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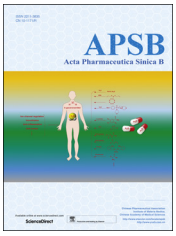

ORIGINAL ARTICLE

Calycosin-7-*O*-*β*-D-glucopyranoside stimulates osteoblast differentiation through regulating the BMP/WNT signaling pathways

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KEY WORDS

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ST2 cells

Abstract The isoflavone calycosin-7-*O*-*β*-D-glucopyranoside (CG) is a principal constituent of *Astragalus membranaceus* (AR) and has been reported to inhibit osteoclast development *in vitro* and bone loss *in vivo*. The aim of this study was to investigate the osteogenic effects of CG and its underlying mechanism in ST2 cells. The results show that exposure of cells to CG in osteogenic differentiation medium increases ALP activity, osteocalcin (*Ocal*) mRNA expression and the osteoblastic mineralization process. Mechanistically, CG treatment increased the expression of bone morphogenetic protein 2 (BMP-2), p-Smad 1/5/8, *β*-catenin and Runx2, all of which are regulators of the BMP- or wingless-type MMTV integration site family (WNT)/*β*-catenin-signaling pathways. Moreover, the osteogenic effects of CG were inhibited by Noggin and DKK-1 which are classical inhibitors of the BMP and WNT/*β*-catenin-signaling pathways, respectively. Taken together, the results indicate that CG promotes the osteoblastic

Abbreviations: ALP, alkaline phosphatase; AR, *Astragalus membranaceus*; BMP, bone morphogenetic protein; CG, calycosin-7-*O*-*β*-D-glucopyranoside; DKK-1, dickkopf-1; ECL, enhanced chemiluminescence; FGF, fibroblast growth factor; HAase, hyaluronidase; IGF1, insulin-like growth factor 1; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OBM, osteogenic differentiation medium; Ocal, osteocalcin; OPN, osteopontin; OVX, ovariectomized; PVDF, polyvinylidine fluoride; TGF-*β*, transforming growth factor *β*; WNT, wingless-type MMTV integration site family

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differentiation of ST2 cells through regulating the BMP/WNT signaling pathways. On this basis, CG may be a useful lead compound for improving the treatment of bone-decreasing diseases and enhancing bone regeneration.

1. Introduction

Regulation of bone mass is controlled by continuous bone remodeling through osteoblastic bone formation and resorption. Disorders of bone remodeling are implicated in a variety of diseases such as osteoporosis, hypercalcemia and rheumatoid arthritis as well as tumor metastasis into bone[1]. Understanding osteoblastic differentiation is therefore crucial to improving the treatment of such disorders.

Osteoblasts are the main bone-forming cells arising from mesenchymal stem cells. They produce alkaline phosphatase (ALP) and bone matrix proteins such as osteocalcin (Ocal) and osteopontin (OPN) which act to induce osteoblastic mineralization[2]. Osteoblast differentiation is regulated by various signaling pathways involving bone morphogenetic proteins (BMPs), wingless-type MMTV integration site family (WNT)/*β*-catenin proteins, transforming growth factor-*β* (TGF-*β*), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), and mitogen-activated protein kinase (MAPK)[3–5]. Of these pathways, the one involving BMPs is key in skeletal development, maintaining adult bone homeostasis and stimulating bone formation in fracture healing[6]. Activation of the WNT/*β*-catenin signaling pathway is essential for proper bone development[7] and, in cooperation with the BMP signaling pathway, regulates osteoblast differentiation and bone formation[8].

Astragalus membranaceus (AR) is one of the most important medicinal plants in traditional Chinese medicine. In recent years, it has received considerable attention because of its immunostimulant effects[9], antibacterial and antiviral properties, hepatoprotective and antiinflammatory activity and beneficial cardiovascular effects[10]. Of particular interest is the observation that AR inhibits osteoclast development *in vitro* and bone loss *in vivo* in ovariectomized (OVX) rats[11]. Moreover, AR combined with calcium has been shown to significantly improve bone mineral density, biomechanical strength, and ash weight of the femur and tibia of OVX rats[12]. However, the main osteogenically active components of AR remain to be identified.

The isoflavone calycosin-7-*O*-*β*-D-glucopyranoside (CG, Fig. 1) is a principal constituent of AR. It is a strong inhibitor of hyaluronidase (HAase)[13] and of matrix degradation caused by IL-1*β* or HAase in human articular cartilage explant and chondrocytes[14]. Since it is known that isoflavones are active in preventing osteoporosis[15,16], it is reasonable to hypothesize that CG may exhibit osteogenic effects. Accordingly this study aimed to investigate the osteogenic effects of CG and its role in the osteogenic differentiation of bone marrow stromal cell.

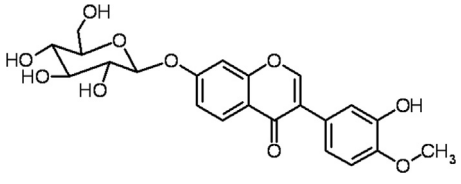

Figure 1 Chemical structure of calycosin-7-*O*-*β*-D-glucopyranoside (CG).

2. Materials and methods

2.1. Cell culture

Bone marrow stromal ST2 cells were seeded at a density of 1×10^5 cells/mL and cultured in regular growth culture medium containing *α*-minimum essential medium supplemented with 15% fetal bovine serum (Biochrom, Australia), 100 units/mL penicillin (Gibco, Australia) and 100 mg/L streptomycin (Gibco) in a humidified atmosphere of 5% CO_2 at 37 °C. At 80% confluence, the cells were cultured in osteogenic differentiation medium (OBM) which consisted of the above culture medium containing 10 nmol/L dexamethasone (Sigma-Aldrich, USA), 10 mmol/L *β*-glycerophosphate (Sigma-Aldrich), 50 μg/mL ascorbic acid (Sinopharm Chemical Reagent, China) and various concentrations of CG (98.3%, National Institutes for Food and Drug Control, CAS 20633-67-4, China) added as a solution in dimethyl sulfoxide (Sigma-Aldrich, final concentration 0.1%).

2.2. Cell viability assay

Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) assay. Briefly, cells (1×10^4 cells/well in 96-well plates) were maintained in OBM at 37 °C for 24 h. Cells were then treated with CG (4, 8, 16 and 32 μmol/L) in OBM for 1, 3 and 6 days at which times 20 μL MTT (5 mg/mL) was added to each well and samples incubated in the dark at 37 °C. After 4 h, medium was discarded and the precipitated formazan dissolved in DMSO (150 μL/well). Absorbance was measured with a microplate

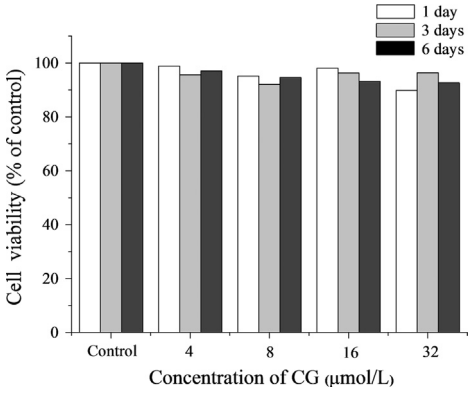

Figure 2 Effect of CG on the proliferation of ST2 cells. Cells were seeded in 96-well plates for 24 h and then treated with different concentrations of CG for 1, 3 and 6 days. Cellular proliferation was determined using the MTT assay. Results are expressed as means ± SD ($n = 3$).

reader (iMARK^{tm}, BIO-RAD) at 570 nm. Cell viability in OBM (without CG) was used as control and designated as 100%.

2.3. ALP activity assay

After incubation with CG (4, 8, 16 and 32 μmol/L) in OBM for 3, 7 and 9 days, ST2 cells were collected and lysed with 0.1% Triton X-100. *p*-Nitrophenyl phosphate (pNPP, Sigma-Aldrich) was used as the substrate to measure the intracellular ALP activity. Briefly, 100 μL lysate supernatant was incubated with 100 μL substrate solution containing 3 mmol/L pNPP, 1 mol/L diethanolamine buffer and 0.5 mmol/L $MgCl_2$ for 30 min at 37 °C. The reaction was stopped by adding 0.2 mol/L NaOH solution and absorbance determined using a microplate reader at 405 nm. Relative ALP activity was normalized to the protein concentration of each sample assayed using the BCA method and then to the control.

2.4. Alizarin red-S staining

After incubation of cells with CG (4, 8, 16 and 32 μmol/L) in OBM for 28 days, mineralized nodule formation was determined using Alizarin red-S (ARS)[17]. Briefly, cells were washed twice with PBS, fixed in ice-cold 70% ethanol for 1 h at room temperature and then stained with 40 mmol/L ARS solution (pH 4.2) at room temperature for 30 min. Images of the stained matrix were acquired using a digital camera. To quantify mineralization, stained cells were dissolved by adding 10% cetylpyridinium chloride for 1 h and transferred to a 96-well plate for measuring absorbance at 570 nm with a microplate reader.

2.5. RNA isolation and real-time PCR (RT-PCR)

After incubation of cells with CG (4, 8, 16 and 32 μmol/L) in OBM for 9 days, the cells were collected and total RNA extracted using TRIzol reagent (Invitrogen, USA). RNA of each sample was reverse transcribed to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen). cDNA was then amplified using GoTaq® DNA Polymerase (Promega, USA) and SYBR Green PCR Master Mix (Applied Biosystems, USA). The expression levels were quantified using a CFX Connect™ Real-Time System (BIO-RAD). The primers used for real-time PCR were as follows: *Runx2:* 5′-TGCTTCAT TCGCCTCACAAA-3′ (sense) and 5′-TTGCAGTCTTCCTGGA-GAAAGTT-3′ (antisense); *Ocal:* 5′-TGCTTGTGACGAGCTAT CAG-3′ (sense) and 5′-TGAACTAGGAGGGACAGGAG-3′ (anti-sense); *BMP2:* 5′-TGAGGATTAGCAGGTCTTTG-3′ (sense) and 5′-

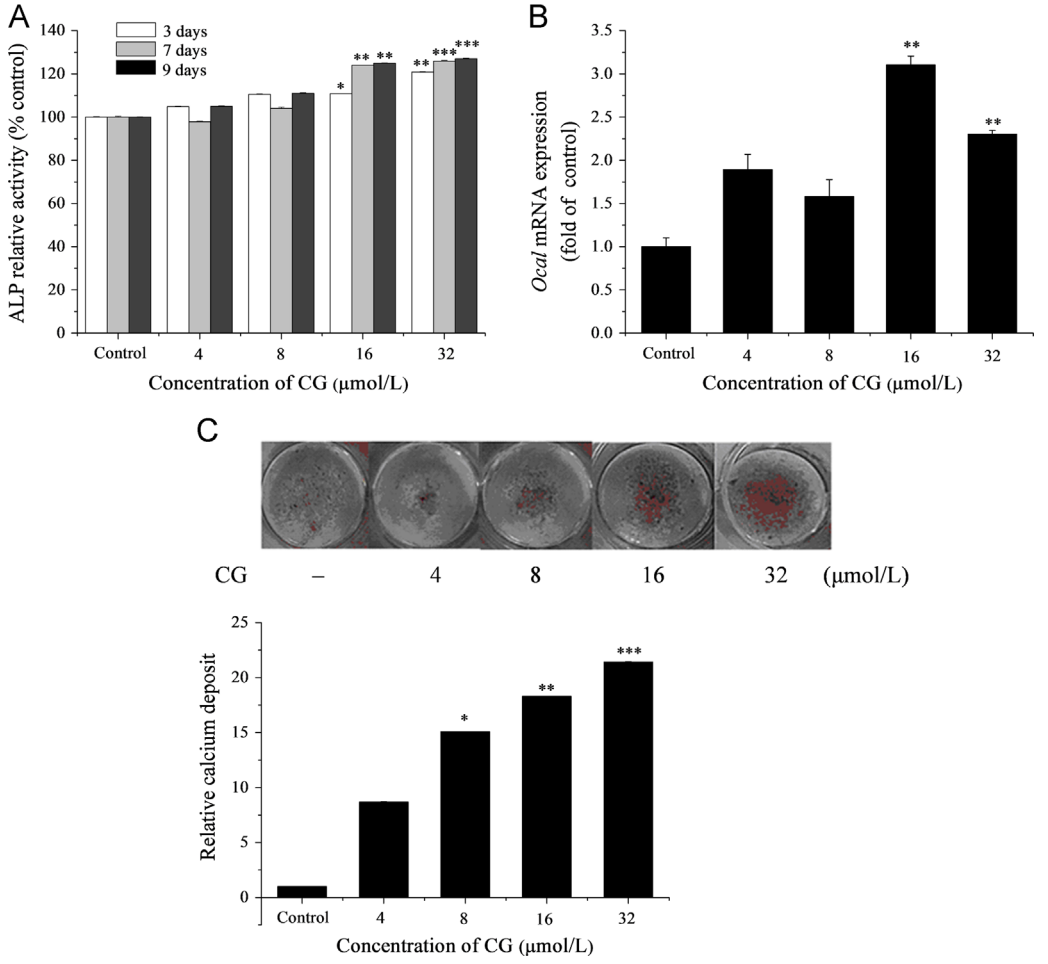

Figure 3 Effect of CG on osteoblast differentiation in ST2 cells as indicated by: (A) ALP activity where cells were cultured in OBM as described in Section 2 and treated with different concentrations of CG for 3, 7 and 9 days prior to determination of ALP activity; (B) expression of the osteoblast marker gene *Ocal* after exposure to CG at the indicated concentrations for 9 days. mRNA was determined by real-time PCR analysis; and (C) osteoblastic mineralization. Cells were cultured in OBM and treated with CG for 28 days after which mineralization deposits were identified by Alizarin red S staining. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control.

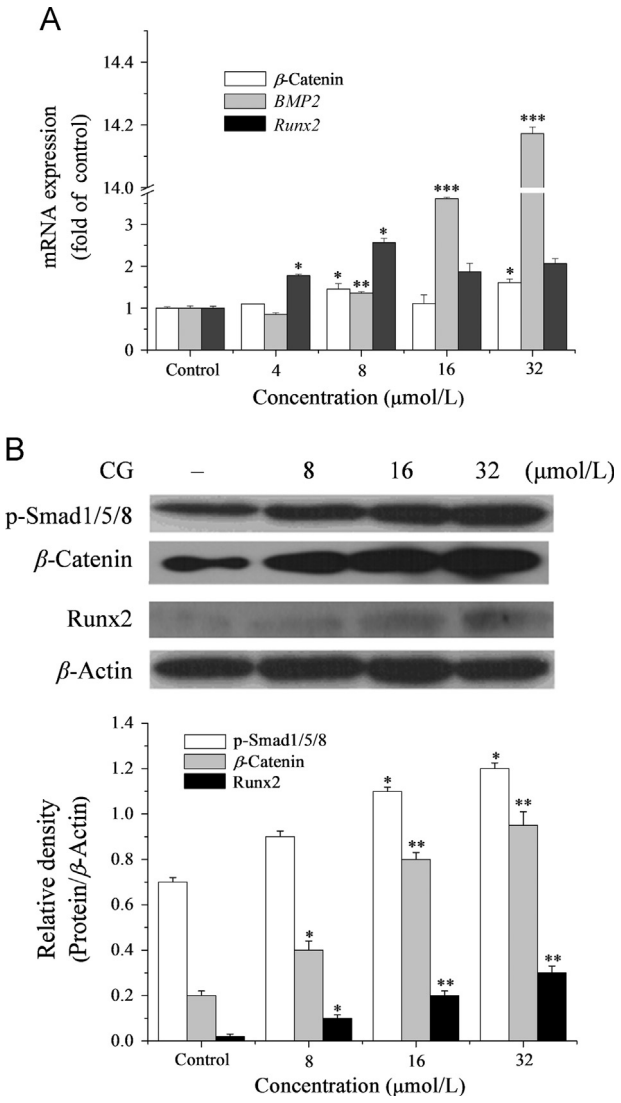

Figure 4 Effect of exposure to CG for 9 days on the BMP/WNT pathway in ST2 cells as indicated by: (A) expression of the osteoblast marker genes, *BMP-2*, *Runx2* and *β*-catenin. mRNAs determined by real-time PCR analysis; (B) expression of the osteoblast markers, p-Smad1/5/8, *β*-catenin and Runx2. Protein expression was determined by Western blot analysis. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control.

CACAACCATGTCCTGATAAT-3′ (antisense); *β*-catenin*:* 5′-CCG-TTCGCCTTCATTATGGA-3′ (sense) and 5′-CCTAACTAAGC-TTTGGAACGG-3′ (antisense); *GAPDH:* 5′-CCGTTCGCCTTCAT-TATGGA-3′ (sense) and 5′-CCTAACTAAGCTTTGGAACGG-3′ (antisense). Values were normalized to that of *GAPDH* using the $2^{-\Delta\Delta CT}$ method.

2.6. *Protein isolation and Western blotting*

After incubation of cells with CG (8, 16 and 32 μmol/L) in OBM for 9 days, the cells were collected and lysed in RIPA buffer (20 mmol/L Tris-HCl, 200 mmol/L NaCl, 1% Triton X-100, 1 mmol/L dithiothreitol) containing 1% protease inhibitor (Roche). The concentration of protein was measured using a Protein Assay Kit (BIO-RAD). Total protein from each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidine fluoride (PVDF) membrane. The blotting membrane was then incubated with primary antibodies of anti-Runx2, anti-*β*-catenin and anti-p-Smad1/5/8 (Santa Cruz Biotechnology). Subsequently, the blots were washed with TBST (10 mmol/L Tris-HCl, 50 mmol/L NaCl, 0.25% Tween 20) and incubated with HRP-conjugated secondary antibody. The blots were visualized with enhanced chemiluminescence (ECL) and exposed to photographic film. *β*-Actin was used as a loading control.

2.7. *Osteogenetic analysis after co-treatment with CG and Noggin or Dickkopf (DKK-1)*

After incubation of cells with 16 μmol/L CG and 0.5 μg/mL Noggin (Sigma-Aldrich) or 0.1 μg/mL DKK-1 (Peprotech, USA) in OBM for 9 days, cells were collected, lysed with 0.1% Triton X-100 and subjected to determination of ALP activity as mentioned above. In addition, cells were collected, lysed in RIPA buffer and protein expression of p-Smad1/5/8 and *β*-catenin determined by Western blotting.

2.8. *Statistical analyses*

Data were analyzed using SPSS 13.0 software. Values are expressed as mean ± S.E.M. unless otherwise indicated. Data analysis was performed by one way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Differences for which $P<0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. *Effect of CG on ST2 cell viability*

ST2 cells are a type of bone marrow stromal cell which can be differentiated into osteoblast-like cells in OBM[18] by inducing the formation of a matrix of type I collagen and, through subsequently activating the BMP signaling pathway[19], be stimulated to further differentiate into mature osteoblasts. Activation of WNT/*β*-catenin signaling induces differentiation of pluripotent mesenchymal cells into osteoblast progenitors. When these osteoprogenitors become osteoblasts, both the BMP and WNT/*β*-catenin pathways can promote further differentiation as evidenced by increased ALP activity and mineralization[20,21]. On this basis, ST2 cells were used in the present study to investigate the osteogenic effect of CG. After incubation of ST2 cells in OBM with up to 32 μmol/L CG for up to 6 days, there was no evidence of cytotoxicity to the cells (Fig. 2). In addition, DMSO was not cytotoxic to ST2 cells at the final concentration of 0.1% (data not shown).

3.2. *CG promotes osteoblastic differentiation of ST2 cells*

The process of bone formation is reported to first involve osteoblast proliferation followed by increased ALP activity. The latter is a well-recognized early marker of osteoblast differentiation, development and maturation of extracellular matrix leading ultimately to mineralization[22]. Our *in vitro* data show that exposure to increasing concentrations of CG for 3, 7 and 9 days caused increases in ALP activity which was significant at 16 μmol/L and more significant at 32 μmol/L (Fig. 3A). Expression of mRNA by the osteogenic marker gene *Ocal* was also increased by treatment with 16 and 32 μmol/L CG for 9 days (Fig. 3B). In addition, exposure to CG treatment for 28 days caused a significant increase in mineralized nodule formation (a well-

recognized late marker of osteoblast differentiation) at concentrations >8 μmol/L (Fig. 3C). These indicators of bone-formation appear to have little correlation with stimulation of cellular proliferation as the growth rate of ST2 cells slowed only slightly with exposure to increasing CG concentrations (Fig. 2).

3.3. *Mechanism of CG-induced osteogenesis*

The BMP pathway is one of the main signaling cascades that stimulate bone formation. The mechanism of receptor activation involves BMP-induced phosphorylation of two sequentially activated kinases, with the type I receptor acting as a substrate for the type II receptor kinase. The activated type I receptor relays the signal to the cytoplasm by phosphorylating its downstream target, Smad1/5/8 protein, which then interacts with Smad4 and translocates into the nucleus[23]. A number of compounds have been found to affect this pathway by increasing the expression of BMPs and/or activating the downstream signaling pathway[24–27].

The results of this study indicate that not only *BMP-2* mRNA expression is dose-dependently increased by CG treatment (4 − 32 μmol/L) for 9 days (Fig. 4A), but also the translational level of phosphorylated Smad1/5/8 (Fig. 4B). We also showed that Noggin (a specific inhibitor of the BMP pathway[28]) significantly inhibited the increase in CG-induced ALP activity and Smad1/5/8 phosphorylation in ST2 cells (Fig. 5A and B). These two findings are the first evidence that BMP signaling is involved in bone metabolism regulated by CG. Also the fact that Noggin significantly inhibited the CG-induced increase in ALP activity and Smad1/5/8 phosphorylation further confirms that CG-induced osteogenic regulation is involved in the BMP pathway.

WNT/β-catenin signaling is another key pathway in osteoblastic differentiation that contributes to regulating bone formation and remodeling[29,30]. The results show that CG treatment (8 − 32 μmol/L) of ST2 cells increased the transcriptional and translational level of β-catenin, and that co-treatment with CG and DKK-1 (a specific inhibitor of the WNT/β-catenin signaling pathway[29]) significantly inhibited increases in ALP activity and β-catenin protein expression (Figs. 4 and 5). Thus it is clear that WNT/β-catenin signaling is also involved in CG-induced osteogenesis in ST2 cells.

As the master osteogenic transcription factor, Runx2, is a downstream regulator of the WNT/BMP pathway and plays a critical role in the process of osteoblast maturation[31] and *Ocal* expression[32]. The results of this study indicate that mRNA and

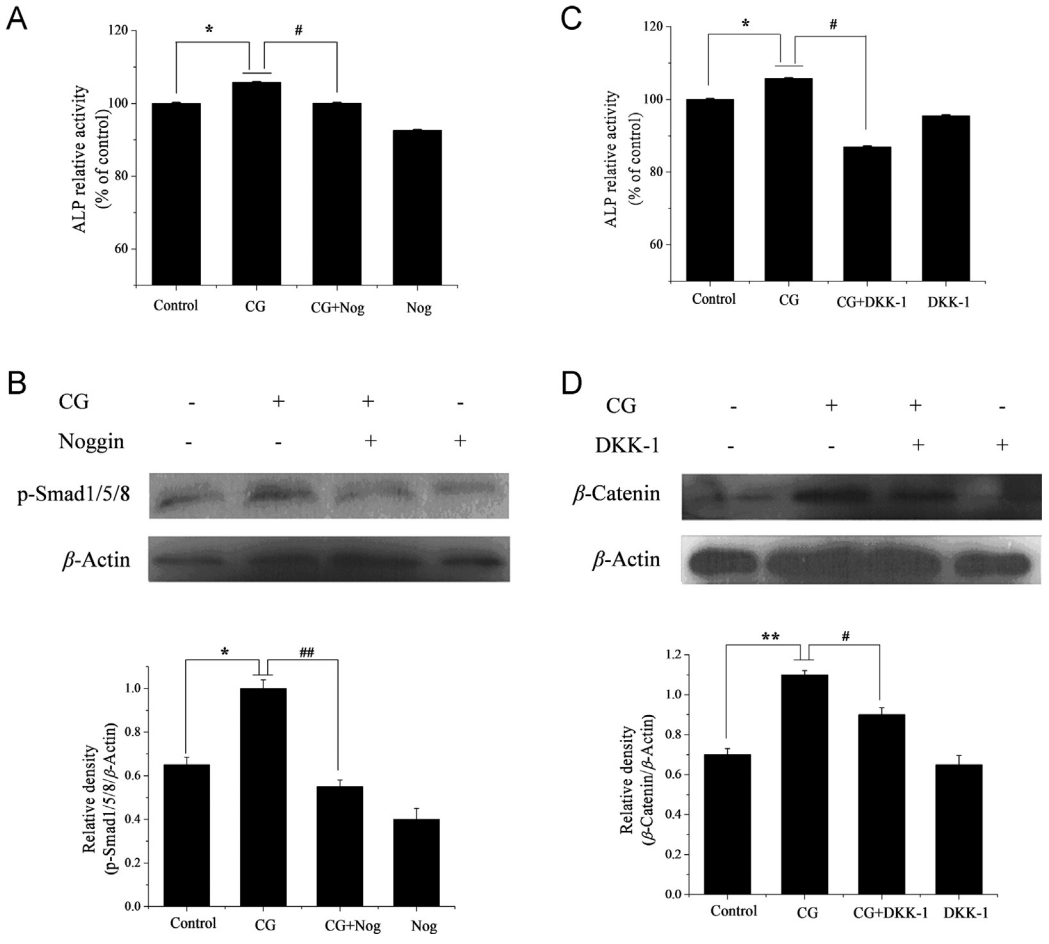

Figure 5 Effect of BMP/WNT pathway inhibitors on the osteogenic effects of CG in ST2 cells after 9 days. (A) Effect of Noggin (Nog) on CG-induced ALP activity. Cells were cultured with Noggin (0.5 μg/mL) in the presence of CG (16 μmol/L) prior to determination of ALP. (B) Effect of Noggin on Smad1/5/8 phosphorylation. (C) Effect of DKK-1 on ALP activity. Cells were cultured with DKK-1 (0.1 μg/mL) in the presence of CG (16 μmol/L) prior to determination of ALP assay. (D) Effect of DKK-1 on β-catenin expression. Protein expression was determined by Western blot analysis. $^{*}P<0.05$, $^{**}P<0.01$ *versus* control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* CG alone treated group.

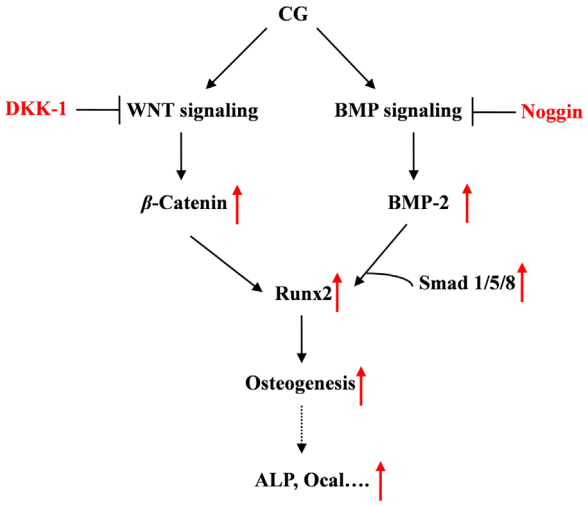

Figure 6 The underlying mechanism of CG-induced osteogenesis in ST2 cells. The scheme indicates the effect of CG on the BMP- and WNT-signaling pathways leading to osteogenesis.

protein expression of Runx2 in ST2 cells are increased by exposure to CG (8 – 32 μmol/L) for 9 days (Fig. 4). On this basis, we speculate that CG-induced Runx2 translocation occurs through regulating the WNT/BMP signaling pathway and subsequently increases ALP activity and *Ocal* expression to perform its osteogenic activity (Fig. 6).

In conclusion, our findings demonstrate that CG can stimulate osteoblastic differentiation of ST2 cells by regulating the WNT/BMP signaling pathways. The underlying osteogenic mechanism of CG in ST2 cells is presented in Fig. 6. Considering safety and cost, CG appears to be an alternative therapeutic agent for osteoporosis and bone-related diseases that merits further investigations.

Acknowledgments

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