



Original Article

(2R,3S)-Pinobanksin-3-cinnamate promotes osteoblast differentiation through cAMP and cGMP pathways

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ABSTRACT

Flavones have the potential of being used as a dietary supplement for bone health promotion beyond calcium and vitamin D. Recent studies have showed that flavones enhanced bone formation and inhibited bone resorption by affecting osteoblast and osteoclast differentiation through various cell signaling pathways. In this study, we investigated the effects of a new flavone (2R,3S)-pinobanksin-3-cinnamate, isolated from the metabolites of the endophytic fungus *Penicillium* sp. FJ-1 of *Acanthus ilicifolius* L., Acanthaceae, on osteoblast differentiation by using MC3T3-E1 cells. It was observed that (2R,3S)-pinobanksin-3-cinnamate promoted osteoblast differentiation, as evidenced by increased mineralization process and alkaline phosphatase activity, as well as expression of genes encoding the bone differentiation. Moreover (2R,3S)-pinobanksin-3-cinnamate treatment upregulated the gene expression of wingless-type MMTV integration site family, bone morphogenetic protein and runt-related transcription factor 2, and protein expression of phosphor-Smad1/5/8, β -catenin and runt-related transcription factor 2 in MC3T3-E1 cells. The osteoblast differentiation effects induced by (2R,3S)-pinobanksin-3-cinnamate were attenuated by the bone morphogenetic protein antagonist Noggin, and wingless-type MMTV integration site family signaling pathway inhibitors Dickkopf-1. Co-treatment with adenosine 30,50-cyclic monophosphate and guanosine 30,50-cyclic monophosphate pathway inhibitors, H89 and KT5823, respectively, reversed the (2R,3S)-pinobanksin-3-cinnamate-induced activations of p-Smad1/5/8, β -catenin, and runt-related transcription factor 2. Our data demonstrated that (2R,3S)-pinobanksin-3-cinnamate promoted the osteoblast differentiation of MC3T3-E1 cells, at least partially through the adenosine 30,50-cyclic monophosphate and guanosine 30,50-cyclic monophosphate signaling pathways, providing the scientific rational to develop (2R,3S)-pinobanksin-3-cinnamate against bone loss-associated diseases.

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Introduction

Skeletal homeostasis is under the control of continuous bone remodeling of osteoclastic bone resorption and osteoblastic bone formation. Imbalances of bone remodeling causes a wide variety of bone-related disorders, including osteoporosis, which is prevalent worldwide (Rodan and Martin, 2000). Osteoporosis is a systemic condition, characterized by low bone mass, decreased bone mineral density and microarchitectural deterioration of bone tissue predisposing to increased fragility of bone and susceptibility to fractures (Niu et al., 2012). Current treatments for osteoporosis include anti-resorptives, anticatabolic drugs, and anabolic agents

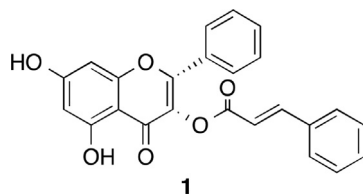
(Riggs and Parfitt, 2005; Augustine and Horwitz, 2013). However, few effective treatments are available and there is a pressing unmet need to search for novel therapeutic strategies.

The intracellular morphogenetic protein BMP-Runx2 and the Wnt/ β -catenin signaling pathways play a crucial role in stimulating osteoblast differentiation and bone formation (Hayashi et al., 2009). Guanosine 30,50-cyclic monophosphate (cGMP) and adenosine 30,50-cyclic monophosphate (cAMP) are known to participate in the signaling of a variety of important events in bone biology (Liou et al., 2015). Furthermore, studies have shown that the cGMP and cAMP signaling pathways played a positive role in osteoblast proliferation and differentiation. It has been observed that elevation of intracellular cAMP enhanced BMP action and increased ALP activity of osteoblastic cells in experimental animals (Kinoshita et al., 2000; Nakagawa et al., 2007).

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Flavones, found in a wide diversity of plant foods, herbs, spices, and beverages, have the potential of dietary components for bone health promotion beyond calcium and vitamin D. Recent studies have indicated that flavone consumption had a stronger association with bone health than general fruit and vegetable consumption (Chen et al., 2006; Prynne et al., 2006). As an active component from the metabolites of the endophytic fungus *penicillium* sp.FJ-1 of *Acanthus ilicifolius* L., Acanthaceae (2R,3S)-pinobanksin-3-cinnamate (PC, **1**) has been demonstrated to exert potent neuroprotective effects on corticosterone-damaged PC12 cells and animal model (Liu et al., 2014, 2015). In our preliminary compound screening, PC showed potent activities to increase osteogenesis. This study was to further explore the effects of PC on the osteoblast differentiation and its underlying mechanisms, developing PC as a therapeutic agent against osteoporosis.



Materials and methods

Cell line, chemicals and reagents

The cell line MC3T3-E1 was purchased from Beijing Cell Bank (Beijing, China). Fetal bovine serum (FBS) and α -minimal essential medium (α -MEM) were from Gibco BRL (Gaithersburg, USA). Alizarin red S and 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium-bromid (MTT) were purchased from Sigma-Aldrich (St. Louis, USA). ALP assay kit was purchased from Jiancheng Biological Engineering (Nanjing, China). Real-time PCR reagents were purchased from Thermo Fisher (Waltham, MA, USA). PC was isolated and identified by Prof. Jie Lu from Institute of Materia Medica, Shandong Academy of Medical Sciences, China (Liu et al., 2014). PC was dissolved in DMSO and diluted with PBS for the experiments.

Cell culture and treatment

MC3T3-E1 cells were cultured in α -MEM supplemented with 10% FBS, 1% streptomycin-penicillin, and osteogenic differentiation medium at 37 °C, 95% air/5% CO₂. MC3T3-E1 cells were treated with PC with or without inhibitors; cells treated with DMSO as the negative control.

Cell viability measurement

Cell proliferation was determined by MTT assay. After treatment, MC3T3-E1 cells were seeded in 96-well plates at 4×10^4 /100 μ l. MTT solution (0.5 mg/ml) was added to each well, and incubated for 4 h at 37 °C. 200 μ l DMSO was added to dissolve the MTT formazan. The absorbance was measured at 570 nm by the ELISA reader.

ALP activity measurement

After treatment, MC3T3-E1 cells were collected and lysed. After centrifuge, the supernatant was collected. The ALP activity was determined by the assay kit according to the manufacturer's instructions.

Alizarin Red S staining

After treatment, MC3T3-E1 cells were washed and fixed with 70% ice-cold ethanol for 1 h. Cells were then stained with 40 mM Alizarin red S (pH 4.2) for 10 min. The stains were eluted with DMSO to quantify the amount of Alizarin red S staining by measuring the absorbance at 540 nm with the ELISA reader.

Real-time RT-PCR

After treatment, the total RNA was extracted from MC3T3-E1 cells with TRIzol reagent. cDNA was synthesized using the reverse transcription kit with 0.5 μ g RNA. Quantitative PCR was run on the ABI Step one Plus System (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix, using comparative C_t value method to quantify the target gene expression. The gene expression was normalized by the housekeeping gene β -actin. The gene-specific primer sequences are the following. For *Alp*, forward: cgcatgacatcccagaaag; reverse: gcctggtagttgtgtgagc, GenBank Reference: X13409.1; For *Opn*, forward: tccaatcgctccacagtcg; reverse: agctgacttgactcatggct, GenBank Reference: AF515708.1; For *Ocn*, forward: acatgagggagacaacaggg; reverse: tcaggag-gaaaagtggggac, GenBank Reference: L24431.1; For *Bmp2*, forward: tggccctcataaagaagca; reverse: acaggtcagagaacaggggtg, NCBI Reference: NM.007553.3; For *Bmp4*, forward: gggtcctgacacatcatca; reverse: ctgattctgacatgctggcc, GenBank Reference: BC034053.1; For *Wnt1*, forward: cgatccatctctcccacctc; reverse: cccacccca-gaagaagaat, NCBI Reference: NM.021279.4; For *Wnt3*, forward: gcactgggaaggggtagatt; reverse: aaagccgtacaccatccaga, NCBI Reference: NM.009521.2; For *Runx2*, forward: gccaggcgattttcatagtg; reverse: ggtaaaggtggctgggtagtg, NCBI Reference: NM.001146038.2; For β -actin, forward: cctctatgccacacagtg; reverse: cctgcttgctgacacatc, NCBI Reference: NM.007393.5.

Western Blot analysis

After treatment, MC3T3-E1 cells were collected and lysed using RIPA buffer to extract protein. The protein concentration was determined by protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of samples (50 μ g protein) were separated by the 4–12% (v/v) SDS-polyacrylamide gel. Protein was then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 1% BSA and incubated with appropriate primary antibodies (anti-phospho Smad1/5/8 (Ser463/465) rabbit pAb, AB3848-I, 1:1000; anti-Smad1/5/8 rabbit pAb, sc-6031-R, 1:1500; anti- β -Catenin (H-102) rabbit pAb, sc-7199, 1:1000; anti-Runx2 (M-70) rabbit pAb, sc-10758, 1:1000; anti- β -actin rabbit pAb, ab16039, 1:3000) at 4 °C overnight. After washing, the membranes were further incubated with corresponding goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (ab6721, 1:5000). The membranes were then exposed to Pierce™ ECL substrates followed by the X-ray film development.

Statistical analysis

Values were presented as mean $\pm$ SD. Data was analyzed by one-way analysis of variance (ANOVA) with SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Dunnett's *t* test was performed for comparing the experimental groups with the control group. *p* < 0.05 was considered as significant difference.

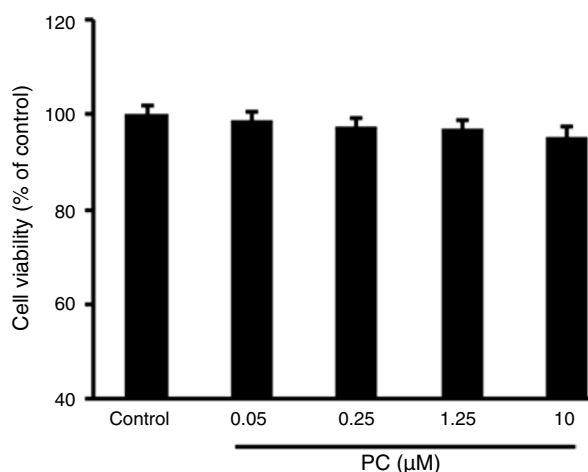


Fig. 1. MC3T3-E1 cells were treated with PC at 0.05, 0.25, 1.25, 10 μM for 72 h and then the cell viability was tested by MTT assay. PC did not cause significant toxicity on MC3T3-E1 cells. Data were expressed as mean ± SD ($n = 3$). Samples were measured in triplicate and experiments were repeated three times.

Results

Effects of PC treatment on MC3T3-E1 cell viability

MC3T3-E1 cells were treated with PC at different concentrations for 72 h. Compared to the DMSO control, PC treatment didn't cause obvious cell damage (Fig. 1).

PC treatment promoted osteoblast differentiation of MC3T3-E1 cells

MC3T3-E1 cells were treated with PC at different concentrations. Results indicated that PC promoted osteoblast differentiation of MC3T3-E1 cells, as evidenced by increased ALP activity and mineralization, and upregulating the gene expression of *Alp*, *Opn* and *Ocn* (Fig. 2).

PC treatment activated BMP/WNT signaling pathway

To investigate the signaling pathways involved in the osteoblast differentiation of MC3T3-E1 cells induced by PC treatment, BMP/WNT pathways were analyzed. Results indicated that PC treatment increased gene expression of *Bmp* and *Wnt* families as well as *Runx2*, and also increased the protein expression of p-Smad1/5/8, β-Catenin and *Runx2*. Pretreating the MC3T3-E1 cells with BMP antagonist *Noggin* and WNT inhibitor *Dkk-1* 2 h before PC significantly attenuated PC-mediated ALP and mineralization increase, as well as the protein expression (Fig. 3).

PC treatment promoted osteoblast differentiation of MC3T3-E1 cells through cAMP and cGMP pathways

To further explore pathways involved in the osteoblast differentiation of MC3T3-E1 cells induced by PC treatment, cAMP and cGMP pathway inhibitors H89 and KT5823 were co-treated with PC. Results showed that PC-induced osteoblast differentiation was significantly reversed (Fig. 4).

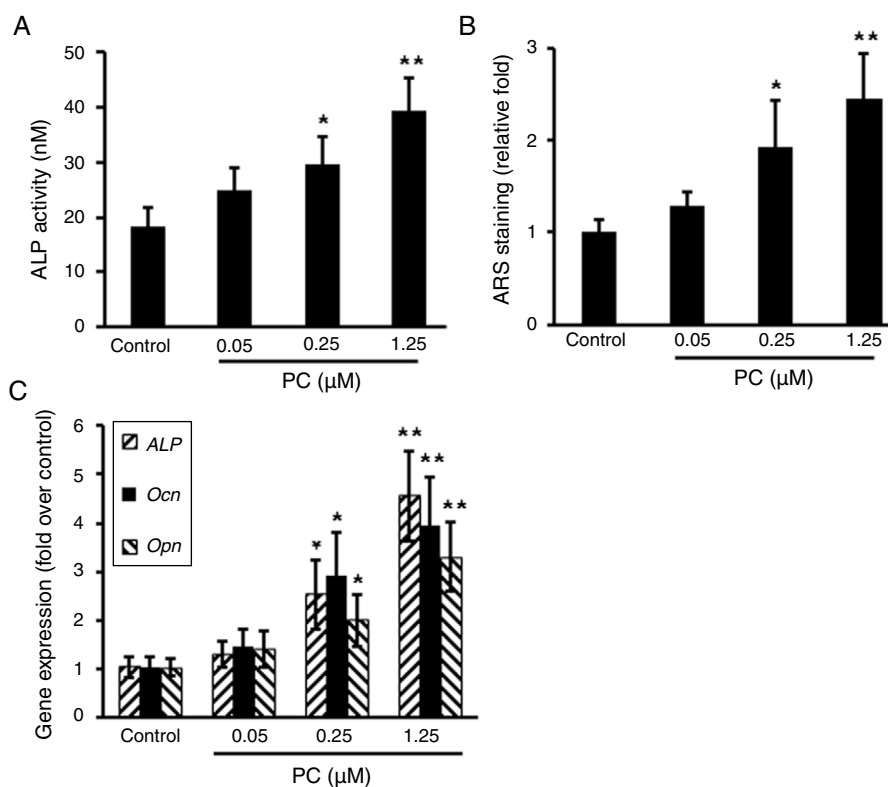


Fig. 2. PC increased osteoblast differentiation of MC3T3-E1 cells. MC3T3-E1 cells were treated with PC at 0.05, 0.25, 1.25 μM for different time points. Differentiation was assessed by ALP activity (A, 5 days), Alizarin red S staining (B, 14 days) and expression of osteoblast differentiation marker genes (C, 7 days). Data were expressed as mean ± SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ vs control. Samples were measured in triplicate and experiments were repeated three times.

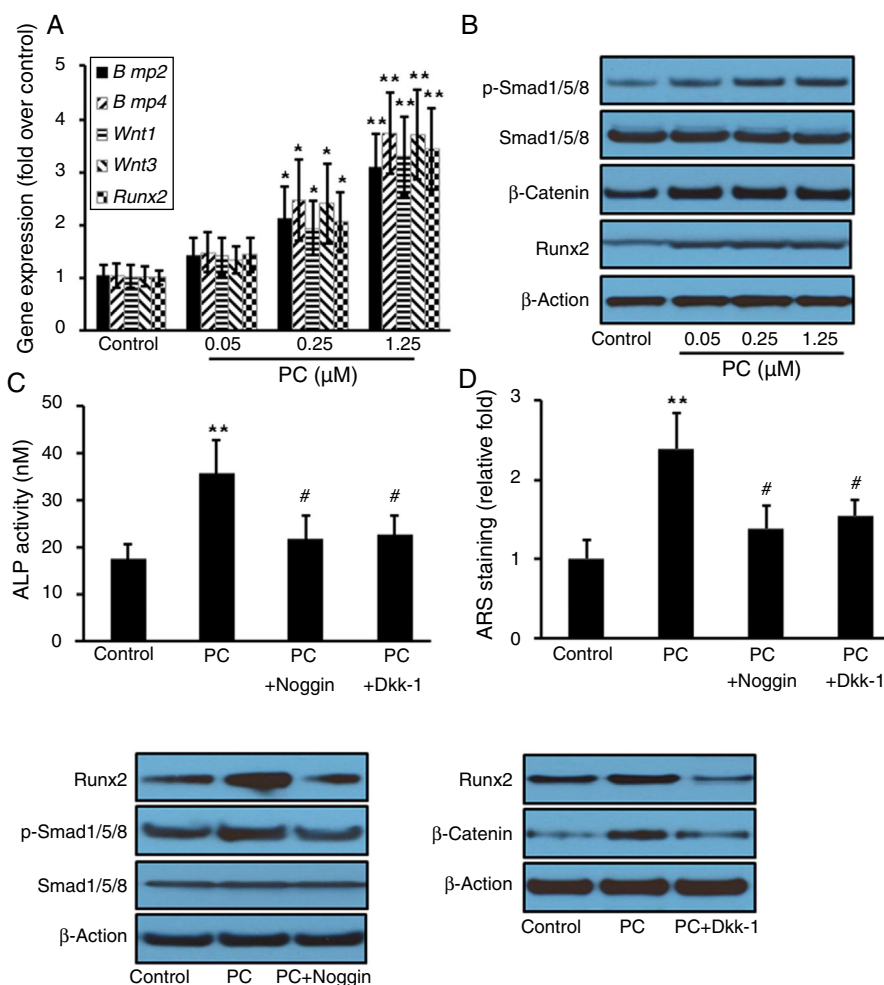


Fig. 3. PC treatment activated BMP/WNT signaling pathways. PC treatment increased gene expression of *Bmps*, *Wnts* and *Runx2* (A), protein expression of p-Smad1/5/8, β -Catenin and *Runx2* (B). BMP antagonist Noggin and WNT inhibitor Dkk-1 pretreatment significantly attenuated PC-mediated (1.25 μ M) ALP activity (C) and mineralization (D), as well as the protein expression (E). Data were expressed as mean $\pm$ SD ($n = 3$). # $p < 0.05$ vs PC alone; * $p < 0.05$, ** $p < 0.01$ vs control. Samples were measured in triplicate and experiments were repeated three times.

Discussion

Bone metabolism is under the control of mutual interaction between osteoclasts and osteoblasts. Osteoblasts promote bone production by regulating the differentiation and proliferation of osteoblast precursors (Corrado et al., 2017). However, osteoblast differentiation is severely compromised in osteoporosis (Marie and Kassem, 2011). Therefore, promoting osteoblast differentiation is an effective strategy to prevent pathological progression. Several key transcription factors, such as *Runx2*, play an important role in regulating osteoblast differentiation, which further stimulate mineralization and lead to bone formation (Yoon et al., 2013). In this study, PC has been demonstrated to induce differentiation in mouse osteoblast MC3T3-E1 cells, without significantly affecting cell growth. PC not only increased ALP activity, but also enhanced BMP/WNT/*Runx2* expression, and mineralization in osteoblasts.

Recent studies on flavones have identified molecular targets in cell signaling pathways that affect bone metabolism. The most studied mechanisms for benefits of flavones to bone have been in the estrogenic actions of phytoestrogens (Tang et al., 2011). Flavones have bone anabolic activity, which has exciting implications beyond merely inhibiting bone resorption through suppressing osteoclast activation. Flavones have also been shown to activate signaling through BMP/WNT, which increases the

expression of the major transcription factor *Runx2* (Chen et al., 2008; Chen et al., 2009).

MC3T3-E1 cell line has been widely used to study osteoblast differentiation because it could differentiate into osteoblast-like cells and further mature osteoblasts (Kim et al., 2014; Liou et al., 2015). As the main bone-forming cells, osteoblasts produce ALP and bone matrix proteins such as osteopontin (OPN) and osteocalcin (OCN), which are related to the osteoblast mineralization (Long, 2011). Therefore, ALP activity is a well-recognized early marker of the osteoblast phenotype, and the mineralization shown by the Alizarin red S staining is a biological marker of the terminal differentiation (Aubin, 1998; Sun et al., 2018). In this study, treating MC3T3-E1 cells with PC significantly increased ALP activity and mineralization, and the gene expression of *Alp*, *Opn* and *Ocn*, suggesting PC stimulated the early and late osteoblast differentiation and maturation.

Osteoblast differentiation is regulated by BMP and WNT pathways. Moreover, these two pathways cooperate to regulate the bone formation. BMP remains inoperative in the cytoplasm as one of the main signaling cascades (Wan and Cao, 2005; Krishnan et al., 2006). Upon activation, BMP is activated by the p-Smad1/5/8 and moves into the nucleus to regulate the target gene transcription (Zhao et al., 2002). Several compounds have been discovered to promote osteoblast differentiation through the BMP pathway

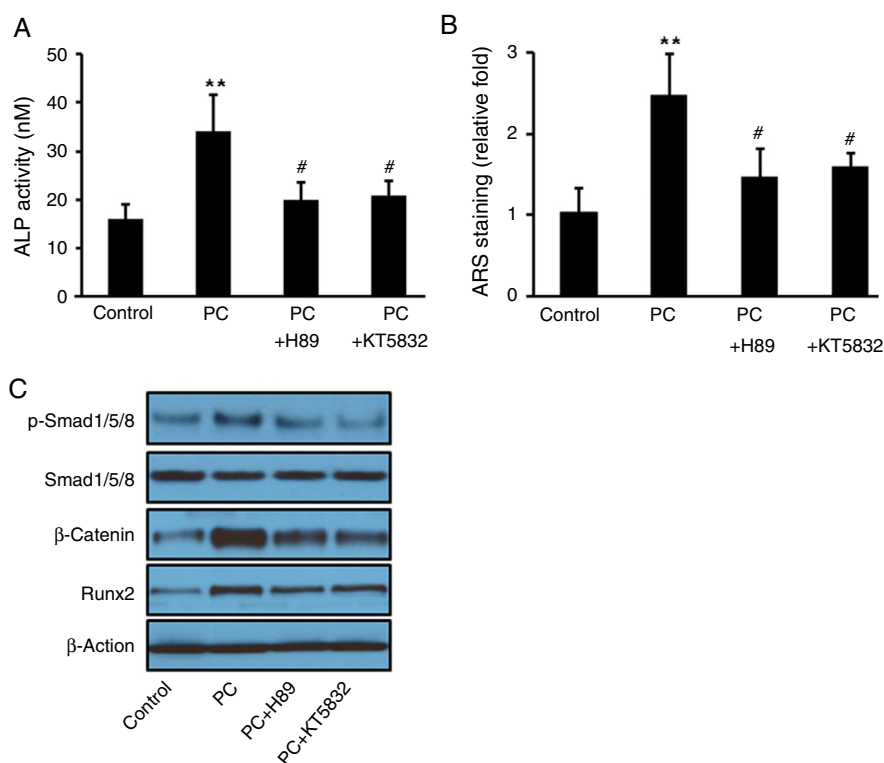


Fig. 4. PC-induced osteoblast differentiation was regulated by cAMP/cGMP pathways. cAMP inhibitor H89 (10 μ M) and cGMP inhibitor KT5832 (3 μ M) pretreatment significantly counteracted PC-mediated (1.25 μ M) ALP activity (A) and mineralization (B), as well as the protein expression (C). Data were expressed as mean $\pm$ SD ($n=3$). # $p < 0.05$ vs PC alone; * $p < 0.05$, ** $p < 0.01$ vs control. Samples were measured in triplicate and experiments were repeated three times.

(Jia et al., 2003; Lo et al., 2010). WNT/ β -Catenin signaling pathway contributes toward osteoblast differentiation as another critical mechanism. After binding with LRP5/6 and Frizzled receptors to stabilize β -catenin in the cytoplasm, β -catenin translocates into the nucleus to regulate osteoblast differentiation-related gene expression (Kikuchi, 2000; MacDonald and He, 2012). As a downstream regulator of WNT/BMP pathway, Runx2 is crucial in the process of osteoblast differentiation (Lee et al., 2003). In this study, PC treatment increased the gene expression of *Bmp*, *Wnt* ligands and *Runx2*, as well as the protein expression of p-Smad1/5/8, β -catenin and Runx2. Moreover, the BMP antagonist Noggin and WNT inhibitor Dkk-1 significantly inhibited PC-induced ALP activity and mineralization, further confirming PC-induced osteoblast differentiation through the functional cross talk between BMP/WNT pathways.

Increasing evidence suggested that cAMP signaling is a major pathway for regulating several osteoblastic genes, such as Runx2 and osteocalcin. These studies substantiated that the cAMP signaling pathway is important in osteoblast recruitment from osteoprogenitor cells (Wakabayashi et al., 2002). As one of the most important osteogenic intracellular second messengers, cGMP signaling pathway increases osteoblast proliferation and differentiation, as well as the osteogenesis-associated gene expression (Wang et al., 2009; Chen et al., 2014). In this study, cAMP and cGMP pathway inhibitors, H89 and KT5823, reversed PC-induced protein expression of osteogenesis and ALP activity, suggesting that the effects of PC in osteoblast differentiation were mediated, at least partially via cAMP/cGMP signaling pathways.

In conclusion, our data demonstrated that PC isolated from the metabolites of the endophytic fungus *penicillium* sp. FJ-1 of *Acanthus ilicifolius* promoted osteoblast differentiation of MC3T3-E1 cells through activations of BMP/WNT pathways via cAMP/cGMP

signaling, providing the scientific rationale to develop PC as a potential agent for the prevention of bone loss.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent

The authors declare that no patient data appear in this article.

Authors' contribution

Study concepts and design: Z-FG; Experimental studies: HZ, G-PZ, HJ; data analysis: HZ, G-PZ; manuscript preparation: HZ, G-PZ, HJ, Z-FG.

Conflicts of interest

The authors declare no conflicts of interest.

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