Original Article
Catalpol promotes proliferation and osteogenic differentiation of rat bone marrow stromal cells

Zhi-Ming Chen, Ming Lu, Fu-Jiang Zhao, Xin Sha, Hua-Song Ma

Department of Orthopaedic Surgery, 306th Hospital, Spinal Surgery Center of PLA, Beijing 100101, China

Abstract: Catalpol, an iridoidglucoside found in the root of RehmanniaglutinosaLibosch. This study was designed to investigate the effects of catalpol on the proliferation and osteogenic differentiation of rat BMSCs in vitro. When rat BMSCs cultivated in the basal medium or the osteogenic induction medium (OS with or without catalpol), cell proliferation was analyzed using an MTT and BrdU assay; and the osteogenic differentiation was assessed by alkaline phosphatase activity detection, mineralization and determination of osteogenic marker genes expression. Here, we demonstrated that rat BMSCs cultured in the basal medium with low dose catalpol (lower than 50 μg/ml) caused a significant increase in proliferation. Furthermore, Catalpol treatment increased the ALP activity, mineralization (Alizarin red S staining) and mRNA levels of ALP, OC, and BSP genes (as osteogenic makers), whereas decreased protein expression of PPARγ2 (as an adipogenic marker) during osteogenic induction. In conclusion, catalpol could stimulate BMSCs proliferation and promote their osteogenic differentiation by upregulating expression of osteogenic marker genes in a dose-dependent manner. Thus, catalpol may play an important therapeutic role in osteoporosis patients by improving osteogenic differentiation of BMSCs.

Keywords: Catalpol, bone marrow stromal cells (BMSCs), proliferation, differentiation, osteogenic cells

Introduction

Osteoporosis, a serious public health issue, is a disease that leads to an increase in bone fragility and susceptibility to bone fracture due to the decrease in bone mass and deterioration in bone structure [1, 2]. Although there are various therapeutic approaches that are available to relieve the progress of this common disease, such as bisphosphonates, hormone replacement therapy, selective oestrogen receptor modulators and calcitonin, unfavorable side effects have limited their clinical use. Furthermore, lost bone mass cannot be regained [2, 3]. Therefore, effective therapeutic strategy is urgently needed, such as developing satisfactory bone building (anabolic) agents that can stimulate new bone formation and correct the imbalance of trabecular microarchitecture characteristic of established osteoporosis [4, 5]. Bone marrow stromal cells (BMSCs) contain a subset of multipotent cells that give rise to osteoblasts, adipocytes, chondrocytes, and myocytes [6]. Since BMSCs are the precursor cells of osteoblast lineage [7, 8], they play an important role in bone modeling and remodeling, where they give rise to the essential osteoblasts for bone formation. They also contribute to maintaining the balance between bone formation and resorption [9]. It's report that panax notoginseng saponins can regulate bone formation by increasing the proliferation and inducing differentiation of the BMSCs [10].

Rehmanniais a genus of six species of flowering plants in the order Lamiales, which is endemic to China. It is frequently prescribed in China and has been reported to have a wide range of biological and pharmacological activities, including anti-tumor, purgative, sedative, liver protective, and anti-aging activities [11]. There is increasing evidence show that the extracts from Rehmanniais are widely applied for osteoporosis treatment [12-14]. In the investigation of the safety and efficacy of herb extracts, AIF (a water soluble extract of three herbs, Panax notoginseng (Burk.) F. H. Chen, Rehmannia glutinosa Libosch and Eleutherococcus senticosus) was used in Korean knee osteoarthritis patients for six weeks, and was found to be

safe, tolerable and effective for symptomatic improvement of pain and physical function [15]. In addition, R. glutinosa extracts may enhance bone metabolism in osteoporosis by stimulating the proliferation and bone-forming activity of osteoblasts as well as inhibiting generation and bone-resorbing activity of osteoclasts. The extract may also increase the expression of bone-related genes. In vivo studies using ovariectomy-induced osteoporotic rats showed an alleviation of decreased trabecular bone mineral density and increased cortical bone thickness and trabeculation of bone marrow spaces [16]. Catalpol, a main active component of Rehmannia Root, could promote proliferation and differentiation of MC3T3-E1 cells in vitro [17]. Comparing to mice, rat is a much more complicated organism and have a closer genetic relationship to human, furthermore the osteogenic effect of catalpol on rat BMSCs, which is the progenitor of the osteoblast, is scarcely available. So we aimed to investigate the effects of catalpol on the proliferation and differentiation of BMSCs and to show the regulatory effects of catalpol on anti-osteoporosis in BMSCs osteogenesis.

Materials and methods

Chemicals and reagents

Catalpol was purchased from China pharmaceutical and biological products inspection (Lot: 110808-201009). Dulbecco's modified eagle medium (DMEM) were purchased from Gibco BRL Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) were purchased from Thermo Scientific (Beijing, China), TRIzol were purchased from invitrogen (USA). Culture flasks and plates were from Nunc (Denmark). ALP colorimetric assay kit was purchased from Renbao Bioengineering Institute (Shanghai, China). MTT was purchased from Nanjing Keygen Biotech. Co., Ltd (Nanjing, China). Dexamethasone, ascorbate acid, β-glycerophosphate and DMSO were purchased from Sigma (USA). Anti-PPARγ2, anti-ALP and anti-OC were purchased from Santa Cruz Biotechnology (Beijing, China), anti-BSP was frome R&D and GADPH antibodies was purchased from Guangzhou Weijia Technology Co., Ltd., (Guangdong, China).

Cell culture

BMSCs were obtained from 4-week-old male Sprague-Dawley rat (The Experimental Animal Center of Medical College, Shantou University, Shantou, China), as previously reported [18]. Briefly, the femurs and tibias were dissected, the ends of the bones were cut, and the bone marrow was flushed out with 2 mL of ice-cold DMEM containing 10% (v/v) FBS by using a needle and syringe (18-gauge). A single-cell suspension of bone marrow cells was obtained by repeated aspiration and blow of the dropper, and then cells were counted with a hemocytometer. Cells were seeded into 25 mL cell culture flasks at a density of 1×10^6 cells/mL and cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (basal medium, as control) and kept in a humidified incubator with 5% CO_2 and 95% air at a temperature of 37°C. After 2 days, the culture medium and non-adherent cells were removed. The medium was changed two or three times weekly. As the culture reached almost complete confluence, cells (passage 3-6) were subcultured or plated for subsequent experiments. This study was approved by the animal ethic committee of Beijing Tiantan Hospital.

MTT assay

Cell viability was determined using a CCK-8 method (Dojindo, Japan). BMSCs were seeded at the density of 1×10^4 cells/cm^2 on 96-well culture plates (Corning Costar, USA) with 200 ml culture medium per well. Then, cells were treated with catalpol at concentrations ranging from 1 to 100 μg/ml and were monitored within 9 days of culture. The absorbance value was measured in a multi-well spectrophotometer (Bio-Rad, USA) at 490 nm. And bromodeoxyuridine assay (Roche, USA) following the manufacturer's instructions.

BrdU assay

BMSCs were plated on coverslips pre-coated with poly-lysine (PLL, Sigma-Aldrich) in six-well plates (Corning Costar, USA) at 3×10^4 cells/ml density. Cells were treated with different concentrations of catapol. Bromodeoxyuridine (BrdU) (Roche, USA) was then added to each medium for 6 h and the cells were fixed with 4% paraformaldehyde. The cells were counterstained by 4, 6-diamino-2-phenylindole (DAPI, Sigma-Aldrich, USA). The percentage of BrdU-positive cells over total DAPI cells was determined by randomly counting 10 nonoverlapping microscopic fields for each coverslip in three

Table 1. Primer sequences and cycle conditions used for PCR

Gene	Primer sequence (forward/reverse)	T (annealing)	Cycles
ALP	5'CCCAAAGGCTTCTTCTTG 3' (1146-1163) 5'CTGGTAGTTGTTGTGAGC 3' (1485-1502)	51	34
BSP	5'GATAGTTCGGAGGAGGAGGG 3' (293-312) 5'CTAACTCCAACTTTCCAGCGT 3' (444-464)	59	29
OC	5'AGGGCAATAAGGTAGTGAA 3' (449-467) 5'GAGGCTCTGAGAAGCATAAA 3' (681-700)	53	35
GAPDH	5'CCTGCACCACCAACTGCTTA 3' (522-541) 5'GGCCATCCACAGTCTTCTGAG 3' (641-661)	52	26

separate experiments as described previously [19].

Differentiation protocol and cell treatments

For the osteogenic-differentiation, BMSCs were seeded at approximately 1×10^4 cells/cm^2 on culture dishes and induced in an osteogenic induction medium (OS: basal medium, 0.1 µM dexamethasone, 50 µM ascorbate acid, and 10 mM β-glycerophosphate sodium) with or without catalpol (final concentration at 1, 10 and 50 µg/ml). The medium was changed two or three times every week and the BMSCs were kept under 5% CO_2 at 37°C in a humidified condition. These cultures and basal medium culture were then tested by ALP activity assay, mineralization assay to examine the influence of the catalpol on osteogenic differentiation of BMSCs.

Alkaline phosphatase (ALP) staining and activity assay

The supernatant was discarded and then subjected to the intra-cellular ALP enzyme assay at 0, 3, 7, 11, and 14 days. For the ALP activity assay, cells were washed twice with PBS followed by trypsinization and then scraped into ddH$_2$O. This was followed by three cycles of freezing and thawing. ALP activity was determined at 405 nm using pNPP as the substrate. A 50 µl sample was mixed with 50 µl pNPP (1 mg/ml) in a 1 M diethanolamine buffer containing 0.5 mM MgCl$_2$, pH 9.8 and incubated at 37°C for 15 min on a bench shaker. There action was stopped by the addition of 25 µl of 3N NaOH/100 µl of reaction mixture. Enzyme activity was quantified by absorbance measurements at 405 nm. Total protein content was determined with the BCA method in aliquots of the same samples with the PIERCE protein assay kit, read at 562 nm, and calcu-

lated according to a series of albumin (BSA) standards. ALP levels were normalized to the total protein content at the end of the experiment.

Calcium deposit analysis based on Alizarin red S staining and quantification

On day 21, the medium was removed, the cells were fixed with ice-cold 70% ethanol (v/v) for 10 min and rinsed thoroughly with distilled water. The cultures were then stained with 40 mM Alizarin red S in deionized water (pH 4.2) for 10 min at room temperature. After removing Alizarin red S solution by aspiration, the cells were rinsed with fresh PBS and dried at room temperature. Calcium deposit was evaluated by means of the cetylpyridinium chloride (CPC) method. The dish area was observed under a light-microscope. Stained cultures were photographed followed by a quantitative destaining procedure using 10% CPC. Alizarin red S concentrations were calculated by comparison with an Alizarin red S dye standard curve and expressed as nmol/ml.

Real-time RT-PCR assay

Cells were cultured and treated as described above. The total RNA was extracted using Trizol reagent. First-strand cDNA synthesis was performed using 1 µg of total RNA and an avianmyeloblastosis virus reverse transcription system. The primers were designed using primer express software (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was performed using the SYBR Green PCR Master Mix and ABI Prism 7300 Sequence Detection System (Applied Biosystems). All data were analysed using the expression of the gene encoding β-actin as a reference. The sequences of the primers (**Table 1**) used are available upon request. The relative expression levels were calculated according to the formula $2^{-\Delta Ct}$, where ΔCt was the difference in threshold cycle (Ct) values between the target gene and the endogenous control.

Transient transfection and luciferase reporter assay

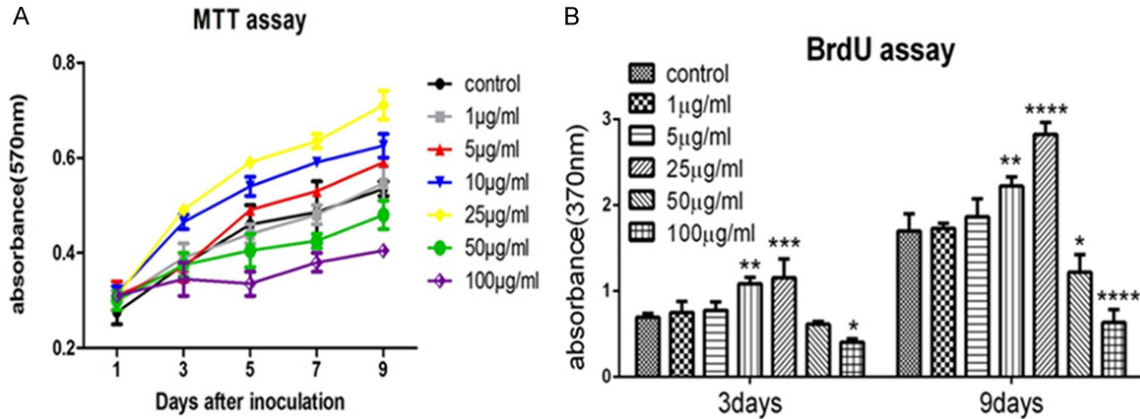The luciferase reporter construct PPRE×3-TK-LUC was transiently transfected into cells gr-

Figure 1. Catalpol promoted BMSCs proliferation. BMSCs were treated with catalpol for 1-9 days, and the proliferation rate was assessed by MTT (A) and BrdU (B) test. Data represent the mean ± SD in five times independent experiments (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001, compared with control group.

own in 24 well plates using the Lipofectamine 2000 reagent according to the manufacturer's instructions. The PPRE-luciferase reporter construct (PPRE×3-TK-LUC) driven by three copies of the PPAR response element (PPRE) was from B. M. Forman (Department of Gene Regulation and Drug Discovery, Beckman Research Institute of City of Hope National Medical Center, Duarte, CA, USA). A plasmid expressing the gene encoding β-galactosidase driven by the cytomegalovirus (CMV) promoter (Clontech Laboratories, Palo Alto, CA, USA) was simultaneously co-transfected as an internal control. The medium was replaced 4 h after transfection. Twenty-four hours after transfection, the cells were treated with the indicated concentrations of agonists or catalpol for an additional 24 h and harvested for luciferase reporter assays as described previously [20].

Western blot analysis

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. Protein concentration was assayed with BCA reagent (Sigma). An equal amount of each protein sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred to PVDF membranes. The membranes were blocked for 2 h in 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBST). And, anti-PPARγ2 and GADPH antibodies were used. Images were analyzed with

Quantity One software (BioRad, Hercules, CA) and band intensity was quantified and normalized against GADPH [21].

Statistical analysis

Data represent Mean ± SD, Statistical significance was determined with two-tailed unpaired Student's t-test between 2 groups. And data analyzed by one-way analysis of variance followed by Dunnett's multiple comparison tests. P < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). All experiments were performed a minimum of three times.

Results

Low dose of catalpol promoted BMSCs proliferation

Firstly, we used the MTT and BrdU assays to determine whether catalpol can affect the proliferation activity of BMSCs. The proliferation of BMSCs exposed to catalpol at concentrations ranging from 1 to 100 µg/ml was monitored within 9 days of culture. The day 1 is the latency period for cell proliferation. Cells started to proliferate at day 3 and catalpol significantly promoted BMSCs proliferation at 10 µg/ml and 25 µg/ml final concentration, and 1 µg/ml catalpol treatment exerted no effects on cell proliferation. Interestingly, we found that high concentration of catalpol stimulation (50 and 100 µg/ml) inversely decreased the BMSCs proliferation after 5 days culture (**Figure 1A**). In agreement with the MTT results, BrdU assay showed
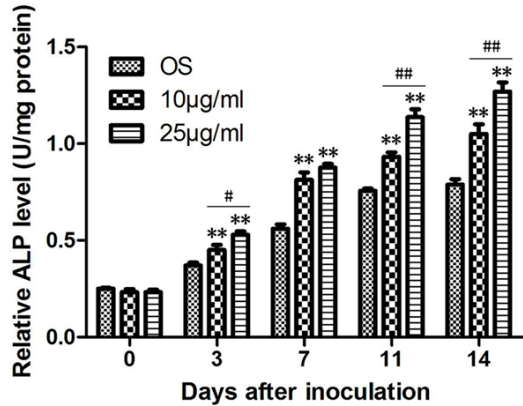
Figure 2. Catalpol induces the ALP (Alkaline phosphatase) activity of BMSCs. ALP activity of the BMSCs cultured in OS or OS and different concentrations of catalpol was determined. Data represent the mean ± SD in four times independent experiments (n = 5). *$P < 0.05$, **$P < 0.01$ compared with OS group, #$P < 0.05$, ##$P < 0.01$.
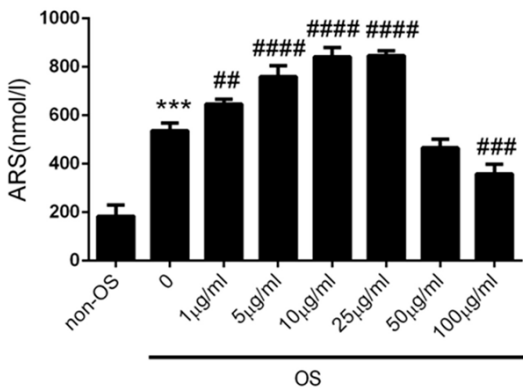

Figure 3. Catalpol enhanced BMSCs mineralization. The mineralization was quantified by Alizarin Red S assay after BMSCs induced in an OS or OS with catalpol (1, 5, 10, 25, 50, 100 μg/ml) for 21 days. Data represent the mean ± SD in five times independent experiments (n = 3). ***$P < 0.001$ compared with non-OS group, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$ compared with OS group.

that low dose ofcatalpol promoted but high dose of catalpol suppressed the cell growth (**Figure 1B**), suggesting that high concentration of catalpol was poisonous to cell growth and which may provide a guidance for clinical usage.

Catalpol induces the ALP (Alkaline phosphatase) activity of BMSCs

Next, we asked whether BMSCs differentiation was affected by catalpol. ALP, a homodimeric-

metalloenzyme which catalyzes the hydrolysis of phosphomonoester with release of inorganic phosphate and alcohol, is one of the most frequently used marker for osteoblasts and is known to be critically involved in the initiation of mineralization during bone formation. So we performed the ALP activity assay to assess the osteogenic differentiation. BMSCs undergoing osteogenesis showed a gradually rising of ALP activity in OS culture. In addition, comparing to the OS culture, catalpol treatment significantly enhanced the ALP activity in a dose-dependent manner, especially in the late stage like day 11 and day 14 (**Figure 2**).

Low dose of catalpol enhanced BMSCs mineralization

To further confirm that catalpol can promote BMSCs differentiate to osteoblasts, we performed the ARS experiment to evaluate the mineralization of BMSCs, Which was indicative of the BMSCs differentiating down the osteoblast lineage.

Alizarin red S (ARS) staining assay has been used for decades to evaluate calcium-rich deposits by cells in culture. We used a destaining procedure to quantify the mineral matrix deposition. The Alizarin red S content was significantly increased in response to the OS culture, what's more, the low dose of catalpol promoted this enhancement in dose-dependent manner, but high dose of catalpol suppressed this effect (**Figure 3**). So it suggests that low dose of catalpol increases the calcium deposit and mineralization of BMSCs and has a positive role on BMSCs differentiating to osteoblast cells.

Catalpol increased the expression of osteoblastic markers but suppressed adipogenic gene expression

During the osteogenesis, BSP is appeared following ALP expression and localized to the matrix of mineralization, which can promote the nucleation of hydroxyapatite mineralization in vitro and increases calcium incorporation and nodule formation [22]. Subsequently, OC appears on the terminal of osteoblastic differentiation. Ducy et al [23]. indicated that OC can bind to Ca^{2+} to regulate calcium ion homeostasis and bone mineralization. So we then carried out the Q-PCR experiment to investigate the

A

B

catalpol(μg/ml)   0   5   10   25

ALP
OC
BSP
GAPDH

C

PPRE-LUC

D
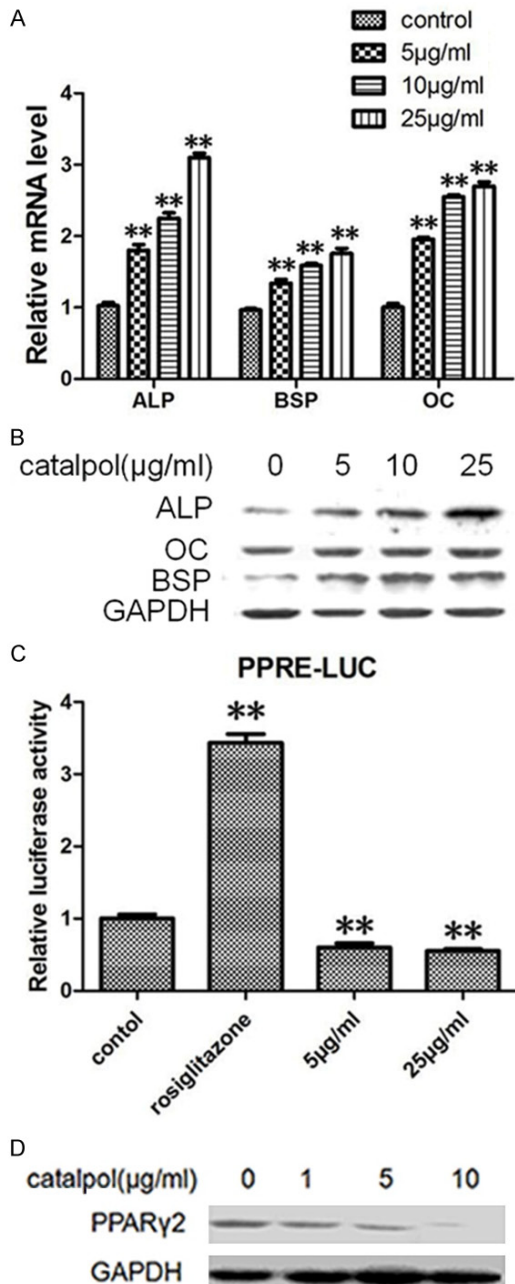
catalpol(μg/ml)   0   1   5   10

PPARγ2
GAPDH

Figure 4. Catalpol increased the expression of osteoblastic markers but suppressed adipogenic gene expression. A. ALP, OC, and BSP mRNA levels were measured by qPCR after cells were induced with or without catalpol for 11 days. B. Western blotting analysis of ALP, OC, and BSP expression. C. Cells transfected transiently with PPRE×3-TK-LUC were treated without (control) or with the indicated concentrations of drugs for 24 h. D. Western blotting analysis of PPARγ2 expression. Data represent the mean ± SD in three times independent experiments. *$P < 0.05$, **$P < 0.01$ compared with control group.

effect of catalpol on the expression of these osteoblast markers. As expected, the dose-

dependent catalpol (1-25 μg/ml) treatment increased the mRNA level of ALP, BSP and osteocalcin (**Figure 4A**), as well as the protein level of ALP, BSP and osteocalcin (**Figure 4B**). Previous studies have demonstrated the reciprocal relationship between adipogenesis and osteogenesis. As PPARγ2 (as an adipogenic marker) inhibits osteogenesis, so we then analyze the effect ofcatalpolon PPARγ2 activation, luciferase reporter assay was performed. The results showed that catalpol decreased the level of PPAR activation (**Figure 4C**). In addition, the protein content of PPARγ2 was obviously decreased with catalpol treatment in a dose-dependent manner (**Figure 4D**). Therefore, we demonstrates that catalpol promotes the osteogenesis by increasing the expression level of osteoblastic markers and suppressing the adipogenic gene expression. Altogether, these data indicate that catalpol positively regulate BMSCs proliferation and differentiation into osteoblasts.

Discussion

BMSCs contain a subset of multipotent cells including a clinically relevant and therapeutically appropriate pool of mesenchymal and progenitor cells with the capacity to differentiate into osteoblasts, adipocytes, myocytes, and chondrocytes [8, 24, 25]. BMSCs are pluripotent cells that have been used to facilitate bone repair because of their capability of differentiating into osteoblasts [26]. Study has shown that BMSCs derived from osteoporosis patients have lower ALP activity compared to the normal controls. The cultures of osteoporotic BMSCs in osteogenic stimulation medium did not show a meaningful of calcium deposition [27]. Therefore, enhancement of BMSCs differentiation in osteogenic process could be an alternative therapeutic target for osteoporosis and related diseases [28].

In traditional Chinese medicine, many herbs are used in the treatment of osteoporosis and bone nonunion. However, due to their complicated components, it is very difficult to find an effective monomer and to prove their therapeutic functions in molecular level and the corresponding mechanism. So it's necessary to analyze their active ingredients and illustrate their functions. To date, many monomers in Chinese herbs have been found effective in their clinical application for the therapy of osteoporosis and bone fracture [29]. Previous studies have dem-

onstrated that catalpolcan be used to treat and prevent osteoporosis. It is thought that catalpol may be a potential candidate for treating osteoporosis. This study utilized BMSCs cultures to systematically analyze the effect of catalpol on proliferation and differentiation in vitro. When BMSCs cultured in the presence of catalpol at the concentrations of 1, 10, and 50 μg/ml could significantly increase the proliferation of BMSCs (**Figure 1**). Our results suggested that catalpol could promote the proliferative capacity of BMSCs to recover the bone mass in osteoporosis patients.

Additionally, we performed many assays to evaluate the capacity of BMSCs differentiate into osteoblasts. As shown in **Figure 2**, the level of ALP activity (one of the most frequently used biochemical markers of osteoblast activity) at days 3, 7, 11 and 14 were significantly higher with OS and catalpol treatment comparing to OS alone, suggesting that catalpol may enhance osteogenic differentiation by increasing ALP activity. Meanwhile, our results also demonstrated that the mineralization of BMSCs was significantly increased by catalpol. Thus, catalpol as a positive indicator, could promote osteogenesis in BMSCs. Our results also showed that catalpol not only significantly increased the expression of BSP and OC (two respective phenotypic markers for the middle and later stage of osteoblast differentiation) but also ultimately accelerated mineralization, suggesting that catalpol could enhance differentiation and maturation of osteoblasts at multiple levels, from early to terminal stages of the cell differentiation process. It's well known that PPARγ2 could enhance adipocyte maturity, regulate lipoprotein metabolism [30] and inhibits osteogenesis suppressing the osteoblastic phenotype [31]. Thus the inhibition of PPARγ2 activity could be an interesting strategy to combat osteoporosis [32] by promoting BMSCs osteogenic differentiation. It is attractive to hypothesize that proper catalpol could be a beneficial therapy by promoting the proliferation and osteogenic differentiation of BMSCs which is associated with the bone mass recovering of osteoporosis.

In summary, our results demonstrate that low dose of catalpol enhanced the BMSCs proliferation, which indicate that catalpol treatment can provide much more precursor cells of osteoblast lineage. Furthermore, low dose of catalpol promote the osteogenic differentiation of BMSCs by upregulating expression of osteogenic marker genes, implying that it could have a beneficial therapy for osteoporosis and related diseases by stimulating BMSCs to osteogenic differentiation. Therefore, low dose of catalpol may be a reasonable and natural active component for the clinical prevention and treatment of osteoporosis and related disease patients.

Disclosure of conflict of interest

None.

Address correspondence to: Hua-Song Ma, Department of Orthopaedic Surgery, 306th Hospital, Spinal Surgery Center of PLA, Beijing 100101, China. Tel: 86-10-66356729; E-mail: mahusong@vip.163.com

References

[1]   Gundert-Remy U. Strong bones in later life: luxury or necessity? The problem of reimbursement. Bull World Health Organ 1999; 77: 434-5.

[2]   Keen R. Osteoporosis: strategies for prevention and management. Best Pract Res Clin Rheumatol 2007; 21: 109-22.

[3]   Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. Science 2000; 289: 1508-14.

[4]   Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 2000; 289: 1501-4.

[5]   Berg C, Neumeyer K, Kirkpatrick P. Teriparatide. Nat Rev Drug Discov 2003; 2: 257-8.

[6]   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: 40-7.

[7]   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.

[8]   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: 143-7.

[9]   Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 2000; 21: 115-37.

[10] Li XD, Wang JS, Chang B, Chen B, Guo C, Hou GQ, Huang DY, Du SX. Panax notoginseng sa-

ponins promotes proliferation and osteogenic differentiation of rat bone marrow stromal cells. J Ethnopharmacol 2011; 134: 268-74.

[11] Zhao JS, Ye ZB, Zhao T, Chen JJ, Zhong JG, Wang L. Clinical and pathological experimental study on deferring senile effects of Liu Wei Di Huang Pill. World Science and Technology/ Modernization of Traditional Chinese Medicine and Materia Medica 2006; 8: 117-22.

[12] Sheng L, Xing GS, Wang Y, Tan ZL, Lou JS. Effects of radix rehmanniae preparataon the biochemical markers of bone metabolism and the bonemineral density in ovariectomized rats. Chin J Osteoporos 2006; 12: 496-8.

[13] Wu MS, Li E, Zhao SZ, Bai X, Li AY. Effect of tonifying kidney contained serum on mRNA and protein expression of estrogen receptor in osteoblast rats. Chin Pharmacol Bull 2008; 24: 1396-7.

[14] Wu MS, Li E, Zhao SZ, Bai X, Li AY. The effects of Kang Gu Song Pellet (KGSKL) on bone quality and biomechanics performances of the lum boverovertebral bone in ovariectomized rats. Chin J Gerontol 2008; 28: 2289-92.

[15] Park SH, Kim SK, Shin IH, Kim HG, Choe JY. Effects of AIF on Knee Osteoarthritis Patients: double-blind, randomized placebo-controlled study. Korean J Physiol Pharmacol 2009; 13: 33-7.

[16] Oh KO, Kim SW, Kim JY, Ko SY, Kim HM, Baek JH, Ryoo HM, Kim JK. Effect of rehmannia glutinosa libosch extracts on bone metabolism. Clin Chim Acta 2003; 334: 185-95.

[17] Wu MS, Zhao SZ, Li E, Bai X. Effects of catalpol from Radix rehmanniae on proliferation, differentiation and matrix mineralization of MC3T3-E1 cells. Chinese Pharmacological Bulletin 2010; 26: 509-13.

[18] Lennon DP, Caplan AI. Isolation of rat marrow-derived mesenchymal stem cells. Exp Hematol 2006; 34: 1606-7.

[19] Meng ZX, Nie J, Ling JJ, Sun JX, Zhu YX, Gao L, Lv JH, Zhu DY, Sun YJ, Han X. Activation of liver X receptors inhibits pancreatic islet beta cell proliferation through cell cycle arrest. Diabetologia 2009; 52: 125-35.

[20] Ma CH, Wang SS, Ma SP. Separation, characterization and dose-effect relationship of the PPARγ-activating bio-active constituents in the Chinese herb formulation 'San-Ao Decoction'. Chinese Journal of Experimental Formulas of Chinese Medicine 2012; 19: 149-53.

[21] Zhao H, Xu Z, Qin H, Gao Z, Gao L. miR-30b regulates migration and invasion of human colorectal cancer via SIX1. Biochem J 2014; 460: 117-25.

[22] Gordon JA, Tye CE, Sampaio AV, Underhill TM, Hunter GK, Goldberg HA. Bone sialoprotein expression enhances osteoblast differentiation and matrix mineralization in vitro. Bone 2007; 41: 462-73.

[23] Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. Nature 1996; 382: 448-52.

[24] Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001; 19: 180-92.

[25] Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997; 276: 71-4.

[26] Kim HJ, Park JB, Lee JK, Park EY, Park EA, Riew KD, Rhee SK. Transplanted xenogenic bone marrow stem cells survive and generate new bone formation in the posterolateral lumbar spine of non-immunosuppressed rabbits. Eur Spine J 2008; 17: 1515-21.

[27] Rodriguez JP, Garat S, Gajardo H, Pino AM, Seitz G. Abnormal osteogenesis in osteoporotic patients is reflected by altered mesenchymal stem cells dynamics. J Cell Biochem 1999; 75: 414-23.

[28] 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: 70-4.

[29] Kou G, Wang S, Cheng C, Gao J, Li B, Wang H, Qian W, Hou S, Zhang D, Dai J, Gu H, Guo Y. Development of SM5-1-conjugated ultrasmall superparamagnetic iron oxide nanoparticles for hepatoma detection. Biochem Biophys Res Commun 2008; 374: 192-7.

[30] Li AC, Glass CK. PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. J Lipid Res 2004; 45: 2161-73.

[31] Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung UI, Kubota N, Terauchi Y, Harada Y, Azuma Y, Nakamura K, Kadowaki T, Kawaguchi H. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest 2004; 113: 846-55.

[32] 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: 1007-12.