

Epigallocatechin-3-gallate protects against secondary osteoporosis in a mouse model via the Wnt/ β -catenin signaling pathway

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Abstract. Epigallocatechin-3-gallate (EGCG) is a polyphenolic compound extracted and isolated from green tea, which has a variety of important biological activities *in vitro* and *in vivo*, including anti-tumor, anti-oxidation, anti-inflammation and lowering blood pressure. The aim of the present study was to investigate the protective effect of EGCG against secondary osteoporosis in a mouse model via the Wnt/ β -catenin signaling pathway. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to analyze runt-related transcription factor 2 and osterix mRNA expression, and the protein expression of cyclin D1, Wnt and β -catenin, and suppressed peroxisome proliferator-activated receptor γ protein expression. The protective effect of EGCG against secondary osteoporosis was examined and its potential mechanism was analyzed. Treatment with EGCG significantly decreased serum calcium, urinary calcium, body weight and body fat, and increased leptin levels in mice with secondary osteoporosis. In addition, EGCG treatment significantly inhibited the structure score of articular cartilage and cancellous bone in proximal tibia metaphysis in mice with secondary osteoporosis. Treatment also significantly decreased alkaline phosphatase activity, runt-related transcription factor 2 and osterix mRNA expression. EGCG also significantly induced the protein expression of cyclin D1, Wnt and β -catenin, and suppressed peroxisome proliferator-activated receptor γ protein expression in mice with secondary osteoporosis. Taken together, these results suggest that EGCG may be a possible new drug in clinical settings.

Introduction

Osteoporosis is a type of systemic bone disease that is characterized by low bone mass, microstructural damage of the bone, increased bone fragility and potential bone fracture (1). Osteoporosis can be divided into two categories, primary type and secondary type (2). The former is further divided into postmenopausal (type I), senile (type II) and idiopathic osteoporosis disease. The latter refers to osteoporosis induced by diseases that influence bone physiology or by drugs, such as osteoporosis induced by long-term high-dose glucocorticoid treatment (3). In general, postmenopausal (type I) osteoporosis occurs within 5-10 years following the menopause in women, senile osteoporosis develops in individuals aged >70 years old and idiopathic osteoporosis is predominantly observed in adolescents; however, its pathogenesis is unknown (3). Due to the wide application of glucocorticoids in clinical practice, the incidence of glucocorticoid-induced osteoporosis has continuously risen in recent years, and as such, it is now considered the third most common type of osteoporosis, following postmenopausal and senile osteoporosis (4).

The role of Wnt signaling in bone metabolism has become a key area of interest in recent years. Previous studies have demonstrated that Wnt can directly affect the pluripotent precursor cell differentiation process into bone cells (4,5). The stable expression of Wnt1 and Wnt3a can promote the proliferation of osteocytes and induce alkaline phosphatase (ALP) activity, which is the early stage marker of osteoblast differentiation (6). However, apart from ALP, the other relevant markers for osteoblast differentiation, including runt-related transcription factor 2 (Runx2), osteocalcin (OC) and type I collagen, are not markedly affected, which indicates that Wnts can promote the growth of precursor osteoblasts and promote osteoblast differentiation at the early stage (7).

β -catenin serves an important role in the Wnt signaling pathway. The bone stem cell lineage can differentiate into osteoblasts, adipocytes and chondrocytes; it can also be differentiated into osteoblasts via the action of bone morphogenetic protein 2 (BMP-2). During this process, BMP-2 can upregulate β -catenin (8). The overexpression β -catenin in the C3H10T1/2 cell line can increase the expression and activity of ALP; however, it had no significant effect on OC, which is a differentiation marker of osteoblasts during the late stage (9).

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This suggests that β -catenin may serve a role in precursor osteoblast and osteoblast proliferation and differentiation, and that it may be regulated by BMP-2 (10).

Tea contains a large amount of polyphenolic compounds, which are collectively known as tea polyphenols, accounting for 18-36% of the dry weight of tea; a number of studies have shown that polyphenols have a strong antioxidant capacity and serve as natural food antioxidant additives (11,12). A previous study investigating the chemical compositions of tea polyphenols demonstrated that the main components of tea polyphenols are catechins, accounting for 70 to 80% of total polyphenols, which mainly included catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin-3-gallate (EGCG) and other active substances; EGCG occupied the highest content percentage of ~50 to 60% of total catechins (12). A large number of studies have revealed that EGCG has biological activities including anti-cancer, anti-mutation, the prevention and treatment of cardiovascular diseases, and regulating the endocrine and immune systems; it also has inhibitory activity associated with metabolic enzymes, which have a marked impact on the liver detoxification function of the body (13,14). The aim of the present study was to investigate the potential anti-osteoporosis effects of EGCG in secondary osteoporosis and the potential underlying mechanism in a mouse model.

Materials and methods

Animal experiments. The animal protocol was approved by the Committee on the Ethics of Animal Experiments of The 309th Hospital of The People's Liberation Army (Beijing, China). A total of 22 6-week-old male C57BLKS/J mice (weight, 20-22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were given free access to food and water. They were caged individually under a controlled temperature (23-25°C) and humidity (50-60%) with a 12 h artificial light/dark cycle. The present study used a dexamethasone-induced model of osteoporosis, as described previously (4). Mice were divided into 3 groups: The control (n=6), model (n=8) and EGCG (0.5 mg/kg/day; IP, n=8; Fig. 1) groups. In the model and EGCG groups, mice were injected with dexamethasone (5 mg/kg/day) into the intraarticular space of the right knee for 4 weeks to establish the osteoporosis model (15). In EGCG groups, mice were injected with 0.5 mg/kg/day of EGCG for 4 weeks. At 0 and 4 week, body weight was record, and blood was were collected from tail vein and used to measure body fat using a commercial kit (A042-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Body weight and body fat measurements. The mice were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium and body weight was measured at the 0 and 4 week time points of EGCG treatment. In addition, Dual-Energy X-ray Absorptiometry (Lunar Prodigy; GE Healthcare, Chicago, IL, USA) was used to measure body fat at the 0 and 4 week time points of EGCG treatment.

Serum calcium, urinary calcium, and bone and energy metabolism. The mice were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium. Then, blood was collected from the tail vein and blood was transferred

to tubes. A TechniconSMAC (Technicon Instruments Corp, Tarrytown, NY, USA) determined the serum calcium level. Urinary calcium/creatinine (cat no. C004-2) and ALP activity (cat no. A059-2) was measured using ELISA kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The leptin serum concentrations were assayed at Novartis International AG (Basel, Switzerland) using a Luminex 200™ Multiplexing Instrument.

Histomorphological analysis. Following treatment with EGCG, mice were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium and sacrificed using decollation. Femoral head tissue was dissected by the axial plane and were fixed in 4% formaldehyde at room temperature for >24 h. These bone samples were then decalcified in 10% ethylene diamine tetraacetic acid solution for 15 days at room temperature and embedded in paraffin. Samples were cut into 4 mm thick sections and stained with hematoxylin and eosin at 5 min at room temperature. The articular cartilage (AC) and cancellous bone in proximal tibia metaphysis (PTM) were then observed using a fluorescent microscope (x20; Zeiss Axioplan 2-300, Carl Zeiss MicroImaging) using the Mankin histological grading system, as described previously (16).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was prepared using TRIzol Reagent (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) from femoral head tissue. Total RNA was then reverse transcribed using a Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR was conducted using a Rotor-Gene 3000 System (Corbett Life Science; Qiagen, Inc., Valencia, CA, USA) and the SYBR Green PCR Master Mix Reagent kit (Qiagen, Inc.). The primers used were as follows: Runx2 forward, 5'-TGTCATGGCGGGTAACGATG-3' and reverse, 5'-CCCTAAATCACTGAGGCGGT-3'; OSX forward, 5'-CCTCTGCGGGACTCAACAAC-3' and reverse, 5'-AGC CCATTAGTGCTTGTAAGG-3'; and GAPDH forward, 5'-CTATAAATTGAGCCCGCAGC-3' and reverse, 5'-GAC CAAATCCGTTGACTCCG-3'. The thermocycling conditions were: 95°C for 10 min, then 40 cycles of 95°C for 30 sec, 60°C for 45 sec, followed by 72°C for 30 sec. The expression was quantified using $2^{-\Delta\Delta C_t}$ method (17).

Western blotting. Total proteins were extracted from femoral head tissue using Radioimmunoprecipitation Assay Lysis Buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and measured using an Enhanced Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). 50 μ g proteins were separated by 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Following incubation in 5% skim milk powder and TBST in 0.1% Tween-20 for 1 h at room temperature, the membrane was hybridized with anti-Cyclin D1 (cat no. sc-717; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Wnt (sc-136163; 1:1,000; Santa Cruz Biotechnology, Inc.), anti- β -catenin (cat no. sc-7199; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-peroxisome proliferator-activated receptor (PPAR)- γ (cat no. sc-81152; 1:1,000; Santa Cruz Biotechnology, Inc.) and anti-GAPDH

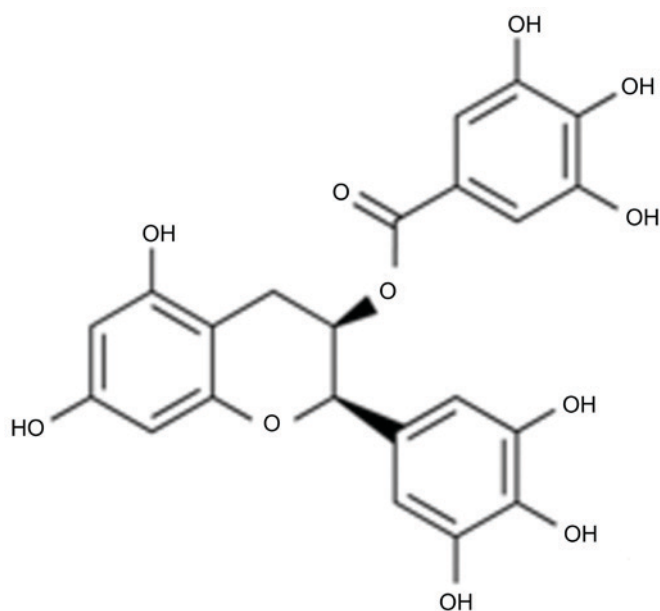


Figure 1. Chemical structure of epigallocatechin-3-gallate.

(cat no. sc-25778; 1:5,000;) primary antibodies overnight at 4°C. The membrane was then incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (cat no. sc-2004 or sc-2005, respectively; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. Proteins were detected with an enhanced chemiluminescence reagent (Amersham; GE Healthcare) and analyzed using Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are presented as the mean \pm standard error of the mean of three independent experiments. The results were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA), and a one- or two-way analysis of variance with a Tukey post hoc test were performed to evaluate the data. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EGCG protects against lipid metabolism. The effect of EGCG on lipid metabolism was evaluated in a mouse model of secondary osteoporosis. Following the 4 week treatment period, the body weight and body fat content in the secondary osteoporosis mouse model group were markedly higher than those of control group (Fig. 2). Treatment with EGCG for 4 weeks effectively reduced this secondary osteoporosis-induced increase in body weight and body fat content (Fig. 2).

EGCG protects against secondary osteoporosis. Under experimental conditions, the levels of leptin, calcium, calcium activity and urine calcium/creatinine (Ca/Cr) were detected. As shown in Fig. 3A, the leptin level was significantly decreased in secondary osteoporosis mouse model group at 4 weeks when compared with the control group. In addition, the levels of calcium, calcium activity and urine Ca/Cr in the secondary osteoporosis mouse model group were significantly increased when compared with the control group (Fig. 3B-D).

Treatment with EGCG effectively enhanced the level of leptin and inhibited the levels of calcium, calcium activity and urine Ca/Cr when compared with the model group (Fig. 3).

EGCG protects bone structure and bone turnover. To determine the anti-osteoporosis effects of EGCG associated with bone structure and bone turnover, the AC and PTM structure scores were analyzed. As shown in Fig. 4, the AC and PTM structure scores in the secondary osteoporosis mouse model group were significantly greater than those of the control group. EGCG treatment significantly decreased the AC and PTM structure scores when compared with the model group (Fig. 4).

EGCG decreases secondary osteoporosis-induced ALP activity, and Runx2 and Sp7 transcription factor osterix (OSX) mRNA expression. To determine whether ALP, Runx2 and OSX are involved in the effects of EGCG on osteoporosis, ALP, Runx2 and OSX mRNA expression were measured using RT-qPCR. A significant increase in ALP, Runx2 and OSX mRNA expression was observed in the secondary osteoporosis mouse model group when compared with the control group (Fig. 5). In addition, treatment with EGCG significantly reduced this secondary osteoporosis-induced increase in ALP, Runx2 and OSX mRNA expression (Fig. 5).

EGCG decreases secondary osteoporosis-induced Runx2, OSX and PPAR γ protein expression. The effects of EGCG on Runx2, OSX and PPAR γ protein expression were further determined by western blotting (Fig. 6A). Osteoporosis significantly induced Runx2 (Fig. 6B), OSX (Fig. 6C) and PPAR γ (Fig. 6D) protein expression in the secondary osteoporosis mouse model group when compared with the control group. In addition, treatment with EGCG significantly suppressed this osteoporosis-induced increase in Runx2, OSX and PPAR γ protein expression.

EGCG increases Wnt, β -catenin and cyclin D1 protein expression in secondary osteoporosis. To investigate the role of Wnt, β -catenin and cyclin D1 in the effect of EGCG on osteoporosis, western blotting was performed (Fig. 7A). Wnt (Fig. 7B), β -catenin (Fig. 7C) and cyclin D1 (Fig. 7D) protein expression in the secondary osteoporosis mouse model group was significantly lower than that of the control group. However, treatment with EGCG significantly increased Wnt, β -catenin and cyclin D1 protein expression when compared with the secondary osteoporosis mouse model group.

Discussion

Osteoporosis is a common degenerative disease that primarily causes increased bone fragility and reduced bone density, which eventually leads to bone fracture (18). China has the greatest aging population in the world, of which at least 90 million people suffer from osteoporosis; it is expected that the number of patients with osteoporosis will increase to 221 million by the year 2050 (19). As osteoporosis severely affects the health of the elderly, identifying prevention strategies and treatments has become a major public health concern. The results of the present study demonstrated that

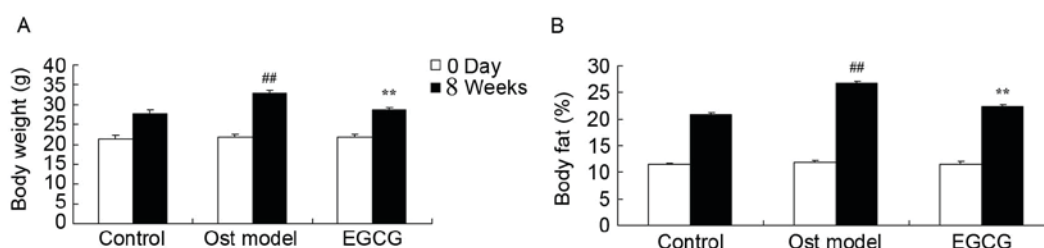


Figure 2. EGCG protects against adverse lipid metabolism. Following 4 weeks of EGCG treatment the (A) body weight and (B) percentage of body fat in mice with secondary osteoporosis was significantly decreased. ^{##}*P*<0.01 vs. control group; ^{**}*P*<0.01 vs. osteoporosis model group. Control, control group; Ost model, secondary osteoporosis model group; EGCG, epigallocatechin-3-gallate group.

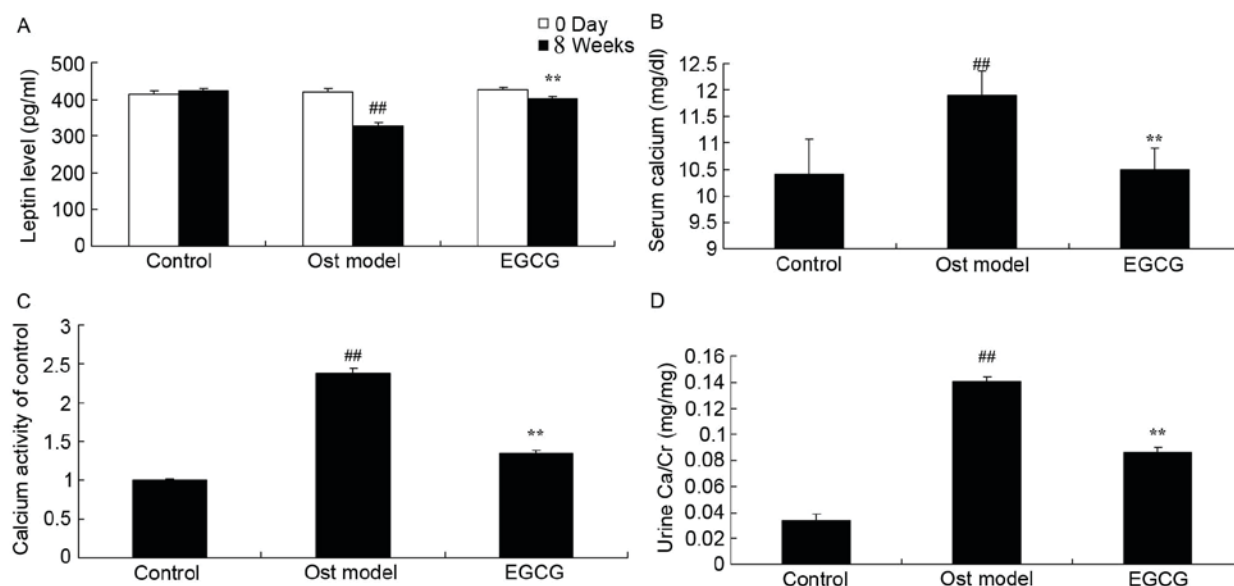


Figure 3. EGCG protects against secondary osteoporosis. EGCG treatment was protective of (A) the levels of leptin, (B) the calcium level, (C) calcium activity and (D) urine Ca/Cr in a mouse with secondary osteoporosis. ^{##}*P*<0.01 vs. control group; ^{**}*P*<0.01 vs. osteoporosis model group. Control, control group; Ost model, secondary osteoporosis model group; EGCG, epigallocatechin-3-gallate group; Ca/Cr, calcium/creatinine.

EGCG is protective against adverse lipid metabolism, and bone structure and turnover in mice with secondary osteoporosis, which suggests that EGCG may exert anti-osteoporosis effects.

Osteoblasts are mainly derived from bone marrow mesenchymal stem cells, the differentiation process of which is dependent on the regulation of a number of transcription factors and cytokines (20). Bone marrow mesenchymal stem cells are pluripotent stem cells that can differentiate into osteoblasts, chondrocytes and adipocytes (21). A previous study revealed that when the cells have high expressions of Runx2 and OSX, mesenchymal stem cells are differentiated into osteoblasts (21). However, when these cells highly express sex-determining region Y-box 9, mesenchymal stem cells are differentiated into chondrocytes, and when they have a high expression of PPAR, they differentiate into chondrocytes (22). The results of the present study demonstrated that EGCG treatment significantly reduced ALP, Runx2 and OSX mRNA expression, and suppressed Runx2 and OSX protein expression in a mouse model of secondary osteoporosis.

Marrow fat cells are derived from mesenchymal stem cells. PPAR γ mediates marrow mesenchymal stem cell differentiation into fat cells. PPAR γ is a subtype of PPARs, which is a

ligand-activated nuclear transcription factor that is involved in cell differentiation, growth and apoptosis (23). In addition, PPAR γ mediates the adipogenesis of marrow mesenchymal stem cells (24). A higher expression of PPAR γ protein in mice indicates a significant increase in bone turnover and lower osteogenic differentiation in marrow (25). These data demonstrated that EGCG significantly inhibited PPAR γ protein expression in secondary osteoporosis. Lee *et al* (26) suggested that EGCG may suppress lipid deposition through PPAR γ and the WNT/ β -catenin signaling pathway.

Runx2 gene expression is regulated by a variety of hormones, cytokines and endogenous active substances (25). A number of signaling pathways have been shown to participate in the regulation of Runx2 expression or activity (25). The Wnt-low-density lipoprotein receptor-related protein 5 (LRP5)- β -catenin signaling pathway serves an important role in osteoblast proliferation and differentiation (27). Wnt and LRP5/6 are combined to form a complex receptor that induces β -catenin activation and migration to the nucleus; β -catenin in the nucleus binds the lymphoid enhancer-binding factor-1/T cell factor 1 transcription factors to form a compound that activates the transcription of the *Runx2* gene (28). The addition of Wnt inhibitors can inhibit the proliferation and differentiation

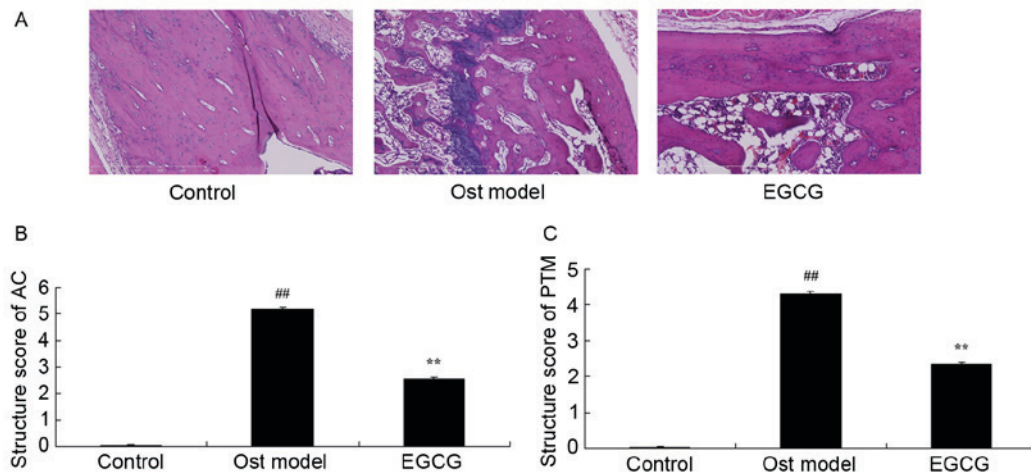


Figure 4. EGCG protects bone structure and turnover in secondary osteoporosis. (A) Hematoxylin and eosin staining of bone tissues to analyze the (B) AC and (C) PTM scores in mice with secondary osteoporosis. EGCG protected mice with secondary osteoporosis against adverse (B) bone structure of AC and (C) PTM of bone turnover in a mouse model of secondary osteoporosis. Magnification, $\times 100$. ^{##} $P < 0.01$ vs. control group; ^{**} $P < 0.01$ vs. osteoporosis model group. Control, control group; Ost model, secondary osteoporosis model group; EGCG, epigallocatechin-3-gallate group; AC, articular cartilage; PTM, proximal tibia metaphysis.

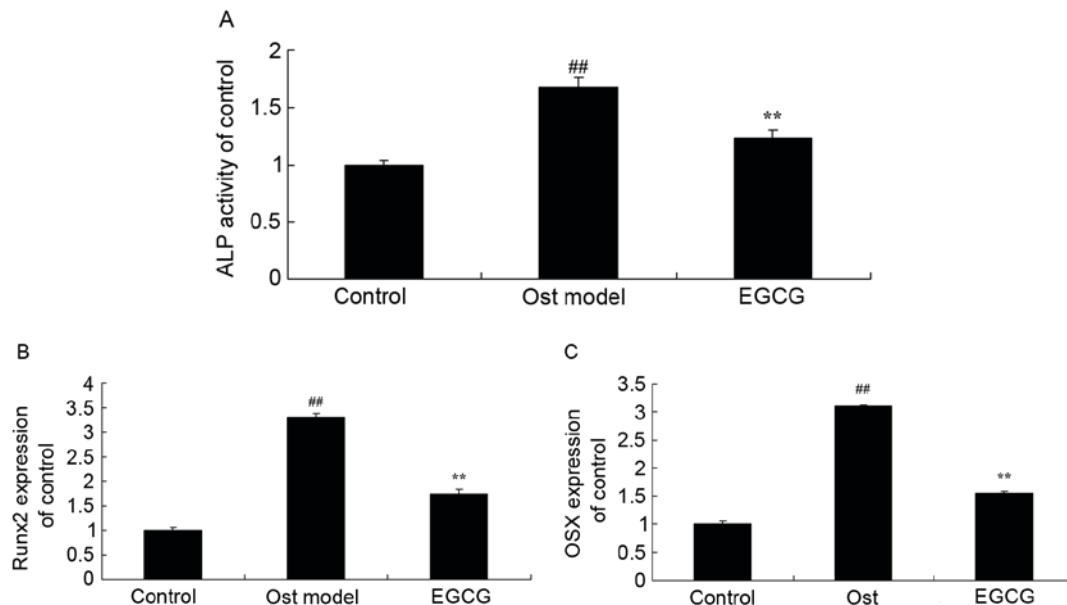


Figure 5. EGCG restores ALP activity, and Runx2 and OSX mRNA expression. Secondary osteoporosis significantly increased (A) ALP activity, and (B) Runx2 and (C) OSX expression, however, EGCG treatment decreased these levels in a mouse model of secondary osteoporosis. ^{##} $P < 0.01$ vs. control group; ^{**} $P < 0.01$ vs. osteoporosis model group. Control, control group; Ost model, secondary osteoporosis model group; EGCG, epigallocatechin-3-gallate group; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; OSX, Sp7 transcription factor osterix.

of osteoblasts. In mice without the secreted frizzled-related protein 1 gene, the Wnt signaling pathway can be significantly activated, Runx2 promoter activity is increased and the mRNA content of the Runx2 gene is increased; this also indicates that the Wnt- β -catenin signaling pathway may regulate the expression of Runx2 (29). Previous studies have revealed that BMP2 can activate mothers against decapentaplegic homolog 1/5 (Smad1/5) in osteoblasts, and induce Smad4 activation and nuclear translocation, thereby activating Runx2 (9,30). Taken together, the experimental results of the present study demonstrated that EGCG significantly induced β -catenin and Wnt3a protein expression in mice with secondary osteoporosis. Thus, β -catenin/Wnt may regulate the expression as well as the

activities of PPAR γ in secondary osteoporosis treated with EGCG.

CyclinD1 synthesis and expression is dependent on growth factors, and following the removal of growth factors, CyclinD1 synthesis is terminated immediately (31). Therefore, it is regarded to have the effect of growth factor sensors; namely, the growth factor-induced signal is associated with the regulation of the cell cycle. Cyclin D1, cyclin dependent kinase 4 (CDK4) and CDK6 combine to form a CDK4/6-CyclinD1 complex that promotes the G1 phase of the cell cycle; CyclinD1 is a key protein for the cell proliferation signals of G1 phase (32). The present study revealed that EGCG treatment significantly induced Cyclin

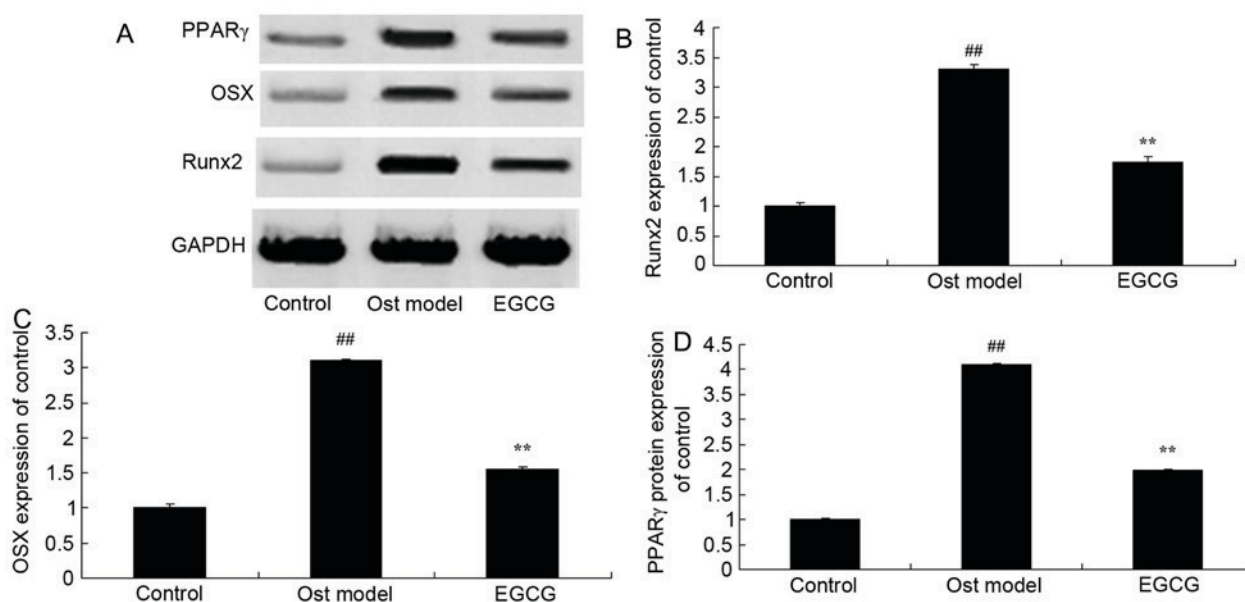


Figure 6. EGCG and Runx2, OSX and PPAR γ protein expression. EGCG protects against Runx2 protein expression as shown by (A) western blot analysis. (B) Runx2, (C) OSX and (D) PPAR γ protein expression increased in the mouse model of secondary osteoporosis, however, treatment with EGCG reversed this effect and decreased the expression of these proteins. ## $P < 0.01$ vs. control group; ** $P < 0.01$ vs. osteoporosis model group. Control, control group; Ost model, secondary osteoporosis model group; EGCG, epigallocatechin-3-gallate group; Runx2, runt-related transcription factor 2; OSX, Sp7 transcription factor osterix; PPAR γ , peroxisome proliferator-activated receptor γ .

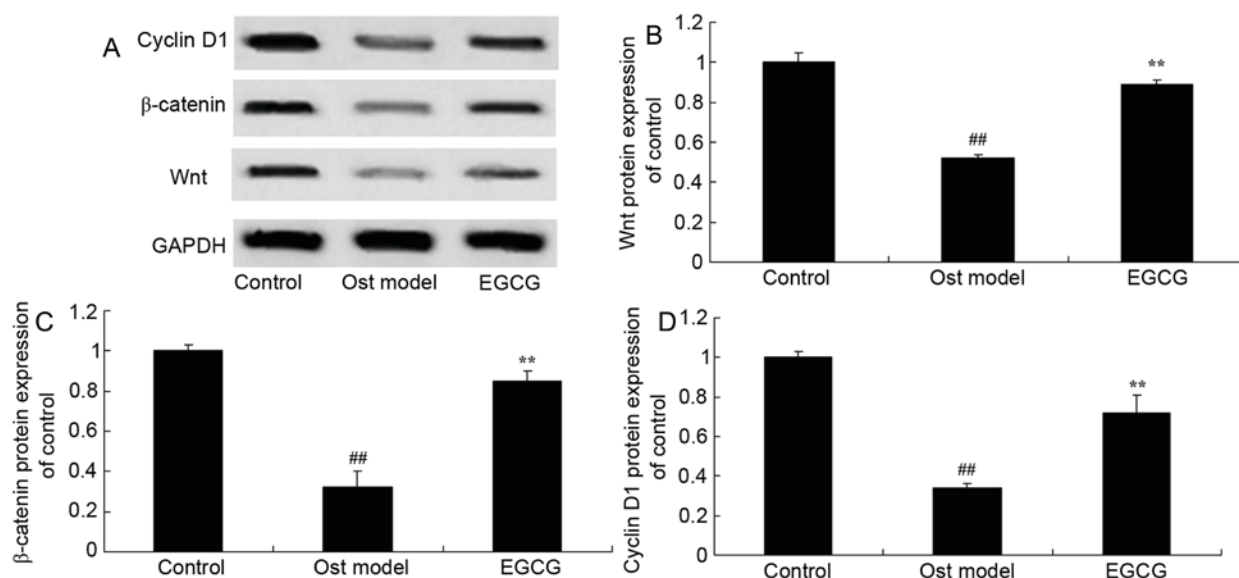


Figure 7. EGCG and Wnt, β -catenin and Cyclin D1 protein expression. EGCG protects against Wnt protein expression as shown by (A) western blot analysis. (B) Wnt, (C) β -catenin and (D) Cyclin D1 protein expression decreased in the mouse model of secondary osteoporosis, however, treatment with EGCG reversed this effect and increased the expression of these proteins. ## $P < 0.01$ vs. control group; ** $P < 0.01$ vs. osteoporosis model group. Control, control group; Ost model, secondary osteoporosis model group; EGCG, epigallocatechin-3-gallate group.

D1 protein expression in mice with secondary osteoporosis. Wang *et al* (33) reported that EGCG treatment protects against the hydrogen peroxide-induced inhibition of osteogenic differentiation through β -catenin and cyclin D1 expression in human bone marrow-derived mesenchymal stem cells.

In conclusion, the results of the present study indicated that EGCG treatment may be protective against lipid metabolism, secondary osteoporosis, and bone structure and turnover in mice with secondary osteoporosis induced by

dexamethasone, as indicated by the analysis of PPAR γ and the Wnt/ β -catenin signaling pathway. These results suggest that PPAR γ and Wnt/ β -catenin may be novel therapeutic targets for future treatments of secondary osteoporosis using EGCG.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QL designed the experiment; JX, XL, JL, LG, HX and GW performed the experiments; QL and JX analyzed the data; QL wrote the manuscript.

Ethics approval and consent to participate

The animal protocol was approved by the Committee on the Ethics of Animal Experiments of the 309th Hospital of The People's Liberation Army (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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