Amino Acids (2010) 39:1217–1226
DOI 10.1007/s00726-010-0557-8

ORIGINAL ARTICLE

Tanshinone IIA enhances BMP-2-stimulated commitment of C2C12 cells into osteoblasts via p38 activation

Hye Joo Kim · Seong Hwan Kim

Received: 1 February 2010 / Accepted: 4 March 2010 / Published online: 19 March 2010

Abstract In this study, we demonstrate a stimulatory effect of tanshinone IIA isolated from the root of *Salvia miltiorrhiza* on the commitment of bi-potential mesenchymal precursor C2C12 cells into osteoblasts in the presence of bone morphogenetic protein (BMP)-2. At low concentrations, tanshinone IIA enhanced BMP-2-stimulated induction of alkaline phosphatase (ALP), an early phase biomarker of osteoblast differentiation, and mRNA expression of BMPs. ALP induction was inhibited by the BMP antagonist noggin, suggesting that tanshinone IIA enhances the osteogenic activity of BMP signaling. Furthermore, considering the tanshinone IIA-mediated enhancement of BMP-2-stimulated Smad-Runx2 activities, tanshinone IIA could enhance the osteogenic activity of BMP-2 via acceleration of Smad-Runx2 activation. Additionally, pharmacologic inhibition studies suggest the possible involvement of p38 in the action of tanshinone IIA. The p38 inhibitor SB202190 strongly and dose-dependently inhibited tanshinone IIA-enhanced ALP induction. SB202190 also dose-dependently inhibited the tanshinone IIA-induced p38 activation and combined tanshinone IIA-BMP-2-induced Smad activation. In conclusion, tanshinone IIA enhances the commitment of C2C12 cells into osteoblasts and their differentiation through synergistic cross talk between tanshinone IIA-induced p38 activation and BMP-2-induced Smad activation. These activations could subsequently induce the activation of Runx2, which induces osteogenesis via regulation of the osteogenic factors BMP and ALP expression.

Keywords Tanshinone IIA · Osteoblast differentiation · BMP · p38 · Smad · Runx2

H. J. Kim · S. H. Kim (✉)
Laboratory of Chemical Genomics, Pharmacology Research Center, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600, Korea
e-mail: hwan@krict.re.kr

H. J. Kim
Department of Chemistry, Seoul National University, Gwanak-gu, Seoul 151-742, Korea

Introduction

Bone homeostasis is maintained by the ongoing dynamic process of bone remodeling, a coordinated balance between osteoblast-mediated bone formation (or deposition) and osteoclast-mediated bone resorption (Harada and Rodan 2003). An imbalance in bone remodeling due to increased bone resorption over bone formation leads to reduced bone mass and consequent skeletal diseases such as osteoporosis. Reduced bone mass increases the risk of fractures and resultant skeletal deformity, pain, functional limitations, increased mortality, and severe economic burden (van der Klift et al. 2005); the prevention and treatment of reduced bone mass is an important means of improving quality of life.

Most agents used to treat osteoporosis are anti-resorptive agents that inhibit the activity of osteoclasts (Rodan and Martin 2000); however, these agents have only modest effects on increasing bone mass (Christiansen and Lindsay 1990; Riggs and Hartmann 2003). Thus, the development of anabolic agents that stimulate or accelerate bone formation and improve bone architecture is an area of interest. However, the FDA-approved anabolic agent parathyroid hormone has limited use because it is comparatively expensive and difficult to administer. Identification of new, effective anabolic agents that are less expensive and simple to use is greatly needed (Garrett 2007).

Osteoblast-mediated bone formation is a highly regulated, sequential process. Mesenchymal stem cells (MSCs) initially commit to preosteoblasts, which mature into osteoblasts, the essential for bone mineralization (Katagiri et al. 1994). Interestingly, bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β superfamily, strongly induce osteogenesis (Phimphilai et al. 2006). Since natural compounds have historically yielded a variety of therapeutic agents, there have been recent efforts to identify bioactive small molecules from natural sources that could prevent and treat bone-related diseases while minimizing adverse side effects. Using the BMP-2 reporter assay, lovastatin has been identified as a natural product that stimulates osteoblast differentiation. This discovery suggests the possibility of other natural products functioning as anabolic agents (Mundy et al. 1999).

Tanshinone IIA (Fig. 1a) is one of the major active phytochemicals isolated from the root of *Salvia miltiorrhiza* (or 'Danshen'), a well-known traditional Oriental medicine used in the treatment of diseases including postmenopausal syndrome. Tanshinone IIA reportedly has a broad range of biological activity, including anti-cancer and anti-inflammatory effects (Wang et al. 2005; Jang et al. 2003). Interestingly, the effect of tanshinone IIA on bone metabolism has been reported in several studies; tanshinone IIA inhibited the formation of multinucleated osteoclasts (Lee et al. 2005) and total tanshinone containing 17% tanshinone IIA and 8% cryptotanshinone prevented ovariectomy-induced cancellous bone loss in rats through inhibition of bone resorption (Cui et al. 2004). The inhibitory effect of tanshinone IIA on osteoclast differentiation has been explained by its potential to suppress the expression levels of c-Fos and NFATc1, transcription factors required for osteoclast differentiation (Kwak et al. 2008). In a study evaluating the effect of *Salvia miltiorrhiza* extracts and its components on osteoporosis, the methanol and ethanol-isolated fractions had a low concentration of tanshinone IIA and completely inhibited osteoclastogenesis, but failed to effect osteoblast differentiation (Kim et al. 2008). Furthermore, tanshinone IIA suppresses inflammatory bone loss by inhibiting the synthesis of prostaglandin E_2 in osteoblasts. The effects of tanshinone IIA on osteoblastogenesis have not been studied. We evaluated the effect of tanshinone IIA on the commitment of bi-potential mesenchymal precursor C2C12 cells into osteoblasts and investigated potential mechanisms explaining its osteogenic activity.

Materials and methods

Materials

Tanshinone IIA was purchased from Sigma (MO, USA). In this study, 10 mM of tanshinone IIA 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 (Seoul, Korea). PI3K inhibitor (LY294002; Cat. No. 440202), Akt inhibitor (Cat. No. 124005), MEK inhibitor (PD98059; Cat. No. 513000) and p38 inhibitor (SB202190; Cat. No. 559389) were purchased from Calbiochem (EMD Biosciences, Inc., La Jolla, CA, USA).

Cell culture

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 and after 1 day, cells were differentiated by

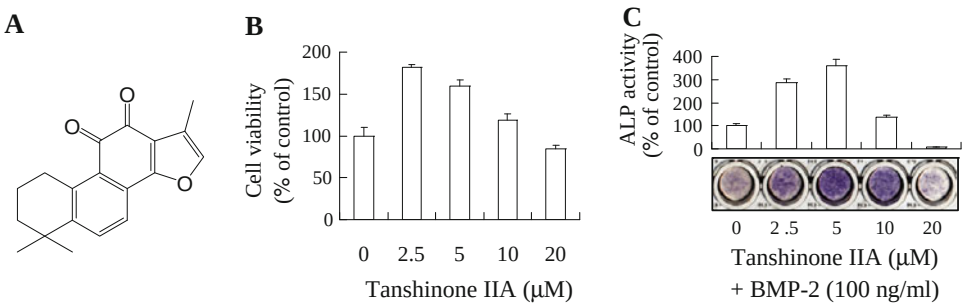

Fig. 1 Tanshinone IIA enhanced BMP-2-induced osteoblast differentiation in C2C12 cells. **a** Structure of tanshinone IIA. **b** Effect of tanshinone IIA on the cell viability. Cells (4×10^3 cells/well) were cultured in a 96-well plate for 1 day and then the medium was replaced with DMEM containing 5% FBS and rhBMP-2 (100 ng/ml) in the presence or absence of tanshinone IIA (differentiation day 0). Medium was changed every 3 days. Cell viability assay was then performed on the differentiation day 6. **c** Effect of tanshinone IIA on BMP-2-stimulated ALP induction. On the differentiation day 6, ALP staining and its activity assay were performed as described in "Materials and methods"

replacing the differentiation medium (DM; DMEM containing 5% FBS and rhBMP-2 50-100 ng/ml). The medium was changed every 3 days.

Cell viability assay

Cells were seeded in a 96-well plate at 4×10^3 cells/well. After 24 h, cells were cultured in DM with serially diluted tanshinone IIA for 6 days. Cell growth was evaluated in triplicate using Cell Counting Kit-8 (Dojindo Molecular Technologies, ML, USA) according to the manufacturer's protocol; absorbance was measured at 450 nm using the Wallac EnVision microplate reader (PerkinElmer, Finland).

Alkaline phosphatase staining and activity assay

C2C12 cells were seeded at 4×10^3 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. The medium was changed every 3 days. After 6 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 plates and cells were scanned with UMAX PowerLook1100 (UMAX, Taiwan) and captured under a microscope equipped with a DP70 digital camera (Olympus Optical, Japan), respectively. 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 $10{,}000 \times g$ 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).

Evaluation of mRNA expression level

Primers were designed using an on-line primer design program (Rozen and Skaletsky 2000) (Table 1). Total RNA was isolated using TRIzol reagent (Life Technologies, MD, USA) according to 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, USA). SYBR green-based quantitative PCR was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA, USA) 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 first segment (95°C for 10 min) activated the polymerase; the second segment included 3-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 $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). GAPDH was used as the control gene. Significance was determined with GAPDH-normalized $2^{-\Delta\Delta CT}$ values.

Western blot analysis

For western analysis, cells were homogenized in buffer consisting of 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 $10{,}000 \times g$ 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, IL, USA). 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

Table 1 Primer sequences used in this study

Target gene	Forward (5′–3′)	Reverse (5′–3′)
ALP	ATGGGCGTCTCCACAGTAAC	TCACCCGAGTGGTAGTCACA
OC	TATGTGTCCTCCGGGTTCAT	GCCCTCTGCAGGTCATAGAG
Runx2	GCCGGGAATGATGAGAACTA	GGACCGTCCACTGTCACTTT
BMP-2	GCTCCACAAACGAGAAAAGC	AGCAAGGGGAAAAGGACACT
BMP-4	CCTGGTAACCGAATGCTGAT	AGCCGGTAAAGATCCCTCAT
BMP-6	TTCTTCAAGGTGAGCGAGGT	TAGTTGGCAGCGTAGCCTTT
BMP-7	CGATACCACCATCGGGAGTTC	AAGGTCTCGTTGTCAAATCGC
BMP-9	CAGAACTGGGAACAAGCATCC	GCCGCTGAGGTTTAGGCTG
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

the Mini Protean 3 Cell (Bio-Rad, CA, USA). 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, USA) with the exception of antibody against p-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad 8 (Ser 426/428) (Cell Signaling, MA, USA). 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).

Runx2 luciferase reporter assay

To measure Runx2 activity, the p6 × osteoblast-specific *cis*-acting element (OSE) 2-luc reporter vector was transiently transfected into C2C12 cells that were seeded 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 tanshinone IIA and/or BMP-2. 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

Significance was determined by Student's *t* test and differences were considered significant when $P < 0.05$.

Results

The effect of tanshinone IIA on the commitment of MSCs (C2C12 cells) into pre-osteoblasts was evaluated by visualization of ALP and measuring expression levels of genes involved in osteoblast differentiation. At non-cytotoxic concentrations (≤10 μM; Fig. 1b), tanshinone IIA significantly enhanced BMP-2-stimulated ALP induction (Fig. 1c). Interestingly, ALP induction by tanshinone IIA was strongly observed at 5 μM; consistent with this result, tanshinone IIA enhanced the BMP-2-stimulated expression of ALP and osteocalcin (OC), as shown in Table 2. ALP and OC are molecular markers for the early and late stage of osteoblastic differentiation, respectively. Since the osteogenic activity of tanshinone IIA in the presence of BMP-2 was strongly exhibited at low concentrations, concentrations of tanshinone IIA up to 5 μM were used in the following experiments.

In the absence of BMP-2, the stimulatory effect of tanshinone IIA on ALP induction was not observed (data not shown) suggesting that the osteogenic activity of tanshinone IIA could be dependent on the presence of BMP-2. This suggestion was confirmed by the co-treatment of noggin with BMP-2 and tanshinone IIA (Fig. 2a); the co-treatment of noggin completely inhibited BMP-2-stimulated ALP induction and the tanshinone IIA-mediated enhancement of BMP-2-stimulated ALP induction.

Furthermore, the possibility that the osteogenic activity of tanshinone IIA might result in the induction of endogenous BMP expression was examined by evaluating the post-treated effect of noggin on tanshinone IIA-enhanced ALP induction. When the commitment of C2C12 cells into osteoblasts was induced by treatment with BMP-2 combined with tanshinone IIA for 4 days and cells were then incubated with noggin for 2 days, noggin significantly inhibited tanshinone IIA-enhanced ALP induction (Fig. 2b).

The inhibition of tanshinone IIA-enhanced ALP induction by post-treatment of noggin suggested that tanshinone IIA could enhance the BMP-2-stimulated expression of endogenous BMPs. Therefore, the effect of tanshinone IIA on the expression of BMPs was evaluated. Real-time PCR revealed that tanshinone IIA could enhance the BMP-2-stimulated expression of endogenous BMPs (Table 2); the expressions of BMP-2, 4, 6, 7, and 9 were significantly induced by the combination of rhBMP-2 and low concentrations (2.5–5 μM) of tanshinone IIA as well as rhBMP-2 alone with the exception of BMP-7.

Since the osteogenic activity of BMP is known to activate Smad-Runx2 (Lian et al. 2003; Gersbach et al. 2004; Phimphilai et al. 2006), the enhancement of the BMP-2-stimulated Smad-Runx2 activities by tanshinone IIA was evaluated. While 100 ng/ml rhBMP-2 did not significantly induce mRNA expression of Runx2, its combination with tanshinone IIA significantly induced Runx2 mRNA expression (Table 2). The strong activity of tanshinone IIA in the induction of Runx2 mRNA expression was also observed at 5 μM. Consistent with this result, tanshinone IIA significantly enhanced BMP-2-stimulated Runx2 activity (Fig. 3a). We further evaluated the effect of tanshinone IIA on Smad activation and found that tanshinone IIA also enhanced the nuclear level of p-Smad1/5/8 (Fig. 3b). Additionally, tanshinone IIA enhancement of p-Smad1/5/8 was inhibited by the co-treatment of noggin with tanshinone IIA and rhBMP-2 (data not shown).

Table 2 Effect of tanshinone IIA on BMP-2-stimulated induction of genes required for osteogenesis. Cells were treated with BMP-2 (100 ng/ml) and/or tanshinone IIA for 3 days and then the mRNA levels were evaluated by quantitative real-time PCR. Fold changes relative to each gene level in the control are presented as mean ± standard deviation

	ALP	OC	Runx2	BMP-2	BMP-4	BMP-6	BMP-7	BMP-9
Control	1.06 ± 0.41	1.03 ± 0.34	1.01 ± 0.13	1.01 ± 0.22	1.00 ± 0.11	1.02 ± 0.25	1.02 ± 0.27	1.00 ± 0.04
rhBMP-2	3.65 ± 0.94[b]	1.60 ± 0.38	1.30 ± 0.35	5.76 ± 0.36[c]	4.55 ± 1.93[a]	1.97 ± 0.47[a]	2.41 ± 1.30[a]	5.41 ± 0.80[c]
rhBMP-2 + Tanshinone IIA 2.5 μM	20.15 ± 4.51[e]	43.58 ± 8.80[f]	3.57 ± 1.11[d]	133.27 ± 17.74[f]	41.47 ± 8.20[f]	17.91 ± 3.98[e]	19.66 ± 1.68[f]	28.37 ± 5.50[e]
rhBMP-2 + Tanshinone IIA 5 μM	18.91 ± 0.91[f]	34.38 ± 4.11[f]	7.57 ± 1.39[f]	220.54 ± 48.27[f]	86.40 ± 20.61[e]	25.85 ± 2.84[f]	49.72 ± 5.48[f]	67.35 ± 1.64[f]
rhBMP-2 + Tanshinone IIA 10 μM	9.25 ± 2.82[d]	3.71 ± 0.13[f]	3.72 ± 0.37[e]	6.21 ± 1.52	4.73 ± 1.13	1.00 ± 0.07	1.87 ± 0.51	4.79 ± 0.23
rhBMP-2 + Tanshinone IIA 20 μM	11.18 ± 1.57[e]	2.52 ± 0.64	3.92 ± 0.31[f]	5.18 ± 1.07	4.47 ± 1.32	1.61 ± 0.37	1.59 ± 0.52	4.67 ± 0.41

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ (compared to group 'Control')

[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$ (compared to group 'rhBMP-2')

Recent studies demonstrated that Akt and mitogen-activated protein (MAP) kinase signaling pathways, both of which are downstream signaling molecules of phosphatidylinositol 3-kinase (PI3K), play key roles in the activation of BMP-2 gene expression (Ghosh-Choudhury et al. 2007). Therefore, in order to identify the signaling pathway required for tanshinone IIA-enhanced ALP induction, the effects of pharmacological inhibitors on tanshinone IIA-enhanced ALP induction were evaluated. As shown in Fig. 4a, tanshinone IIA-enhanced ALP induction was shown to be strongly and weakly inhibited by p38 inhibitor and MEK inhibitor, respectively, but not inhibitors for PI3K or AKT. ALP activity assay revealed the dose-dependent inhibition of ALP induction by p38 inhibitor, but MEK inhibitor did not exhibit dose-dependent inhibitory activity on ALP induction (Fig. 4b). The involvement of p38 and MEK signaling pathways in tanshinone IIA-enhanced ALP induction was confirmed by Western blot analysis (Fig. 4c). Treatment with rhBMP-2 increased phosphorylated levels of p-38 and ERK but not JNK, and these inductions were enhanced by the addition of tanshinone IIA. These results suggested that p38 and ERK could be the downstream signaling molecules of BMP. In subsequent studies, we focused our investigation on the functional involvement of p38 in the process of tanshinone IIA-enhanced osteogenic action of BMP-2 using the p38 inhibitor SB202190.

The hypothesis that p38 could be involved in the downstream of BMP signaling led us to evaluate whether BMP-2 and/or tanshinone IIA per se could induce activation of p38 and Smad. Interestingly, even in the absence of rhBMP-2, tanshinone induced p38 phosphorylation (Fig. 5a). Additionally, tanshinone IIA enhanced BMP-2-induced activations of p38 as well as Smad1/5/8, but the p38 inhibitor SB202190 dose-dependently inhibited enhancement of p38 activation by tanshinone IIA and inhibited activation of Smad activation by BMP-2 alone or in combination with tanshinone IIA.

Since the induction of Runx2 activity (as shown in Fig. 3a) and p38 activation by tanshinone IIA per se (Fig. 5a) suggested the possibility that tanshinone IIA alone could activate Runx2 via a p38 pathway, we used the Runx2 luciferase activity assay to investigate this. As shown in Fig. 5b, tanshinone IIA-induced Runx2 activity was inhibited by SB202190 independent of the presence of BMP-2.

Discussion

The induction of osteogenic marker (such as ALP and OC) and factor (such as BMPs) expression is important in the differentiation of MSCs into pre-osteoblasts. In C2C12

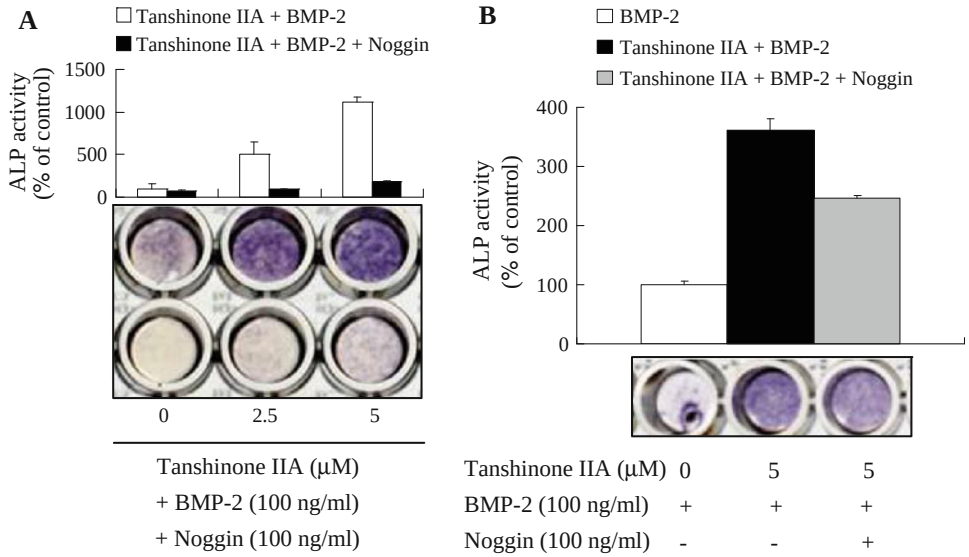

Fig. 2 Effect of noggin on tanshinone IIA-mediated enhancement of BMP-2-stimulated ALP induction. **a** Noggin (100 ng/ml) was co-treated with BMP-2 (100 ng/ml) and/or tanshinone IIA on the differentiation day 0 and 3. **b** Osteogenesis was induced by the treatment of BMP-2 and/or tanshinone IIA on the differentiation day 0 and 2, and then noggin was treated alone on the differentiation day 4. On the differentiation day 6, ALP staining and its activity assay were performed

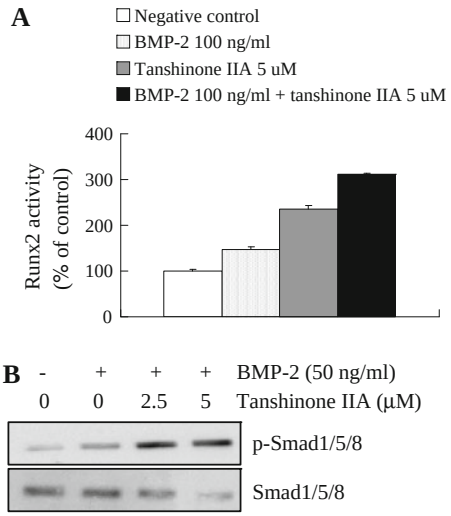

Fig. 3 Effects of tanshinone IIA on Runx2 and Smad activation. **a** Runx2 activation was measured by luciferase reporter assay using 6 × OSE2-luciferase plasmid-transfected C2C12 cells. Cells (4×10^3 cells/well) were cultured in a 96-well plate for 1 day and then cells were incubated with DMEM containing 5% FBS in the presence or absence of BMP-2 and/or tanshinone IIA for 1 day. **b** The effect of tanshinone IIA on the activation of Smad was evaluated by Western blot analysis. Cells (1×10^5 cells/well) were cultured in a 6-well plate for 1 day and then cells were incubated with DMEM containing 5% FBS in the presence or absence of BMP-2 (50 ng/ml) and/or tanshinone IIA for 10 min. After protein fractionation, Smad1/5/8 and p-Smad1/5/8 were detected in the cytosolic and nuclear fractions, respectively

cells, a mouse bi-potential mesenchymal precursor line, the early osteogenic marker ALP activity and the late osteogenic marker OC expression were strongly induced by osteogenic factors such as BMP-2, 4, 6, 7, and 9 (Cheng et al. 2003). Apparently, BMP-2 exhibits 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). Interestingly, simultaneous gene transfer of both 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); these results suggest that the ability to enhance expression of other BMP genes may play an important role in bone formation. BMP-4, 6, and 9 are also reported to have osteogenic potential in C2C12 cells (Ebisawa et al. 1999; Li et al. 2003; Bessa et al. 2009).

In this study, we found that the BMP-2-stimulated commitment of bi-potent mesenchymal stem cells, C2C12, into osteoblasts was enhanced by low concentrations of tanshinone IIA coincident with induction of BMPs expression in the transcript level; in the presence of BMP-2, tanshinone IIA significantly increased the transcription of osteogenic BMP isoforms BMP-2, 4, 6, 7, and 9 at low concentrations. Considering that the simultaneous transfer of BMP-2 and BMP-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 increased BMP levels induced by tanshinone IIA act synergistically to promote osteogenesis. Furthermore, tanshinone IIA-enhanced ALP induction was prevented by co-treatment or post-treatment with the BMP antagonist noggin. Since silencing of noggin enhances osteoblastic differentiation of BMP-responding cells in vitro and rhBMP-2-induced new bone formation in vivo (Takayama et al. 2009), these results suggested that tanshinone IIA could enhance the osteogenic activity of BMP signaling.

BMP-2 can exhibit its osteogenic activity via activation of the Smad-Runx2 signaling pathway. The phosphorylation

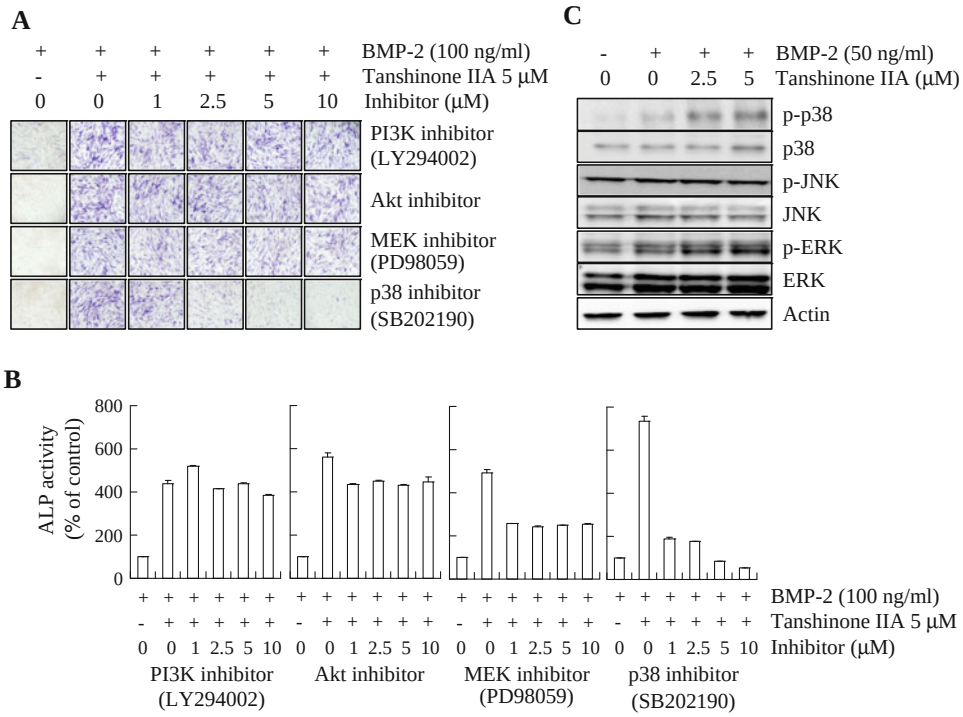

Fig. 4 Involvement of p38 in the tanshinone IIA-induced enhancement of BMP-2-stimulated ALP induction. Cells (4×10^3 cells/well) in a 96-well plate were treated with each inhibitor for 2 h and then BMP-2 and/or tanshinone IIA were added into cells. After 3 days, cells were treated with each inhibitor for 2 h and then BMP-2 and/or tanshinone IIA were added into cells. On the differentiation day 6, ALP staining (**a**) and its activity assay (**b**) were preformed as described in "Materials and methods". **c** The effect of tanshinone IIA on the activation of MAP kinases was evaluated by Western blot analysis. Cells (1×10^5 cells/well) were cultured in a 6-well plate for 1 day and then cells were incubated with DMEM containing 5% FBS in the presence or absence of BMP-2 and/or tanshinone IIA for 10 min

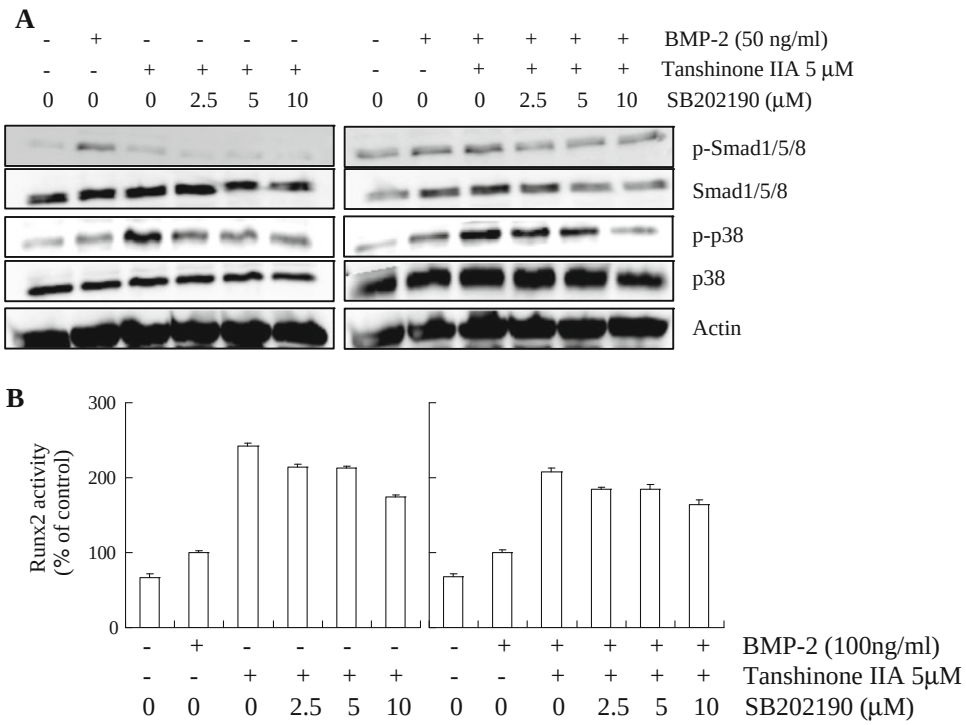

Fig. 5 SB202190 inhibits p38-Runx2 and p38-Smad-Runx2 activations induced by tanshinone IIA and its combination with BMP-2, respectively. **a** Western blot analysis was performed in order to evaluate the effect of SB202190 on the activation of p38 and p38-Smad induced by tanshinone IIA and its combination with BMP-2, respectively. Samples were prepared as described in "Materials and methods". **b** In order to evaluate the effect of SB202190 on the activation of Runx2 induced by tanshinone IIA or its combination with BMP-2, the luciferase activity assay was performed as described in "Materials and methods"

and nuclear translocation of Smad induces transcriptional activity of Runx2, a key mediator protein of the BMP-2 signaling pathway for osteoblastic precursor cell differentiation via the regulation of osteogenic gene expression (Lian et al. 2003; Gersbach et al. 2004; Phimphilai et al. 2006; Bessa et al. 2009). The binding of BMP-2 to its receptor triggers the association of phosphorylated Smad1/5 with Smad4; this complex then migrates into the nucleus where it interacts with Runx2 to activate transcription of OSE2-containing genes required for MSC commitment and osteoblast differentiation (Ducy and Karsenty 1995; Merriman et al. 1995). Interestingly, promoters of osteoblast-specific genes, such as OC and osteopontin, contain OSE2s (Phimphilai et al. 2006; Hanai et al. 1999; Guicheux et al. 2003). Here, we demonstrated tanshinone IIA-mediated enhancement of BMP-2-stimulated Smad-Runx2 activities; the combination of tanshinone IIA with rhBMP-2 significantly induced Runx2 mRNA expression and activity. In addition, tanshinone IIA enhanced BMP-2-stimulated activation of Smad1/5/8. These results suggested that the enhanced osteogenic activity of BMP-2 induced by tanshinone IIA could be a result of accelerating Smad-Runx2 activation.

Recently, the activations of Akt and MAP kinases, both of which lie downstream in the PI3K signaling pathway, have been 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 also essential for the induction of 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 to the ERK pathway (Chae et al. 2002). As described above, PI3K may play a role in regulating activation of Akt and MAP kinases to trigger osteoblast differentiation. 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).

In this study, tanshinone IIA-enhanced ALP induction was shown to be strongly and dose-dependently inhibited by a p38 inhibitor, suggesting the possible involvement of p38 in the action of tanshinone IIA. The MEK inhibitor PD98059, which sequentially inhibits ERK activation, weakly inhibited the tanshinone IIA-enhanced ALP induction, but did not exhibit dose-dependent inhibitory activity on ALP induction. We could not exclude the functional involvement of ERK in the process of tanshinone IIA-enhanced osteogenic action of BMP-2, but here we focused on the role of p38 in that process. Apparently, BMP-2-induced ALP induction has been reported to be mediated through Smad-independent activation of p38 (Nohe et al. 2002). The possible involvement of p38 in the action of tanshinone IIA was experimentally confirmed; even in the absence of rhBMP-2, tanshinone induced p38 phosphorylation. In addition, tanshinone IIA enhanced BMP-2-induced activations of p38 as well as Smad1/5/8. Furthermore, the p38 inhibitor SB202190 dose-dependently inhibited tanshinone IIA-induced p38 activation and tanshinone IIA plus BMP-2-induced Smad activation. These results suggest that p38 signaling could play a crucial role in the enhancement of tanshinone IIA-induced p38 activation and BMP-2-induced Smad activation by the combination of tanshinone IIA and BMP-2.

In conclusion, tanshinone IIA could enhance the commitment of C2C12 cells into osteoblasts and their differentiation through synergistic cross talk between tanshinone IIA-induced p38 activation and BMP-2-induced Smad activation. These activations could subsequently induce the activation of Runx2, an inducer of osteogenesis via the regulation of several osteogenic factor and marker expression (Fig. 6). Furthermore, considering that osteogenic BMPs such as BMP-2 and BMP-7 have recently been approved for clinical applications in spinal fusion, fracture healing, and dental tissue engineering, anabolic agents that stimulate BMP expression or its signaling pathway could be used to treat osteoblast-related diseases via bone formation or regeneration (Nakashima and Reddi 2003; Seeherman and Wozney 2005). From this point of view, the identification of phytochemicals such as tanshinone IIA with the potential to enhance the commitment of mesenchymal protenitors into osteoblasts and their differentiation by activating signaling pathways to induce BMP gene expression might lead to the development of therapeutics that benefit bone regeneration and fracture healing.

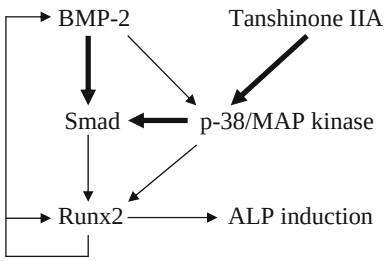

Fig. 6 Suggested model for tanshinone IIA-induced enhancement of BMP-2-stimulated osteogenesis. Tanshinone IIA could enhance the BMP-2-induced Smad-Runx2 activation via its potential to induce the p38 activation; tanshinone IIA-induced the p38-Smad activation could enhance the BMP-2-mediated Smad-Runx2 activation

Acknowledgments We thank Professor Hyun-Mo Ryoo (Department of Cell and Developmental Biology, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, Korea) for providing the p6 × OSE2-luc reporter vector. This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry of Health, Welfare & Family Affairs, Republic of Korea (A080216).

References

Bessa PC, Cerqueira MT, Rada T, Gomes ME, Neves NM, Nobre A, Reis RL, Casal M (2009) Expression, purification and osteogenic bioactivity of recombinant human BMP-4, -9, -10, -11 and -14. Protein Expr Purif 63(2):89–94

Boden SD, Zdeblick TA, Sandhu HS, Heim SE (2000) The use of rhBMP-2 in interbody fusion cages. Definitive evidence of osteoinduction in humans: a preliminary report. Spine 25(3):376–381

Chae HJ, Jeong BJ, Ha MS, Lee JK, Byun JO, Jung WY, Yun YG, Lee DG, Oh SH, Chae SW, Kwak YG, Kim HHm Lee ZH, Kim HR (2002) ERK MAP kinase is required in 1, 25(OH)2D3-induced differentiation in human osteoblasts. Immunopharmacol Immunotoxicol 24(1):31–41

Chen D, Harris MA, Rossini G, Dunstan CR, Dallas SL, Feng JQ, Mundy GR, Harris SE (1997) Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. Calcif Tissue Int 60(3):283–290

Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC (2003) Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 85-A(8):1544–1552

Christiansen C, Lindsay R (1990) Estrogens, bone loss and preservation. Osteoporos Int 1(1):7–13

Cui L, Wu T, Liu YY, Deng YF, Ai CM, Chen HQ (2004) Tanshinone prevents cancellous bone loss induced by ovariectomy in rats. Acta Pharmacol Sin 25(5):678–684

Ducy P, Karsenty G (1995) Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol 15(4):1858–1869

Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T (1999) Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112(Pt 20):3519–3527

Garrett IR (2007) Anabolic agents and the bone morphogenetic protein pathway. Curr Top Dev Biol 78:127–171

Gersbach CA, Byers BA, Pavlath GK, García AJ (2004) Runx2/Cbfa1 stimulates transdifferentiation of primary skeletal myoblasts into a mineralizing osteoblastic phenotype. Exp Cell Res 300(2): 406–417

Ghosh-Choudhury N, Abboud SL, Nishimura R, Celeste A, Mahimainathan L, Choudhury GG (2002) Requirement of BMP-2-induced phosphatidylinositol 3-kinase and Akt serine/threonine kinase in osteoblast differentiation and Smad-dependent BMP-2 gene transcription. J Biol Chem 277(36):33361–33368

Ghosh-Choudhury N, Mandal CC, Choudhury GG (2007) Statin-induced Ras activation integrates the phosphatidylinositol 3-kinase signal to Akt and MAPK for bone morphogenetic protein-2 expression in osteoblast differentiation. J Biol Chem 282(7):4983–4993

Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G, Caverzasio J (2003) Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. J Bone Miner Res 18(11):2060–2068

Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, Imamura T, Ishidou Y, Fukuchi M, Shi MJ, Stavnezer J, Kawabata M, Miyazono K, Ito Y (1999) Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. J Biol Chem 274(44):31577–31582

Harada S, Rodan GA (2003) Control of osteoblast function and regulation of bone mass. Nature 423(6937):349–355

Jang SI, Jeong SI, Kim KJ, Kim HJ, Yu HH, Park R, Kim HM, You YO (2003) Tanshinone IIA from Salvia miltiorrhiza inhibits inducible nitric oxide synthase expression and production of TNF-α, IL-1β and IL-6 in activated RAW 264.7 cells. Planta Med 69(11):1057–1059

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127(6 Pt 1):1755–1766

Kawai M, Bessho K, Maruyama H, Miyazaki J, Yamamoto T (2006) Simultaneous gene transfer of bone morphogenetic protein (BMP)-2 and BMP-7 by in vivo electroporation induces rapid bone formation and BMP-4 expression. BMC Musculoskelet Disord 7:62

Kawamura N, Kugimiya F, Oshima Y, Ohba S, Ikeda T, Saito T, Shinoda Y, Kawasaki Y, Ogata N, Hoshi K, Akiyama T, Chen WS, Hay N, Tobe K, Kadowaki T, Azuma Y, Tanaka S, Nakamura K, Chung UI, Kawaguchi H (2007) Akt1 in osteoblasts and osteoclasts controls bone remodeling. PLoS ONE 2:e1058

Kim HJ, Park HD, Kim JH, Cho JY, Choi JY, Kim JK, Kim HJ, Shin HI, Ryoo HM (2004) Establishment and characterization of a stable cell line to evaluate cellular Runx2 activity. J Cell Biochem 91(6):1239–1247

Kim HK, Woo ER, Lee HW, Park HR, Kim HN, Jung YK, Choi JY, Chae SW, Kim HR, Chae HJ (2008) The correlation of Salvia miltiorrhiza extract-induced regulation of osteoclastogenesis with the amount of components tanshinone I, tanshinone IIA, cryptotanshinone, and dihydrotanshinone. Immunopharmacol Immunotoxicol 30(2):347–364

Kwak HB, Sun HM, Ha H, Kim HN, Lee JH, Kim HH, Shin HI, Lee ZH (2008) Tanshinone IIA suppresses inflammatory bone loss by inhibiting the synthesis of prostaglandin E2 in osteoblasts. Eur J Pharmacol 601(1–3):30–37

Lee SY, Choi DY, Woo ER (2005) Inhibition of osteoclast differentiation by tanshinones from the root of Salvia miltiorrhiza bunge. Arch Pharm Res 28(8):909–913

Li JZ, Li H, Sasaki T, Holman D, Beres B, Dumont RJ, Pittman DD, Hankins GR, Helm GA (2003) Osteogenic potential of five different recombinant human bone morphogenetic protein adenoviral vectors in the rat. Gene Ther 10(20):1735–1743

Lian JB, Stein JL, Stein GS, van Wijnen AJ, Montecino M, Javed A, Gutierrez S, Shen J, Zaidi SK, Drissi H (2003) Runx2/Cbfa1 functions: diverse regulation of gene transcription by chromatin remodeling and co-regulatory protein interactions. Connect Tissue Res 44(Suppl 1):141–148

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408

Merriman HL, van Wijnen AJ, Hiebert S, Bidwell JP, Fey E, Lian J, Stein J, Stein GS (1995) The tissue-specific nuclear matrix protein, NMP-2, is a member of the AML/CBF/PEBP2/runt domain transcription factor family: interactions with the osteocalcin gene promoter. Biochemistry 34(40):13125–13132

Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G (1999) Stimulation of bone formation in vitro and in rodents by statins. Science 286(5446):1946–1949

Nakashima M, Reddi AH (2003) The application of bone morphogenetic proteins to dental tissue engineering. Nat Biotechnol 21(9):1025–1032

Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P (2002) The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 277(7):5330–5338

Phimphilai M, Zhao Z, Boules H, Roca H, Franceschi RT (2006) BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. J Bone Miner Res 21(4):637–646

Riggs BL, Hartmann LC (2003) Selective estrogen-receptor modulators-mechanisms of action and application to clinical practice. N Engl J Med 348(7):618–629

Rodan GA, Martin TJ (2000) Therapeutic approaches to bone diseases. Science 289(5484):1508–1514

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386

Seeherman H, Wozney JM (2005) Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. Cytokine Growth Factor Rev 16(3):329–345

Suzuki A, Guicheux J, Palmer G, Miura Y, Oiso Y, Bonjour JP, Caverzasio J (2002) Evidence for a role of p38 MAP kinase in expression of alkaline phosphatase during osteoblastic cell differentiation. Bone 30(1):91–98

Takayama K, Suzuki A, Manaka T, Taguchi S, Hashimoto Y, Imai Y, Wakitani S, Takaoka K (2009) RNA interference for noggin enhances the biological activity of bone morphogenetic proteins in vivo and in vitro. J Bone Miner Metab 27(4):402–411

Valentin-Opran A, Wozney J, Csimma C, Lilly L, Riedel GE (2002) Clinical evaluation of recombinant human bone morphogenetic protein-2. Clin Orthop Relat Res 395:110–120

van der Klift M, de Laet CD, Pols HA (2005) Assessment of fracture risk: who should be treated for osteoporosis? Best Pract Res Clin Rheumatol 19(6):937–950

Wang X, Wei Y, Yuan S, Liu G, Lu Y, Zhang J, Wang W (2005) Potential anticancer activity of tanshinone IIA against human breast cancer. Int J Cancer 116(5):799–807

Yeh LC, Tsai AD, Lee JC (2002) Osteogenic protein-1 (OP-1, BMP-7) induces osteoblastic cell differentiation of the pluripotent mesenchymal cell line C2C12. J Cell Biochem 87(3):292–304