ERα and Wnt/β-catenin signaling pathways are involved in angelicin-dependent promotion of osteogenesis

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Abstract. Reports of the ameliorative effect of angelicin on sex hormone deficiency-induced osteoporosis have highlighted this compound as a candidate for the treatment of osteoporosis. However, the molecular mechanisms of action of angelicin on osteoblast differentiation have not been thoroughly researched. The aim of the present study was to evaluate the effect of angelicin on the proliferation, differentiation and mineralization of rat calvarial osteoblasts using a Cell Counting Kit-8, alkaline phosphatase activity and the expression of osteogenic genes and proteins. Treatment with angelicin promoted the proliferation, matrix mineralization and upregulation of osteogenic marker genes including collagen type I α 1 and bone γ-carboxyglutamate in fetal rat calvarial osteoblasts. Furthermore, angelicin promoted the expression of β-catenin and runt related transcription factor 2, which serve a vital role in the Wnt/β-catenin signaling pathway. Consistently, the osteogenic effect of angelicin was attenuated by the use of a Wnt inhibitor. Moreover, angelicin increased the expression of estrogen receptor α (ERα), which also serves a key role in osteoblast differentiation. Taken together, these results demonstrated that angelicin may promote osteoblast differentiation through activation of ERα and the Wnt/β-catenin signaling pathway.

Introduction

Osteoporosis is a metabolic skeletal disorder characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility (1). It has become a considerable public health burden owing to its association with age-associated fractures. Bone metabolism is a lifelong process and involves bone formation and bone resorption. Osteoporosis is the outcome of an imbalance in the coupling of bone resorption and formation, favoring the former. Therefore, safe and effective anti-osteoporosis drugs are necessary to increase bone formation and/or reduce bone resorption (2).

The more commonly used anti-osteoporosis drugs are bisphosphonates (alendronate), receptor activator of nuclear factor-κB ligand inhibitors (denosumab), calcitonin, parathyroid hormone therapy, selective estrogen receptor modulators and others (3). However, bisphosphonate-associated osteonecrosis of the jaws and other potential non-skeletal effects including hypocalcemia and worsening of renal impairment, may affect the clinical choices (4,5). Traditional Chinese medicines (TCM) have been widely used to prevent bone disorders (6). Natural products used in TCM may be of use in the discovery of anti-osteoporosis drugs.

Angelicin (Fig. 1) is a natural coumarin compound in *Psoralea corylifolia*, which is widely used for the treatment of various conditions, including eczema, psoriasis, diabetes, cancer and osteoporosis (7,8). Angelicin was reported to stimulate the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into osteoblasts and suppress the differentiation into adipocytes (9). An *in vivo* study demonstrated that angelicin increases bone strength in a sex hormone deficiency-induced osteoporosis model (10). However, its specific molecular mechanisms of action are not fully understood.

In the present study, the effect of angelicin on osteoblasts was investigated using fetal rat calvarial osteoblasts. The results suggested that angelicin may promote osteogenic differentiation via estrogen receptor α (ERα) and the canonical Wnt/β-catenin pathways. This study describes the mechanism of action of angelicin, which may be of use for the control of osteoporosis.

Materials and methods

Culture of fetal rat calvarial osteoblasts. A total of 5 female Wistar rats (1-day-old) weight 5-6 g were purchased from Shandong University Laboratory Animal Center. The animal care and experiments were approved by the Ethics Committee for Animal Care and Use of Shandong Academy of Medical Sciences. Culture of fetal rat calvarial

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osteoblasts was performed as described previously (11). The growth medium was supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (Beyotime, Haimen, China) in H-DMEM (Gibco, Waltham, MA, USA). The osteoblast differentiation medium (OBM) was supplemented with 10 mM β-glycerophosphate disodium salt hydrate and 50 μg/ml ascorbic acid (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for the growth medium.

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Haimen, China) was performed to evaluate the cell proliferation. The cells ($2x10^3$ cells/well in a 96-well plate) were maintained in growth medium for 24 h at 37˚C in the presence of 5% CO_2. Once the cells reached 60% confluence, they were treated with various concentrations (0, 0.1, 1 or 10 μM) of angelicin for 24, 48 or 72 h. Following treatment, 10 μl of CCK-8 solution was added to each well of the plate, and the samples were incubated in the dark for 1 h at 37˚C. The absorbance was measured with a microplate reader (ELx808; BioTek Instruments, Inc., Winooski, VT USA) at 450 nm.

Measurement of alkaline phosphatase (ALP) activity. Osteoblasts were seeded in 24-well culture plates at a density of $1x10^5$ cells/well and incubated with various concentrations of angelicin (0, 0.1, 1 or 10 μM) for 7 days. The cells were washed with PBS three times once the culture medium was removed. The cells were lysed in 0.1% Triton X-100 buffer on ice, and the samples centrifuged at 13,500 x g for 15 min at 4˚C. P-nitrophenyl phosphate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used as the substrate to evaluate ALP activity. The absorbance was measured at 405 nm and normalized to the total protein determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Haimen, China).

ALP and Alizarin red S staining. Osteoblasts were seeded in 24-well culture plates ($5x10^3$ cells/well) and incubated with various concentrations of angelicin (0, 0.1, 1 or 10 μM) to perform ALP and Alizarin red S staining at day 9 and 12 respectively. The cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The ALP stain was performed with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase color development kit (Beyotime Institute of Biotechnology). Alizarin red S staining was performed with 0.5% Alizarin red S solution (pH 4.2) for 1 h at room temperature. Images of the stained cells were captured with a digital camera (Canon 600D, Canon, Inc., Tokyo, Japan). To quantify the level of mineralization, the stained cells were dissolved in 10% cetylpyridinium chloride for 1 h at room temperature. Subsequently, the dissolved matter was transferred to a 96-well plate, and the absorbance at 560 nm was measured with a microplate reader (ELx808; BioTek Instruments, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Osteoblasts were seeded in 6-well plates ($8x10^3$ cells/well) and treated with angelicin (0, 0.1, 1 or 10 μM). TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) was added to the plate at 24 h after treatment to extract the total RNA, according to the manufacturer's instructions. The RNA samples were reverse transcribed into cDNAs using a ReverTra Ace® qPCR RT Kit (Toyobo Life Science, Osaka, Japan) at 37˚C for 15 min and 98˚C for 5 min. qPCR was performed for ALP, runt related transcription factor 2 (RUNX2), bone γ-carboxyglutamate (OCN), collagen type I α 1 (COL1A1) and the internal control GAPDH under standard enzyme and cycling conditions on a LightCycler® 480II real-time PCR system (Roche Applied Science, Penzberg, Germany) using Taq SYBR® Green qPCR Premix (NOVA, Yugong Biolabs. Co., Lianyungang, China). The thermocycling conditions were as follows: 94˚C for 3 min followed by 40 cycles of 94˚C for 15 sec and 64˚C for 1 min. All reactions were performed in triplicate, and the mRNA expression level was calculated using the $2^{-\Delta\Delta Cq}$ method with normalization to GAPDH (12). The primer sequences are given in Table I. The experiments were replicated three times.

Laser confocal microscopy. Cells were seeded in 48-well plates ($2x10^3$ cells/well), incubated, and fixed with 4% paraformaldehyde for 15 min at room temperature following treatment with angelicin (10 μM) in the presence or absence of Dickoppf-1 (Dkk-1; 0.1 μg/ml) for 24 h. The cells were washed three times with PBS, and permeabilized with 0.3% Triton X-100 in PBS for 30 min. This step was followed by blocking with Immunol Staining Blocking Buffer (Beyotime Institute of Biotechnology) for 1 h at room temperature. The cells were incubated with anti-β-catenin antibody (51067-2-AP; 1:100; Proteintech Group, Inc., Chicago, IL, USA) overnight at 4˚C, and rewarmed at 37˚C for 1 h the following day. The cells were incubated with Alexa Fluor® 488-conjugated secondary antibody (SA00013-2; 1:500; Proteintech Group, Inc) for 50 min at 37˚C, stained with DAPI (Beyotime Institute of Biotechnology) at room temperature for 5 min, washed three times, and imaged using a laser scanning confocal microscope (magnification, x40; Olympus Corporation, Tokyo, Japan).

Western blotting analysis. The cells were seeded in 25 cm^2 flasks ($2x10^4$ cells/well) and treated with angelicin (0, 0.1, 1 or 10 μM) for 24 h. The cells were lysed in cell lysis buffer (Beyotime Institute of Biotechnology) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM phenylmethane sulfonyl fluoride (freshly added). The total protein contents were quantified by a BCA assay kit. Each sample (30 μg) was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (0.45 μm). The membranes were incubated in blocking solution (5% non-fat milk) for 1 h at room temperature and incubated overnight at 4˚C with the following primary antibodies: β-catenin (51067-2-AP; 1:2,000; Proteintech Group, Inc.), bone morphogenetic protein 2 (BMP2; 18933-1-AP; 1:1,000, Proteintech Group, Inc.), RUNX2 (12556; 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA), ERα (21244-1-AP; 1:2,000; Proteintech Group, Inc.) and GAPDH (60004-1-Ig; 1:3,000, Proteintech Group, Inc.). Following three washes with a TBS and tween 20 solution, the membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (A0208; 1:2,000; Beyotime Institute of Biotechnology) for 1 h at room temperature. The immunoreaction signals were scanned using Image

Table I. Primer sequences used in this study.

Name	Sequences
COL1A1-FP	5'-GCATGGCCAAGAAGACATCC-3'
COL1A1-RP	5'-CCTCGGGTTTCCACGTCTC-3'
ALP-FP	5'-ACGAGGTCACGRCCATCCT-3'
ALP-RP	5'-CCGAGTGGTGGTCACGAT-3'
RUNX2-FP	5'-CCACAGAGCTATTAAAGTGACAGTG-3'
RUNX2-RP	5'-ACAAACTAGGTTTAGAGTCATCAAGC-3'
OCN-FP	5'-GGACATTACTGACCGCTCCT-3'
OCN-RP	5'-TTTTCAGTGTCTGCCGTGAG-3'
GAPDH-FP	5'-TGGGAAGCTGGTCATCAAC-3'
GAPDH-RP	5'-GCATCACCCCATTTGATGTT-3'

FP, forward primer; RP, reverse primer; COL1A1, collagen type I α 1; ALP, alkaline phosphatase; RUNX2, runt related transcription factor 2; OCN, bone γ-carboxyglutamate.

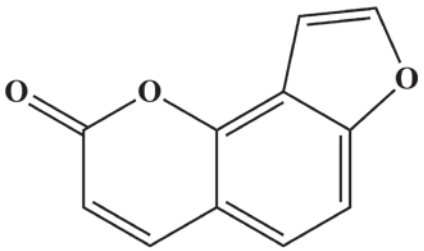

Figure 1. Chemical structure of angelicin.

Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and enhanced chemiluminescent reagent (Beyotime Institute of Biotechnology). The densities of the product bands were quantified using ImageJ (version 1.51j8; National Institutes of Health, Bethesda, MD, USA) and standardized against the GAPDH band of each sample.

Statistical analysis. Each treatment group had at least three replicates (n=3), and each experiment was repeated three times. All values are expressed as the mean ± standard deviation. Differences between groups were assessed using one-way analysis of variance and the Newman-Keuls multiple-comparison test when appropriate. P<0.05 was considered to indicate a stastically significant difference.

Results

Angelicin promotes the proliferation of osteoblasts. Osteoblasts derive from BMSCs present underneath the periosteum. The bone matrix, composed of collagen and specialized proteins, are primarily synthesized by osteoblasts (13). During the process of bone formation, mineralization of the collagen matrix is formed by groups of connected osteoblasts (14). Thus, improving the number of osteoblasts can promote bone formation (15). The effects of angelicin (0, 0.1, 1 or 10 μM) on the proliferation of osteoblasts following 24, 48 or 72 h of treatment were examined using the CCK-8 assay. Compared with the control group, the angelicin-treated osteoblasts had significantly higher numbers of viable cells as the concentration and incubation time increased (Fig. 2), with 10 μM angelicin having the maximum effect (P<0.05).

Angelicin promotes the osteogenic differentiation of osteoblasts. The effect of angelicin on the osteogenic differentiation of osteoblasts was evaluated by ALP activity, ALP staining, Alizarin red S staining and osteoblast-specific gene expression. Osteoblasts were cultured in OBM with different concentrations of angelicin (0, 0.1, 1, or 10 μM). As exhibited in Fig. 3A, angelicin promoted the expression of ALP in a dose-dependent manner in osteoblasts, an effect that was also confirmed by an ALP activity assay (Fig. 3B). ALP may aid in the matrix mineralization process mediated by osteoblasts (16). Extracellular matrix mineralization constitutes a major part of bone formation, and is considered a functional *in vitro* endpoint reflecting advanced cell differentiation. Therefore, the effect of angelicin treatment in stimulating matrix mineralization was assessed using Alizarin red S staining. After angelicin treatment for 12 days, the mineralized nodule formation was significantly increased compared with that in the control groups (Fig. 3C). Quantitative results obtained using cetylpyridinium chloride demonstrated that there was no difference between the control group and the 0.1 μM angelicin group. However, 1 and 10 μM angelicin, particularly the 10 μM treatment, promoted the formation of mineralized nodules.

Furthermore, the expression of osteoblastic genes was assessed by RT-qPCR. Compared with the control group, the groups treated with 1 and 10 μM angelicin had significantly higher expression levels of ALP, OCN, RUNX2 and COL1A1 (Fig. 3D-G).

Effect of angelicin on the Wnt signaling pathway in osteoblasts. To investigate the underlying mechanism of angelicin-induced osteogenesis, the effects of angelicin on the protein levels of β-catenin, Runx2 and BMP2 were analyzed. The western blotting results demonstrated that angelicin promoted the expression of β-catenin, Runx2 and BMP2 in a dose-dependent manner (Fig. 4A). Immunostaining revealed that β-catenin was located in the cytoplasm and nucleus, and β-catenin levels in the nucleus appeared to increase following treatment with 10 μM angelicin (Fig. 4B). The Wnt/β-catenin signaling pathway serves a key role in the regulation of bone rebuilding. To clarify whether angelicin stimulated bone formation through the Wnt/β-catenin signaling pathway, cells were co-treated with angelicin and Dkk-1, a specific antagonist of the Wnt/β-catenin signaling pathway. Upregulation of β-catenin induced by angelicin was significantly inhibited following treatment with Dkk-1 (Fig. 5A). Moreover, angelicin-induced matrix mineralization and ALP activity was also inhibited following exposure to Dkk-1 (Fig. 5B and C). To confirm this result, immunostaining was performed. Consistently, this also indicated that the angelicin-induced increase of β-catenin translocation to the nucleus may have been inhibited by Dkk-1 (Fig. 6).

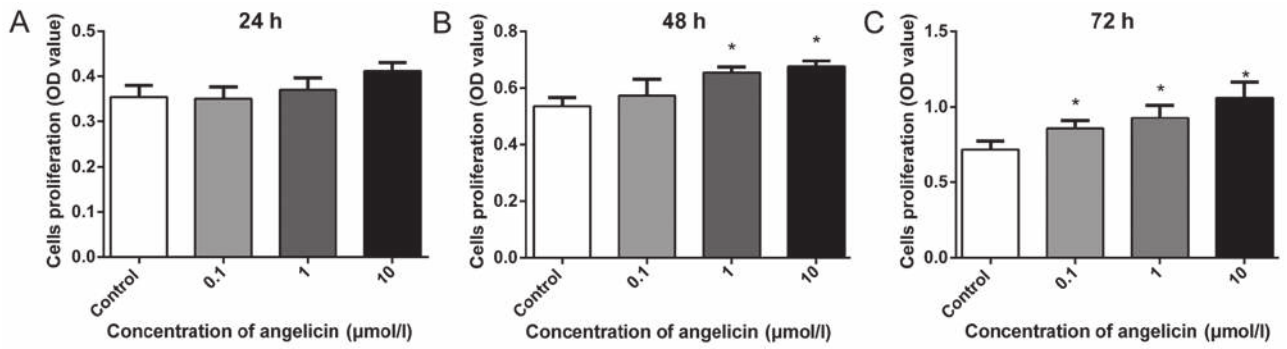

Figure 2. Angelicin promotes the proliferation of osteoblasts. The cells were seeded in 96-well plates for 24 h and treated with different concentrations of angelicin for a further (A) 24, (B) 48 or (C) 72 h. Cellular proliferation was determined via a Cell Counting Kit-8 assay. Data are expressed as the mean ± standard deviation (n=6). *P<0.05 vs. respective control group. OD, optical density.

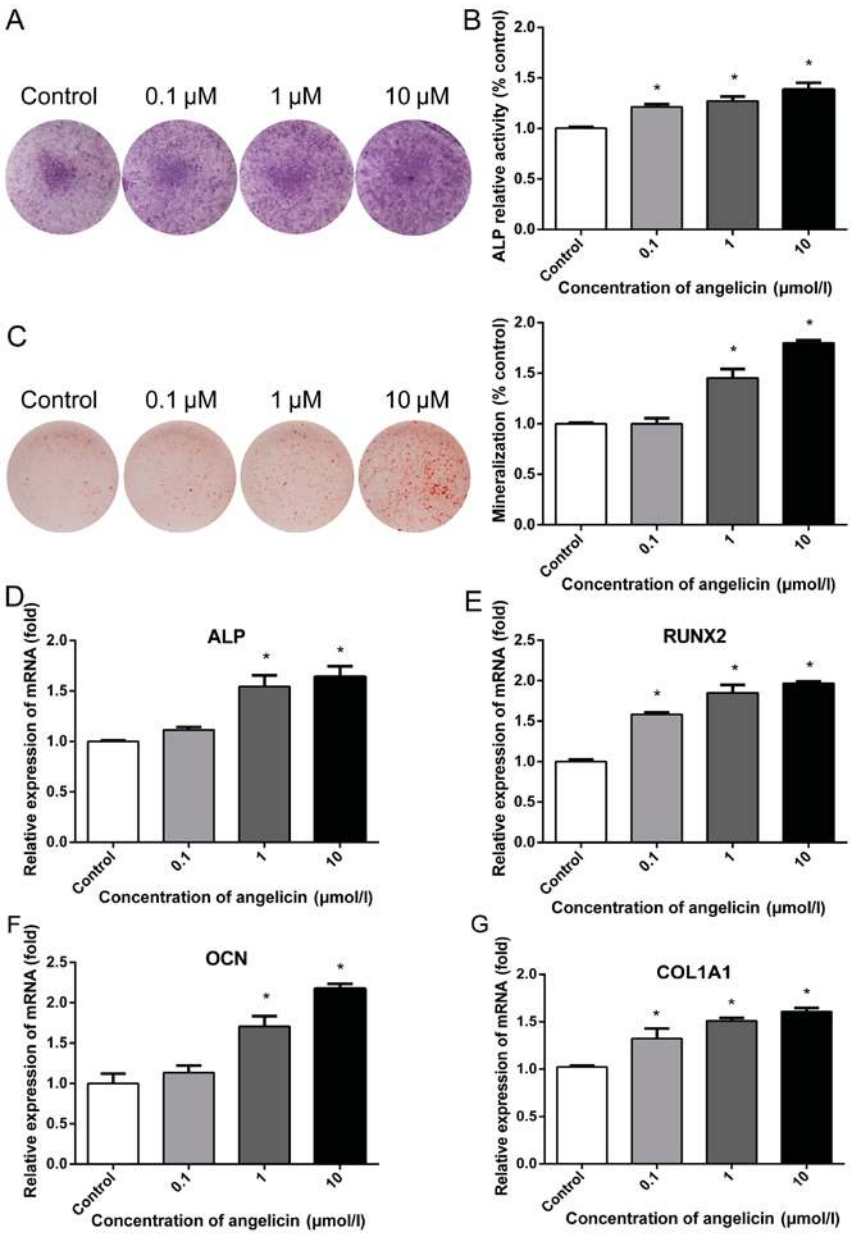

Figure 3. Angelicin promotes the osteogenic differentiation of osteoblasts. Cells were seeded in 24-well culture plates and incubated with various doses of angelicin (0, 0.1, 1 or 10 μM). (A) ALP staining and (B) ALP activity measurement were performed at day 9 and day 7, respectively. (C) Alizarin red S staining was performed at day 12 and 10% cetylpyridinium chloride was used to quantify the mineralization levels. Effects of angelicin treatments on gene expression in osteoblasts were measured by reverse transcription-quantitative polymerase chain reaction for (D) ALP, (E) RUNX2, (F) OCN and (G) COL1A1 mRNA levels. All data are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. respective control group. ALP, alkaline phosphatase; RUNX2, runt related transcription factor 2; OCN, bone γ-carboxyglutamate; COL1A1, collagen type I α 1.

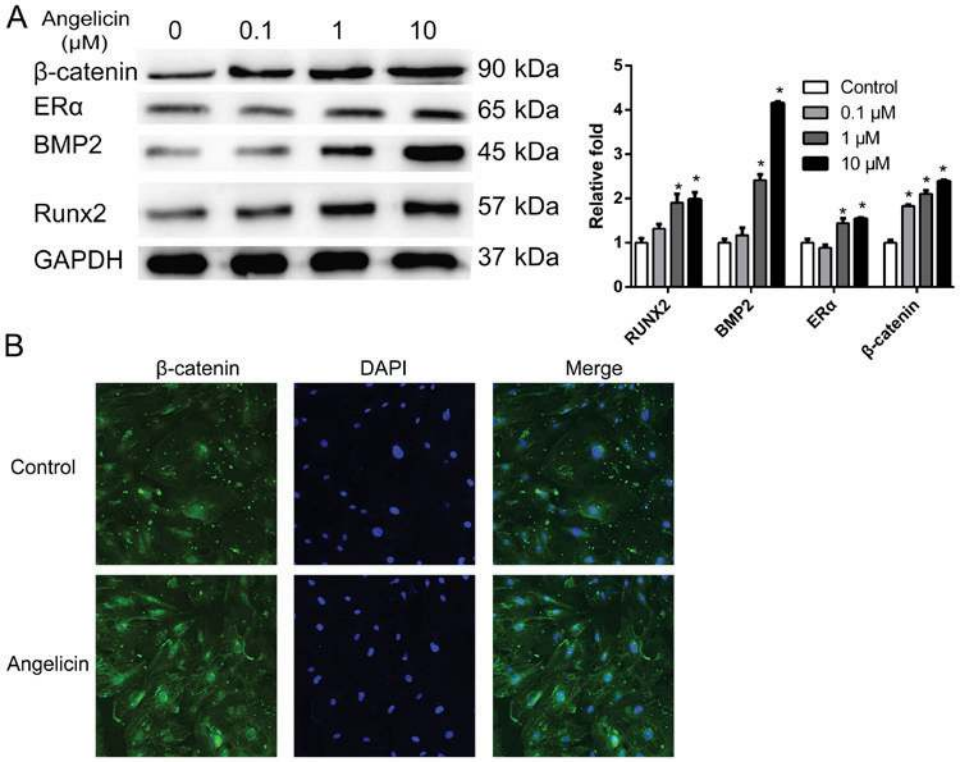

Figure 4. Effects of treatment with angelicin on protein expression in osteoblasts. (A) The total protein levels of RUNX2, BMP2, ERα and β-catenin in osteoblasts were subjected to western blot analysis, and densitometry data were normalized to GAPDH. (B) Immunofluorescence analysis of β-catenin treated with 10 μM angelicin for 24 h. Magnification, x400. Results are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. respective control group. RUNX2, runt related transcription factor 2; BMP2, bone morphogenetic protein 2; ERα, estrogen receptor α.

Effect of angelicin on ERα in osteoblasts. Previous studies suggested angelicin was involved in estrogen-like neuroprotection in an animal model of spinal cord injury, and morphological data indicated ERα level was upregulated by angelicin (17). Furthermore, ERα also serves an important role in bone formation (18). To investigate whether ERα may be involved the process of angelicin-induced osteoblast differentiation, ERα protein levels were also detected via western blotting. This demonstrated that ERα protein also increased following treatment with angelicin (Fig. 4A).

Discussion

The Wnt/β-catenin signaling pathway serves a notable role in osteogenic differentiation. The present study demonstrated that angelicin induced β-catenin upregulation and may have promoted its accumulation in the nucleus, which suggested that angelicin sustained the stabilization of β-catenin. β-catenin is a pivotal signaling molecule in the Wnt/β-catenin signaling pathway, and is upregulated when Wnt signaling is activated. Increased β-catenin in the cytoplasm induces β-catenin translocation into the nucleus, which subsequently binds to lymphoid enhancer binding factor/transcription factor family of proteins and regulates the expression of Wnt target genes, including Runx2.

Bone formation may be divided into two stages (19). In the first stage, osteoblast proliferation serves an important role (20). Bone formation is directly linked with the osteoblast number, and therefore a decreased osteoblast number correlates with a lower bone formation rate (21). Thus, primary rat osteoblast viability/proliferation was assessed via CCK-8 assay to determine whether angelicin affects the number of osteoblasts. The results demonstrated that angelicin significantly stimulated osteoblast proliferation.

The second stage is characterized by extracellular matrix synthesis and matrix mineralization (22). ALP expression and activity increases in the initial period of this stage. In the present study, angelicin was demonstrated to potentiate the activity of ALP, as observed with the activity test and ALP staining. In the second stage, RUNX2 also serves a notable role and is a target gene of the Wnt/β-catenin signaling pathway (23). RUNX2 is upregulated in the early stages of osteogenesis and may promote the expression of bone matrix-associated genes, including COL1A1. Moreover, the skeleton of RUNX2-deficient mice is formed of cartilage due to the absence of osteoblasts (24). In the present study, RUNX2 mRNA and protein levels were significantly upregulated following exposure to angelicin, suggesting that angelicin-induced upregulation of β-catenin may have promoted the expression of its downstream target genes in cultured osteoblasts.

Angelicin was also involved in the upregulation of BMP2, which has the ability to induce bone formation and is frequently used in bone regeneration and repair treatments (25). BMP2 also serves a role in increasing ALP activity and osteocalcin synthesis (26). The BMP signaling pathway closely interacts with Wnt/β-catenin signaling pathways, and they cooperatively regulate osteoblast differentiation (27). However, a recent study has suggested that BMP2 may be a downstream target of the Wnt/β-catenin signaling pathway in osteoblasts (28).

To verify the importance of Wnt/β-catenin signaling pathway in angelicin-induced osteogenesis, osteoblasts were

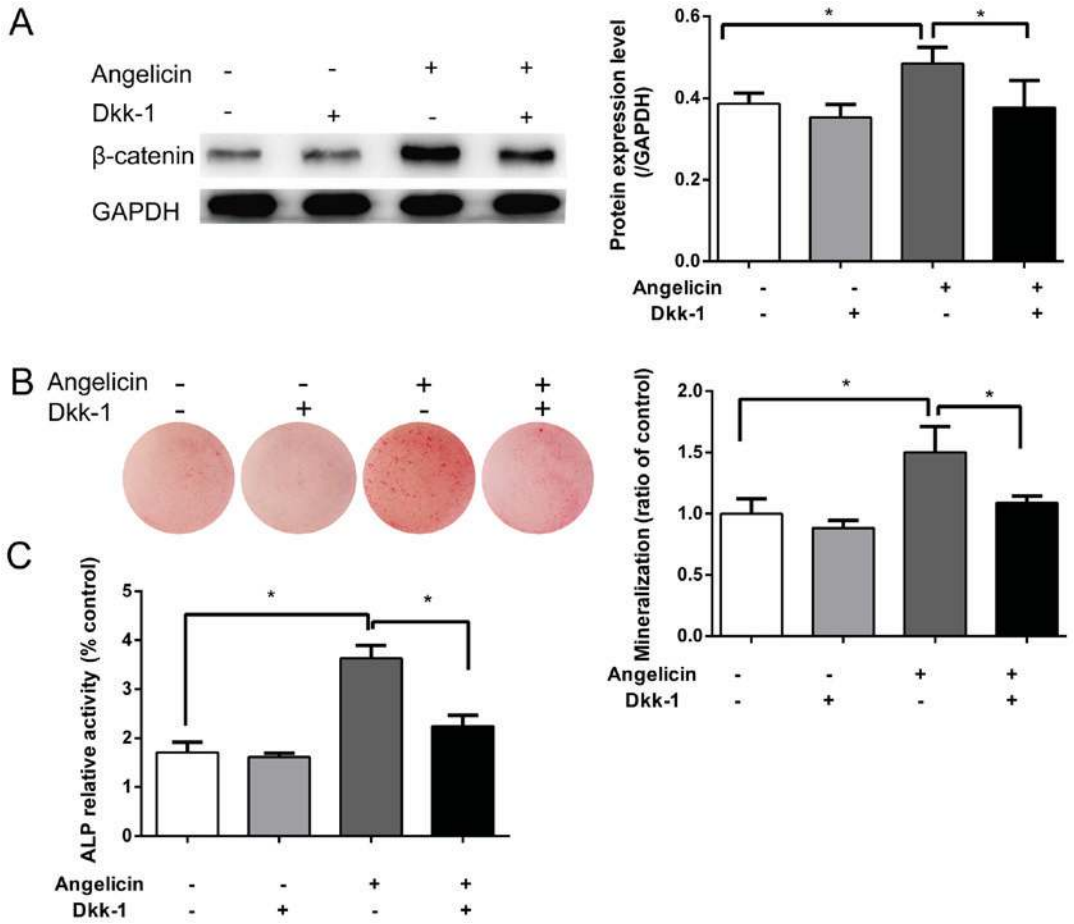

Figure 5. Effects of treatment with Dkk-1 on protein expression and osteogenesis in cultured osteoblasts. The cells were cultured with Dkk-1 (0.1 μg/ml) and angelicin (10 μM). (A) β-catenin expression, (B) mineralization and (C) ALP activity were performed to detect the effect of Dkk-1. Results are presented as the mean ± standard deviation from three independent experiments. *P<0.05. Dkk-1, Dickoppf-1; ALP, alkaline phosphatase.

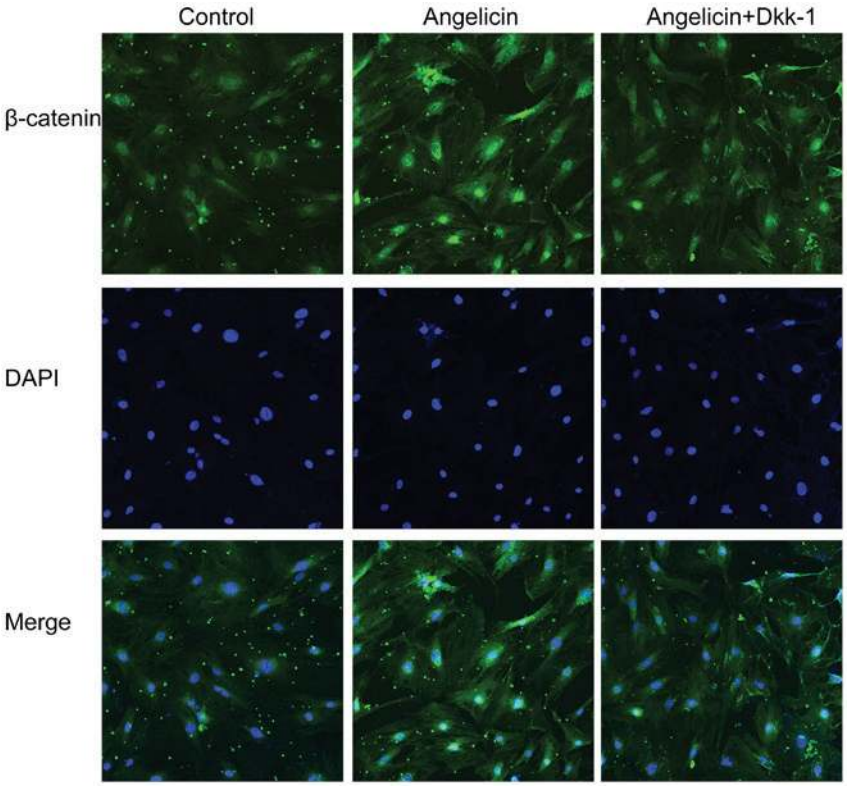

Figure 6. Immunofluorescence analysis of β-catenin treated with angelicin (10 μM) and Dkk-1 (0.1 μg/ml) for 24 h. Magnification, x400. Dkk-1, Dickoppf-1.

co-treated with angelicin and Dkk-1, a Wnt antagonist that may specifically inhibit the Wnt/β-catenin pathway by isolating the LRP5/6 receptor (29). The β-catenin protein level, mineralization nodules and ALP activity were evaluated following the co-treatment with angelicin and Dkk-1. Dkk-1 treatment significantly lowered the angelicin-induced β-catenin expression, mineralization nodules and ALP activity, which further confirmed that angelicin-induced osteogenic regulation occurred via the Wnt/β-catenin pathway.

Previous studies have demonstrated that the Wnt/β-catenin pathway may cooperate with ERα in bone development (30,31). ERs are activated by estrogen and are divided into two classes, ERα and ERβ, and have been detected in osteoblasts, osteocytes and osteoclasts (32). Studies have confirmed that ERα and ERβ serve different roles in bone and in other tissues (33,34). Estrogen acts in bone-residing cells primarily through ERα, and lower levels of ERα in osteoblasts may lead to compromised bone strength and bone mass in female mice (35). ERα potentiates bones response to mechanical strain, and regulates trabecular bone formation and thereby trabecular bone volume in female and male mice (35,36). A recent study demonstrated that angelicin exhibits estrogen-like activity and induces the upregulation of ERα in a spinal cord injury model (17). In the present study, angelicin was associated with an increase in ERα protein levels in rat primary osteoblasts.

In conclusion, angelicin may promote osteogenic differentiation via ERα and the canonical Wnt/β-catenin signaling. This study described the mechanism of action of angelicin, which may constitute a novel method for the control of osteoporosis. However, the direct targets of angelicin remain to be elucidated. Future studies are required to predict and confirm the potential binding targets of angelicin, and confirm its therapeutic potential.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JH and YC conceived and designed the experiments. LG performed the experiments. BL, JP and XY analyzed the data. LG and JP wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed according to the guidelines of the Chinese Experimental Animals Administration Legislation and were approved by the Ethics Committee for Animal Care and Use of Shandong Academy of Medical Sciences.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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