

(R)-dehydroxyabscisic alcohol β -D-apiofuranosyl-(1'' \rightarrow 6')- β -D-glucopyranoside enhances the osteoblastic differentiation of ST2 cells via the BMP/WNT pathways

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Abstract. *Lonicera japonica* has been used in traditional Chinese medicine as an important medicinal plant, with the ability to inhibit osteoclast development and bone loss. However, it is not clear which active ingredient exerts these effects. (R)-dehydroxyabscisic alcohol β -D-apiofuranosyl-(1'' \rightarrow 6')- β -D-glucopyranoside (DAG) is an active constituent isolated from *Lonicera japonica*. In the present study, the ST2 bone marrow stromal cell line was treated by DAG at different concentrations and the osteoblastic differentiation was explored by ELISA assay, Von Kossa staining, Alizarin Red S staining, reverse transcription-quantitative polymerase chain reaction and western blot analysis. The results revealed that DAG promoted osteoblastic differentiation, as evidenced by increasing mineralization and alkaline phosphatase (ALP) activity, as well as the expression of genes encoding bone differentiation markers, including *Alp*, osteopontin (*Opn*) and osteocalcin (*Ocn*). In addition, DAG upregulated the gene expression of bone morphogenetic protein (*Bmp*)-2, *Bmp4*, Wnt family member (*Wnt*)-1, *Wnt3* and runt-related transcription factor 2 (*Runx2*), as well as the protein expression of phosphorylated-mothers against decapentaplegic homolog (Smad) 1, Smad5 Smad8, β -catenin and Runx2 in ST2 cells. The osteogenic effects induced by DAG were attenuated by the BMP antagonist Noggin and the WNT signaling pathway inhibitor Dickkopf related protein-1. The data indicated that DAG promoted the osteoblastic differentiation of ST2 cells,

at least partially through regulating the BMP/WNT signaling pathways. This provides scientific rationale for the development of DAG as a treatment for bone loss-associated diseases, such as osteoporosis.

Introduction

Bone mass regulation is under the control of continuous bone remodeling, which is a balance of osteoclastic bone resorption and osteoblastic bone formation. Disorders of bone remodeling cause a variety of bone-associated diseases, including osteoporosis (1). Osteoporosis occurs when the body makes too little bone, loses too much bone, or a combination of these two factors. Osteoporotic bones have lost density or mass, and contain abnormal tissue structure. As a result, bones become weak and may easily break following a fall. Current therapies include anti-catabolic drugs, anti-resorptives and anabolic agents (2,3). Unfortunately, the treatments available for individuals with osteoporosis are ineffective due to adverse effects (4). Therefore, it is important to identify novel therapeutic strategies.

As the main bone-forming cells, osteoblasts produce alkaline phosphatase (ALP), and bone matrix proteins such as osteocalcin (OCN) and osteopontin (OPN), which are associated with osteoblastic mineralization (5). Osteoblast differentiation is regulated by bone morphogenetic proteins (BMPs) and WNT family member (WNT) pathways (6,7). BMPs are critically involved in bone formation processes for the maintenance of bone homeostasis and skeletal development (8). The WNT pathway is also essential for bone development (9). In addition, these two pathways cooperate to regulate osteoblast differentiation and bone formation.

Lonicera japonica has been used in traditional Chinese medicine to treat various diseases, including osteoporosis (10,11). As an active component obtained from the flower buds of *Lonicera japonica*, (R)-dehydroxyabscisic alcohol β -D-apiofuranosyl-(1'' \rightarrow 6')- β -D-glucopyranoside (DAG; Fig. 1) (12), was identified to have potent activity in improving osteoblastic differentiation in our preliminary

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compound screening. The present study aimed to further investigate the effects of DAG on osteoblastic differentiation and its underlying mechanisms, in order to support the potential development of DAG as a therapeutic agent against bone-associated diseases, such as osteoporosis.

Materials and methods

Materials. The ST2 bone marrow stromal cell line was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT, Alizarin Red S and silver nitride were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Recombinant Noggin was purchased from Sigma-Aldrich (Merck KGaA) and Dickkopf-related protein 1 (Dkk-1) was purchased from PeproTech, Inc. (Rocky Hill, USA). The ALP assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Polymerase chain reaction (PCR) reagents were purchased from Thermo Fisher Scientific, Inc. DAG was provided by Professor Han (Tongji University, Shanghai, China) and was isolated and identified using spectroscopic and HPLC methods with purity >98% (12). All other reagents including DMSO, paraformaldehyde and Triton X-100 were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). DAG was dissolved in dimethyl sulfoxide (DMSO) for the *in vitro* experiments.

Cell culture and treatment. ST2 cells were cultured in RPMI-1640 medium supplemented with 15% FBS, 1% streptomycin-penicillin and osteogenic differentiation medium (cat. no. A1007201; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 37°C and 5% CO₂ incubator. In the cell viability assays, ST2 cells were treated with DAG at 0.1, 0.5 or 2.5 μM for 72 h at 37°C; in other treatments, ST2 cells were treated with DAG at 0.1, 0.5 or 2.5 μM at 37°C for different time periods according to the assays (14 days for von Kossa staining; 5 days for ALP activity; 14 days for ARS staining; 7 days for gene expression); in the mechanistic studies, ST2 cells were pretreated with Noggin (10 μg/ml) or Dkk-1 (0.5 μg/ml) for 1 h at 37°C, and then treated with DAG at 2.5 μM for different time periods according to the assays at 37°C (14 days for von Kossa staining; 5 days for ALP activity; 14 days for ARS staining; 7 days for gene expression; 10 days for protein expression); cells were treated with DMSO as the negative control.

Cell viability measurement. An MTT assay was performed to determine cell viability. Treated ST2 cells were seeded into 96-well plates at a density of 3x10⁴/100 μl. MTT solution (10 μl) was added to each well, and incubated for 4 h at 37°C. DMSO (200 μl) was subsequently added into each well to dissolve the formazan. The absorbance was measured by a plate reader at 570 nm.

ALP activity measurement. Following treatment, ST2 cells were collected and lysed using 0.1% Triton X-100 (Sinopharm Chemical Reagent Co., Ltd.). ALP activity was measured using the cALP stain kit (cat. no. D001-1; Nanjing Jiancheng

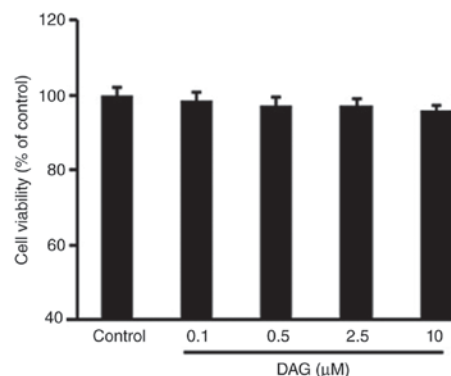


Figure 1. ST2 cell viability was determined with an MTT assay. Cells were treated with DAG at 0.1, 0.5, 2.5 and 10 μM for 72 h and the cell viability was subsequently determined. DAG did not cause any significant toxic effects. Data are expressed as the mean ± standard deviation (n=3). Samples were measured in triplicate and experiments were repeated three times. DAG, (*R*)-dehydroxyabscisic alcohol β-D-apiofuranosyl-(1'→6')-β-D-glucopyranoside.

BioEngineering Institute Co., Ltd.) according to the manufacturer's instructions.

Von Kossa staining. Treated ST2 cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at 4°C. Following washing with PBS, cells were stained with 5% silver nitride under UV radiation for 30 min at room temperature. The stained cells were washed twice and observed using an Olympus BX60 light microscope (magnification, x40; Olympus Corporation, Tokyo, Japan).

Alizarin Red S staining. Treated ST2 cells were washed twice with PBS and fixed in 70% ice-cold ethanol for 1 h. Following another wash, cells were stained with 40 mM Alizarin Red S (pH 4.2) for 10 min at room temperature. The stains were eluted with DMSO to quantify the amount of Alizarin Red S staining, by measuring the absorbance at 570 nm.

Reverse transcription-quantitative PCR (RT-qPCR). Following treatment, ST2 cells were collected and total RNA was extracted with TRIzol[®] reagent. RNA (0.4 μg) was used to generate cDNA using an iScript[™] Reverse Transcription Supermix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 25°C for 5 min, 46°C for 20 min and 95°C for 1 min. qPCR was run with the SYBR-Green ER[™] qPCR SuperMix Universal (cat. no. 11762100; Thermo Fisher Scientific, Inc.) on a MX3000p system (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) (95°C for 10 min, 95°C for 15 sec and 60°C for 60 sec; 40 cycles), using the comparative C_q value method to quantify the target gene expression in different samples (13). The gene expression was normalized using the housekeeping gene β-actin. The gene-specific primer sequences were as follows: *Alp* forward, 5'-GCTGATCATCC CACGTTTT-3' and reverse, 3'-CTGGGCCTGGTAGTTGTT GT-5' (GenBank reference, X13409.1); *Ocn* forward, 5'-CTT GGGTTCTGACTGGGTGT-3' and reverse, 3'-GCCCTCTGC AGGTCATAGAG-5' (GenBank reference, L24431.1); *Opn* forward, 5'-TGCACCCAGATCCTATAGCC-3' and reverse, 3'-CTCCATCGTCATCATCATCG-5' (GenBank reference,

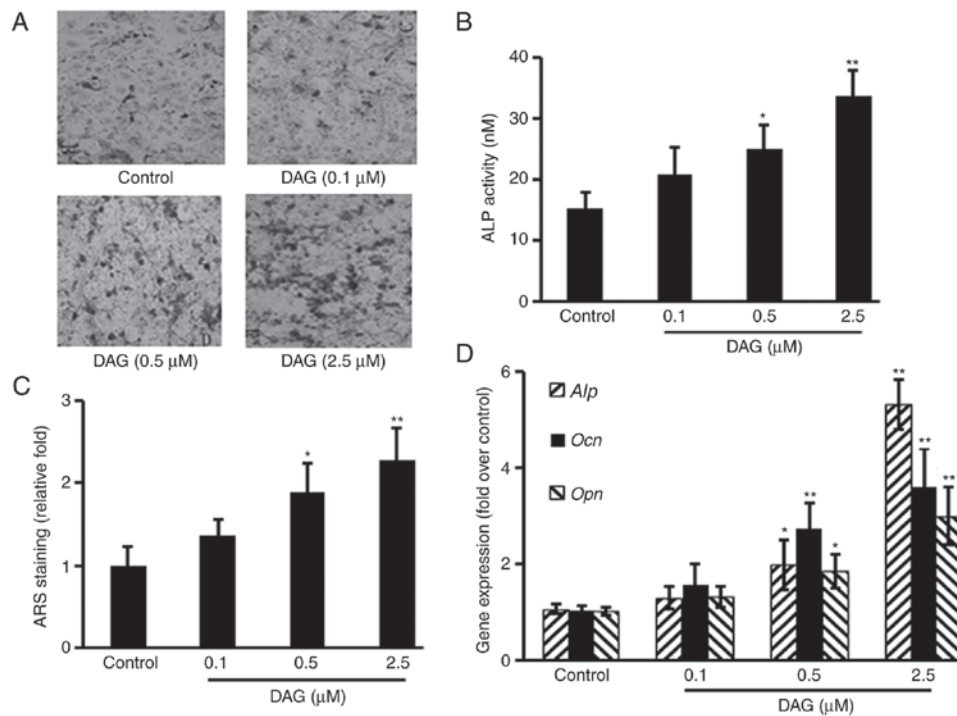


Figure 2. DAG increases the osteoblastic differentiation of ST2 cells. ST2 cells were treated with DAG at 0.1, 0.5 and 2.5 μM for different time periods. Differentiation was assessed by (A) mineralized nodule formation with von Kossa staining (14 days; magnification, $\times 40$), (B) ALP activity (5 days), (C) ARS staining (14 days) and (D) expression of osteoblast marker genes (7 days). Data are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$ and ** $P<0.01$ vs. control. Samples were measured in triplicate and experiments were repeated three times. DAG, (*R*)-dehydroxyabscisic alcohol β -D-apiofuranosyl-(1'' \rightarrow 6'')- β -D-glucopyranoside; ALP, alkaline phosphatase; Ocn, osteocalcin; Opn, osteopontin; ARS, Alizarin Red S.

AF515708.1); *Bmp2* forward, 5'-CCCCAAGACACAGTTCCCTA-3' and reverse, 3'-GAGACCGCAGTCCGTCTAAG-5' (NCBI reference: NM_007553.3); *Bmp4* forward, 5'-TCTAGAGGTCCCCAGAAGCA-3' and reverse, 3'-CTTCCCGGTCTCAGGTATCA-5' (GenBank reference, BC034053.1); *Wnt1* forward, 5'-ACAGCAACCACAGTCGTCAG-3' and reverse, 3'-GAATCCGTCAACAGGTTTCGT-5' (NCBI reference, NM_021279.4); *Wnt3* forward, 5'-GCGACTTCCTCAAGGACAAG-3' and reverse, 3'-AAAGTTGGGGGAGTTCTCCTGT-5' (NCBI reference, NM_009521.2); runt related transcription factor 2 (*Runx2*) forward, 5'-CCCAGCCACCTTTACTACA-3' and reverse, 3'-TATGGAGTGCTGCTGGTCTG-5' (NCBI reference, NM_001145920.2); β -actin forward, 5'-AGCATGTACGTAGCCATCC-3' and reverse, 3'-CTCTCAGCTGTGGTGGTGAA-5' (NCBI reference, NM_007393.5).

Western blot analysis. Treated ST2 cells were collected to extract protein with RIPA lysis buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.). The protein concentration was determined by a bicinchoninic acid protein assay kit. Protein samples of equal quantity (40 $\mu\text{g}/\text{lane}$) were subjected to 4-12% (v/v) SDS-PAGE. Proteins were subsequently transferred onto polyvinylidene fluoride membranes. Following blocking with 5% dried skimmed milk for 1 h at room temperature, membranes were washed three times with PBS/T (PBS containing 0.1%/Tween-20) and incubated with the following primary antibodies: Anti-mothers against decapentaplegic homolog (*Smad1*)/5/8 (cat. no. sc-6031-R; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phosphorylated (p)-*Smad1*/5/8 (cat. no. AB3848-I; 1:500; EMD Millipore, Billerica, MA, USA), anti- β -catenin (cat. no. sc-7199; 1:500,

Santa Cruz Biotechnology, Inc.), anti-Runx2 (cat. no. sc-10758; 1:500; Santa Cruz Biotechnology, Inc.) and anti- β -actin (cat. no. ab8227; 1:5,000, Abcam, Cambridge, UK) at 4°C overnight. Following washing with PBS, the membranes were further incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. ab6721; 1:8,000; Abcam) for 1 h at room temperature. Following washing, membranes were exposed to Pierce™ enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.) followed by X-ray film development.

Statistical analysis. Data was analyzed by one-way analysis of variance with SAS version 9.1 software (SAS Institute, Inc., Cary, NC, USA) followed by Dunnett's t-test. Values were expressed as the mean \pm standard deviation. $P<0.05$ was considered to indicate as statistically significant difference.

Results

DAG does not affect ST2 cell viability. ST2 cells were treated with DAG at different concentrations for 72 h. DAG treatment did not result in any significant cytotoxic effects when compared with the negative control, as presented in Fig. 1.

DAG treatment induces ST2 cell osteoblastic differentiation. To study the effects of DAG on osteoblastic differentiation, ST2 cells were treated with DAG at various concentrations. The results demonstrated that DAG increased the osteoblastic differentiation of ST2 cells, as evidenced by increased mineralized nodule formation measured by von Kossa

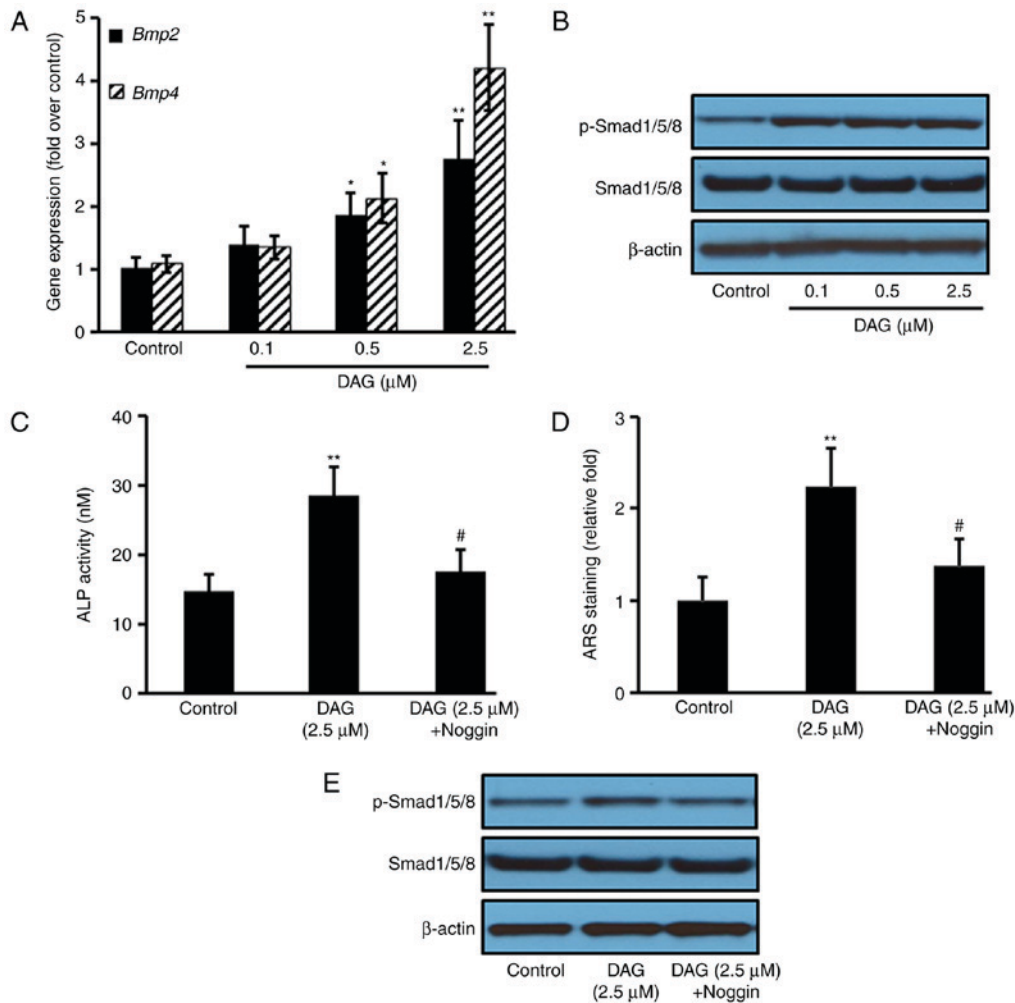


Figure 3. DAG treatment activates the BMP signaling pathway. (A) DAG treatment increased the gene expression of *Bmp2* and *Bmp4*, as well as the (B) protein expression of p-Smad1/5/8. (C) BMP antagonist Noggin pretreatment significantly attenuated the DAG-mediated increase in ALP activity and (D) mineralization, as well as the (E) protein expression of p-Smad1/5/8. Data are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$ vs. DAG alone; # $P<0.05$ and ** $P<0.01$ vs. control. Samples were measured in triplicate and experiments were repeated three times. DAG, (*R*)-dehydroxyabscisic alcohol β -D-apiofuranosyl-1-(1'' \rightarrow 6')- β -D-glucopyranoside; BMP, bone morphogenetic protein; ALP, alkaline phosphatase; p-, phosphorylated; Smad, mothers against decapentaplegic homolog; ARS, Alizarin Red S.

staining (Fig. 2A), ALP activity (Fig. 2B) and calcium deposits measured by ARS staining (Fig. 2C) in a dose-dependent manner, as well as by the upregulation of *Alp*, *Ocn* and *Opn* gene expression (Fig. 2D).

DAG treatment activates the BMP signaling pathway. To investigate the signaling pathways involved in the osteoblastic differentiation of ST2 cells induced by DAG, the BMP pathway was analyzed by RT-qPCR and western blotting. The results demonstrated that compared with the control group, DAG treatment increased the gene expression of *Bmp2* and *Bmp4* (Fig. 3A), as well as the protein expression of p-Smad1, 5 and 8 (Fig. 3B). Pretreating the ST1 cells with the BMP antagonist Noggin for 1 h prior to DAG treatment significantly reduced the DAG-mediated increase in ALP levels (Fig. 4C) and mineralization (Fig. 4D), as well as the protein expression of p-Smad1, 5 and 8 (Fig. 4E).

DAG treatment activates the WNT signaling pathway. To further investigate the signaling pathways involved in the

osteoblastic differentiation of ST2 cells induced by DAG, WNT pathway gene and protein expression was analyzed. The results revealed that DAG treatment increased the gene expression of *Wnt1* and 3 (Fig. 4A), as well as the protein expression of β -catenin (Fig. 4B). Pretreating the ST1 cells with the WNT inhibitor Dkk-1 for 1 h prior to DAG treatment significantly attenuated the DAG-mediated increase in ALP levels (Fig. 4C) and mineralization (Fig. 4D), as well as the protein expression of β -catenin (Fig. 4E).

DAG treatment increases Runx2 expression. To further confirm that the DAG-induced osteoblastic differentiation of ST2 cells occurred through the BMP and WNT signaling pathways, the expression of Runx2, a key transcription factor in osteoblastic differentiation, was measured following DAG treatment. The results demonstrated that DAG treatment increased the gene (Fig. 5A) and protein (Fig. 5B) expression of Runx2. Noggin and Dkk-1 pretreatment significantly inhibited the DAG-stimulated increase in Runx2 protein expression (Fig. 5C).

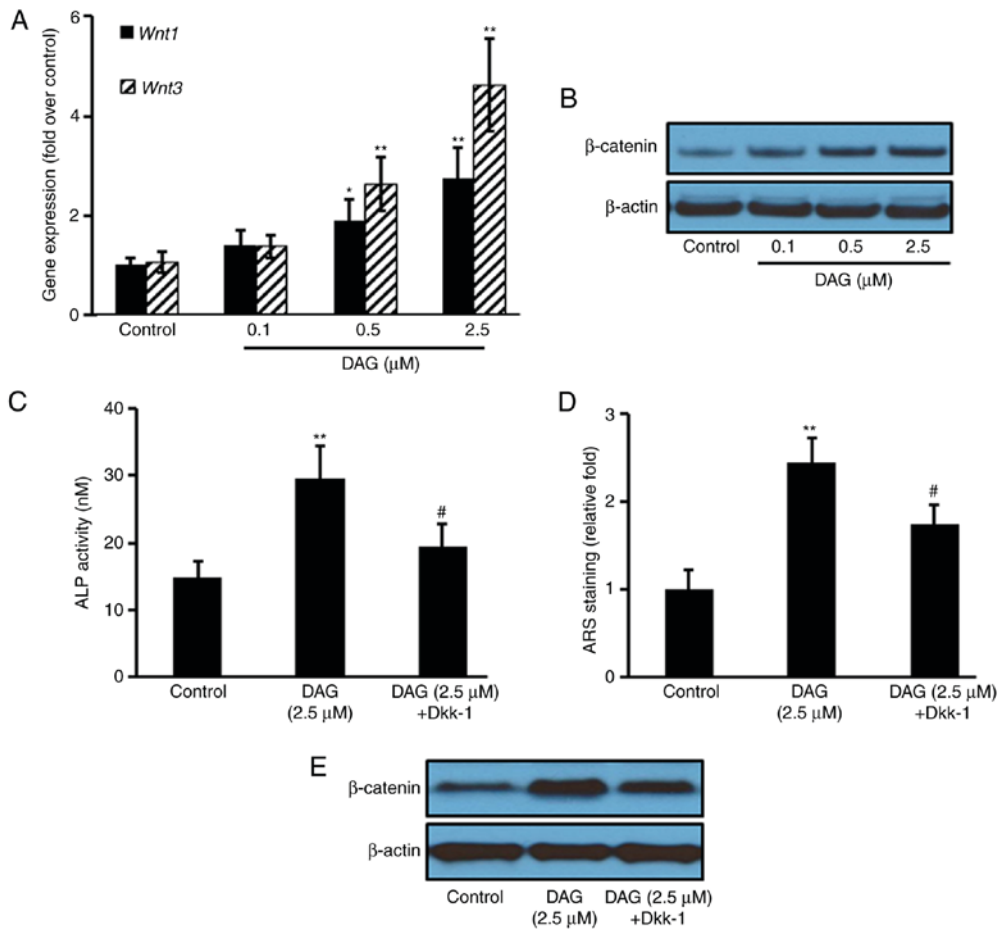


Figure 4. DAG treatment activates the WNT signaling pathway. (A) DAG treatment increased the gene expression of *Wnt1* and *Wnt3*, as well as the (B) protein expression of β -catenin. (C) WNT inhibitor Dkk-1 pretreatment significantly attenuated the DAG-mediated increase in ALP activity and (D) mineralization, as well as the (E) protein expression of β -catenin. Data are expressed as the mean \pm standard deviation (n=3). #P<0.05 vs. DAG alone; *P<0.05 and **P<0.01 vs. control. Samples were measured in triplicate and experiments were repeated three times. DAG, (*R*)-dehydroxyabscisic alcohol β -D-apiofuranosyl-(1'' \rightarrow 6')- β -D-glucopyranoside; WNT, Wnt family member; Dkk-1, Dickkopf-related protein 1; ALP, alkaline phosphatase; ARS, Alizarin Red S.

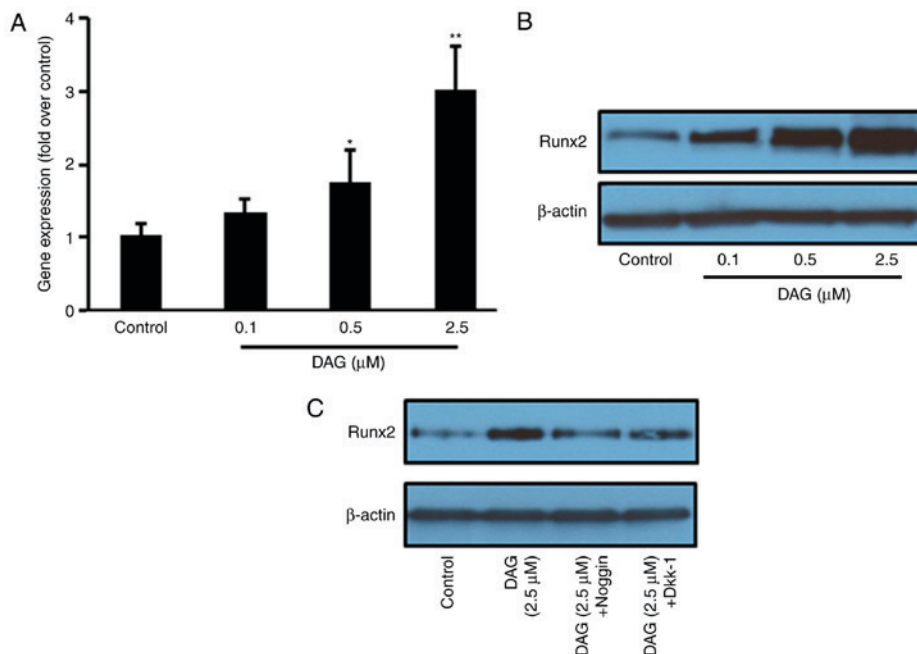


Figure 5. DAG treatment increases Runx2 expression. DAG treatment increased the (A) gene and (B) protein expression of Runx2. (C) Noggin and Dkk-1 pretreatment significantly inhibited the DAG-mediated increase in Runx2 protein expression. Data are expressed as the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01 vs. control. Samples were measured in triplicate and experiments were repeated three times. DAG, (*R*)-dehydroxyabscisic alcohol β -D-apiofuranosyl-(1'' \rightarrow 6')- β -D-glucopyranoside; Runx2, runt-related transcription factor 2; Dkk-1, Dickkopf-related protein 1.

Discussion

Osteoporosis is the most common bone-associated disease, characterized by increased bone fragility and decreased bone mass (14). As an important process of bone formation, osteoblastic differentiation is severely compromised in osteoporosis. Therefore, promoting osteoblastic differentiation is an effective strategy to prevent pathological progression. In the present study, it was demonstrated that DAG, a novel compound from the flower buds of *Lonicera japonica*, had no toxicity on bone marrow stromal cells and increased osteoblastic differentiation. DAG differs from WIN-34B, another compound isolated from *Lonicera japonica*, which has been demonstrated to increase osteogenesis and decrease osteoclastogenesis via promoting ALP activity and mineralization of human mesenchymal stem cells through inhibition of the NF- κ B, JNK and p38 MAPK pathways (10). The present study demonstrated that DAG exerted its effects by activating the BMP/WNT signaling pathways.

The ST2 cell line has been widely used for the study of osteoblastic differentiation, as these differentiate into osteoblast-like cells and further mature into osteoblasts (15,16). The process of new bone formation involves osteoblast differentiation and proliferation, and finally extracellular matrix mineralization. ALP activity is a well-recognized marker of early osteoblast differentiation, and the mineralization may be detected by von Kossa staining and Alizarin Red S staining, as a biological marker of the terminal differentiation (17,18). In the present study, ST2 cells treated with DAG significantly increased ALP activity and mineralization, and upregulated the gene expression of *Alp*, *Ocn* and *Opn*, suggesting that DAG stimulated early and late osteoblastic differentiation, as well as maturation.

BMP family proteins serve an important role in the regulation of bone remodeling and formation, as one of the main signaling cascades (19). Inactive BMP resides in the cytoplasm, and upon activation by p-Smad1/5/8, it translocates to the nucleus to regulate the transcription of target genes (20). Several compounds have been reported to promote osteoblastic differentiation through activating the BMP signaling pathway (21,22). In the present study, DAG treatment increased the gene expression of *Bmp*, as well as the protein expression of phosphorylated Smad1/5/8. Furthermore, the BMP antagonist Noggin significantly inhibited DAG-induced ALP activity and mineralization, further confirming that DAG induced osteoblastic differentiation through the BMP signaling pathway.

WNT/ β -catenin signaling contributes to osteoblastic differentiation, as another critical pathway that regulates bone remodeling and formation (23). WNT ligands bind with Frizzled, low density lipoprotein receptor-related protein (LRP) 5 and LRP6 receptors to stabilize β -catenin in the cytoplasm, resulting in the translocation of β -catenin into the nucleus to regulate osteoblastic differentiation-associated gene expression (24,25). The results of the present study revealed that treating ST2 cells with DAG increased the gene expression of *Wnt* ligands and the protein expression of β -catenin. Furthermore, the DAG-induced osteogenic effects were attenuated by the WNT inhibitor Dkk-1, indicating that WNT/ β -catenin signaling was involved in DAG-induced osteoblastic differentiation.

Runx2 is a downstream regulator of the WNT/BMP signaling pathways, which is essential in the process of osteoblastic differentiation (26). The present results indicated that DAG treatment increased Runx2 gene and protein expression in ST2 cells, implying that functional cross-talk existed between the WNT/BMP pathways.

In conclusion, the present study demonstrated that DAG isolated from the flower buds of *Lonicera japonica* stimulated the osteoblastic differentiation of ST2 cells through the WNT/BMP signaling pathways. This provides scientific rationale for the development of DAG as a therapeutic agent against bone diseases, such as osteoporosis.

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Availability of data and materials

The data and materials are available from the corresponding author upon reasonable request.

Authors' contributions

Study concept and design was by WZ; experimental studies by YL, TY, TC and JH; data analysis was by YL, TC and YG; manuscript preparation by YL, TY, TC, JH, YG and WZ. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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