

Curcumin alleviates glucocorticoid-induced osteoporosis through the regulation of the Wnt signaling pathway

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Abstract. It is known that prolonged glucocorticoid (GC) treatment results in osteoporosis. This study aimed to evaluate the protective effects of curcumin on the bones of rats with dexamethasone (DXM)-induced osteoporosis. In the present study, rats were administered DXM for 60 days to induce osteoporosis, and they were then treated with curcumin (100 mg/kg/day) for a further 60 days. H&E staining was used to observe the pathological changes in the femurs. Serum osteocalcin levels and collagen-type I fragments (CTX) were examined as bone metabolism markers. The results revealed that treatment with curcumin attenuated DXM-induced bone injury in femurs, increased the serum levels of osteocalcin and decreased the levels of CTX. In addition, in *in vitro* experiments, primary rat osteoblasts treated with curcumin at 0.5, 1 and 2 μ M were exposed to 100 nM DXM. An MTT assay was used to determine the proliferative ability of the cells. Alkaline phosphatase activity, and the mRNA expression levels of runt-related transcription factor 2 (Runx2), osterix, osteocalcin, collagen, type 1, alpha 1 (Col1A1) and osteonectin were detected to assess transcription factor-associated osteogenic differentiation. The mRNA and protein expression levels of osteoprotegerin (OPG) and receptor activator for nuclear factor-kappa B ligand (RANKL) were detected to assess cytokine-associated osteoclastogenesis. The results demonstrated that curcumin prevented the DXM-induced inhibition of the proliferative ability of the osteoblasts in a dose-dependent manner. In addition, curcumin upregulated the mRNA expression levels of transcription factors that favor osteoblast differentiation and increased the ratio of OPG to RANKL. Moreover, the effects of curcumin on the Wnt signaling pathway were also investigated. RT-qPCR and

western blot analysis demonstrated that the Wnt signaling pathway, which was inhibited by DXM, was re-activated upon treatment with curcumin. Immunofluorescence staining revealed that curcumin restored the intranuclear staining of β -catenin in the DXM-stimulated osteoblasts. Collectively, our data demonstrate that curcumin may be a potential therapeutic agent for the treatment of GC-induced osteoporosis.

Introduction

Glucocorticoids (GCs) are widely used in the treatment of various diseases, such as asthma, rheumatoid arthritis, and systemic lupus erythematosus for its anti-immune and anti-infectious effects (1,2). However, GCs also have a number of adverse effects, including osteoporosis. GC therapy is the most common cause of osteoporosis, leading to osteonecrosis of the femoral head and fractures, which may also be associated with fracture-related morbidity and a decreased quality of life (3-5). Osteoporosis guidelines recommend that patients who are administered chronic GC therapy should also be treated for osteoporosis (6-9). Therefore, the development of compounds that attenuate GC-induced osteoporosis (GIOP) is of clinical significance.

Curcumin is the main active ingredient of turmeric (*Curcuma Longa*), which is a traditional Chinese medicine with a long history of use as a treatment for inflammatory conditions (10). Curcumin is a highly pleiotropic molecule which is effective in treating a number of chronic diseases, such as inflammatory bowel disease, pancreatitis, arthritis and certain types of cancer (11). Moreover, previous studies have found that curcumin also protects against ovariectomy-induced bone loss and decreases osteoclastogenesis in rodent models (12-15). In addition, Yang *et al* demonstrated that curcumin improved bone microarchitecture and enhanced mineral density in APP/PS1 transgenic mice (16). In our previous study, we demonstrated that curcumin attenuated GIOP by inhibiting osteocytic apoptosis (17). In the present study, we aimed to continue the investigation of the possible mechanisms responsible for the protective effects of curcumin against GIOP.

The Wnt/ β -catenin signaling pathway is an important pathway that is involved in the growth, development and maintenance of a number of tissues, including bone tissue (18). Osteoblasts and osteoclasts are two cell types that are critical for bone formation and maintenance. Wnt/ β -catenin signals in osteoblasts induce the expression of osteoprotegerin (OPG)

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and thereby inhibit osteoclast differentiation (19). In a study on pre-osteoblast-specific β -catenin conditional knockout mice, osteoblast differentiation was shown to be suppressed, whereas adipocyte differentiation was enhanced in bone marrow stromal cells, which indicates that the Wnt/ β -catenin signal is the determinant of the cell fate of pre-osteoblasts (20). In addition, loss-of-function mutation of the low-density lipoprotein receptor-related protein 5 (LRP5), an important protein in the Wnt/ β -catenin signaling pathway (21,22), has been shown to correlate with a decrease in bone mass (23), and gain-of-function mutations in LRP5 have also been shown to cause increased bone density at certain locations (24,25). Therefore, compounds that induce the activation of Wnt/ β -catenin signaling are beneficial for the treatment of osteoporosis. Curcumin has been shown to activate the Wnt/ β -catenin signaling pathway in *in vivo* and *in vitro* studies (26-28). However, other studies have demonstrated that curcumin suppresses this pathway (29,30). In the present study, as a possible pharmacological mechanism responsible for the bone-protective effects of curcumin, the regulatory effects of curcumin on the Wnt/ β -catenin signaling pathway were investigated using *in vivo* and *in vitro* models of dexamethasone (DXM)-induced osteoporosis.

Materials and methods

Animals. Female 5-month-old Sprague-Dawley rats were obtained from the Experimental Animal Centre of China Medical University (Shenyang, China). The animals were housed under standard laboratory conditions at a stable temperature (22-24°C) and a 12/12-h light/dark cycle. This study was approved by the Ethics Committee of China Medical University (Shenyang, China).

Induction of osteoporosis and treatments. The rats were randomly divided into 3 groups (n=6 per group) as follows: the control group, the DXM group and the DXM + curcumin group. The rats in the DXM and DXM + curcumin groups received subcutaneous injections of dexamethasone (0.1 mg/kg/day, Tianjin Pharmaceutical Group Xinzheng Co., Ltd., Zhengzhou, China) for 60 days. The rats in the control group were injected subcutaneously with the vehicle only. Bone mineral density (BMD) was measured at the proximal tibia to confirm that the model had been successfully established. The rats in the DXM + curcumin group received an intragastric administration of curcumin (100 mg/kg/day, Dalian Meilun Biology Technology Co., Ltd., Dalian, China) for a further 60 days. The rats in the control and DXM groups received the vehicle only [0.5% sodium carboxymethyl cellulose (CMC-Na)]. Following treatment, BMD was measured again, and the rats were euthanized by an overdose of pentobarbital. Blood was collected from the inferior vena cava. The muscle around the femurs was removed using surgical scissors and the femurs were collected.

Measurement of BMD. BMD was measured at the femurs by dual-energy X-ray absorptiometry bone densitometry using a Hologic QDR 4500 machine (Hologic, Bedford, MA, USA) and the accompanying experimental animal assessment software. BMD was represented as g/cm². All samples were measured 3 times, and the mean values were calculated. The coefficient of variation for the BMD measurements was 1.02%.

Determination of the activities of serum bone-specific alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP). The activities of bone-specific ALP and TRAP were determined using commercial kits for ALP and TRAP activity assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. Briefly, the serum samples were mixed with the test solutions and at 37°C for the indicated periods of time. Finally, the absorbance at 520 nm (ALP) or 530 nm (TRAP) was read using a microplate reader (ELX-800; Bio-Tek Instruments, Winooski, VT, USA).

Determination of levels of serum osteocalcin and serum collagen type I fragments (CTX). The serum levels of osteocalcin and CTX were determined using commercial ELISA kits specific to osteocalcin and CTX (USCN Life Science, Wuhan, China) according to the manufacturer's instructions. Briefly, the serum samples were diluted according to the manufacturer's instructions and incubated with antibody solutions specific for osteocalcin and CTX at 37°C for 1 h. After washing, streptavidin-HRP was added followed by incubation at 37°C for 30 min. The absorbance was measured following the addition of stop solution by a microplate reader (ELX-800; Bio-Tek Instruments) at 450 nm.

Histological examination. The femoral samples were fixed in 4% paraformaldehyde for 24 h, dehydrated using a series of ethanol and embedded in paraffin. Paraffin blocks were cut into 5- μ m-thick slices, deparaffinized in xylene and hydrated using a series of ethanol. The femoral tissue sections were then stained with hematoxylin and eosin (H&E) solution (Solarbio Science & Technology Co., Ltd., Beijing, China) and observed under a light microscope (DP73; Olympus, Tokyo, Japan).

Cell culture. Calvarial bones from 40 24-h-old neonatal Sprague Dawley rats (Experimental Animal Centre of China Medical University, Shenyang, China) were used for primary osteoblast cell culture. Following euthanasia, the calvarial bones were removed and cut into 1-3-mm³ sections and digested in 1% trypsin and 0.2% collagenase type II at 37°C for 30 min. The digestion was then terminated, and the mixture was filtered and centrifuged at 300 x g for 10 min. The supernatant was discarded and the cells were washed twice using Tris-buffered saline (PBS) and resuspended in Dulbecco's modified essential medium (DMEM, Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM l-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were plated into the indicated culture plates and maintained in a humidified atmosphere of 5% CO₂-95% air at 37°C.

MTT assay. The cells were seeded into a 96-well plate at 4x10³ cells/well. The cells were exposed to DXM (0, 1, 10, 100 and 1,000 nM) for 24 h. Subsequently, the cells were seeded in a 96-well plate at 4x10³ cells/well and treated with curcumin (0, 0.5, 1 and 2 μ M) for 24 h. The cells were then seeded in a 96-well plate at 4x10³ cells/well and pre-treated with curcumin (0.5, 1 and 2 μ M) for 2 h, and then exposed to DXM (100 nM) for 24 h. Finally, the cells were seeded in a 96-well plate at 4x10³ cells/well and treated with curcumin (0.5, 1 and 2 μ M and DXM 100 nM simultaneously for 24 h.

Table I. Sequences of primers used for RT-qPCR.

Gene	Primer sequences	
	Upstream	Downstream
Wnt1	CGAAACCGCCGCTGGAAC	GGAGGTGATTGCGAAGATAAACG
LRP5	GCAGAGCCACCATCCACAG	TCTTGCCCATCCAATCCAC
SOST	GTGCAAGTGCAAGCGCCTCA	GCTCCGCCTGGTTGGCTTIG
Osteoclastin	TAAGGTGGTGAATAGACTCCG	GCCATAGATGCGCTTGTAG
Osterix	GCCTACTTACCCGTCTGACTTTG	ACTGCCTTGGGCTTATAGACATC
DKK1	TATGAGGGCGGGAACAAGTA	AAATGGCTGTGGTCAGAGGG
Col1A1	CAAGGACTATGAAGTTGATGC	ACCAGTAGAGAAATCGCAGT
Osteonectin	GGGCAGACCAATACCTCACTA	CCGACCATTCCCTCCGTTG
Runx2	CCATAACGGTCTTCACAAATC	GAGGCGGTCAGAGAACAACCT
OPG	GACCCAGAGCGAAACACG	GGCACAGCAAACCTGAAGAA
RANKL	CATCGGGTCCCATAAAG	GAAGCAAATGTTGGCGTA
β -catenin	AGTCCTTATGAGTGGGAGCAA	GTTTCAGCATCTGTGACGGTTC
β -actin	GGAGATTACTGCCCTGGCTCCTAGC	GGCCGGACTCATCGTACTCCTGCTT

Wnt1, wingless-type MMTV integration site family, member 1; Runx2, runt-related transcription factor 2; Col1A1, collagen, type 1, alpha 1; OPG, osteoprotegerin; RANKL, receptor activator for nuclear factor-kappa B ligand; LRP5, low-density lipoprotein receptor-related protein 5; DKK1, Dickkopf WNT signaling pathway inhibitor 1; SOST, sclerostin.

Following treatment, MTT (0.2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well followed by incubation at 37°C for 4 h. The supernatant was then removed, and 200 μ l dimethyl sulfoxide (DMSO) were added to solve the precipitate, and the absorbance at 570 nm was read using a microplate reader (ELX-800, Bio-Tek Instruments).

mRNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total mRNA from the femoral tissues or primary rat osteoblasts was isolated using an RNA Simple Total RNA kit (Tiangen Biotech, Co., Ltd., Beijing, China) according to the manufacturer's instructions. cDNA was reverse transcribed with oligonucleotide primer using super Moloney Murine Leukemia Virus (M-MLV) (BioTeke, Beijing, China) in a 20- μ l system. The qPCR reactions were performed in a 20- μ l system containing 10 μ l SYBR-Green Master Mix (Tiangen Biotech Co., Ltd.), 0.5 μ M of forward and reverse primers, and 1 μ l template cDNA on an Exicycler™ 96 real-time quantitative thermal block (Bioneer, Daejeon, Korea). The sequences of the primers are listed in Table I. Data were normalized to β -actin and analyzed using the comparative threshold cycle (CT) method.

Western blot analysis. The bone tissues or cells were homogenized in ice-cold radioimmunoprecipitation (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). Following centrifugation at 12,000 x g at 4°C for 10 min, the supernatant was collected and the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). Proteins were then separated on 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and transferred electrophoretically

onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using the wet transfer method. The membranes with target proteins were blocked with 5% non-fat milk at room temperature for 1 h, and then incubated with the primary antibodies at 4°C overnight. After a washing stage, the membranes were subsequently incubated with horseradish peroxidase-labeled goat anti-rabbit or donkey anti-goat IgG (1:5,000, Beyotime Institute of Biotechnology) at 37°C for 45 min. The protein blots were visualized using enhanced chemiluminescence (7 Sea Pharmtech, Shanghai, China) and exposed to Fuji Rx 100 X-ray film (Fuji Photo Film, Tokyo, Japan). The gray values of the blots were analyzed using Gel-Pro-Analyzer (Media Cybernetics, Bethesda, MD, USA). β -actin was used as a loading control. The primary antibodies used in this study were as follows: phosphorylated glycogen synthase kinase (p-GSK-3 β antibody (1:200, sc-11757; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), GSK-3 β antibody (1:200, sc-9166; Santa Cruz), OPG antibody (1:500, bs-0431R; Bioss, Beijing, China) and receptor activator for nuclear factor-kappa B ligand (RANKL) antibody (1:500, bs-0747R; Bioss, Beijing, China).

Immunofluorescence staining. The cells were cultured on glass coverslips and fixed in 4% paraformaldehyde for 15 min. Slips were washed in PBS 3 times and incubated with 0.1% Triton X-100 for 30 min at room temperature. After a washing stage, the slips were blocked in goat serum for 15 min at room temperature. The cells were then incubated with anti- β -catenin antibody (1:200, BA0426; Boster, Wuhan, China) at 4°C overnight. Subsequently, the cells were washed with PBS and incubated with cy3-labeled goat anti-rabbit secondary antibody (1:200, Beyotime Institute of Biotechnology) at room temperature for 1 h. The cells were then co-stained with DAPI

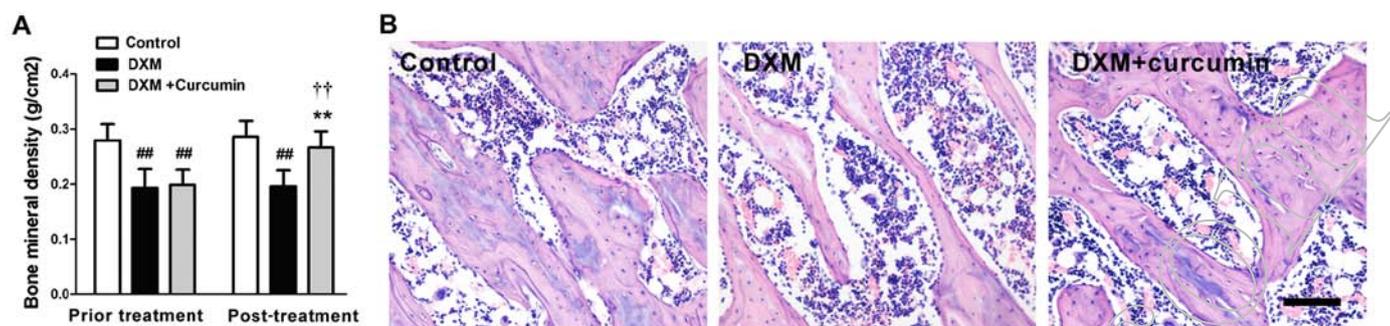


Figure 1. Effects of curcumin on bone mineral density (BMD) and histological changes in rats with dexamethasone (DXM)-induced osteoporosis. (A) Changes in BMD before and after curcumin treatment. DXM induced a significant decrease in BMD, and curcumin reversed this change. (B) H&E staining of femurs. DXM caused obvious damage to the femurs of rats and curcumin attenuated the damage. Scale bar, 100 μ m. Data are represented as the means \pm SD, n=6. ##P<0.01 vs. the control group on the same day, **P<0.01 vs. the DXM group on the same day, **P<0.01 vs. the DXM + curcumin group prior to treatment.

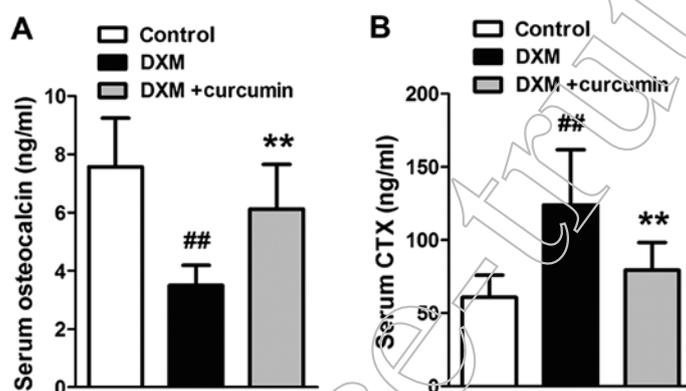


Figure 2. Effects of curcumin on the bone metabolism of rats with dexamethasone (DXM)-induced osteoporosis. Rats with DXM-induced osteoporosis exhibited (A) significantly decreased osteocalcin levels and (B) increased collagen-type I fragments (CTX) serum levels. The administration of curcumin reversed these changes. Data are presented as the means \pm SD, n=6. ##P<0.01 vs. the control group, **P<0.01 vs. the DXM group.

and then observed under a fluorescence microscope (BX53; Olympus).

Statistical analysis. Statistical analyses were conducted with SPSS19 software (IBM, New York, NY, USA). Data are expressed as the means \pm standard deviation (SD). Differences between groups were evaluated by one-way ANOVA followed by Fisher's least significant difference (LSD) test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Effects of curcumin on BMD and histological changes in the femurs of rats with DXM-induced osteoporosis. We measured the BMD of rats before and after treatment with curcumin. As illustrated in Fig. 1A, after 60 days of DXM administration, the BMD of the rats was significantly decreased compared with the rats in the control group (P<0.01). After a further 60 days of treatment with curcumin, the BMD of the femurs increased significantly (P<0.01 vs. DXM group and DXM + curcumin group post-treatment), and the value was similar to that of the control group (P>0.05).

Histological changes were examined by H&E staining. In the control group, the femurs exhibited a complete trabecular structure and ordered arrangement of the trabeculae. In the

DXM group, significantly reduced and thinning trabeculae and small numbers of empty bone lacunae were observed. Treatment with curcumin markedly reversed these changes (Fig. 1B).

Effects of curcumin on the bone metabolism of rats with DXM-induced osteoporosis. The osteocalcin content was examined as a bone formation biomarker, and the CTX content was examined as a bone resorption biomarker. As shown in Fig. 2, the osteocalcin levels were markedly decreased and the CTX levels were markedly increased in the serum following the administration of DXM. However, treatment with curcumin significantly reversed these changes.

Effect of curcumin on the Wnt signaling pathway. The mRNA expression levels of key proteins in the Wnt signaling pathway were measured by RT-qPCR. Following exposure to DXM, the mRNA expression levels of Wnt, β -catenin and LRP5 were significantly downregulated, whereas the mRNA expression levels of the two receptor inhibitors, sclerostin (SOST) and Dickkopf-1 were upregulated (Fig. 3A). In addition, the phosphorylation of GSK-3 β , a cytosolic Wnt signaling inhibitor, was inhibited by DXM (Fig. 3B). However, treatment with curcumin effectively reversed these changes. These results indicated that DXM induced the inhibition of Wnt signaling in the femurs of rats, and that curcumin, at least partly, reversed this inhibition.

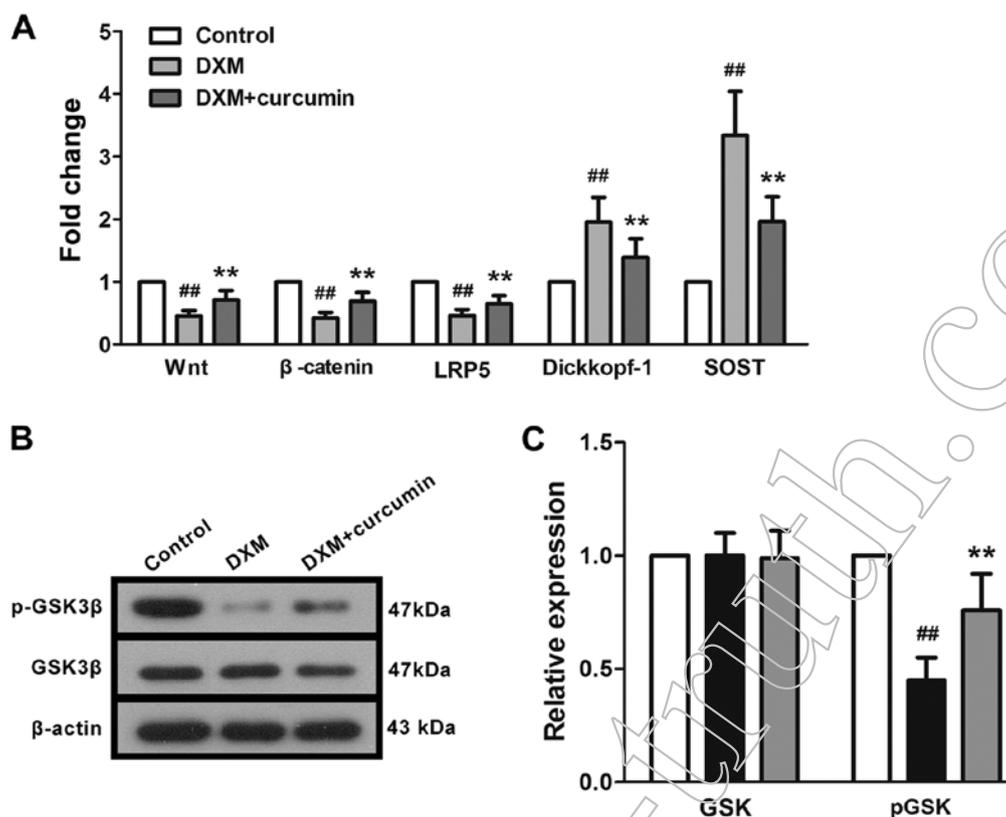


Figure 3. Effects of curcumin on the Wnt signaling pathway. (A) The mRNA expression levels of Wnt, β -catenin, low-density lipoprotein receptor-related protein 5 (LRP5), sclerostin (SOST) and Dickkopf-1 in the femurs of rats with dexamethasone (DXM)-induced osteoporosis. (B and C) The phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) in the femurs of rats with DXM-induced osteoporosis. DXM significantly inhibited the Wnt signaling pathway, and curcumin prevented this inhibition. Data are presented as the means \pm SD, n=6. ##P<0.01 vs. the control group, **P<0.01 vs. the DXM group.

Effects of DXM and curcumin on the viability of primary osteoblasts. The effects of DXM and curcumin on the viability of primary osteoblasts were evaluated by MTT assay. As shown in Fig. 4A and B, exposure to DXM at 100 and 1,000 nM markedly decreased the viability of the osteoblasts, whereas at all concentrations (0.5, 1 or 2 μ M) curcumin caused no toxicity to osteoblasts. Subsequently, the effects of curcumin on the viability of DXM-stimulated osteoblasts were evaluated. In the first experiment, the cells were pre-treated using the indicated concentrations of curcumin for 2 h and then exposed to 100 nM DXM for 24 h. In this experiment, curcumin at 1 and 2 μ M significantly increased cell viability in a dose-dependent manner (Fig. 4C). In the second experiment, the cells were treated with the indicated concentrations of curcumin and 100 nM DXM simultaneously. In this experiment, curcumin also inhibited the toxic effects of DXM on primary osteoblasts in a dose-dependent manner, although the values were slightly lower than those in the first experiment (Fig. 4D).

Effects of curcumin on the differentiation and maturation of primary osteoblasts. Based on the results of MTT assay, we treated the cells with curcumin and DXM simultaneously in order to perform the following experiments. As expected, DXM at 100 nM induced a marked decrease in ALP activity in the primary osteoblasts. Curcumin at 2 μ M significantly reversed this decrease, but exerted little effect on normal cells (cells not stimulated with DXM; Fig. 5A). In addition, the mRNA expres-

sion levels of osteoblast differentiation-associated proteins were measured. As illustrated in Fig. 5B, the mRNA expression levels of osteocalcin, collagen, type 1, alpha 1 (Col1A1), osteonectin, runt-related transcription factor-2 (Runx2) and osterix were markedly downregulated following stimulation with DXM. We also examined the expression of two crucial factors related to osteogenesis, OPG and RANKL. DXM induced the marked downregulation of OPG and the upregulation of RANKL mRNA and protein expression (Fig. 5C and D). Curcumin at 2 μ M partly, but still significantly, reversed the effects of DXM on the expression of these genes.

Effects of curcumin on the Wnt signaling pathway in primary osteoblasts. The effects of curcumin on the Wnt signaling pathway were examined *in vitro*. In line with our *in vivo* experiments, DXM downregulated the mRNA expression of Wnt, β -catenin and LRP5, and upregulated the mRNA expression of Dickkopf-1 and SOST (Fig. 6A). In addition, GSK phosphorylation was inhibited by stimulation with DXM (Fig. 6B). These changes were partly reversed by treatment with curcumin. In addition, we used immunofluorescence staining in order to examine β -catenin trafficking in osteoblasts (Fig. 6C). In the control primary osteoblasts (no treatment), we observed a strong intranuclear staining of β -catenin, reflecting activated Wnt/ β -catenin signaling. Exposure to DXM reduced the intranuclear staining of β -catenin, representing the significant inhibition of Wnt/ β -catenin signaling. Treatment with curcumin markedly,

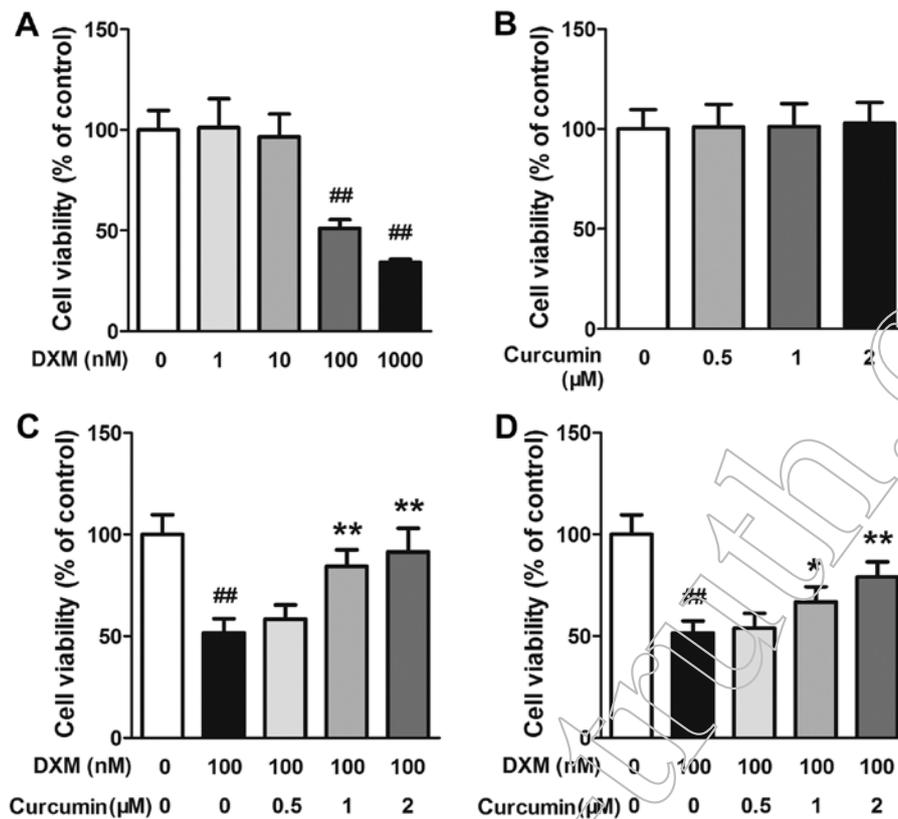


Figure 4. Effects of dexamethasone (DXM) and curcumin on cell viability. (A) Effect of DXM on cell viability. (B) Effect of curcumin on cell viability. (C) Effect of treatment with curcumin on the viability of DXM-exposed cells. (D) Effects of treatment of curcumin and DXM simultaneously on cell viability. Data are presented as the means \pm SD, n=3. ##P<0.01 vs. the control group, *P<0.05 and **P<0.01 vs. the DXM (100 nM) group.

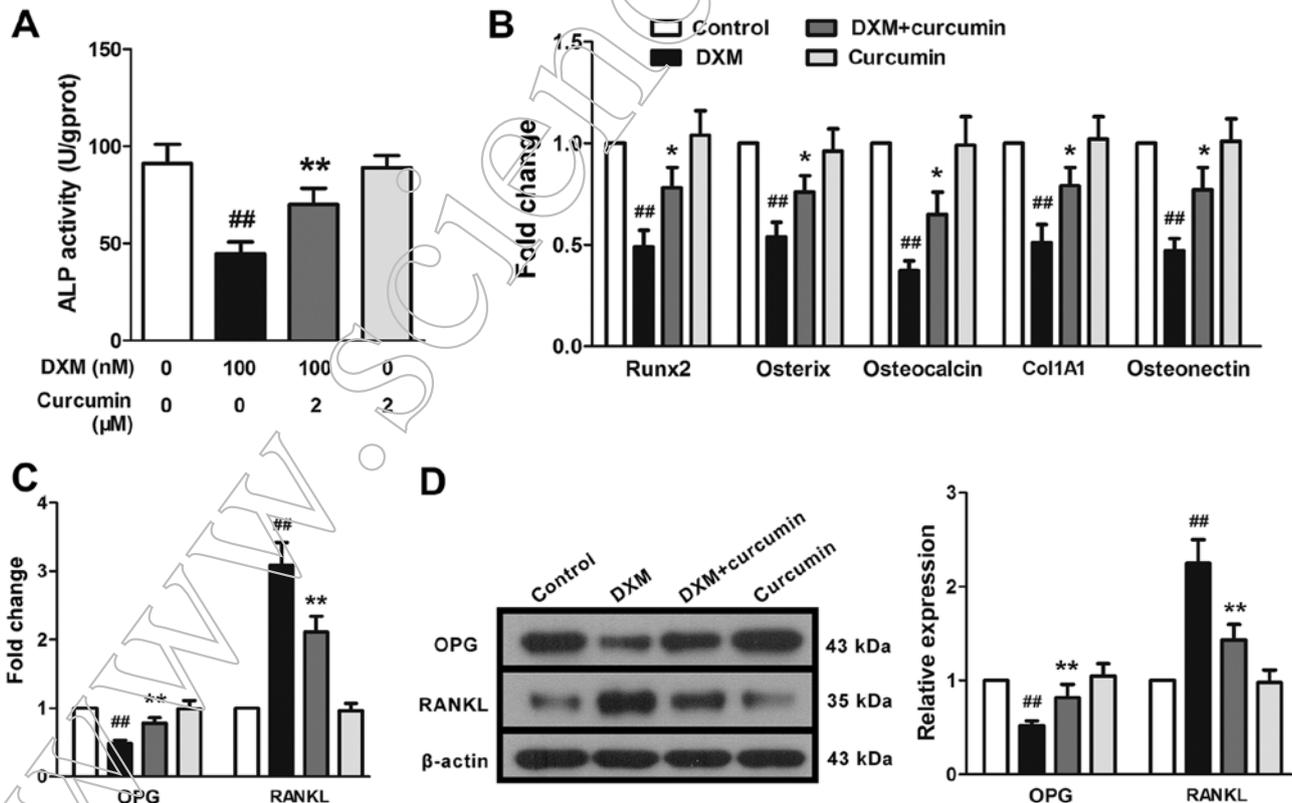


Figure 5. Effects of curcumin on the differentiation of primary osteoblasts. Following exposure to dexamethasone (DXM), (A) alkaline phosphatase (ALP) activity was markedly reduced, and (B) the mRNA expression levels of osteocalcin, collagen, type 1, alpha 1 (Col1A1), osteonectin, runt-related transcription factor 2 (Runx2) and osterix were significantly downregulated, and (C and D) osteoprotegerin (OPG) expression was diminished and receptor activator for nuclear factor-kappa B ligand (RANKL) expression was enhanced. Curcumin reversed these changes. Data are presented as the means \pm SD, n=3. ##P<0.01 vs. the control group, **P<0.05 vs. the DXM (100 nM) group.

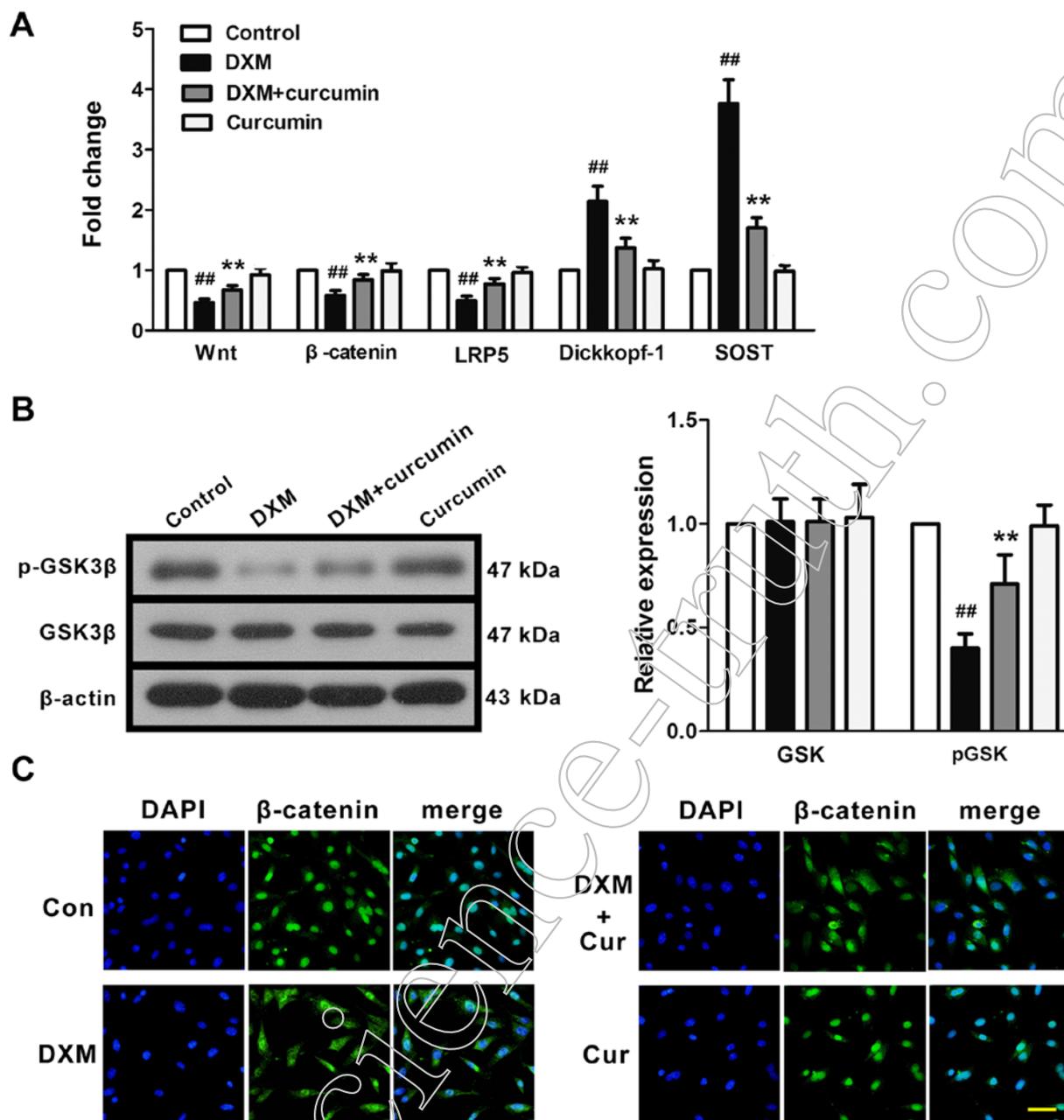


Figure 6. Effects of curcumin on the Wnt signaling pathway in primary osteoblasts. (A) The mRNA expression levels of Wnt, β -catenin, low-density lipoprotein receptor-related protein 5 (LRP5), sclerostin (SOST) and Dickkopf-1 in DXM-stimulated primary osteoblasts. (B) The phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) in dexamethasone (DXM)-stimulated primary osteoblasts. (C) β -catenin trafficking in DXM-stimulated primary osteoblasts. DXM significantly inhibited the Wnt signaling pathway, and curcumin prevented the inhibition. Scale bar, 50 μ m. Data are presented as the means \pm SD, n=3. ^{##}P<0.01 vs. the control group, ^{**}P<0.01 vs. the DXM (100 nM) group. Con, control; Cur, curcumin.

although not completely, restored the intranuclear staining of β -catenin. Curcumin did not affect β -catenin nuclear staining in the normal osteoblasts.

Discussion

In the present study, curcumin was found to be effective at attenuating DXM-induced osteoporosis both *in vivo* and *in vitro*, as evidenced through its restoration of BMD and the serum levels of the bone metabolic biomarkers, osteocalcin and CTX, in rats, and its regulation of bone differentiation and mature-associated proteins in primary osteoblasts. In addition,

the Wnt/ β -catenin signaling pathway was found to be re-activated by curcumin, which may be related to the bone-protective effects of curcumin. These results indicate that curcumin exerts protective effects against GIOP.

GIOP is the most common cause of iatrogenic osteoporosis (31). Osteoporosis is characterized by reduced bone mass and deterioration of the bone microarchitecture (32,33). In previous clinical studies, bone loss was observed in proximal femurs following treatment with GC (34,35). In previous studies, GC-treated animals also exhibited decreased BMD and bone mineral content (BMC) (36,37). In the present study, in line with these findings, DXM caused a significant reduc-

tion in BMD. However, following treatment with curcumin, this reduction in BMD was reversed. Trabecular bone is one of the types of bone which is most susceptible to the effects of GCs (38-40), and the proximal femur is rich in trabecular bone. In the present study, the structure of femurs was damaged upon DXM treatment, as represented by the occurrence of bone lacunae and thinned trabeculae, and these injuries were attenuated by treatment with curcumin. These results indicate the therapeutic effects of curcumin against GIOP.

The disruption of the bone formation-resorption balance plays a key role in osteoporosis (41). Serum osteocalcin is known as a marker of bone formation, and CTX is known as a marker of bone resorption (42). In the present study, the reduced levels of osteocalcin and the increased levels of CTX indicated the imbalance of bone formation-resorption in the rats with DXM-induced osteoporosis. In addition, in our *in vitro* experiments, DXM induced a marked reduction in ALP, a marker of osteoblast differentiation, in primary rat osteoblasts. Osteoblastic differentiation was also investigated at the gene level. A significant downregulation in the mRNA expression levels of Runx2, osterix, osteocalcin, CollA1 and osteonectin was observed in the DXM-stimulated osteoblasts. Runx2 and osterix are two major transcription factors that play an essential role in the formation of adult bones and the expression of osteoblast genes (43,44). Runx2 is the upstream controller of osterix (45,46). A number of bone matrix protein genes, such as CollA1, osteoponin and osteocalcin, are regulated by Runx2 (45). Osterix is a zinc finger-containing transcription factor. The conditional deletion of osterix postnatally severely disrupts the maturation, morphology and function of osteocytes (47). We found that curcumin recovered the serum osteocalcin and CTX levels and significantly reversed the DXM-induced decrease in Runx2 and osterix genes, thereby upregulating downstream gene expression and restoring ALP activity. These data suggest that the bone-protective effects of curcumin are mediated through the regulation of Runx2 and osterix expression.

Osteoblasts and osteoclasts are also important participants in the regulation of adult bone remodeling. OPG and RANKL are two key cytokines produced by osteoblasts that are involved in this process. RANKL stimulates bone resorption by binding with the receptor RANK on osteoclasts (48). OPG interacts with RANKL and inhibits its binding with the receptor (49). Therefore, the balance of RANKL and OPG production determines the rate of bone resorption and affects bone maturation (50). In this study, following the exposure of osteoblasts to DXM, we noted that the expression of OPG decreased and that of RANKL increased, indicating the abnormal enhancement of bone resorption. However, this induction of bone resorption was abolished by treatment with curcumin to some extent. This result indicates that the regulation of OPG and RANKL also contributes to the bone-protective effects of curcumin.

The canonical Wnt/ β -catenin signaling pathway is involved in a number of physiological processes throughout development and adult life, including bone formation (51). Wnt binds with specific cell-surface receptors Frizzled and LRP5/6, thereby leading to binding with Axin, which in turn mediates the proteolysis of β -catenin. LRP5 dysfunction leads to the development of diseases associated with the loss of bone mass,

such as osteoporosis pseudoglioma syndrome (OPS) (23). By contrast, the hyperfunction of LRP5 by mutations leads to the development of diseases associated with increased bone mass, such as sclerosteosis and osteopetrosis. In our study, we noted that Wnt, β -catenin and LRP5 gene expression was significantly diminished following exposure to DXM both *in vivo* and *in vitro*, which suggests that this signaling pathway was inhibited. SOST and Dickkopf-1 are receptor inhibitors which play a key role in the regulation of the Wnt signaling pathway in bone formation (52). SOST binds to LRP6 to inhibit its association with Wnt. Previous studies have indicated that serum SOST is positively correlated with BMD and BMC (53,54). The effects of GCs on SOST expression appear complex and remain to be clarified in detail, as SOST expression was found to be both decreased (55) and increased (56) in bone cells following exposure to GCs. In our study, consistent with the findings of Yao *et al* (56), DXM induced the marked upregulation of SOST expression. Dickkopf (Dkk-1) is also known to inhibit Wnt signaling by binding to LRP5/6 (57). The transient overexpression of Dkk-1 has been shown to markedly decrease ALP activity induced by Wnt3a and reduce the mineralizing capacity in mouse pre-osteoblastic MC3T3-E1 cells (58). In agreement with the findings of previous studies (59-61), we also noted a significant increase in Dkk-1 mRNA expression following exposure to DXM.

GSK-3 β is another negative regulator of the Wnt signaling pathway at the β -catenin level (57). GSK-3 β phosphorylates β -catenin in the cytosol and targets it for proteolysis, thereby inhibiting its nuclear translocation. The induction of the phosphorylation of GSK-3 β inhibits its activity and allows β -catenin to translocate to the nucleus and interact with T-cell factor/lymphocyte enhancer-binding factor (TCF/LEF) to regulate the transcription of target genes (62). Previous studies have demonstrated that the GC-induced activation of GSK-3 β in osteoblasts and the inhibition of GSK-3 β attenuated bone loss (63-65). In the present study, the phosphorylation of GSK-3 β was reduced following exposure to DXM, which was accompanied by the decreased nuclear expression of β -catenin. These data demonstrate that the inhibition of the entire Wnt signaling pathway, from the upstream cell surface receptor to the downstream β -catenin nuclear translocator, was inhibited by exposure to DXM. As expected, treatment with curcumin recovered the activity of this signaling pathway. These results suggest that the Wnt/ β -catenin signaling pathway is involved in the bone-protective effects of curcumin against DXM-induced osteoporosis.

In conclusion, in this study, we demonstrated that curcumin attenuated DXM-induced osteoporosis *in vivo* and osteoblast differentiation dysfunction *in vitro*. Furthermore, the mechanisms underlying the bone-protective effects of curcumin against DXM-induced osteoporosis involved the activation of the Wnt/ β -catenin pathway. In future, curcumin may become a potential bone-protective therapeutic agent for the treatment of GIOP.

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References

- Buttgereit F, Burmester GR and Lipworth BJ: Optimised glucocorticoid therapy: the sharpening of an old spear. *Lancet* 365: 801-803, 2005.
- den Uyl D, Bultink IE and Lems WF: Advances in glucocorticoid-induced osteoporosis. *Curr Rheumatol Rep* 13: 233-240, 2011.
- Hallberg I, Bachrach-Lindström M, Hammerby S, Toss G and Ek AC: Health-related quality of life after vertebral or hip fracture: a seven-year follow-up study. *BMC Musculoskelet Disord* 10: 135, 2009.
- Cauley JA, Thompson DE, Ensrud KC, Scott JC and Black D: Risk of mortality following clinical fractures. *Osteoporos Int* 11: 556-561, 2000.
- Weinstein RS: Glucocorticoid-induced osteoporosis. *Rev Endocr Metab Disord* 2: 65-73, 2001.
- Recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis: 2001 update. American College of Rheumatology Ad Hoc Committee on Glucocorticoid-Induced Osteoporosis. *Arthritis Rheum* 44: 1496-1503, 2001.
- Adler RA and Hochberg MC: Suggested guidelines for evaluation and treatment of glucocorticoid-induced osteoporosis for the Department of Veterans Affairs. *Arch Intern Med* 163: 2619-2624, 2003.
- Nawata H, Soen S, Takayanagi R, Tanaka I, Takaoka K, Fukunaga M, Matsumoto T, Suzuki Y, Tanaka H, Fujiwara S, *et al*: Guidelines on the management and treatment of glucocorticoid-induced osteoporosis of the Japanese Society for Bone and Mineral Research (2004). *J Bone Miner Metab* 23: 105-109, 2005.
- Sambrook PN, Diamond T, Ferris L, Fiatarone-Singh M, Flicker L, MacLennan A, Nowson C, O'Neill S, Greville H: Osteoporosis Australia; National Asthma Campaign: Corticosteroid induced osteoporosis. Guidelines for treatment. *Aust Fam Physician* 30: 793-796, 2001.
- Lestari ML and Indrayanto G: Curcumin. *Profiles Drug Subst Excip Relat Methodol* 39: 113-204, 2014.
- Jurenka JS: Anti-inflammatory properties of curcumin, a major constituent of *Curcuma longa*: a review of preclinical and clinical research. *Altern Med Rev* 14: 141-153, 2009.
- Kim WK, Ke K, Sul OJ, Kim HJ, Kim SH, Lee MH, Kim HJ, Kim SY, Chung HT and Choi HS: Curcumin protects against ovariectomy-induced bone loss and decreases osteoclastogenesis. *J Cell Biochem* 112: 3159-3166, 2011.
- Folwarczna J, Zych M and Trzeciak HI: Effects of curcumin on the skeletal system in rats. *Pharmacol Rep* 62: 900-909, 2010.
- Hussan F, Ibraheem NG, Kamarudin TA, Shuid AN, Soelaiman IN and Othman F: Curcumin protects against ovariectomy-induced bone changes in rat model. *Evid Based Complement Alternat Med* 2012: 174916, 2012.
- Cho DC, Jung HS, Kim KT, Jeon Y, Sung JK and Hwang JH: Therapeutic advantages of treatment of high-dose curcumin in the ovariectomized rat. *J Korean Neurosurg Soc* 54: 461-466, 2013.
- Yang MW, Wang TH, Yan PP, Chu LW, Yu J, Gao ZD, Li YZ and Guo BL: Curcumin improves bone microarchitecture and enhances mineral density in APP/PS1 transgenic mice. *Phytomedicine* 18: 205-213, 2011.
- Chen Z, Xue J, Shen T, Ba G, Yu D and Fu Q: Curcumin alleviates glucocorticoid-induced osteoporosis by protecting osteoblasts from apoptosis in vivo and in vitro. *Clin Exp Pharmacol Physiol*: Oct 30, 2015 (Epub ahead of print).
- Cadigan KM and Nusse R: Wnt signaling: a common theme in animal development. *Genes Dev* 11: 3286-3305, 1997.
- Glass DA II, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA and Karsenty G: Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell* 8: 751-764, 2005.
- Song L, Liu M, Ono N, Bringham FR, Kronenberg HM and Guo J: Loss of wnt/ β -catenin signaling causes cell fate shift of preosteoblasts from osteoblasts to adipocytes. *J Bone Miner Res* 27: 2344-2358, 2012.
- Pinson KI, Brennan J, Monkley S, Avery BJ and Skarnes WC: An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407: 535-538, 2000.
- Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeanet JP and He X: LDL-receptor-related proteins in Wnt signal transduction. *Nature* 407: 530-535, 2000.
- Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, *et al*: Osteoporosis-Pseudoglioma Syndrome Collaborative Group: LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 107: 513-523, 2001.
- Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, *et al*: A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am J Hum Genet* 70: 11-19, 2002.
- Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K and Lifton RP: High bone density due to a mutation in LDL-receptor-related protein 5. *N Engl J Med* 346: 1513-1521, 2002.
- Tiwari SK, Agarwal S, Seth B, Yadav A, Nair S, Bhatnagar P, Karmakar M, Kumari M, Chauhan LK, Patel DK, *et al*: Curcumin-loaded nanoparticles potently induce adult neurogenesis and reverse cognitive deficits in Alzheimer's disease model via canonical Wnt/ β -catenin pathway. *ACS Nano* 8: 76-103, 2014.
- Tiwari SK, Agarwal S, Tripathi A and Chaturvedi RK: Bisphenol-A mediated inhibition of hippocampal neurogenesis attenuated by curcumin via canonical Wnt pathway. *Mol Neurobiol*: May 12, 2015 (Epub ahead of print).
- Chen F, Wang H, Xiang X, Yuan J, Chu W, Xue X, Zhu H, Ge H, Zou M, Feng H and Lin J: Curcumin increased the differentiation rate of neurons in neural stem cells via wnt signaling in vitro study. *J Surg Res* 192: 298-304, 2014.
- He M, Li Y, Zhang L, Li L, Shen Y, Lin L, Zheng W, Chen L, Bian X, Ng HK and Tang L: Curcumin suppresses cell proliferation through inhibition of the Wnt/ β -catenin signaling pathway in medulloblastoma. *Oncol Rep* 32: 173-180, 2014.
- Cui L, Jia X, Zhou Q, Zhai X, Zhou Y and Zhu H: Curcumin affects β -catenin pathway in hepatic stellate cell in vitro and in vivo. *J Pharm Pharmacol* 66: 1615-1622, 2014.
- Albaum JM, Levesque LE, Gershon AS, Liu G and Cadarette SM: Glucocorticoid-induced osteoporosis management among seniors, by year, sex, and indication, 1996-2012. *Osteoporos Int* 26: 2845-2852, 2015.
- Baofeng L, Zhi Y, Bei C, Guolin M, Qingshui Y and Jian L: Characterization of a rabbit osteoporosis model induced by ovariectomy and glucocorticoid. *Acta Orthop* 81: 396-401, 2010.
- Castañeda S, Calvo E, Largo R, González-González R, de la Piedra C, Díaz-Curiel M and Herrero-Beaumont G: Characterization of a new experimental model of osteoporosis in rabbits. *J Bone Miner Metab* 26: 53-59, 2008.
- Reid IR, Evans MC, Wattie DJ, Ames R and Cundy TF: Bone mineral density of the proximal femur and lumbar spine in glucocorticoid-treated asthmatic patients. *Osteoporos Int* 2: 103-105, 1992.
- Sambrook P, Birmingham J, Kempler S, Kelly P, Eberl S, Pocock N, Yeates M and Eisman J: Corticosteroid effects on proximal femur bone loss. *J Bone Miner Res* 5: 1211-1216, 1990.
- Feng M, Zhang R, Gong F, Yang P, Fan L, Ni J, Bi W, Zhang Y, Wang C and Wang K: Protective effects of necrostatin-1 on glucocorticoid-induced osteoporosis in rats. *J Steroid Biochem Mol Biol* 144B: 455-462, 2014.
- Samir SM and Malek HA: Effect of cannabinoid receptors 1 modulation on osteoporosis in a rat model of different ages. *J Physiol Pharmacol* 65: 687-694, 2014.
- Ehrlich PJ and Lanyon LE: Mechanical strain and bone cell function: a review. *Osteoporos Int* 13: 688-700, 2002.
- Laan RF, Buijs WC, van Erning LJ, Lemmens JA, Corstens FH, Ruijs SH, van de Putte LB and van Riel PL: Differential effects of glucocorticoids on cortical appendicular and cortical vertebral bone mineral content. *Calcif Tissue Int* 52: 5-9, 1993.
- Reid IR and Heap SW: Determinants of vertebral mineral density in patients receiving long-term glucocorticoid therapy. *Arch Intern Med* 150: 2545-2548, 1990.
- Kim HJ, Zhao H, Kitaura H, Bhattacharyya S, Brewer JA, Muglia LJ, Ross FP and Teitelbaum SL: Glucocorticoids suppress bone formation via the osteoclast. *J Clin Invest* 116: 2152-2160, 2006.
- Miyazaki T, Matsunaga T, Miyazaki S, Hokari S and Komoda T: Changes in receptor activator of nuclear factor- κ B, and its ligand, osteoprotegerin, bone-type alkaline phosphatase, and tartrate-resistant acid phosphatase in ovariectomized rats. *J Cell Biochem* 93: 503-512, 2004.
- Wysokinski D, Pawlowska E and Blasiak J: RUNX2: A master bone growth regulator that may be involved in the DNA damage response. *DNA Cell Biol* 34: 305-315, 2015.

44. Sinha KM and Zhou X: Genetic and molecular control of osterix in skeletal formation. *J Cell Biochem* 114: 975-984, 2013.
45. Komori T: Regulation of skeletal development by the Runx family of transcription factors. *J Cell Biochem* 95: 445-453, 2005.
46. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR and de Crombrughe B: The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108: 17-29, 2002.
47. Zhou X, Zhang Z, Feng JQ, Dusevich VM, Sinha K, Zhang H, Darnay BG and de Crombrughe B: Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice. *Proc Natl Acad Sci USA* 107: 12919-12924, 2010.
48. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Goto M, Mochizuki SI, Tsuda E, Morinaga T, Udagawa N, *et al*: A novel molecular mechanism modulating osteoclast differentiation and function. *Bone* 25: 109-113, 1999.
49. Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T and Higashio K: Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun* 234: 137-142, 1997.
50. Buckley KA and Fraser WD: Receptor activator for nuclear factor kappaB ligand and osteoprotegerin: regulators of bone physiology and immune responses/potential therapeutic agents and biochemical markers. *Ann Clin Biochem* 39: 551-556, 2002.
51. Kim JH, Liu X, Wang J, Chen X, Zhang H, Kim SH, Cui J, Li R, Zhang W, Kong Y, *et al*: Wnt signaling in bone formation and its therapeutic potential for bone diseases. *Ther Adv Musculoskelet Dis* 5: 13-31, 2013.
52. Rossini M, Gatti D and Adami S: Involvement of WNT/ β -catenin signaling in the treatment of osteoporosis. *Calcif Tissue Int* 93: 121-132, 2013.
53. Amrein K, Amrein S, Drexler C, Dimai HP, Dobnig H, Pfeifer K, Tomaschitz A, Pieber TR and Fahrleitner-Pammer A: Sclerostin and its association with physical activity, age, gender, body composition, and bone mineral content in healthy adults. *J Clin Endocrinol Metab* 97: 148-154, 2012.
54. Sheng Z, Tong D, Ou Y, Zhang H, Zhang Z, Li S, Zhou J, Zhang J and Liao E: Serum sclerostin levels were positively correlated with fat mass and bone mineral density in central south Chinese postmenopausal women. *Clin Endocrinol (Oxf)* 76: 797-801, 2012.
55. Sutherland MK, Geoghegan JC, Yu C, Winkler DG and Latham JA: Unique regulation of SOST, the sclerosteosis gene, by BMPs and steroid hormones in human osteoblasts. *Bone* 35: 448-454, 2004.
56. Yao W, Cheng Z, Pham A, Busse C, Zimmermann EA, Ritchie RO and Lane NE: Glucocorticoid-induced bone loss in mice can be reversed by the actions of parathyroid hormone and risedronate on different pathways for bone formation and mineralization. *Arthritis Rheum* 58: 3485-3497, 2008.
57. Bafico A, Liu G, Yaniv A, Gazit A and Aaronson SA: Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol* 3: 683-686, 2001.
58. Rawadi G, Vayssière B, Dunn F, Baron R and Roman-Roman S: BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J Bone Miner Res* 18: 1842-1853, 2003.
59. Mak W, Shao X, Dunstan CR, Seibel MJ and Zhou H: Biphasic glucocorticoid-dependent regulation of Wnt expression and its inhibitors in mature osteoblastic cells. *Calcif Tissue Int* 85: 538-545, 2009.
60. Ohnaka K, Taniguchi H, Kawate H, Nawata H and Takayanagi R: Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis. *Biochem Biophys Res Commun* 318: 259-264, 2004.
61. Wang FS, Ko JY, Yeh DW, Ke HC and Wu HL: Modulation of Dickkopf-1 attenuates glucocorticoid induction of osteoblast apoptosis, adipocytic differentiation, and bone mass loss. *Endocrinology* 149: 1793-1801, 2008.
62. Lin X, Farooqi AA and Ismail M: Recent progress in fungus-derived bioactive agents for targeting of signaling machinery in cancer cells. *Drug Des Devel Ther* 9: 1797-1804, 2015.
63. Wang FS, Chuang PC, Lin CL, Chen MW, Ke HJ, Chang YH, Chen YS, Wu SL and Ko JY: MicroRNA-29a protects against glucocorticoid-induced bone loss and fragility in rats by orchestrating bone acquisition and resorption. *Arthritis Rheum* 65: 1530-1540, 2013.
64. Smith E and Frenkel B: Glucocorticoids inhibit the transcriptional activity of LEF/TCF in differentiating osteoblasts in a glycogen synthase kinase-3 β -dependent and -independent manner. *J Biol Chem* 280: 2388-2394, 2005.
65. Wang FS, Ko JY, Weng LH, Yeh DW, Ke HJ and Wu SL: Inhibition of glycogen synthase kinase-3 β attenuates glucocorticoid-induced bone loss. *Life Sci* 85: 685-692, 2009.