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Panax Notoginseng Saponins Promote Osteogenic Differentiation of Bone Marrow Stromal Cells Through the ERK and P38 MAPK Signaling Pathways

Xue-dong Li[1], Zhao-yong Liu[1], Bo Chang[1], Dong-xin Liu[1], Bin Chen[1], Chun Guo[1], Yun-guo Wang[1], Jian-kun Xu[1], Dong-yang Huang[2] and Shi-xin Du[1]

[1]Department of Orthopedics, the First Affiliated Hospital, Shantou University Medical College, Shantou, [2]Center for Molecular Biology, Shantou University Medical College, Shantou

Key Words

Panax notoginseng saponins • Bone marrow stromal cells • Mitogen activated protein kinase (MAPK) • Osteogenic differentiation • Osteoporosis

Abstract

The Chinese medicinal herb, Panax notoginseng, has long been used to treat bone fractures and Panax notoginseng saponins (PNS) could promote bone formation. Here, we investigated whether PNS could promote osteogenesis of bone marrow stromal cells (BMSCs) through modulating the MAPK signaling pathways, which are implicated in BMSC osteogenesis. We found that PNS markedly increased the mineralization of BMSCs by alizarin red S assays and stimulate alkaline phosphatase activity of these cells. Additionally, PNS significantly increased the mRNA levels of alkaline phosphatase, core-binding factor a1, and bone sialoprotein while decreasing PPARγ2 mRNA levels. Furthermore, inhibitors of ERK, PD98059, and p38, SB203580 inhibited the osteogenesis-potentiating effects by PNS. PNS stimulated the activation of ERK and p38 as evidenced by increased phosphorylation of these proteins, which was inhibited by PD98059 and SB203580. Our findings indicate that PNS could promote BMSC osteogenesis by activating the ERK and p38 signaling pathways.

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Introduction

Osteoporosis is a disorder in which there is a net bone loss with an increased risk of bone fracture because of an imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation [1]. The disorder poses a major public health threat in societies where aging has become a significant issue for the society [2]. Currently, there are several therapeutic options available to combat osteoporosis with an aim to inhibit bone resorption by osteoclasts and/or increase bone formation by osteoblasts [3]. Estrogen replacement therapy has been the most popular treatment for osteoporosis in the last decade [4]. However, prolonged use of estrogen increases the risk of breast cancer and endometrial cancer, coronary heart disease or stroke, and

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Dr. Shi-xin Du
Department of Orthopedics, The First Affiliated Hospital
Medical College of Shantou University, Shantou
Guangdong 515041 (R.P. China)
Tel. +86-754-88524420, E-Mail xdl622@yahoo.com.cn

venous thromboembolic diseases [5-8]. Biophosphonate therapy though it inhibits bone resorption by osteoclasts, could cause severe incapacitating bone, joint, and/or muscle pain [9, 10]. Noticeably, the suppression of bone turnover by bisphosphonates may eventually lead to an accumulation of fatigue-induced damage, thus potentially offsetting their beneficial effects on the osteoporotic bone [11, 12]. Therefore, development of agents with an anabolic effect on the osteoporotic bone could provide a new alternative for treating osteoporosis [3]. The proliferation and osteogenic differentiation of bone marrow stromal cells were found to associate with bone healing capacity such as for the osteoporotic bone [13]. Osteoblasts originate from bone marrow stromal cells, which have the potential to differentiate into several different lineages, including osteoblasts, chondroblasts, adipocytes and myoblasts. Of these lineages, the osteogenic and adipogenic lineages are closely related [14, 15]. An inverse relationship has been found between the trabecular bone volume and the amount of adipose tissue in the bone marrow [16-18]. An imbalance between osteoblasts and adipocytes is present in the osteoporotic bone, especially in the age-related osteoporotic bone that is accompanied by an increase in the number of adipocytes in the bone marrow [17]. Therefore, it can be inferred that specific inhibition of bone marrow adipogenesis and a concomitant enhancement of osteogenesis of bone marrow stromal cells may provide a novel therapeutic approach to the treatment of osteopenic disorders, like osteoporosis.

Phytoestrogen is derived from plants and possesses estrogenic activities and have minimal side effects. Panax notoginseng is a traditional Chinese herbal medicine and has been used to promote bone healing after fracture since ancient time. The main bioactive constituents of Panax notoginseng are Panax notoginseng saponins, which are phytoestrogens [19]. The three major components of Panax notoginseng saponins are ginsenoside Rg1, Rb1, and notoginsenoside R1 [20]. Panax notoginseng saponins could produce a myriad array of pharmacological responses such as antioxidative [21] and anti-inflammatory response [22]. Ginsenoside Rg1 could stimulate alkaline phosphatase activities and increase the number of osteoblasts *in vitro* [23] and was found to promote the proliferation of bone marrow stromal cells [24]. In addition, when incorporated into polypropylene fumarate and calcium phosphate bone cement composite, ginsenoside Rg1, could promote angiogenesis of osteonecrosed femoral head with disrupted bone blood supply [25]. These findings suggest that Panax notoginseng saponins could be of potential therapeutic uses for such conditions as bone nonunion, osteoporosis and osteonecrosis. Recent studies have shown that Panax notoginseng saponins could improve the development of osteoblasts [26, 27], however, the underlying signaling pathway(s) whereby Panax notoginseng saponins promote the osteogenesis of bone marrow stromal cells remain unelucidated.

The mitogen-activated protein kinases (MAPKs) control multiple cellular processes such as cell proliferation, division, and differentiation, apoptosis and embryogenesis [28, 29]. At least three different MAPK pathways have been described: the extracellular signal related kinases (ERK), Jun amino-terminal kinase (JNK), and p38 MAPK pathway. The ERK pathway has been reported to regulate the proliferation and differentiation of bone cells and bone marrow stromal cells during osteogenic differentiation [30]. ERK has also been demonstrated as an important signaling mechanism in the differentiation of the preadipocyte cell line 3T3-L1 into mature adipocytes [31]. The p38 MAPK and JNK pathways were also shown to be involved in enhancing the osteoblastic differentiation of bone marrow stromal cells by alendronate [32]. We hypothesized that Panax notoginseng saponins could promote the differentiation of the osteoblastic lineage of bone marrow stromal cells through the MAPK signaling pathways. Here, we sought to investigate whether Panax notoginseng saponins could activate the MAPK signaling pathways to potentiate the osteogenesis of bone marrow stromal cells *in vitro*.

Materials and Methods

Preparation of primary rat bone marrow stromal cells

Four-week-old male Sprague-Dawley rats were purchased from the Experimental Animal Center of Shantou University Medical College, Shantou, China, and were housed in environmentally controlled conditions (22°C, a 12-h light/dark cycle with the light cycle from 6:00 to 18:00 and the dark cycle from 18:00 to 6:00) with *ad libitum* access to standard laboratory chow. The study protocol was approved by the local Institution Review Board and all animal experiments were performed according to the guidelines of Institutional Animal Care and Use Committee of Shantou University Medical College. Primary bone marrow stromal cells were collected from the epiphyseal regions of the femora and tibia as previously described [32, 33]and primary cells at passage three to six were typically used for experiments.

Cell proliferation and osteogenic differentiation assays

Cells were plated in a 96-well plate at a density of 1×10^4 cells per well and after a 24-h incubation were treated with

Gene and Genebank accession no.	Primer sequence (forward/reverse)	Tm (°C)	Product size (bp)
ALP (J03572)	5'-AAGGTGGTGGACGGTGAACGGGAGAACG-3' 5'-CGGGCGGAAGTGAGGCAGGTAGCAAAC-3'	65.5	455
BSP (NM_012587)	5'-GCTATGAAGGCTACGAGGGTCAGGATTAT-3' 5'-GGGTATGTTAGGGTGGTTAGCAATGGTGT-3'	59.1	386
Cbfa1 (AF053950)	5'-CCTCACAAACAACCACAGAAC CA-3' 5'-AACTGA AAATACAAA CCATACCC-3'	60	325
PPARγ2 (NM_013124)	5'-CAGGCTTGCTGAACGTGAAG-3' 5'-ACGTGCTCTGTGACAATCTGC-3'	60	177
GADPH (AB017801)	5'-TGCTGAGTATGTCGTGGAG-3' 5'-GCATCAAAGGTGGAAGAAT-3'	52.8	618

Table 1. PCR primer sequences and cycle conditions. Note : ALP, alkaline phosphatase; BSP, bone sialoprotein; Cbfa1, core-binding factor a1; PPARγ2, peroxisome proliferator activated receptor gamma 2 ; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

Panax notoginseng saponins (The contents of the five main ingredients of Panax notoginseng saponins are notoginsenoside R1 10.0%, ginsenoside Rg1 37.9%, ginsenoside Re 5.8%, ginsenoside Rb1 38.4%, and ginsenoside Rd 4.9%, respectively [26]. Chemical purity about 97% was provided from Wu-Zhou Pharmaceutical Group, Wuzhou, China) at 10, 50, or 100 μg/ml. Cell viability was assessed by the tetrazolium-based semi-automated colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Sangon Biotech, Shanghai, China) and absorbance was read at 570 nm using a microtiter plate reader (KHB Labsystems Wellscan K3, Finland). For induction of osteogenic differentiation, bone marrow stromal cells were seeded at a density of 1×10^4 cells/cm^2 in 10-cm culture dishes and were grown under osteogenic induction medium(OIM) in Dulbecco's modified eagle medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 0.1 μM dexamethasone, 50 μM ascorbate acid, and 10 mM β-glycerophosphate sodium. In addition, cells were treated with Panax notoginseng saponins at 10, 50, or 100 μg/mL. Furthermore, bone marrow stromal cells under osteogenic induction were treated with 100 μg/ml Panax notoginseng saponins in the presence or absence of 25 μM PD98059, 10 μM SB203580, or 10 μM SP600125.

For determination of alkaline phosphatase activity, bone marrow stromal cells under osteogenic induction were treated with 10,50 or 100 μ g/mL Panax notoginseng saponins and alkaline phosphatase activity at day 0, 3, 7, 11 and 14 post treatment was determined as previously described [34] by measuring p-nitrophenyl phosphate and was normalized against total cellular protein content and expressed as nmol / min/mg protein. Additionally, mineralization of bone marrow stromal cells was determined by alizarin red S staining at day 21 post treatment with 10,50 or 100μ g/mL Panax notoginseng saponins as previously described [35].Calculation of Alizarin red S concentrations were modified as previously described [27] by comparison with an alizarin red S dye standard curve and expressed as nmol/mL after normalization against the total cellular protein and expressed as nmol/μg protein. The above experiments were performed at least three times independently and each experiment was carried out in quadruplicate.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from bone marrow stromal cells using Total RNA Kit following the manufacturer's recommended protocol (Tiangen, Beijing, China). First-strand cDNA synthesis was carried out by reverse transcription using oligo (dT) from isolated RNA samples and DNA was then used as templates for polymerase-chain reaction (PCR). The PCR conditions and the sequences of primers are listed in Table 1 for the genes encoding the following proteins: rat alkaline phosphatase, core-binding factor a1, bone sialoprotein, peroxisome proliferator activated receptor gamma 2 (PPARγ2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR products were resolved by 2% agarose gel electrophoresis and visualized with ethidium bromide. The relative expression of each gene was quantifed by densitometry using the Gel Image Ver. 3.74 System (Tianon, Shanghai, China) and normalized against GAPDH.

Immunoblotting studies

Cells were lysed using the RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. Anti-ERK, phospho-ERK, p38, phospho-p38 and GADPH antibodies were used (Boster Biological Technology, Wuhan, China). Images were analyzed with Quantity One software (BioRad, Hercules, CA) and band intensity was quantified and normalized against GADPH.

Statistical analysis

The data were expressed as mean ± standard deviation (s.d.) for three or more independent experiments. Statistical significance was estimated by one-way ANOVA with Bonferroni's post test (multiple comparisons), and Student-Newman-Keuls test (comparisons between two groups) was used where appropriate.

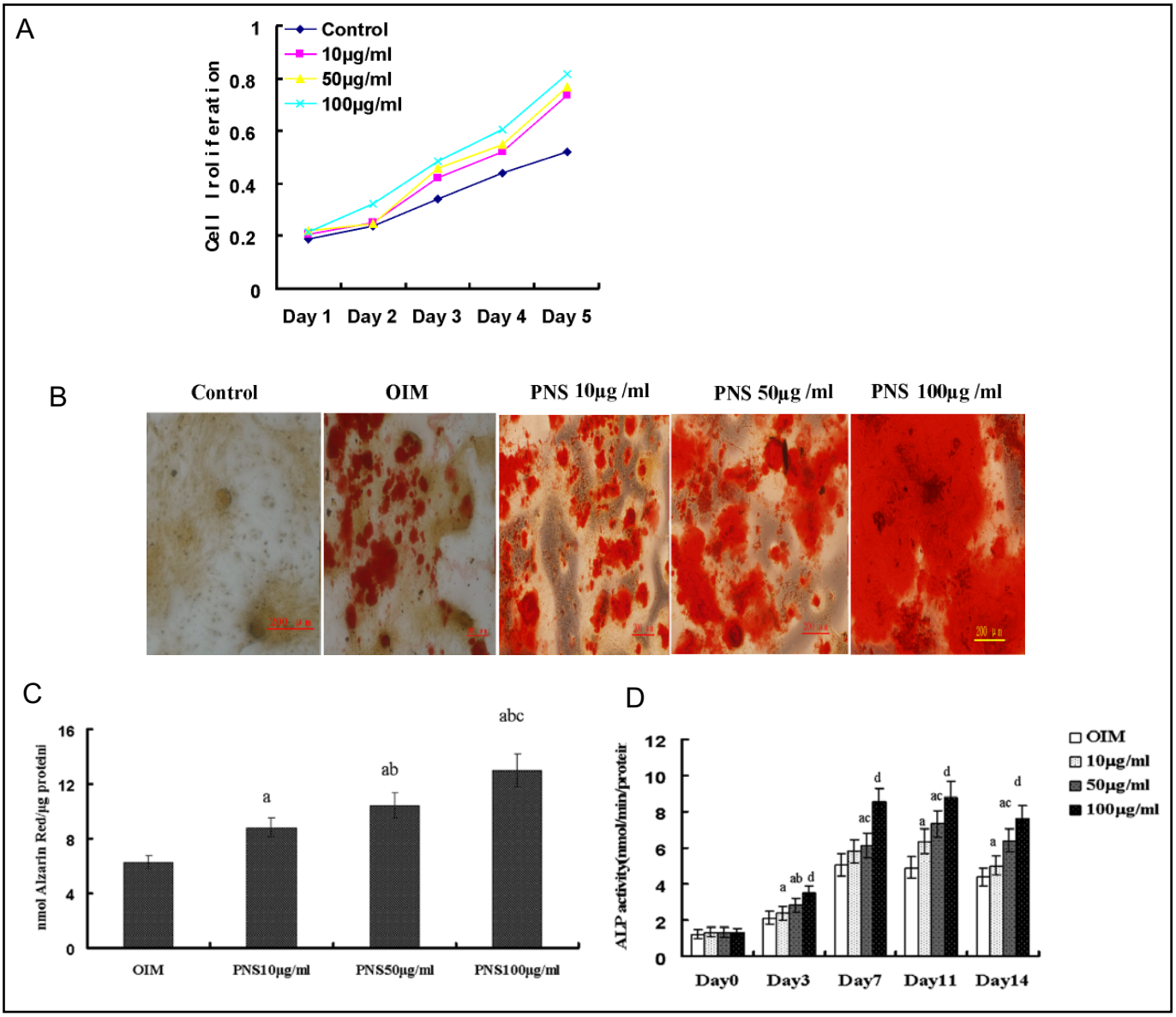

Fig. 1. Panax notoginseng saponins potentiated the osteogenesis of bone marrow stromal cells. (A) Bone marrow stromal cells were treated with Panax notoginseng saponins at 10, 50, or 100 μg /mL for 1-5 days, and cell viability was assessed by the MTT test. Data is expressed as mean ± SD and the experiments were done in quadruplicate (n=4). (B) Bone marrow stromal cells were treated with Panax notoginseng saponins at 10, 50, or 100 μg /mL for 21 days. Mineralization of bone marrow stromal cells was quantified by alizarin red S assays. Data in (C) represents mean ± SD of experiments done in pentaplicate (n=5). ^{a}P<0.01 Versus OIM group; ^{b}P<0.01 Versus 10 μ g/ml group; ^{c}P<0.01 Versus 50 μ g/ml group.(D) Panax notoginseng saponins enhanced alkaline phosphatase activities of bone marrow stromal cells. Alkaline phosphatase activities were measured by conventional method and the data represent the mean ± SD in quadruplicate (n=4). $^{a}P < 0.01$ versus bone marrow stromal cells undergoing simple osteogenic induction; $^{b}P < 0.05$ versus the 10 μg/mL group; $^{c}P < 0.01$ versus the 10 μg/mL group; $^{d}P < 0.01$ versus the 50 μg/mL group.

Results

Panax notoginseng saponins promote the proliferation and mineralization of bone marrow stromal cells

We isolated primary bone marrow stromal cells from the femora and tibia of rats and culture them under osteogenic conditions. We first investigated whether Panax notoginseng saponins could promote the proliferation of bone marrow stromal cells *in vitro*. Our MTT assays showed that, at one week after the start of incubation, 10, 50, and 100 μg/mL Panax notoginseng saponins markedly increased the proliferation of bone marrow stromal cells compared with control

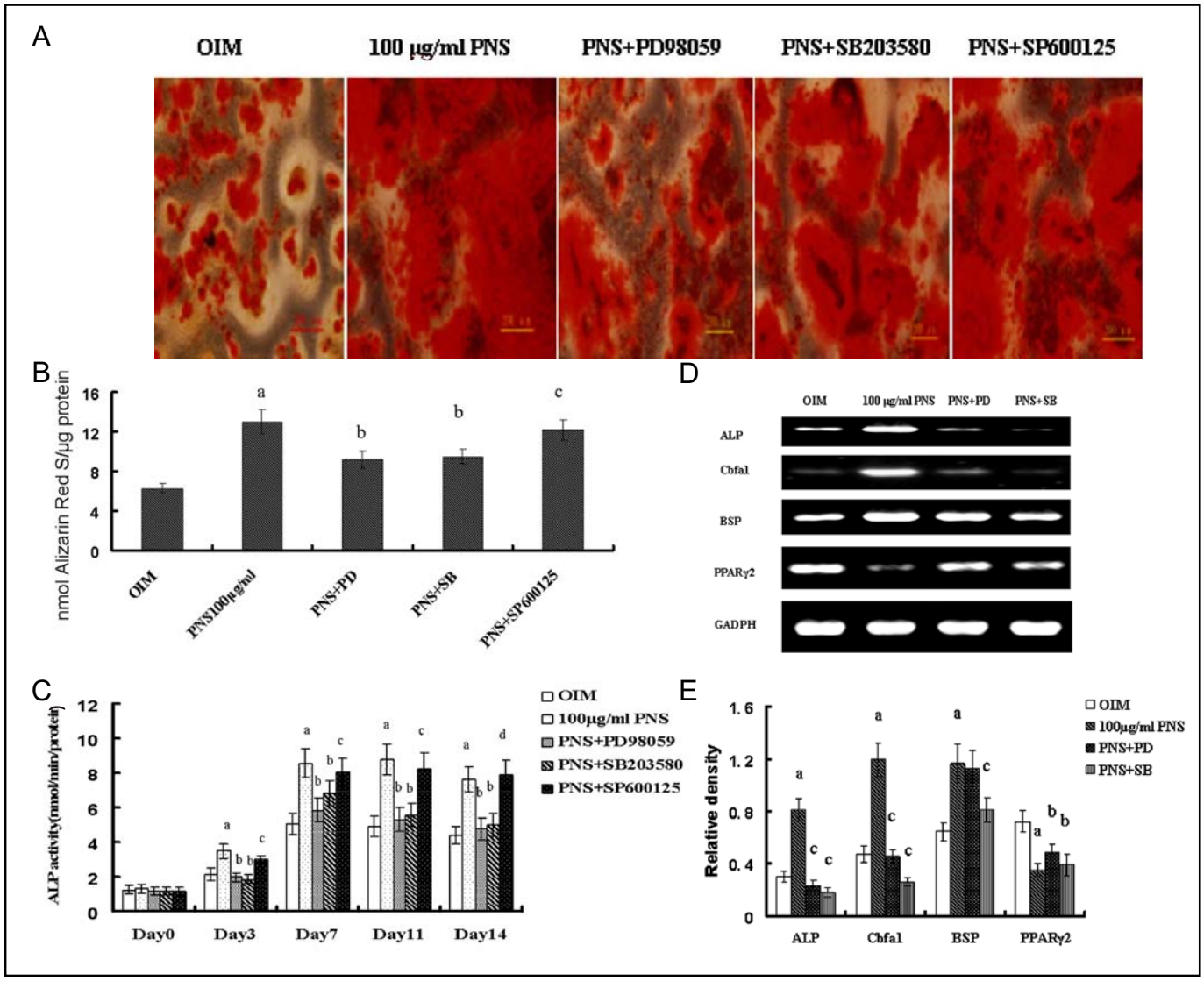

Fig. 2. Panax notoginseng saponins potentiated osteogenesis of bone marrow stromal cells via the ERK and p38 signaling pathways. (A) Alizarin red S assays showed that PD98059 and SB203580, but not SP600125, inhibited Panax notoginseng saponins-potentiated mineralization of bone marrow stromal cells. (B) The content of calcium deposit was quantified and statistically analyzed (n=5). $^{a}P < 0.01$ versus bone marrow stromal cells undergoing simple osteogenic induction; $^{b}P < 0.01$ versus 100μg/ml group. $^{c}P > 0.05$ versus 100μg/ml group.(C) PD98059 and SB203580, but not SP600125, inhibited Panax notoginseng saponins- enhanced alkaline phosphatase activity of bone marrow stromal cells (n=4). $^{a}P < 0.01$ versus OIM ; $^{b}P < 0.01$ versus 100 μg/ml PNS group; c no significant difference versus 100 μg/ml PNS group; $^{d}P < 0.05$ versus 100 μg/ml PNS group.(D) The expression of genes involved in osteogenesis of bone marrow stromal cells was quantified by RT-PCR with or without Panax notoginseng saponins in the absence or presence of PD98059 or SB203580 for 14 days. (E) Densitometric analysis of the genes described in (D). $^{a}P < 0.01$ versus bone marrow stromal cells undergoing simple osteogenic induction; $^{b}P < 0.05$ versus the 100 μg/mL group; $^{c}P < 0.05$ versus 100 μg/ml PNS group.

cells (Fig. 1A). We further studied whether Panax notoginseng saponins could stimulate the mineralization of bone marrow stromal cells by examining these cells using alizarin red S assays. We found that, three weeks after the start of incubation, bone marrow stromal cells growing in osteogenic medium showed apparent mineralization while bone marrow stromal cells growing in non-osteogenic medium showed no noticeable mineralization (Fig. 1B). Furthermore, Panax notoginseng saponins dose-dependently increased the mineralization of bone marrow stromal cells (Fig. 1B and 1C). We then examined the effect of Panax notoginseng saponins on alkaline phosphatase activity of bone marrow stromal cells under osteogenic induction. Determination of

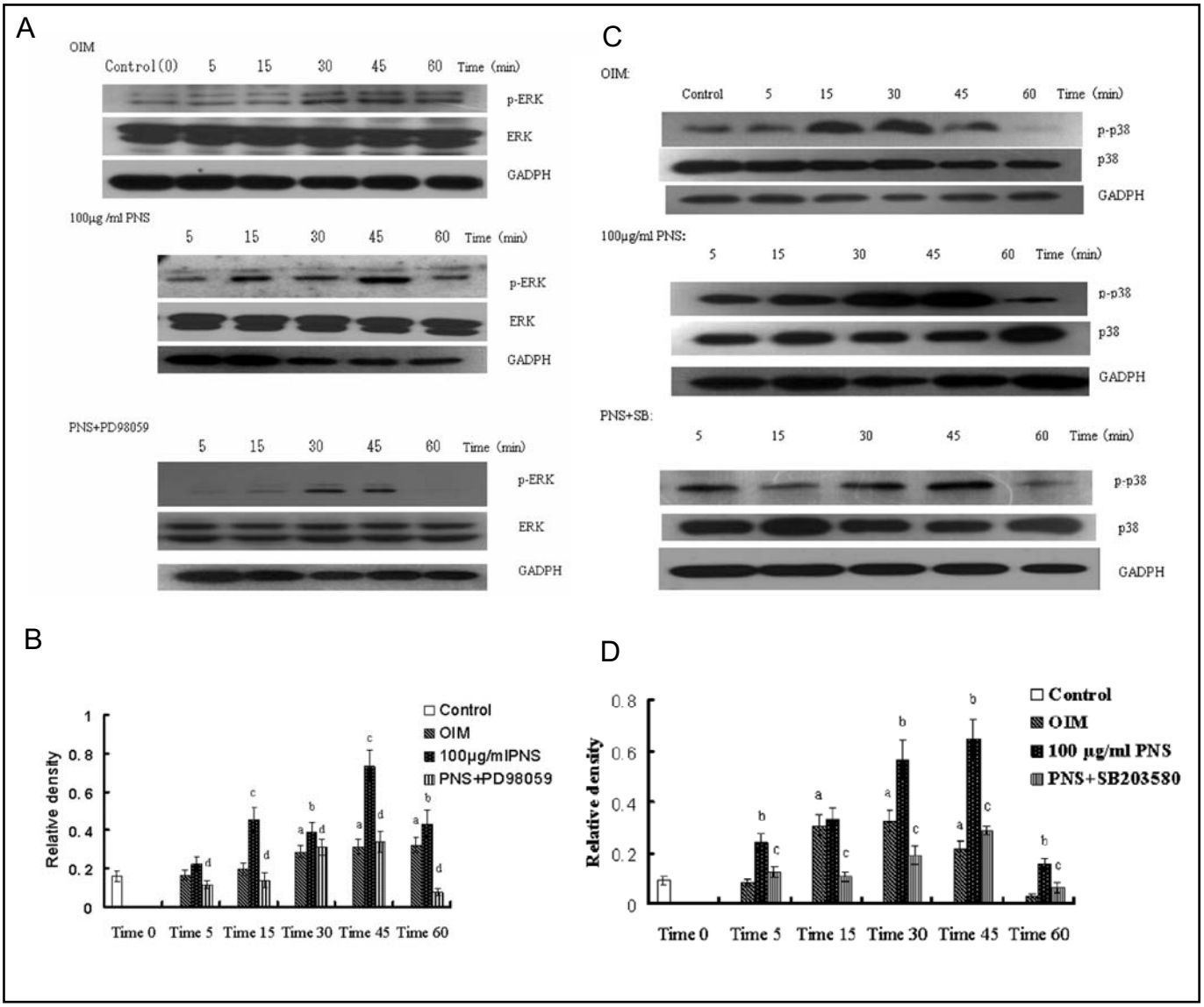

Fig. 3. Panax notoginseng saponins promote the activation of ERK and p38 of bone marrow stromal cells. (A) and (C) Bone marrow stromal cells were serum deprived for 12 h before treatment under osteogenic conditions with 100 μg/mL Panax notoginseng saponins in the absence or presence of PD98059 or SB203580 for the indicated time following pretreatment with 25 μM PD98059 or 10 μM SB203580 for 1 h. Western blotting analysis was performed with antibodies against ERK and p38 or their phosphorylated forms (p-ERK and p-p38). (B) and (D) Densitometric quantification of activated p-ERK and p-p38 was performed. (B) ^{a}P <0.01 versus bone marrow stromal cells undergoing simple osteogenic induction; ^{b}P <0.05 versus OIM group; ^{c}P <0.01 versus Versus OIM group; ^{d}P <0.01 versus 100μg/ml PNS group.(D) ^{a}P <0.01 versus bone marrow stromal cells undergoing simple osteogenic induction; ^{b}P <0.01 versus OIM group; ^{c}P <0.01 versus 100μg/ml PNS group.

alkaline phosphatase activity of bone marrow stromal cells revealed that osteogenic induction caused a time-dependent increase in alkaline phosphatase activity (Fig. 1D), which could be further significantly enhanced by Panax notoginseng saponins (Fig. 1D) ($P < 0.01$).

Panax notoginseng saponins enhanced the osteogenesis of bone marrow stromal cells by activating the ERK and p38 MAPK signaling pathways

The ERK pathway, the p38 MAPK and the JNK pathways have been shown to be intimately involved in

the proliferation and osteoblastic differentiation of bone marrow stromal cells[30]. We investigated whether Panax notoginseng saponins promoted osteogenesis through the ERK and p38 MAPK pathways. We pretreated bone marrow stromal cells with inhibitors of ERK (PD98059, 25 μM), p38 (SB203580, 10 μM) or JNK (SP600125, 10 μM), respectively. We then treated these cells with 100 μg /mL Panax notoginseng saponins for 21 days. Alizarin red S assays showed that PD98059 and SB203580 could markedly suppress Panax notoginseng saponins-mediated increase in calcium deposit (Fig. 2A and 2B), Alkaline phosphatase activity assays also showed that PD98059 and SB203580 could significantly inhibit Panax notoginseng saponins-mediated increase in alkaline phosphatase activities (Fig. 2C). We further investigated the expression of genes involved in osteogenesis in bone marrow stromal cells under osteogenic induction. Our RT-PCR assays showed that, compared with bone marrow stromal cells under simple osteogenic induction, bone marrow stromal cells under osteogenic induction that were treated with Panax notoginseng saponins for 14 days exhibited increased mRNA transcript levels of alkaline phosphatase, core-binding factor a1, and bone sialoprotein (Fig. 2D and 2E). On the other hand, Panax notoginseng saponins caused a reduction in the PPARγ2 mRNA transcript levels. Furthermore, RT-PCR assays revealed that PD98059 and SB203580 markedly suppressed Panax notoginseng saponins-mediated increase in the mRNA transcript levels of alkaline phosphatase, core-binding factor a1, and bone sialoprotein and attenuated Panax notoginseng saponins-mediated reduction of PPARγ2 mRNA transcript levels (Fig. 2D and 2E). On the other hand, JNK inhibitor, SP 600125, did not exert any effect on Panax notoginseng saponins-mediated increase in mineralization and alkaline phosphatase activity. These findings indicated that the ERK and p38 signaling pathways could play critical roles in Panax notoginseng saponins-potentiated osteogenesis of bone marrow stromal cells. These data indicate that Panax notoginseng saponins could modulate the expression of multiple genes involved in osteogenesis.

We then investigated whether Panax notoginseng saponins-mediated potentiated osteogenesis of bone marrow stromal cells under osteogenic induction by activating the ERK and p38 signaling pathways. We examined the phosphorylation levels of ERK and p38 by immunoblotting assays using phosphor-specific antibodies against ERK and p38. We found that ERK and p38 became activated in bone marrow stromal cells under osteogenic induction, which was further enhanced by Panax notoginseng saponins as evidenced by increased levels of phosphorylation of ERK and p38 (Fig. 3A and 3C). ERK and p38 inhibitors, PD98059 and SB203580, could markedly attenuate Panax notoginseng saponins-enhanced ERK and p38 activation (Fig. 3B and 3D). These results together suggested that Panax notoginseng saponins-potentiated osteogenesis involves stimulating the activation of the ERK and p38 signaling pathways.

Discussion

Osteoporosis is associated with osteoblast insufficiency during continuous bone remodeling [1]. Bone marrow stromal cells are regarded as putative osteoblast progenitors and could be induced to differentiate into osteoblasts *in vitro* [36]. Bone remodeling underlies the process of bone repair or osteogenesis in many important bone diseases. Defective osteogenesis is characterized by reduced bone mass and deteriorated bone microstructures with noticeably increased risk of bone fractures [1]. Defective osteoblast differentiation may contribute to osteoporosis [4, 37]. Promotion of proliferation and induction of differentiation of bone marrow stromal cells could offer a promising alternative therapeutic method for bone diseases in which there are significant ongoing bone remodeling activities. We showed here that osteogenesis of primary rat bone marrow stromal cells could be effectively induced *in vitro*, demonstrating that these cells could be expanded *in vitro* to provide a ready source of early passage primary bone marrow stromal cells. We further showed that Panax notoginseng saponins could dose-dependently promote the proliferation and potentiate the osteogenesis of bone marrow stromal cells.

The mechanisms whereby Panax notoginseng saponins promote osteogenesis of bone marrow stromal cells have hitherto remained undefined. Bone formation is the differentiation of bone marrow stromal cells into osteo-progenitor cells and then preosteoblasts and osteoblasts followed by matrix mineralization in a defined spatial cascade of events[38]. Commitment of primitive pluripotential cells to specific lineages is marked by the activation of key transcription factors, which, in turn, turn on the expression of downstream tissue-specific genes [39]. We examined here whether Panax notoginseng saponins promoted osteogenesis of primary bone marrow stromal cells by modulating the expression of osteogenesis-associated genes such as the gene encoding core-binding factor a1, which is a master regulatory

protein in bone marrow stromal cells and the predominant transcriptional activator of osteoblast-associated genes [40-42].We found that Panax notoginseng saponins markedly increased the mRNA transcript levels of genes encoding proteins involved in osteogenesis such as alkaline phosphatase, core-binding factor a1, and bone sialoprotein while depressing the mRNA levels of PPARγ2, a key transcription factor that has been shown to inhibit osteogenesis [43]. Osteoblasts and adipocytes differentiate from a common precursor, the pluripotent mesenchymal stem cells in bone marrow and regulation of PPARγ2 activity has been shown to control the fate of these cells towards osteogenesis or adipogenesis. Suppression of PPARγ2 activity was associated with enhanced osteogenesis [44]. Our findings indicate that attenuate PPARγ2 activity of bone marrow stromal cells is the potential mechanism of Panax notoginseng saponins stimulated the osteogenesis.

The effects of Panax notoginseng saponins on these osteogenesis-associated genes could be through modulating the MAPK signaling pathways. MAPK signaling pathways are important for osteogenesis [30, 45-47]. Inhibitor of ERK, PD98059, could significantly inhibit osteogenic differentiation of bone marrow stromal cells and caused these cells to develop into fully mature adipocytes [31]. Alendronate was found to stimulate osteogenic differentiation and inhibit adipogenic differentiation of bone marrow stromal cells by the ERK and JNK signaling pathways [32]. p38 inhibitor, SB203580, remarkably blocked Tigoenin-induced osteogenesis of bone marrow stromal cells [48]. In the present study, we showed that the ERK and p38 MAPK signaling pathways became phosphorylated in bone marrow stromal cells undergoing osteogenic differentiation. Our results further showed that inhibition of the ERK and p38 MAPK signaling pathways attenuated Panax notoginseng saponins-induced phosphorylation of ERK and p38 in bone marrow stromal cells under osteogenic induction. These data indicate that both the ERK and p38 signaling pathways are involved in Panax notoginseng saponins-potentiated osteogenic differentiation of bone marrow stromal cells. It has been reported that ERK can directly result in the phosphorylation of PPARγ2 and reduces the levels of PPARγ2 [49]. p38 MAPK plays a negative role in regulating PPARγ2 transcriptional activities[50]; inhibition or disruption of p38 leads to increased PPARγ2 expression and transactivation. We also showed here that inhibition of the ERK and p38 signaling pathways was associated with increased PPARγ2 mRNA transcript levels in bone marrow stromal cells undergoing osteogenic induction in the presence of Panax notoginseng saponins.

In summary, our study showed that Panax notoginseng saponins potentiated the osteogenic differentiation of bone marrow stromal cells with enhanced mineralization and increased alkaline phosphatase activities of bone marrow stromal cells. We further demonstrated that Panax notoginseng saponins enhanced the phosphorylation of ERK and p38 in bone marrow stromal cells under osteogenic induction. Our findings shed light on the mechanisms whereby Panax notoginseng saponins potentiate the osteogenesis of bone marrow stromal cells.

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References

1 Raisz LG: Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest 2005;115:3318-3325.

2 Deal C: Potential new drug targets for osteoporosis. Nat Clin Pract Rheumatol 2009;5:20-27.

3 Songlin P, Ge Z, Yixin H, Xinluan W, Pingchung L, Kwoksui L, Ling Q: Epimedium-derived flavonoids promote osteoblastogenesis and suppress adipogenesis in bone marrow stromal cells while exerting an anabolic effect on osteoporotic bone. Bone 2009;45:534-544.

4 Zhang JF, Li G, Chan CY, Meng CL, Lin MC, Chen YC, He ML, Leung PC, Kung HF: Flavonoids of Herba Epimedii regulate osteogenesis of human mesenchymal stem cells through BMP and Wnt/beta-catenin signaling pathway. Mol Cell Endocrinol 2010;314:70-74.

5 Shifren JL, Schiff I: Role of hormone therapy in the management of menopause. Obstet Gynecol 2010;115:839-855.
6 Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N: Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97:1652-1662.
7 Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, McNabb MA, Wenger NK: Raloxifene Use for The Heart (RUTH) Trial Investigators. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006;355:125-137.
8 Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, Goldstein S, Sriram U, Lee A, Thompson J, Armstrong RA, Thompson DD, Powles T, Zanchetta J, Kendler D, Neven P, Eastell R: PEARL Study Investigators.Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010;362:686-696.
9 Licata AA: Discovery, clinical development, and therapeutic uses of bisphosphonates. Ann Pharmacother 2005;39:668-677.
10 Rotella DP: Osteoporosis: challenges andnewopportunities for therapy. Curr Opin Drug Discov Devel 2002;5:477-486.
11 Armamento-Villareal R, Napoli N, Panwar V, Novack D: Suppressed bone turnover during alendronate therapy for high-turnover osteoporosis. N Engl J Med 2006;355:2048-2050.
12 Odvina CV, Zerwekh JE, Rao DS, Maalouf N, Gottschalk FA, Pak CY: Severely suppressed bone turnover: a potential complication of alendronate therapy. J Clin Endocrinol Metab 2005;90:1294-1301.
13 Egermann M, Schneider E, Evans CH, Baltzer AW: The potential of gene therapy for fracture healing in osteoporosis. Osteoporos Int 2005;16:S120-128.
14 Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71-74.
15 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-147.
16 Meunier P, Aaron J, Edouard C, Vingnon G: Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies. Clin Orthop Relat Res 1971;80:147-154.
17 Burkhardt R, Kettner G, Böhm W, Schmidmeier M, Schlag R, Frisch B, Mallmann B, Eisenmenger W, Gilg T: Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. Bone 1987;8:157-164.
18 Verma S, Rajaratnam JH, Denton J, Hoyland JA, Byers RJ: Adipocytic proportion of bone marrow is inversely related to bone formation in osteoporosis. J Clin Pathol 2002;55:693-698.
19 Ng TB: Pharmacological activity of sanchi ginseng (Panax notoginseng). J Pharm Pharmacol 2006;58:1007-1719.
20 Chen W, Dang Y, Zhu C: Simultaneous determination of three major bioactive saponins of Panax notoginseng using liquid chromatography-tandem mass spectrometry and a pharmacokinetic study. Chin Med 2010;5:12.
21 Chen XC, Zhou YC, Chen Y, Zhu YG, Fang F, Chen LM: Ginsenoside Rg1 reduces MPTP-induced substantia nigra neuron loss by suppressing oxidative stress. Acta Pharmacol Sin 2005;26:56-62.
22 Rhule A, Navarro S, Smith JR, Shepherd DM: Panax notoginseng attenuates LPS-induced pro-inflammatory mediators in RAW264.7 cells. J Ethnopharmacol 2006;106:121-128.
23 Gong YS, Chen J, Zhang QZ, Zhang JT: Effect of 17beta-oestradiol and ginsenoside on osteoporosis in ovariectomised rats. J Asian Nat Prod Res 2006;8:649-656.
24 Lu XZ, Wang JH, Wu X, Zhou L, Wang L, Zhang XW, Cao KJ, Huang J: Ginsenoside Rg1 promotes bone marrow stromal cells proliferation via the activation of the estrogen receptor-mediated signaling pathway. Acta Pharmacol Sin 2008;29:1209-1214.
25 Chang CH, Liao TC, Hsu YM, Fang HW, Chen CC, Lin FH: A poly(propylene fumarate)—calcium phosphate based angiogenic injectable bone cement for femoral head osteonecrosis. Biomaterials 2010;31:4048-4055.
26 Li XD, Wan JS, Chang B, Chen B,Guo C, Hou GQ, Huang DY, Du SX: Panax notoginseng saponins promotes proliferation and osteogenic differentiation of rat bone marrow stromal cells. J Ethnopharmacol 2011;134:268-274.
27 Li XD, Chang B, Chen B, Liu ZY, Liu DX, Wang JS, Hou GQ, Huang DY, Du SX: Panax notoginseng saponins potentiate osteogenesis of bone marrow stromal cells by modulating gap junction intercellular communication activities. Cell Physiol Biochem 2010;26:1081-1092.
28 Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH: Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153-183.
29 Johnson GL, Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002;298:191-192.
30 Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF: Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated bymitogen-activated protein kinase. J Biol Chem 2000;275:9645-9652.
31 Sale EM, Atkinson PG, Sale GJ: Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J 1995;14:674-684.
32 Fu L, Tang T, Miao Y, Zhang S, Qu Z, Dai K: Stimulation of osteogenic differentiation and inhibition of adipogenic differentiation in bone marrow stromal cells by alendronate via ERK and JNK activation. Bone 2008;43:40-47.
33 Oliveira JM, Sousa RA, Kotobuki N, Tadokoro M, Hirose M, Mano JF, Reis RL, Ohgushi H: The osteogenic differentiation of rat bone marrow stromal cells cultured with dexamethasone-loaded carboxymethylchitosan/poly (amidoamine) dendrimer nanoparticles. Biomaterials 2009;30:804-813.
34 Li SH, Guo DZ, Li B, Yin HB, Li JK, Xiang JM, Deng GZ: The stimulatory effect of insulin-like growth factor-1 on the proliferation, differentiation, and mineralisation of osteoblastic cells from Holstein cattle. Vet J 2009;179:430-436.
35 Guan CC, Yan M, Jiang XQ, Zhang P, Zhang XL, Li J, Ye DX, Zhang FQ: Sonic hedgehog alleviates the inhibitory effects of high glucose on the osteoblastic differentiation of bone marrow stromal cells. Bone 2009;45:1146-1152.
36 Krause U, Harris S, Green A, Ylostalo J, Zeitouni S, Lee N, Gregory CA: Pharmaceutical modulation of canonical Wnt signaling in multipotent stromal cells for improved osteoinductive therapy. Proc Natl Acad Sci USA 2010;107:4147-4152.

37 Rodríguez JP, Garat S, Gajardo H, Pino AM, Seitz G: Abnormal steogenesis in osteoporotic patients is reflected by altered mesenchymal stem cells dynamics. J Cell Biochem 1999;75:414-423.
38 Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP: Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. J Cell Biochem 1997;64:295-312.
39 Rasmussen TP: Embryonic stem cell differentiation: a chromatin perspective. Reprod Biol Endocrinol 2003;1:100.
40 Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G: Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 1997;89:747-754.
41 Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M: Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 1999;274:6972-6978.
42 Karsenty G: Role of Cbfa1 in osteoblast differentiation and function. Semin Cell Dev Biol 2000;11:343-346.
43 Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung UI, Kubota N, Terauchi Y, Harada Y, Azuma Y, Nakamura K, Kadowaki T, Kawaguchi H: PPAR γ insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest 2004;113:846-855.
44 Cock TA, Back J, Elefteriou F, Karsenty G, Kastner P, Chan S, Auwerx J: Enhanced bone formation in lipodystrophic PPARgamma(hyp/hyp) mice relocates haematopoiesis to the spleen. EMBO Rep 2004;5:1007-1012.
45 Higuchi C, Myoui A, Hashimoto N, Kuriyama K, Yoshioka K, Yoshikawa H, Itoh K: Continuous inhibition of MAPK signaling promotes the early osteoblastic differentiation and mineralization of the extracellular matrix. J Bone Miner Res 2002;17:1785-1794.
46 Viñals F, López-Rovira T, Rosa JL, Ventura F: Inhibition of PI3K/p70 S6K and p38 MAPK cascades increases osteoblastic differentiation induced by BMP-2. FEBS Lett 2002;510:99-104.
47 Hu Y, Chan E, Wang SX, Li B: Activation of p38 mitogen activated protein kinase is required for osteoblast differentiation. Endocrinology 2003;144:2068-2074.
48 Zhou H, Yang X, Wang N, Zhang Y, Cai G: Tigogenin inhibits adipocytic differentiation and induces osteoblastic differentiation in mouse bone marrow stromal cells. Mol Cell Endocrinol 2007;270:17-22.
49 Hu E, Kim JB, Sarraf P, Spiegelman BM: Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. Science 1996;274:2100-2103.
50 Aouadi M, Laurent K, Prot M, Le Marchand-Brustel Y, Binétruy B, Bost F: Inhibition of p38MAPK increases adipogenesis from embryonic to adult stages. Diabetes 2006;55:281-289.