45101; Santa Cruz) in 5% nonfat milk-PBS-Tween 20. The membranes were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.), and analyzed using a Gel-Doc 2000 imaging scanner (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are expressed as the mean ± standard error. Statistical analysis of data was performed using one-way analysis of variance and the statistical software package SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Table I. Design of primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
Runx2	5'-CAGTTCCTAACGGGCACCAT-3'	5'-TTAGGGTCTCGGAGGGAAGG-3'
Col I	5'-TGACCTCAAGATGTGCCACT-3'	5'-GGGAGTTTCCATGAAGCCAC-3'
OC	5'-CATGAGAGCCCTCACA-3'	5'-AGAGCGACACCCTAGAC-3
β-actin	5'-GCTCTCCAGAACATCACTCCTGCC-3'	5'-CGTTGTCATACCAGGAAATGAGCTT-3'

Runx2, runt-related transcription factor 2; Col I, type I collagen; OC, osteocalcin.
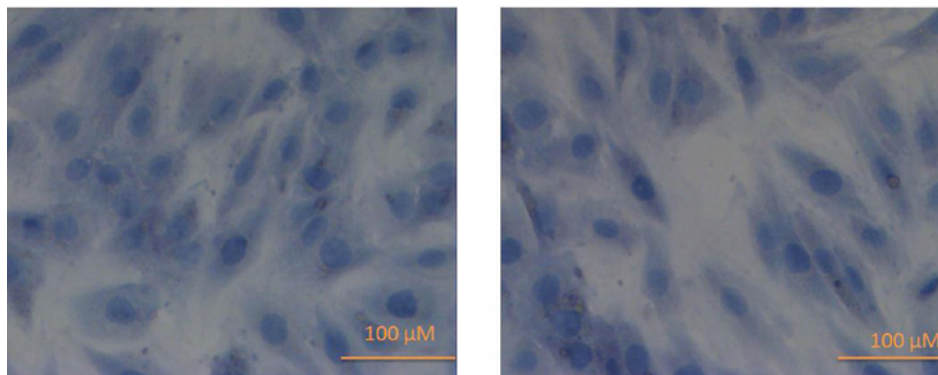

Figure 2. Authentication of bone marrow mesenchymal stem cells. The two panels are alkaline phosphatase stains of the same cell.

Results

Authenticating of BMSCs. The chemical structure of genistein is presented in Fig. 1. Fig. 2 demonstrates that the morphology of cultured BMSCs are spindle-shaped with serial subcultivation, homogeneity and multiplicity. The cell nucleus of stained BMSCs appeared dark blue (Fig. 2).

Effect of genistein on cell proliferation in BMSCs. Analysis indicated that genistein increased BMSC cell proliferation in a time- and dose-dependent manner (Fig. 3). When cells were treated with 20 μm genistein for 2 and 3 days, and 10 μm genistein for 3 days, BMSC cell proliferation was significantly increased compared with untreated BMSC cells (P<0.01; Fig. 3).

Effect of genistein on Runx2, Col I and OC mRNA expression in BMSCs. As presented in Fig. 4, the expression of Runx2, Col I and OC mRNA were significantly reduced following treatment with genistein. In BMSCs, the expression of Runx2 mRNA was significantly inhibited following treatment with 20 μm genistein for 2 days, the expression of Col I mRNA was significantly reduced following treatment with 5, 10 and 20 μm genistein for 2 days, and the expression of OC mRNA was significantly reduced following treatment with 10 and 20 μm genistein for 2 days (P<0.01; Fig. 4).

Effect of genistein on ALP and TG activity in BMSCs. Compared with BMSCs in the absence of genistein, the activity of ALP was significantly inhibited and the activity of TG was significantly enhanced following treatment with 20 μm genistein (P<0.01; Fig. 5).

Effect of genistein on PPARγ protein expression in BMSCs. To observe the mechanism of genistein on BMSCs, the protein expression of PPARγ was analyzed using western blotting. The results demonstrated that the protein expression of PPARγ was significantly increased following BMSC pretreatment with 20 μm genistein for 2 days (P<0.01; Fig. 6).

Effect of PPARγ downregulation on genistein-induced BMSC cell proliferation. GW9662 significantly inhibited PPARγ protein expression in BMSCs treated with 20 μm genistein for 2 days, compared with BMSCs treated only with 20 μm genistein (P<0.01; Fig. 7A and B). In addition, GW9662 significantly reduced the effect of 20 μm genistein on BMSC cell proliferation compared with cells treated only with 20 μm genistein (P<0.01; Fig. 7C).

Effect of PPARγ downregulation on Runx2, Col I and OC mRNA expression and the activity of ALP and TG in BMSCs. As presented in Fig. 8A, the effect of 20 μm genistein on the expression of Runx2, Col I and OC mRNAs was significantly reduced following pretreatment with a PPARγ inhibitor for 2 days (P<0.01), compared with cells treated only with 20 μm genistein for 2 days. In addition, the genistein-induced increase in TG activity and reduction in ALP activity were significantly inhibited by a PPARγ inhibitor (P<0.01; Fig. 8B and C).

Discussion

A number of local factors and hormones are required to transmit signals to transcription factors when BMSCs differentiate in osteogenesis, and these control the expression of specific genes during each stage of BMSC differentiation, thus controlling the
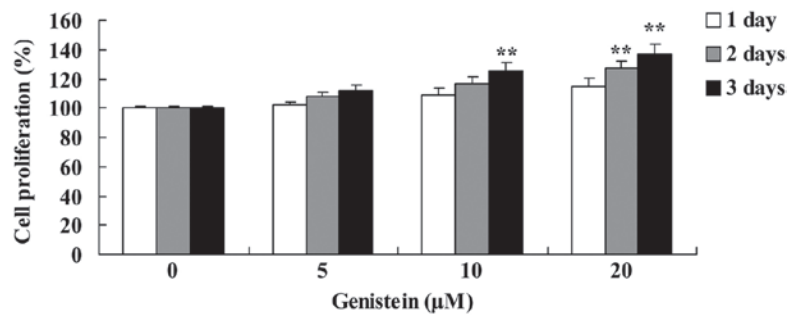
Figure 3. Genistein promotes cell proliferation of bone marrow mesenchymal stem cells. [**]P<0.01 vs. the 0 μM genistein group.
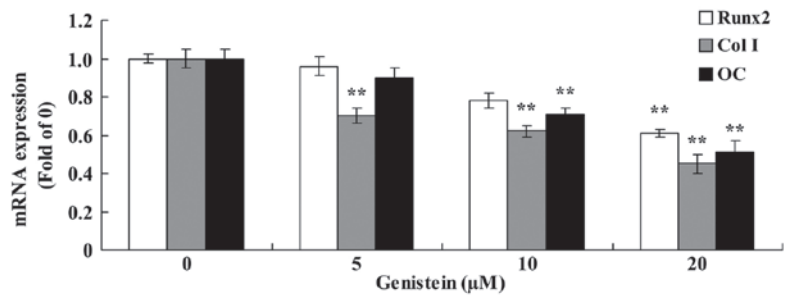

Figure 4. Effect of genistein on Runx2, Col I and OC mRNAs expression of bone marrow mesenchymal stem cells. [**]P<0.01 vs. the 0 μM genistein group. Runx2, runt-related transcription factor 2; Col I, type I collagen; OC, osteocalcin.
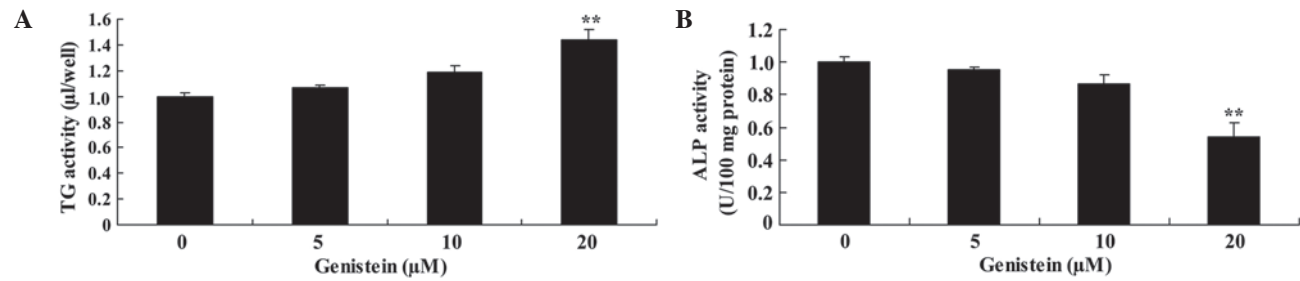

Figure 5. Effect of genistein on (A) TG and (B) ALP activities in bone marrow mesenchymal stem cells. [**]P<0.01 vs. the 0 μM genistein group. TG, triglyceride; ALP, alkaline phosphatase.
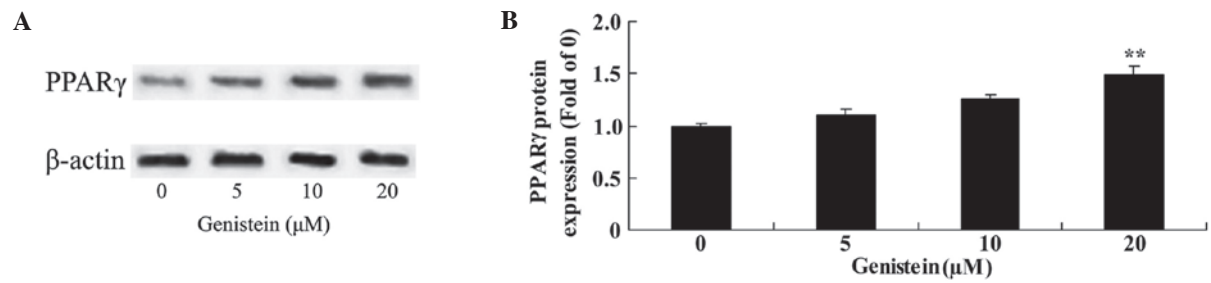

Figure 6. Effect of genistein on PPARγ protein expression in BMSCs. (A) Western blot analysis of the effect of genistein on PPARγ protein expression and (B) statistical analysis of PPARγ protein expression in BMSCs. [**]P<0.01 vs. the 0 μM genistein group. PPARγ, peroxisome proliferator-activated receptor γ; BMSCs, bone marrow mesenchymal cells.

osteoblast at each stage (1,2). Once mesenchymal stem cells become osteoblasts, the osteogenic cells proliferate as a result of mitotic growth factors, which ensure that a sufficient quantity of osteoblasts are generated for osteogenesis (19). Meanwhile, osteogenic cells undergo differentiation into mature osteoblasts during osteogenesis (20).

OC, ALP, Runx2 and Col I are derived during the synthesis of osteoblasts. At different stages of osteogenesis, only part of the aforementioned proteins and factors may be generated (21). With the generation of OC, Runx2 and Col I, osteoblasts will enter into different stages of differentiation (22). Thus, the synthesis and secretion of such proteins are significant markers
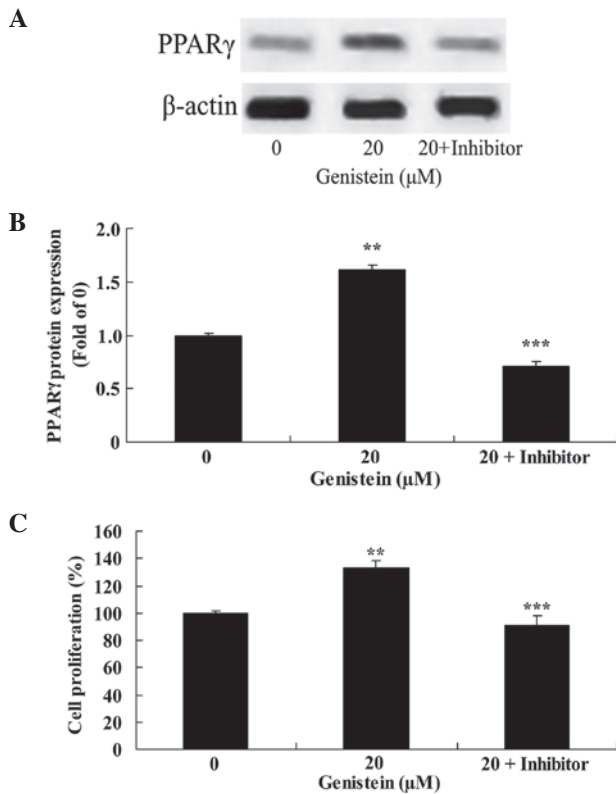
Figure 7. Effects of PPARγ downregulation on genistein-induced cell proliferation of BMSCs. (A) Western blot analysis to analyze the effect of genistein on PPARγ protein expression, (B) statistical analysis of PPARγ protein expression and (C) the effect of genistein on cell proliferation of BMSCs. **P<0.01 vs. 0 μM genistein group; ***P<0.01 vs. 20 μM genistein group. PPARγ, peroxisome proliferator-activated receptor γ; BMSCs, bone marrow mesenchymal cells.

Figure 8. Effect of PPARγ downregulation on (A) Runx2, Col I and OC mRNA expression and (B) TG and (C) ALP activity in bone marrow mesenchymal stem cells. **P<0.01 vs. the 0 μM genistein group; ***P<0.01 vs. 20 μM genistein group. PPARγ, peroxisome proliferator-activated receptor γ; Runx2, runt-related transcription factor 2; Col I, type I collagen; OC, osteocalcin; TG, triglyceride; ALP, alkaline phosphatase.

of osteoblast differentiation, and affect the biological function and performance of osteoblasts (23).

In the present study, genistein significantly accelerated BMSC cell proliferation, reduced Runx2, Col I and OC mRNA expression, and inhibited the activity of ALP and increased the activity of TG, which suggests that genistein has the potential to be used as a BMSC inductive agent. In particular, Relic et al (24) reported that genistein induces adipogenesis through activating PPARγ pathway.

BMSCs possess a multipotent differentiation potential that allows them to differentiate into a variety of cell osteoblasts, including chondrocytes, adipocytes and myoblasts, by a number of induction pathways, and this directional differentiation can alter when the differentiation induction system changes (25). When osteogenesis- and chondrogenesis-inducing factors are present in BMSC culture systems, the expression of PPARγ is significantly increased and an increased number of adipocytes are generated (3). Osteoblasts are able to generate a variety of cytokines that regulate the differentiation and apoptosis of osteoclasts in various stages of differentiation proliferation, maturation and mineralization (26). Therefore, the effect of the PPARγ gene and its ligand on osteoblasts may alter the levels of cytokines in the bone marrow microenvironment, resulting in a direct or indirect influence on the differentiation and function of osteoclasts (3,8).
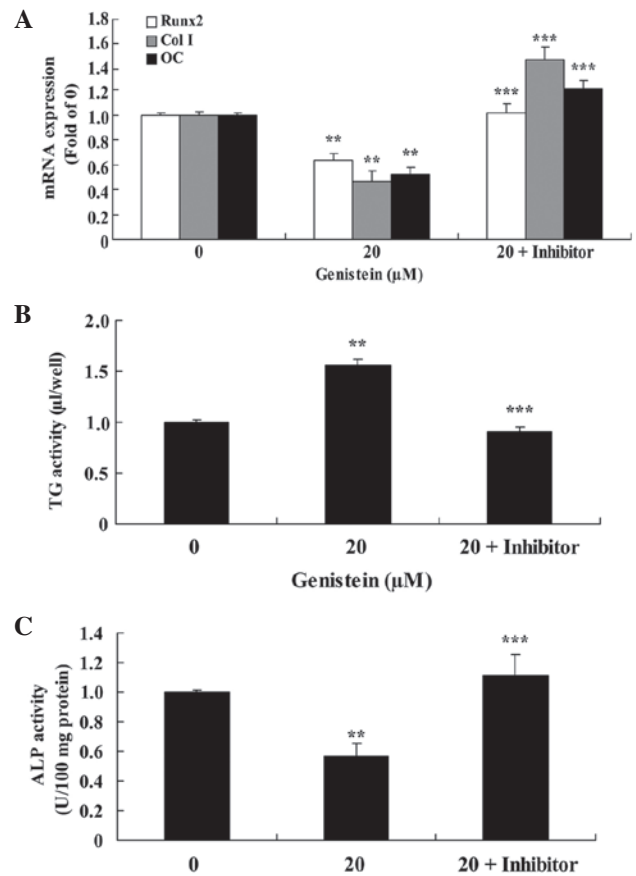
Results from the present study demonstrated that BMSC pretreatment with 20 μm genistein significantly activates PPARγ protein expression. Similarly, a previous report observed that genistein inhibits human osteosarcoma MG-63 cells by activating the PPARγ signaling pathway (27). In addition, Chatterjee et al (28) demonstrated that genistein prevents Alzheimer's disease-associated inflammation through increasing PPARγ expression in cultured astrocytes (29).

PPAR was identified as a substance that may be activated by a peroxysome proliferation stimulator of a fatty acid-like compound (30). It was observed in vitro and in vivo that PPAR served an important role in the regulation of BMSC differentiation (31). A number of studies report that the PPARγ genes possesses an influence on the pathogenesis of osteoporosis induced by microgravity; under simulated microgravity conditions, the expression of PPARγ is increased, thus enhancing the activity of PPARγ, as well as its target gene adipsin and recombinant human leptin (32,33). The present study observed that downregulation of PPARγ reduced the effect of genistein-induced BMSC cell growth, inhibited genistein-induced adipogenic differentiation and suppressed the osteogenic potential of BMSCs.

In conclusion, the results from the current study demonstrate that genistein promotes cell growth, induces adipogenic differentiation and suppresses the osteogenic potential of BMSCs by

upregulating PPARγ expression. To conclude, genistein may be a potential therapeutic agent for the treatment of orthopedic diseases.

Acknowledgements

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