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Physcion-8-*O*-β-D-Glucopyranoside Enhances the Commitment of Mouse Mesenchymal Progenitors Into Osteoblasts and Their Differentiation: Possible Involvement of Signaling Pathways to Activate BMP Gene Expression

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ABSTRACT

Here, we show the involvement of signaling pathways to induce the gene expression of bone morphogenetic protein (BMP) in the osteogenic activity of physcion-8-*O*-β-D-glucopyranoside (physcion-Glu); it stimulated osteoblast differentiation in mouse osteoblast MC3T3-E1 subclone 4 cells and induced BMP-2 gene expression and activation of Akt and ERK/MAP kinases. Physcion-Glu-induced BMP-2 expression and mineralization were attenuated by LY294002, an inhibitor of PI3K that lies upstream of Akt and MAP kinases, suggesting that physcion-Glu induces osteoblast differentiation via PI3K-Akt/MAP kinase signaling pathways, which play important roles in inducing BMP-2 gene expression. Physcion-Glu also enhanced BMP-2-induced commitment of mouse bi-potential mesenchymal precursor C2C12 cells into osteoblasts while inducing the transcription of several osteogenic BMP isoforms, such as BMP-2, -4, -7, and -9. Osteogenic synergy between BMP-2 and physcion-Glu was supported by the fact that noggin inhibited BMP-2 and physcion-Glu-induced alkaline phosphatase expression and activity. Considering that physcion-Glu induced Runx2 activity and the nuclear translocation of p-Smad, physcion-Glu could act by enhancing the BMP signaling pathway that induces Smad activation and translocation to activate Runx2. In conclusion, physcion-Glu could enhance the commitment of mesenchymal progenitors into osteoblasts and their differentiation by activating signaling pathways to induce BMP gene expression. J. Cell. Biochem. 109: 1148–1157, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: PHYSCION-8-*O*-β-D-GLUCOPYRANOSIDE; OSTEOBLAST DIFFERENTIATION; BMP

The homeostasis of bone mass is regulated by the coordinated balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation, referred to as bone remodeling [Harada and Rodan, 2003]. An increase in bone resorption over bone formation leads to an imbalance in bone remodeling that is typical of most skeletal diseases. In case of osteoporosis, the reduction in bone mass increases the risk for fractures followed by skeletal deformity, pain, functional limitations, increased mortality, and

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severe economic burden [van der Klift et al., 2005]. Currently, agents used to treat osteoporosis are antiresorptive agents (bisphosphonates, selective estrogen receptor modulators, and vitamin D analogues) that inhibit the activity of osteoclasts [Rodan and Martin, 2000], but their effects on increasing the bone mass are modest [Christiansen and Lindsay, 1990; Riggs and Hartmann, 2003]. Thus, recent studies have investigated the ability of anabolic agents to stimulate (or accelerate) bone formation, but the FDA-approved anabolic agent parathyroid hormone has limited use because it is comparatively expensive and difficult to use. Therefore, it is important to identify new effective anabolic agents that are less expensive and simple to use [Garrett, 2007].

Bone formation is a highly regulated, sequential process. Following the commitment of mesenchymal stem cells (MSCs) to preosteoblasts, preosteoblasts mature into osteoblasts and then bone mineralization ensues [Katagiri et al., 1994]. Interestingly, bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β superfamily, are powerful inducers of osteogenesis [Phimphilai et al., 2006]. Genetic disruptions of BMPs result in various skeletal and extraskeletal developmental abnormalities [Zhao, 2003]. Recombinant human BMPs (rhBMPs) have potential clinical applications in spinal fusion, fracture healing, and dental tissue engineering [Nakashima and Reddi, 2003; Seeherman and Wozney, 2005]. Among the 14 BMP family members, BMP-2 plays a crucial role in the process of MSC commitment to differentiate into preosteoblasts through bone mineralization [Wozney et al., 1988; Zhao et al., 2002]. Importantly, rhBMP-2 has been approved by the FDA for application in spinal fusion and the treatment of long bone fractures [McKay et al., 2007; White et al., 2007]. Thus, there has been increasing interest in identifying anabolic agents that regulate the BMP pathway. For instance, BMP-2 reporter assays have identified lovastatin as a natural product that stimulates osteoblast differentiation, suggesting that the BMP-2 promoter could be useful for identifying novel agents that enhance osteoblast differentiation [Mundy et al., 1999].

Here, we evaluated the effect of physcion-8-*O*-β-D-glucopyranoside (physcion-Glu; Fig. 1A) on the differentiation of MC3T3-E1 subclone 4 cells and the commitment of bi-potential mesenchymal precursor C2C12 cells into osteoblasts and investigated the potential mechanism to explain the osteogenic activity of physcion-Glu.

MATERIALS AND METHODS

MATERIALS

Physcion-Glu was isolated from the root extract of *Polygonum multiflorum* [Kim et al., 2008]. In brief, the dried roots of *P. multiflorum* (12 kg) were extracted twice with methanol (MeOH) by maceration at room temperature for 7 days. The MeOH solution was combined and evaporated in distilled water (30 L) and partitioned with an equal volume of methylene chloride (MC, 3 × 30 L), ethylacetate (EtOAc, 3 × 30 L), and *n*-butanol (*n*-BuOH, 3 × 30 L) successively, which afforded 130 g of MC fraction, 359 g of

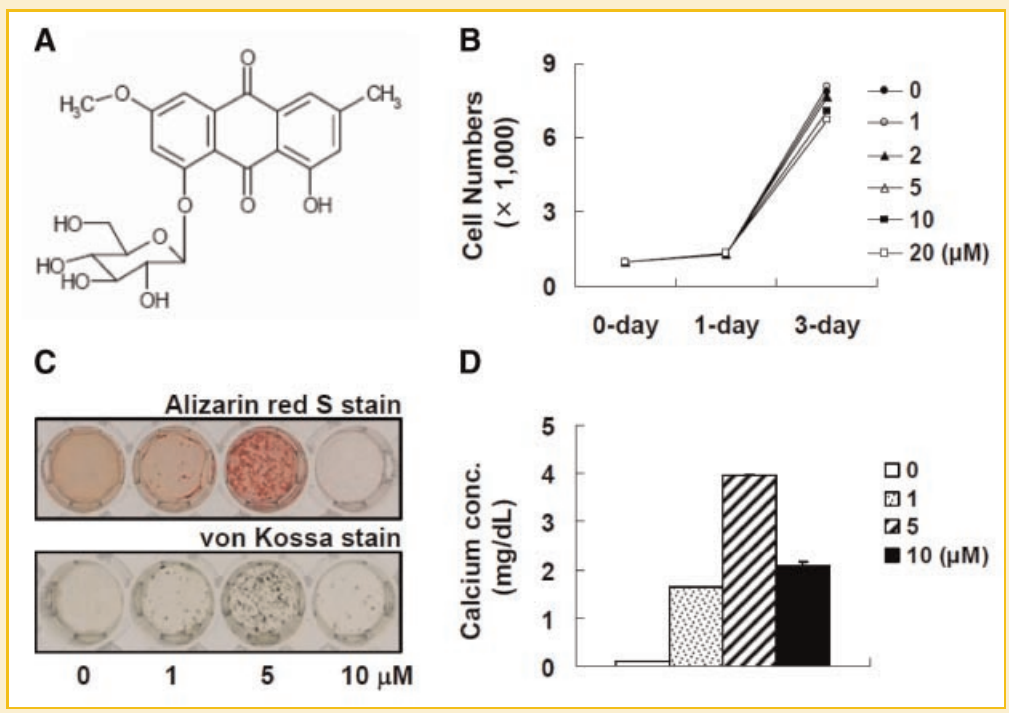

Fig. 1. Physcion-Glu stimulates differentiation and mineralization in MC3T3-E1 subclone 4 cells. A: Structure of physcion-Glu. B: Effect of physcion-Glu on the cell growth. Cells (1×10^3 cells/well) were cultured in a 96-well plate for 1 day and then treated with physcion-Glu. Cell growth was evaluated after 1 or 3 days. C: The effect of physcion-Glu on mineralization was evaluated by Alizarin red S and von Kossa staining. Cells (1.5×10^4 cells/well) were plated in a 24-well plate and cultured in growth medium (GM). After reaching confluence, cells were cultured in differentiation medium (DM) in the absence or presence of physcion-Glu. The mineralized nodules were stained with Alizarin red S (upper) and von Kossa stain (lower) on differentiation day 15. D: Deposited calcium content (mg/dl) was measured on differentiation day 15. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

EtOAc fraction, 477 g of *n*-BuOH fraction, and 810 g of aqueous fraction, respectively. The *n*-BuOH fraction was then chromatographed on silica gel (70–23 mesh) eluted with a gradient solvent system (MeOH in MC 1–50%), from which physcion-Glu (20 mg) was isolated. For measuring the purity of physcion-Glu, HPLC analysis was carried out using Shiseido nanospace SI-1 pump (Tokyo, Japan), Thermo Model 200 Detector (MA), and Shimadzu ODS column; Shim-pack PREP-ODS (H)-Kit, 4.6 mm × 250 mm, 5 μm particle size (Tokyo, Japan). The column temperature was set to 40°C, and solvent system consisted of methanol–H_2O = 70:30 at a 0.5 μl/min flow rate. The sample injection volume was 20 μl, and the UV detection was performed at 254 nm. Physcion-Glu's purity was 87% (t_R = 11.2 min). Physcion-Glu exhibits good water solubility (>500 μM) with 5% DMSO. In this study, 10 mM of physcion-Glu in DMSO was used as a stock solution and diluted with culture medium. Therefore, 0.2% DMSO was used as a vehicle control in all experiments. Recombinant human BMP-2 (rhBMP-2) and noggin were purchased from PeproTech (Korea). LY294002 was purchased from Merck Biosciences Calbiochem (Germany).

CELL CULTURE

Mouse osteoblast MC3T3-E1 subclone 4 cells with high differentiation potential [Wang et al., 1999] and mouse bi-potential mesenchymal precursor C2C12 cells were purchased from American Type Culture Collection (VA). MC3T3-E1 subclone 4 cells were cultured in the growth medium [GM, α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml streptomycin] in humidified 5% CO_2 at 37°C; the medium was changed every 3 days. Cells (1.5×10^4 cells/well) were plated in a 24-well plate and cultured in GM. After reaching confluence, the cells were cultured in differentiation medium [DM, GM with 50 μg/ml of ascorbic acid (Fluka, Germany) and 10 mM of β-glycerophosphate (Sigma, MO)]; the medium was changed every 3 days. C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) containing 10% FBS, 100 U/ml of penicillin, and 100 μg/ml streptomycin. Cells were seeded at 2×10^4 cells/cm^2 and after 1 day, cells were differentiated by replacing the medium with DMEM containing 5% FBS and rhBMP-2 (100 ng/ml). The medium was changed every 3 days.

CELL GROWTH ASSAY

For growth assays, cells were plated in a 96-well plate in GM at 1×10^3 cells/well. After 24 h, cells were incubated in serially diluted compound for 1 or 3 days. Cell growth was evaluated in triplicate using Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol; absorbance was measured at 450 nm using the Wallac EnVision microplate reader (Perkin-Elmer, Finland). Absorbance was converted to cell number using a standard curve.

ALIZARIN RED S STAINING

At differentiation day 15, cells were washed twice with PBS, stained with 40 mM Alizarin red S solution (pH 4.2) for 10 min at room temperature, and washed twice with deionized water. Images of stained cells were captured under a microscope equipped with a DP70 digital camera.

VON KOSSA STAINING

At differentiation day 15, cells were washed with PBS and fixed with 2.5% glutaraldehyde in PBS for 30 min. After washing three times with deionized water, cells were incubated with 5% silver nitrate at room temperature under UV light until calcium turned black. After washing with deionized water three times, images of stained cells were captured using a microscope equipped with a DP70 digital camera.

MEASUREMENT OF CALCIUM AMOUNT

To determine calcium content, cells were washed twice with PBS, fixed in 3.7% formaldehyde in PBS for 15 min, and decalcified with 300 μl of 1 N HCl for 24 h. Calcium content was measured using the Calcium C Kit (Wako Pure Chemicals Industries, Japan) according to the manufacturer's protocol.

HIGH-CONTENT ASSAY FOR EVALUATING BMP-2 PROMOTER ACTIVATION

Cloning of the BMP-2 promoter, its transfection, and the high-content assay for evaluating activation of BMP-2 promoter were performed as described previously [Lee et al., 2008]. In brief, the mouse BMP-2 promoter (−472 to −1562 bp; referred to as clone 4) with distal start sites and NF-κB response element and that with the proximal start site (−553 to +355 bp; referred to as clone 5) were amplified by genomic DNA PCR and cloned into pGlow-TOPO (Invitrogen, CA) according to the manufacturer's protocols. BMP-2 promoter-cloned plasmids were transfected into MC3T3-E1 subclone 4 cells using Lipofectamine 2000 (Invitrogen) and transfected cells were selected using Geneticin Selective Antibiotics (Invitrogen). Cells were plated in a 384-well plate at 0.5×10^3 cells/well and cultured in GM. When the cells were confluent (>95%), the medium was switched to DM. At differentiation day 2, nuclei were stained with SYTO59 (1.25 μM/well; Molecular Probes, OR) for 30 min and then BMP-2 promoter-GFP signals were analyzed using the script "CytoNucMulfields script" that was available in the Acapella CytoNuc Analysis package installed in Opera high-content screening instrument (Evotec Technologies, Germany). BMP-2 promoter GFP signals of cytoplasm were measured by the intensity of ring area as simply described in http://las.perkinelmer.com/Content/RelatedMaterials/Brochures/BRO_AcapellaSoftware.pdf. Measured GFP intensity was presented as % of control.

EVALUATION OF mRNA EXPRESSION LEVEL

Primers were designed using an online primer design program [Rozen and Skaletsky, 2000] (Table I). Total RNA was isolated using TRIzol reagent (Life Technologies, MD) according to the manufacturer's protocol. The concentration and purity of total RNA were calculated by measuring absorbance at 260 and 280 nm. First-strand cDNA was synthesized using 2 μg of total RNA and 1 μM of oligo-dT_{18} primer and Omniscript Reverse Transcriptase (Qiagen, CA). SYBR green-based quantitative PCR was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA) with 3 μl of first-strand cDNA diluted 1:50 and 20 pmol of primers, according to the manufacturer's protocols. The PCR reaction consisted of three segments. The

TABLE I. Primers Used in This Study

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
OPN	CGATGATGATGACGATGGAG	TGGCATCAGGATACTGTTCATC
OCN	AAGCAGGAGGGCAATAAGGT	TTTGTAGGCGGTCTTCAAGC
COLI	ACGTCCTGGTGAAGTTGGTC	CAGGGAAGCCTCTTTCTCCT
BMP-2	GCTCCACAAACGAGAAAAGC	AGCAAGGGGAAAAGGACACT
BMP-4	CCTGGTAACCGAATGCTGAT	AGCCGGTAAAGATCCCTCAT
BMP-6	TTCTTCAAGGTGAGCGAGGT	TAGTTGGCAGCGTAGCCTTT
BMP-7	CGATACCACCATCGGGAGTTC	AAGGTCTCGTTGTCAAATCGC
BMP-9	CAGAACTGGGAACAAGCATCC	GCCGCTGAGGTTTAGGCTG
RUNX2	GCCGGGAATGATGAGAACTA	GGACCGTCCACTGTCACTTT
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

first segment (95°C for 10 min) activated the polymerase; the second segment included three-step cycling (40 cycles) at 94°C for 40 s (denaturation), 60°C for 40 s (annealing), and 72°C for 1 min (extension); the third segment was performed to generate PCR product temperature dissociation curves ("melting curves") at 95°C for 1 min, 55°C for 30 s, 95°C for 30 s. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen, 2001]. GAPDH was used as the control gene. Significance was determined with GAPDH-normalized $2^{-\Delta\Delta C_t}$ values.

WESTERN BLOT ANALYSIS

For Western blot analysis, cells were homogenized in buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20, 1 mM PMSF, and one protease inhibitor cocktail tablet (Roche, Germany) at 4°C and then centrifuged at 10,000*g* for 15 min. The supernatant was used as the cytoplasmic protein fraction and nuclear proteins were extracted using NucBuster Protein Extraction kit (Novagen, Germany). Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, IL). Samples (20 μg) were mixed with sample buffer (100 mM Tris–HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 min, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using the Mini Protean 3 Cell (Bio-Rad, CA). The resolved proteins were transferred to a nitrocellulose membrane (Scheicher & Schnell BioScience, Germany). To ascertain the amount of protein loaded and the transfer efficiency, the membranes were stained with Ponceau S staining solution. For immunoanalysis, the membranes were washed and incubated in blocking buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20, 3% nonfat dry milk) and then incubated with diluted primary antibodies (1:1,000) for 2 h at room temperature. Antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) with the exception of antibody against p-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad 8 (Ser 426/428) (Cell Signaling, MA). Following the primary antibody reactions, the membranes were washed with blocking buffer three times (15 min each) and then probed with diluted secondary antibodies (1:2,000) for 1 h. The membranes were washed three times (15 min each) and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd, Japan).

ALKALINE PHOSPHATASE STAINING AND ACTIVITY ASSAY

C2C12 cells were plated at 5×10^3 cells/well in a 96-well plate and incubated for 24 h. Then, the medium was replaced with DMEM containing 5% FBS and 100 ng/ml rhBMP-2. After 3 days, the cells were washed twice with PBS, fixed with 10% formalin in PBS for 30 s, rinsed with deionized water, and stained using the alkaline phosphatase (ALP) Kit (Sigma) under protection from direct light. Images of stained cells were captured under a microscope equipped with a DP70 digital camera (Olympus Optical, Japan). To measure ALP activity, cells were washed twice with PBS and sonicated in lysis buffer (10 mM of Tris–HCl, pH 7.5, 0.5 mM of $MgCl_2$, and 0.1% Triton X-100). After centrifugation at 10,000*g* for 20 min at 4°C, ALP activity in the supernatant was measured in triplicate using the LabAssay ALP Kit (Wako Pure Chemicals Industries). Protein concentration was measured using the BCA Protein Assay kit (Pierce Biotechnology).

Runx2 LUCIFERASE REPORTER ASSAY

To measure Runx2 activity, the p6xosteoblast-specific *cis*-acting element (OSE) 2-luc reporter vector was transiently transfected into C2C12 cells that were plated in a 96-well plate at 5×10^3 cells/well, as described previously with modifications [Kim et al., 2004]. After 24 h, medium was replaced with DMEM containing 5% FBS with or without physcion-Glu. After 24 h, the transfected cells were lysed and luciferase activity was measured using the luciferase reporter assay system (Promega) and the Wallac EnVision microplate reader.

STATISTICAL ANALYSIS

One-way ANOVA with Tukey's multiple comparison test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Sofware, CA).

RESULTS

PHYSCION-Glu STIMULATES DIFFERENTIATION OF MC3T3-E1 SUBCLONE 4 CELLS

In this study, we evaluated the effect of physcion-Glu on osteoblast differentiation and BMP-2 expression. Firstly, we investigated whether noncytotoxic concentrations of physcion-Glu ($\leq$20 μM; Fig. 1B) affected osteoblast differentiation by observing phenotypic changes that reflect mineralization. As shown in Figure 1C, the level of mineralization in cells cultured with 5 μM physcion-Glu was greater than that in the control cells. The levels of calcium deposited in cells were significantly increased by physcion-Glu; $0 < 1$, 5, or 10 μM, $1 < 5$ or 10 μM, and $10 < 5$ μM ($P < 0.001$; Fig. 1D).

PHYSCION-Glu INDUCES mRNA EXPRESSION OF BMP-2

We investigated the ability of physcion-Glu to induce BMP-2 gene expression via high-content assay using a BMP-2 reporter construct and MC3T3-E1 subclone 4 cells. On differentiation day 2, the fluorescent images and quantitative measurement of GFP signal density clearly revealed that physcion-Glu significantly induced BMP-2 expression (Fig. 2A, B); in clone 4, $0 < 2$, 5, or 10 μM, $1 < 2$,

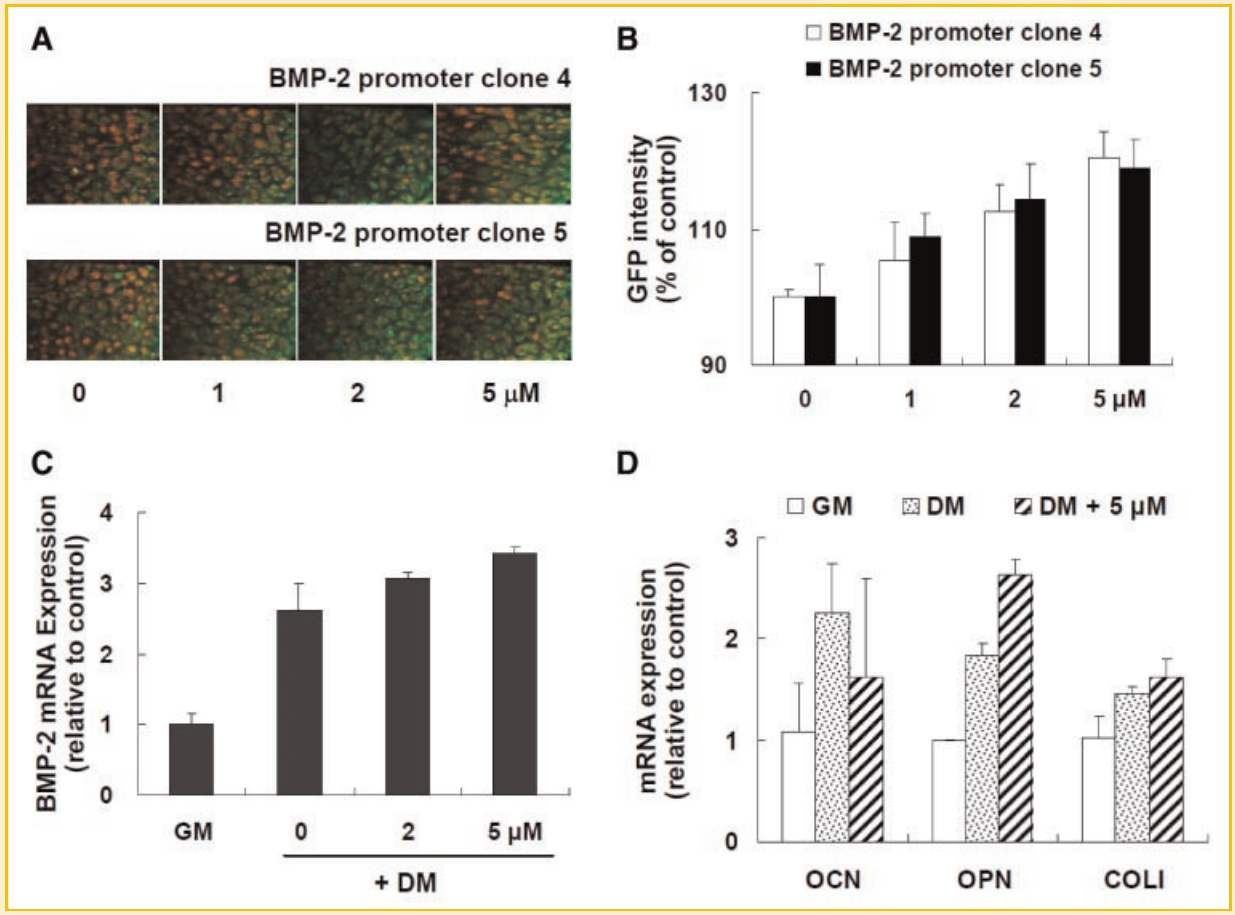

Fig. 2. Physcion-Glu induces expression of BMP-2 gene and osteoblast differentiation-related genes in MC3T3-E1 subclone 4 cells. A: Images of cells expressing BMP-2 promoter-GFP after 2 days of activation with physcion-Glu. B: BMP-2 promoter-GFP signal intensities were measured using an Opera high-content screening instrument. C: Effect of physcion-Glu on BMP-2 mRNA expression was evaluated by real-time quantitative-PCR. Cells (1.5×10^4 cells/well) were plated in a 24-well plate and cultured in growth medium (GM). After reaching confluence, the cells were cultured in differentiation medium (DM) in the absence or presence of physcion-Glu. After 2 days, BMP-2 mRNA expression was evaluated. D: Expression levels of genes related to osteoblast differentiation and mineralization were evaluated by real-time quantitative-PCR. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

5, or 10 μM, 2 < 5 or 10 μM, and 5 < 10 μM ($P < 0.05$), and in clone 5, 0 < 2, 5, or 10 μM, 1 < 5 or 10 μM, 2 < 10 μM, and 5 < 10 μM ($P < 0.01$). Additionally, the effect of physcion-Glu on mRNA expression of BMP-2 was evaluated by real-time quantitative PCR (Fig. 2C). BMP-2 mRNA expression was significantly increased following treatment with 5 μM physcion-Glu; GM < DM, DM + physcion-Glu 2.5 or 5 μM, and DM < DM + physcion-Glu 5 μM ($P < 0.01$). These results suggested that physcion-Glu could induce osteoblast differentiation and mineralization by inducing BMP-2 gene expression.

The osteogenic activity of physcion-Glu was also confirmed by evaluating markers of osteoblast differentiation, such as osteopontin (OPN), osteocalcin (OCN), and type I collagen (COLI; Fig. 2D). The mRNA expression levels of OPN (GM < DM or DM + physcion-Glu 5 μM and DM < DM + physcion-Glu 5 μM, $P < 0.001$) and COLI (GM < DM or DM + physcion-Glu 5 μM, $P < 0.05$) were significantly increased on differentiation day 15; however, treatment with physcion-Glu did not induce a significant increase in the mRNA expression of OCN at that time point.

PHYSCION-Glu-INDUCED BMP-2 EXPRESSION AND MINERALIZATION ARE ATTENUATED BY THE PHOSPHATIDYLINOSITOL 3-KINASE INHIBITOR LY294002

Recent studies demonstrated that Akt and mitogen-activated protein (MAP) kinase signaling pathways play key roles in the activation of BMP-2 gene expression [Ghosh-Choudhury et al., 2007]. Interestingly, the phosphorylation of Akt and ERK, both of which are downstream signaling molecules of phosphatidylinositol 3-kinase (PI3K), was induced by physcion-Glu; this phosphorylation was attenuated by co-treatment with the PI3K inhibitor LY294002, as shown in Figure 3A. LY294002 also inhibited physcion-Glu-induced BMP-2 gene expression (Fig. 3B). Importantly, LY294002 exhibited dose-dependent inhibition of physcion-Glu-induced mineralization in cells that had been differentiated for 15 days (Fig. 3C). Additionally, the time-course phosphorylation of AKT and ERK depending on the presence of LY294002 was examined. As shown in Figure 3D, AKT phosphorylation was time dependently increased by physcion-Glu up to 30 min after treatment, but it conversely decreased by the co-treatment with physcion-Glu and

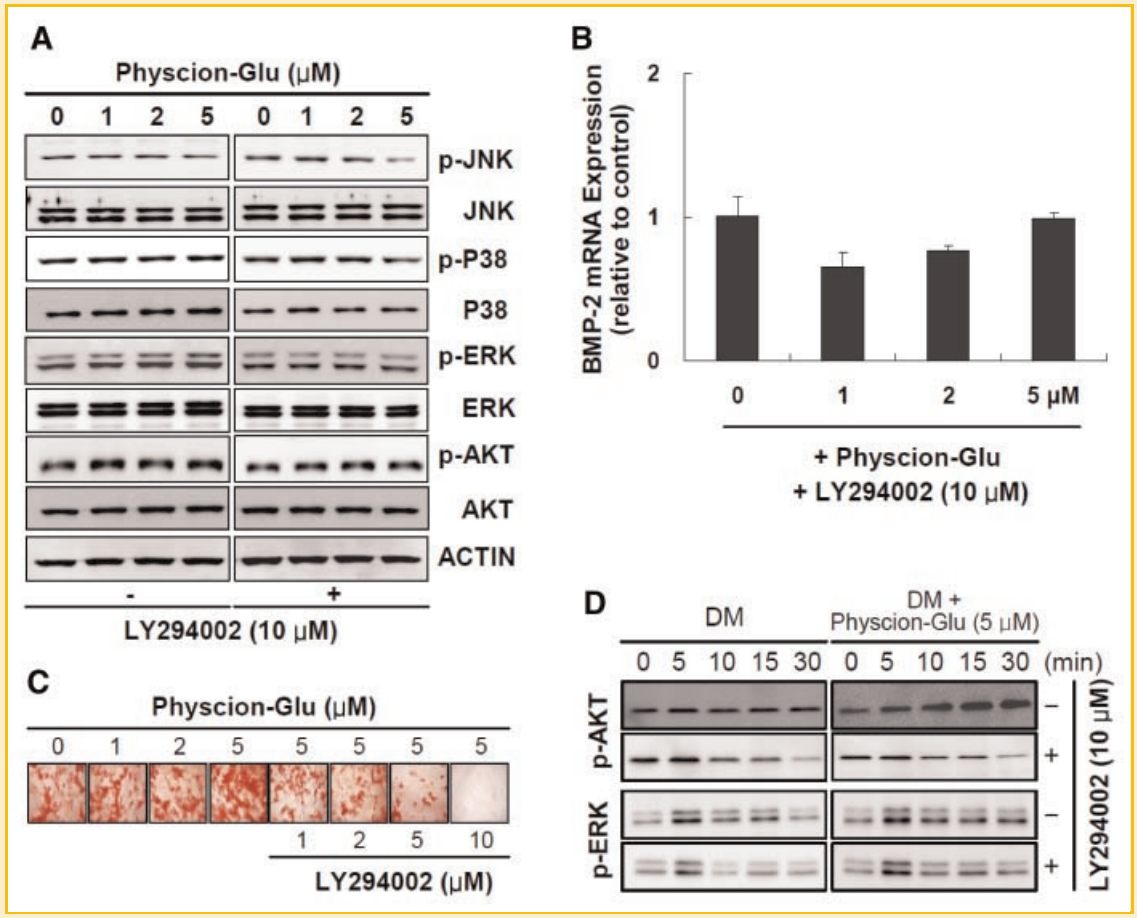

Fig. 3. Involvement of PI3K and ERK activation in the physcion-Glu-induced differentiation of MC3T3-E1 subclone 4 cells. A: Effect of LY294002 on physcion-Glu-induced activation of signaling molecules. Cells (5×10^5 cells) were plated in a 100 mm dish and cultured in growth medium (GM). After reaching confluence, cells were incubated in differentiation medium (DM) in the absence or presence of physcion-Glu. The activation of Ras, PI3K, Akt, and MAP kinases in cells incubated with physcion-Glu for 30 min was evaluated by Western blot analysis. Cells were pretreated with LY294002 for 2 h before physcion-Glu treatment, followed by Western blot analysis. B: The effect of LY294002 on physcion-Glu-induced BMP-2 mRNA expression. Cells were pretreated with LY294002 for 2 h prior to treatment with physcion-Glu; then, BMP-2 mRNA expression was evaluated on differentiation day 2, as described in Figure 2. C: Effect of LY294002 on physcion-Glu-induced mineralization. Cells were co-treated with LY294002 and physcion-Glu every 3 days; mineralized nodules were stained with Alizarin red S on differentiation day 15. D: The time-course phosphorylation of Akt and ERK by physcion-Glu in the presence of LY294002 was evaluated by Western blot analysis. Cells were treated with physcion-Glu in the presence or absence of LY294002 and then homogenized at the indicated time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

LY294002. ERK phosphorylation was done by physcion-Glu 5 min after treatment. Physcion-Glu-induced ERK phosphorylation was maintained up to 30 min, but it was shown to be attenuated by the co-treatment with physcion-Glu and LY294002. These results suggested that the osteogenic activity of physcion-Glu could result from its potential to induce the expression of BMP-2 via PI3K-mediated activation of Akt and ERK.

PHYSCION-Glu ACCELERATES MESENCHYMAL STEM CELL COMMITMENT INTO OSTEOBLASTS BY ACTIVATING BMP EXPRESSION AND ITS SIGNALING PATHWAY

The induction of BMP-2 gene expression is also important in the commitment of MSCs into preosteoblasts. Therefore, we evaluated the effect of physcion-Glu on the commitment of MSCs (C2C12 cells) into preosteoblasts in the presence of BMP-2. Physcion-Glu alone (without BMP-2) did not induce ALP expression (data not shown), but physcion-Glu enhanced the BMP-2-stimulated ALP expression in a dose-dependent manner relative to cells treated BMP-2 alone (Fig. 4A). In C2C12 cells, the effect of physcion-Glu on the mRNA induction of BMPs was also evaluated by quantitative real-time PCR (Table II). BMP-2 mRNA expression was significantly induced by the combination of rhBMP-2 and low concentrations (5–10 μM) of physcion-Glu (control or rhBMP-2 < rhBMP-2 + physcion-Glu 5 or 10 μM and rhBMP-2 + physcion-Glu 5 μM < rhBMP-2 + physcion-Glu 10 μM; $P < 0.001$) but not rhBMP-2 alone. The mRNA expression of BMP-4 that was expressed to a greater extent than were other BMP isoforms was significantly induced by rhBMP-2 alone or its combination with physcion-Glu (control < rhBMP-2 or rhBMP-2 + physcion-Glu 5, 10, or 20 μM; $P < 0.001$), and additionally, the rhBMP-2-stimulated BMP-4 mRNA induction was significantly enhanced by high concentrations (10–20 μM) of physcion-Glu (rhBMP-2 < rhBMP-2 + physcion-Glu 10 or 20 μM, and rhBMP-2 + 5 or 10 μM < rhBMP-2 + physcion-Glu 20 μM; $P < 0.05$). BMP-6 mRNA expression was significantly induced by

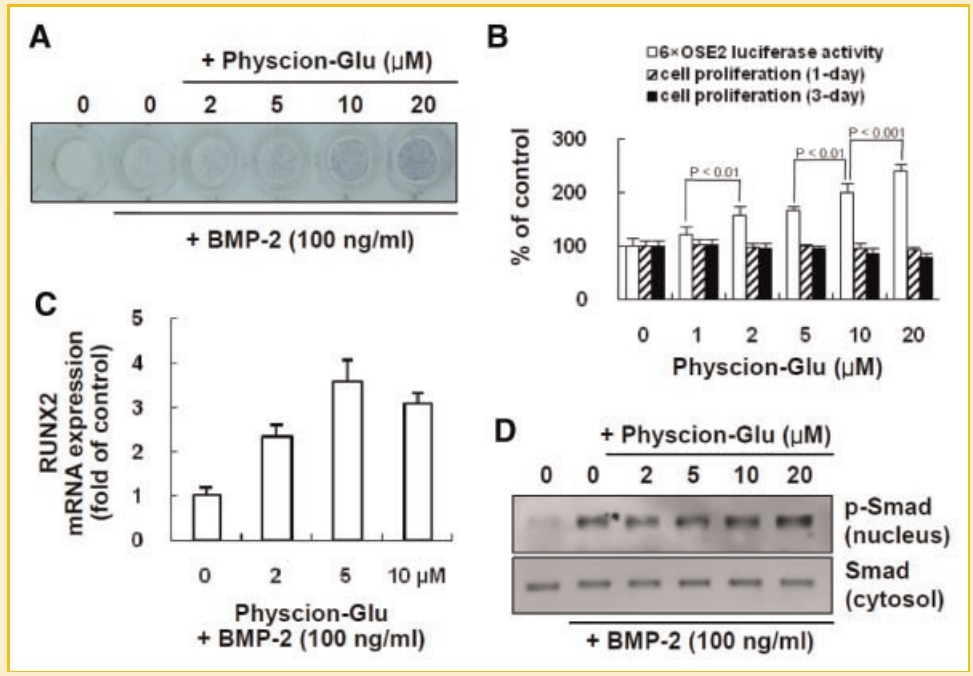

Fig. 4. Physcion-Glu enhances rhBMP-2-induced commitment of C2C12 cells into osteoblasts with Runx2/Smad activation. A: C2C12 cells were plated at 2×10^4 cells/cm^2. After an overnight incubation, the medium was replaced with DMEM containing 5% FBS, rhBMP-2 (100 ng/ml), and physcion-Glu. After 3 days, the commitment of C2C12 cells into osteoblasts was visualized by ALP staining. B: Effects of physcion-Glu on Runx2 activity and cell proliferation. Runx2 activation was measured by luciferase reporter assay using 6×OSE2-luc-stably transfected C2C12 cells; the effect of physcion-Glu on the growth of C2C12 was evaluated as described in Figure 1B. C: Effect of physcion-Glu on Runx2 mRNA expression. Cells were treated with physcion-Glu and rhBMP-2 (100 ng/ml) for 3 days, and Runx2 mRNA level was evaluated by quantitative real-time PCR. D: Effect of physcion-Glu on Smad1/5/8 activation. The cytosolic level of Smad and the nuclear level of p-Smad1/5/8 in cells co-treated physcion-Glu with rhBMP-2 for 30 min were evaluated by Western blot analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rhBMP-2 alone or its combination with low concentrations (5–10 μM) of physcion-Glu (control < rhBMP-2 or rhBMP-2 + physcion-Glu 5 or 10 μM; $P < 0.05$). BMP-7 mRNA expression was significantly induced by the combination of rhBMP-2 and low concentrations (5–10 μM) of physcion-Glu (control or rhBMP-2 < rhBMP-2 + physcion-Glu 5 or 10 μM; $P < 0.001$), but not rhBMP-2 alone. Interestingly, BMP-7 mRNA was strongly induced by the combination of rhBMP-2 and physcion-Glu at 5 μM. Similar to BMP-7 expression pattern in a response to rhBMP-2 and/or its combination with physcion-Glu, BMP-9 mRNA expression was significantly induced by the combination of rhBMP-2 and low concentrations (5–10 μM) of physcion-Glu (control or rhBMP-2 < rhBMP-2 + physcion-Glu 5 or 10 μM; $P < 0.01$), but not rhBMP-2 alone. BMP-9 mRNA was also strongly induced by the combination of rhBMP-2 and physcion-Glu at 5 μM.

Furthermore, physcion-Glu significantly promoted the activation of Runx2 without cytotoxicity (Fig. 4B). The mRNA level of Runx2 was also significantly induced by the addition of physcion-Glu ($P < 0.05$; Fig. 4C) with no difference between physcion-Glu 5 μM and physcion-Glu 10 μM ($P > 0.05$).

Because phosphorylation and nuclear translocation of Smad has been shown to induce Runx2 transcriptional activity as a key mediator protein of BMP-2 signaling pathway [Bessa et al., 2008], we examined the effect of physcion-Glu on the translocation of Smad1/5/8 and found that physcion-Glu enhanced the BMP-2-induced level of nuclear p-Smad1/5/8 in a dose-dependent manner (Fig. 4D).

The inhibitory effect of noggin, a BMP antagonist, on physcion-Glu-enhanced ALP activity in the presence of BMP-2 supported the hypothesis that the stimulatory effect of physcion-Glu on the

TABLE II. Effect of Physcion-Glu on the mRNA Expression Levels of BMPs in C2C12 Cells

	BMP-2	BMP-4	BMP-6	BMP-7	BMP-9
Control	1.00 ± 0.03	377.23 ± 16.99 (1.00 ± 0.05)	0.24 ± 0.01 (1.00 ± 0.02)	1.04 ± 0.42	0.76 ± 0.10 (1.01 ± 0.13)
rhBMP-2	2.28 ± 0.19	1027.90 ± 68.99 (2.73 ± 0.18)	2.02 ± 0.42 (8.28 ± 1.74)	3.70 ± 0.94	3.55 ± 0.49 (4.72 ± 0.67)
rhBMP-2 + physcion-Glu 5 μM	5.32 ± 0.06	1164.10 ± 68.48 (3.09 ± 0.18)	1.40 ± 0.17 (5.74 ± 0.71)	37.16 ± 2.59	31.30 ± 4.91 (41.64 ± 6.54)
rhBMP-2 + physcion-Glu 10 μM	8.42 ± 0.81	1284.26 ± 13.57 (3.41 ± 0.04)	2.23 ± 0.54 (9.16 ± 2.21)	17.16 ± 0.71	12.43 ± 0.56 (16.54 ± 0.76)
rhBMP-2 + physcion-Glu 20 μM	3.42 ± 0.89	1459.90 ± 84.75 (3.87 ± 0.26)	0.84 ± 0.50 (3.44 ± 2.06)	1.57 ± 0.53	3.54 ± 0.07 (4.71 ± 0.10)

Cells were treated with physcion-Glu and rhBMP-2 (100 ng/ml) for 3 days, and the BMP mRNA levels were evaluated by quantitative real-time PCR. Fold changes relative to BMP-2 mRNA expression in control cells are presented as mean ± standard deviation. Fold changes relative to each BMP gene level in control cell are presented in parentheses.

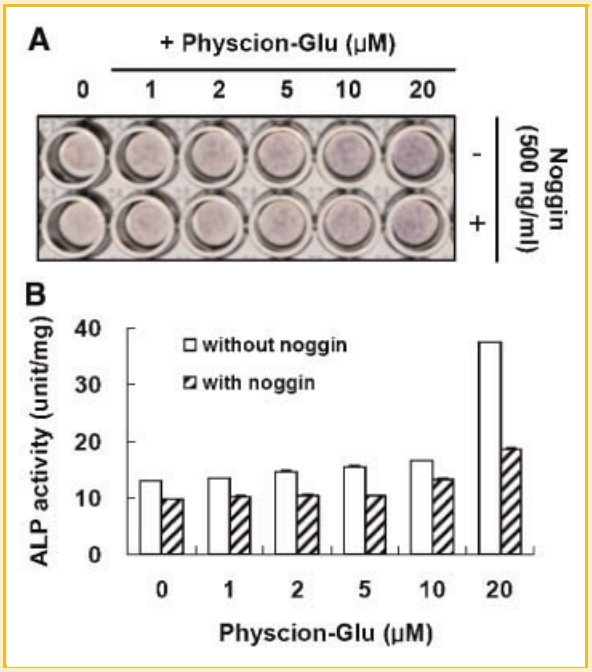

Fig. 5. Noggin inhibits physcion-Glu-enhanced ALP expression and activity in C2C12 cells. Cells were plated at 2×10^4 cells/cm^2. The next day, physcion-Glu and rhBMP-2 were added. After 3 days, the cells were treated with noggin (500 ng/ml) for an additional 3 days and then the commitment of C2C12 cells into osteoblasts was visualized by ALP staining (A) or evaluated by ALP activity assay (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

commitment of MSCs into osteoblasts resulted from its ability to enhance BMP expression and activate its signaling pathway. Cells were plated and the next day, physcion-Glu and rhBMP-2 were added. After 3 days, the cells were treated with noggin for an additional 3 days and then the commitment of C2C12 cells into osteoblasts was visualized by ALP staining (Fig. 5A) or evaluated by ALP activity assay (Fig. 5B). Interestingly, physcion-Glu-enhancements of BMP-2-stimulated ALP expression and activity were dramatically inhibited by the additional treatment of noggin.

DISCUSSION

BMPs have strong osteogenic activity. In fact, BMP-2 and -7 have recently been approved for clinical applications in spinal fusion, fracture healing, and dental tissue engineering, suggesting that anabolic agents that stimulate BMP expression or its signaling pathway, as well as BMPs per se, could be used to treat osteoblast-related diseases via bone formation or regeneration [Nakashima and Reddi, 2003; Seeherman and Wozney, 2005].

Physcion-Glu is an anthraquinone isolated from *P. multiflorum*, a traditional Chinese herbal medicine with several biological activities that include anti-allergic and anti-tumor activities [Kim et al., 2008]. However, its effect on bone metabolism has not been investigated before. In this study, we showed the osteogenic activity of physcion-Glu by activating signaling pathways required for BMP expression. We found that in mouse osteoblast MC3T3-E1 subclone 4 cells, physcion-Glu stimulated osteoblast differentiation and induced BMP-2 gene expression. In addition, physcion-Glu activated Akt and ERK/MAP kinases, but physcion-Glu-induced BMP-2 expression and mineralization were attenuated by LY294002, a specific inhibitor of PI3K, suggesting that physcion-Glu could induce osteoblast differentiation via the PI3K-Akt/MAP kinase signaling pathway that plays an important role in the induction of BMP-2 gene expression.

BMP-2 is a key stimulator of osteoblast differentiation. Recently, the activation of Akt and MAP kinases (especially ERK), both of which lie downstream in the PI3K signaling pathway, were shown to enhance osteoblast differentiation and bone formation by inducing BMP-2 gene expression [Ghosh-Choudhury et al., 2007]. The importance of Akt in bone formation was demonstrated in Akt-deficient mice, in which both bone mass and bone formation were decreased and osteoblast differentiation-related genes were down-regulated [Kawamura et al., 2007]. MAP kinases are essential for inducing osteoblast differentiation-related genes via the activation of distinct cascades that target specific transcription factors. The activation of p38 appeared to be critical for the control of ALP expression during the differentiation of MC3T3-E1 cells [Suzuki et al., 2002]. In primary human osteoblasts, 1,25(OH)$_2$D3-stimulated ALP activity was shown to be directly related with ERK pathway [Chae et al., 2002]. The flow induction of OPN is also mediated through the ERK pathway, and COLI has been shown to be regulated by both ERK and JNK signaling pathways [Wu et al., 2006]. As described above, the activation of Akt and MAP kinases to trigger osteoblast differentiation could be regulated by PI3K. Inhibition of PI3K activity by the specific inhibitor LY294002 has been shown to prevent BMP-2-induced expression of ALP, an early marker of osteoblast differentiation [Ghosh-Choudhury et al., 2002].

The induction of BMP-2 gene expression is also important in the commitment of MSCs to differentiate into preosteoblasts. In C2C12 cells, a mouse bi-potential mesenchymal precursor line, the early osteogenic marker ALP activity, and the late osteogenic marker OCN expression were strongly induced by BMP-2, -4, -6, -7, and -9 [Cheng et al., 2003]. BMP-2 has also exhibited potent osteogenic activity in human clinical trials [Boden et al., 2000; Valentin-Opran et al., 2002], and BMP-7 has shown osteogenic activity in C2C12 cells [Yeh et al., 2002]. As well, BMP-4, -6, and -9 have been reported to have osteogenic potential in C2C12 cells [Ebisawa et al., 1999; Li et al., 2003; Bessa et al., 2009]. Interestingly, simultaneous gene transfer of BMP-2 and -7 induced rapid bone formation and increased expression of endogenous BMP-4 [Kawai et al., 2006], and BMP-2 enhanced the expression of other BMP genes during bone cell differentiation [Chen et al., 1997], suggesting that the ability to enhance expression of other BMP genes could be important in bone formation.

Here, in the presence of BMP-2, physcion-Glu enhanced the commitment of C2C12 cells into osteoblasts and significantly increased the transcription of several osteogenic BMP isoforms, including BMP-2, -4, -7, and -9. Although the basal expression level of BMP-4 was higher than that of other BMPs, the fold induction by physcion-Glu was greatest for BMP-7 and -9. Interestingly, physcion-Glu induced a dose-dependent increase in BMP-4 mRNA expression, whereas BMP-2, -7, and -9 were significantly induced at

low concentrations of physcion-Glu. Considering that the simultaneous transfer of BMP-2 and -7 genes induced more rapid bone formation than that induced by the transfer of either gene alone [Kawai et al., 2006], it is possible that BMPs act synergistically to promote osteogenesis. Furthermore, the results showing the inhibitory effect of noggin on ALP expression and activity induced by the combination of BMP-2 and physcion-Glu suggested to the possible involvement of physcion-Glu-induced BMP expression in its osteogenic action. Noggin is a BMP antagonist and its silencing has been shown to enhance osteoblastic differentiation of BMP-responding cells in vitro and rhBMP-2-induced new bone formation in vivo [Takayama et al., 2009].

BMPs can trigger osteoblast commitment and differentiation in MSCs through the activation of Runx2, which subsequently regulates osteoblast-differentiation-related genes [Gersbach et al., 2004; Phimphilai et al., 2006]. The fact that Runx2 is a major transcription factor controlling osteoblast commitment and differentiation was revealed in Runx2 knockout mice, which have cartilaginous skelectons and complete absence of ossification [Ducy et al., 1997; Otto et al., 1997]. The activity of Runx2 is modulated by Smad, which is an important mediator of BMP signaling [Lian et al., 2003]. The binding of BMP-2 to its receptor can trigger the association of phosphorylated Smad1/5 with Smad4. This complex migrates into the nucleus where it interacts with Runx2 and activates the transcription of OSE2-containing genes that are required for MSC commitment and osteoblast differentiation [Ducy and Karsenty, 1995; Merriman et al., 1995]. Interestingly, promoters of osteoblast-specific genes, such as OCN and OPN, contain OSE2s [Hanai et al., 1999; Guicheux et al., 2003; Phimphilai et al., 2006]. Here, we found that physcion-Glu induced the Runx2 activity with the nuclear translocation of phosphorylated Smad, suggesting that physcion-Glu could enhance the BMP signaling pathway, resulting in Smad activation/phosphorylation and translocation and Runx2 activation.

Physcion-Glu exhibited the osteogenic activity in both preosteoblastic MC-3T3-E1 subclone 4 and bi-potential mesenchymal progenitor C2C12 cells with the induction of BMP expression, but its doses to achieve osteogenic action in both cells were different as well as cell culture condition; in MC3T3-E1 subclone 4 cells, physcion-Glu exhibited the highest osteogenic activity at 5 μM in the differentiation condition (or in the presence of ascorbic acid and β-glycerophosphate) and in C2C12 cells, 20 μM of physcion-Glu was required to exhibit its osteogenic activity in the presence of BMP-2. These results suggested that physcion-Glu could synergistically exhibit its osteogenic activity with osteogenic differentiation factors such as ascorbic acid and BMP-2. Additionally, the difference of osteogenic mechanisms between preosteoblastic MC3T3-E1 subclone 4 cells and bi-potential mesenchymal C2C12 cells might result in the cell type-dependent difference of physcion-Glu's concentration required to exhibit its osteogenic activity suggesting that physcion-Glu might be needed for optimizing its concentration to enhance osteoblast differentiation depending on cell type or its applications.

In conclusion, physcion-Glu could enhance the commitment of mesenchymal progenitors into osteoblasts and their differentiation by activating signaling pathways to induce BMP gene expression. Similar studies may help to elucidate the molecular mechanisms of compounds with osteogenic activity and lead to the development of therapies that benefit bone regeneration and fracture healing by regulating BMP signaling.

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