

Original Research Article

Effect of polygonimitin C on bone formation and resorption in human osteoblast-like MG63 cells

Hua-Li Shi, Xiao-Feng Yang*, Xin-Wei Li, Zhuo-Ling Fu, Xue-Qing Jin and Hai-Lian Ji

Department of Medicine, Hangzhou Medical College, Hangzhou 310053, Zhejiang, PR China

*For correspondence: **Email:** xfyhzm@163.com

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Abstract

Purpose: To investigate the effect of polygonimitin C (PC) on bone formation and resorption in human osteoblast-like MG63 cells.

Methods: MG63 cells were treated with PC at doses of 0, 20, 40 or 80 µg/mL for 48 h, with an untreated group as control. The effect of PC on alkaline phosphatase (ALP) activity in MG63 cells was investigated by p-nitrophenyl phosphate disodium hexahydrate assay. Western blot assay was used to evaluate the effect of PC on the expressions of osterix (OSX), bone morphogenetic protein-2 (BMP-2), runt-related transcription factor 2 (RUNX-2), osteocalcin (OC), fibronectin (FN), type I collagen (COL I), osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) proteins in MG63 cells.

Results: ALP relative activity in MG63 cells treated with PC at 20, 40 or 80 µg/mL (123.58, 137.74 or 159.62 %, respectively) was significantly ($p < 0.05$ or 0.01) higher than that in control group (99.37 %). Expressions of OSX, BMP-2, RUNX-2, OC, FN, COL I and OPG proteins in MG63 cells treated with PC at 20, 40 or 80 µg/mL were significantly ($p < 0.01$) higher than those in control group. However, there were no statistically significant differences in RANKL protein expression between PC-treated MG63 cells and control group.

Conclusion: These results show that PC exerts protective effects against osteoporosis by promoting bone formation and inhibiting bone resorption. Thus, PC may be useful in the development of new anti-osteoporosis drugs.

Keywords: Polygonimitin C, MG63 cells, Bone formation, Bone resorption, Osteoporosis

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INTRODUCTION

Osteoporosis (OP) is characterized by weak bone, which manifests clinically in bone fractures [1]. It is a major public health problem which affects numerous people worldwide, and imposes significant mental stress and economic burden on family and society [2]. The etiology of OP may be primary, secondary or idiopathic [3]. Typical primary OP is sub-divided into postmenopausal and senile OP, while secondary OP is sub-divided into endocrine, metabolic,

drug-induced and deficiency OP [3, 4]. The pathogenesis of idiopathic OP is related to genetic factors [5]. Generally, OP results from imbalance between bone formation and bone resorption, which is controlled by bone-forming osteoblasts and bone-resorbing osteoclasts [6]. In OP patients, the bone-forming function of osteoblasts is lower than the bone-resorbing function of osteoclasts [7]. Therefore, the fundamental principle involved in the treatment of OP is to regulate the imbalance between the

bone-forming function of osteoblasts and bone-resorbing function of osteoclasts.

Studies have shown that the roots of *Polygonum multiflorum* Thunb. (Polygonaceae), also named as *Polygoni multiflori Radix*, have protective effects against OP [8-10]. It has been reported that stilbene glucoside, the main active ingredient of *Polygoni multiflori Radix*, increased bone mineral density and bone strength in rats, suggesting that stilbene glucoside may exhibit a protective effect against OP [11]. The chemical structure of polygonimitin C (PC) from *Polygoni multiflori Radix* is similar to that of stilbene glucoside [12]. This suggests that PC may also exhibit some effects on OP.

Therefore, the aim of this work was to investigate the effect of PC on the bone formation and resorption functions of human osteoblast-like MG63 cells.

EXPERIMENTAL

Plant material

Polygoni multiflori Radix was purchased from Tongrentang Drugstore (Beijing, China) in 2013. It was authenticated by Xiao-Feng Yang, a taxonomist in the Department of Medicine, Hangzhou Medical College. A voucher specimen (no. HZMC 2013DM08) was deposited in the herbarium of Department of Medicine, Hangzhou Medical College for future reference.

Chemicals and reagents

Analytical grade ethanol, chloroform, ethyl acetate, n-butyl alcohol and methanol were purchased from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). Macroporous resin D101 and sephadex LH-20 were obtained from Qingshi Science and Technology Co., Ltd. (Zhengzhou, China) and Merck Millipore (Darmstadt, Germany), respectively. MEM medium and fetal bovine serum (FBS) were products of GIBCO (Waltham, MA, USA). Penicillin and streptomycin were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). p-Nitrophenyl phosphate disodium hexahydrate (pNPP), enhanced BCA protein assay kit and polyvinylidene difluoride (PVDF) membrane were purchased from Sigma-Aldrich (Shanghai, China), Beyotime (Nantong, China) and Merck Millipore (Darmstadt, Germany), respectively.

Primary antibodies for β -actin, osterix (OSX), bone morphogenetic protein-2 (BMP-2), runt-related transcription factor 2 (RUNX-2), osteocalcin (OC), fibronectin (FN), type I

collagen (COL I), osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANKL) were purchased from Bioss (Beijing, China), Cell Signaling Technology (Beverly, MA, USA) or Abgent (San Diego, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence detection kit for HRP was obtained from Biological Industries (Kibbutz Beit Haemek, Israel).

Isolation of PC

Air-dried and ground *Polygoni multiflori Radix* (30 kg) was extracted by refluxing 95 % ethanol three times. The combined ethanol solvent was evaporated under reduced pressure to yield the ethanol extract (1964 g), which was dissolved in water and then successively partitioned with chloroform, ethyl acetate and n-butyl alcohol. The n-butyl alcohol solvent was evaporated under reduced pressure to yield the n-butyl alcohol extract (206 g), which was dissolved in water for further separation. The water-soluble fraction was subjected to macroporous resin D101 column, eluting with a gradient solvent system of ethanol-water to yield 30 % ethanol fraction.

The 30 % ethanol fraction was chromatographed on Sephadex LH-20 column, eluting with 30 % methanol to yield fractions 1 - 7. Fraction 5 (14 g) was chromatographed over silica gel column chromatography, eluting with chloroform-ethyl acetate-methanol (volume ratio = 5: 3: 2) solvent system to yield PC (56 mg). The purity and chemical structure of PC were determined by area normalization method of high performance liquid chromatography (HPLC), mass spectrum (MS) data and nuclear magnetic resonance (NMR) data. PC was dissolved in 0.5 % DMSO to obtain different concentrations used in subsequent assays.

Cell culture

MG63 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in MEM medium supplemented with 1 % penicillin, 1 % streptomycin and 10 % FBS at 37 °C in a humidified atmosphere containing 5 % CO₂.

Grouping and treatment

MG63 cells were seeded on 96-well culture plates at a density of 5×10^5 cells/well and divided into control and three doses of PC groups. After culture for 24 h, cells in control

group were treated with PC at a dose of 0 µg/mL for 48 h, and cells in three doses of PC groups were treated with PC at doses of 20, 40 or 80 µg/mL for 48 h, respectively. All treatments were carried out in triplicate.

Assay of alkaline phosphatase (ALP) activity

After treatment with PC, MG63 cells were washed three times with PBS. Then the supernatant of MG63 cells were obtained with the aid of cell lysis buffer, ultrasound and centrifugation. According to the existing method in the literature [13], ALP activity in the supernatant was determined by pNPP assay. After reactions were completed, the absorbance (A) of each sample was read at 405 nm in a Multiskan MK3 microplate reader (Thermo, USA). Meanwhile, enhanced BCA protein assay kit was used to determine the protein concentration in the supernatant, and the absorbance value of each sample adjusted based on relatively stable protein concentration. The effect of PC on ALP activity in MG63 cells was evaluated by ALP relative activity, calculated as in Eq 1.

$$\text{ALP relative activity (\%)} = (A_t/A_c)100 \dots\dots (1)$$

Where A_t and A_c are the absorbance of the test and control samples, respectively.

Western blot assay

MG63 cells were harvested after treatment with PC, and then their total proteins were extracted with the aid of cell lysis buffer, ultrasound and centrifugation. Enhanced BCA protein assay kit was used to determine the concentration of total proteins. Equal amounts of total proteins (about 30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. After blocking with 5 % non-fat milk, the PVDF membranes were incubated with primary antibodies for β -actin, OSX, BMP-2, RUNX-2, OC, FN, COL I, OPG and RANKL overnight at 4°C. Subsequently, the PVDF membranes were washed with Tris-buffer saline-Tween (TBS-T) and incubated with HRP-conjugated goat anti-rabbit secondary antibody in TBS-T at room temperature for 2 h. Also, after being washed with TBS-T, the proteins on the PVDF membranes were detected on X-ray film by using enhanced chemiluminescence detection kit for HRP. β -actin was used as internal reference protein for equalizing protein loading. The expression level of target protein was expressed relative to β -actin i.e. protein level/ β -actin level.

Statistical analysis

All data are presented as mean \pm standard deviation (SD). Differences among different groups were analyzed by one-way ANOVA on SPSS 21.0 software (Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$ or 0.01 .

RESULTS

Purity and identification of PC

Results of area normalization method in HPLC indicated that the purity of PC was 99.6 %. The chemical structure of PC (Figure 1) was identified by comparing its MS and NMR data with existing literature [12].

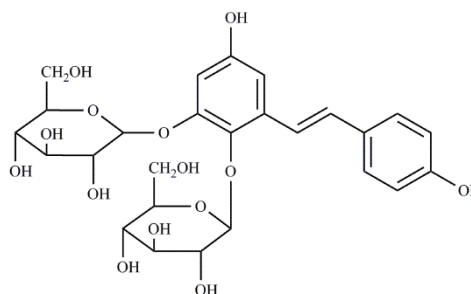


Figure 1: Chemical structure of PC

Effect of PC on ALP activity in MG63 cells

As shown in Figure 2, after treatment with PC (20, 40 or 80 µg/mL), the ALP relative activities (123.58 ± 7.38 %, 137.74 ± 10.25 % or 159.62 ± 12.17 %) in MG63 cells were significantly ($p < 0.05$ or 0.01) higher than that in the control group (99.37 ± 5.49 %).

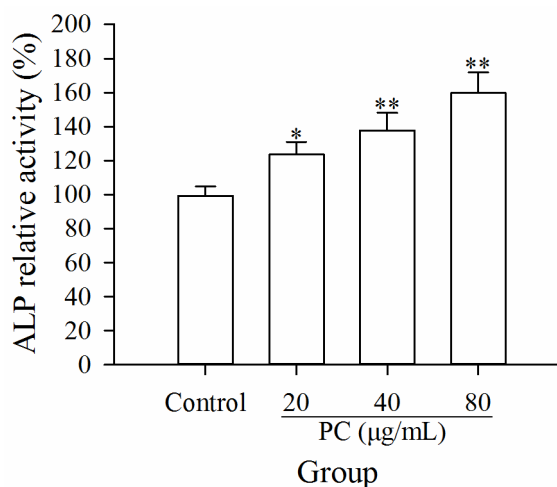


Figure 2: Up-regulative effect of PC on ALP activity in MG63 cells; * $p < 0.05$, ** $p < 0.01$, compared with that in the control group

Effects of PC on expressions of bone formation-related proteins in MG63 cells

As shown in Figure 3, after treatment with PC (20, 40 or 80 µg/mL), expressions of OSX, BMP-2, RUNX-2, OC, FN and COL I proteins in MG63 cells were significantly ($p < 0.01$) higher than those in the control group.

Effects of PC on expressions of bone resorption-related proteins in MG63 cells

As shown in Figure 4, after treatment with PC (20, 40 or 80 µg/mL), the expression of OPG protein in MG63 cells was significantly ($p < 0.01$) higher than that in the control group, while the expression of RANKL protein in MG63 cells was not significantly different from that in the control group.

DISCUSSION

MG63 cells are classic osteoblast-like cells for investigation of the effects of agents on bone formation and bone resorption [14,15]. In the present study, MG63 cells were used to assess the effect of PC on bone formation and bone resorption with the aid of pNNP and Western blot assays.

The differentiation of pre-osteoblasts into mature osteoblasts is promoted by OSX and RUNX-2 [16,17]. RUNX-2 plays an important role in early osteoblasts differentiation and promotes the formation of bone matrix proteins, such as ALP and COL I [16]. OSX is an important transcription factor in terminal osteoblasts differentiation and regulates the expressions of bone formation-related proteins, such as OC and COL I [18,19].

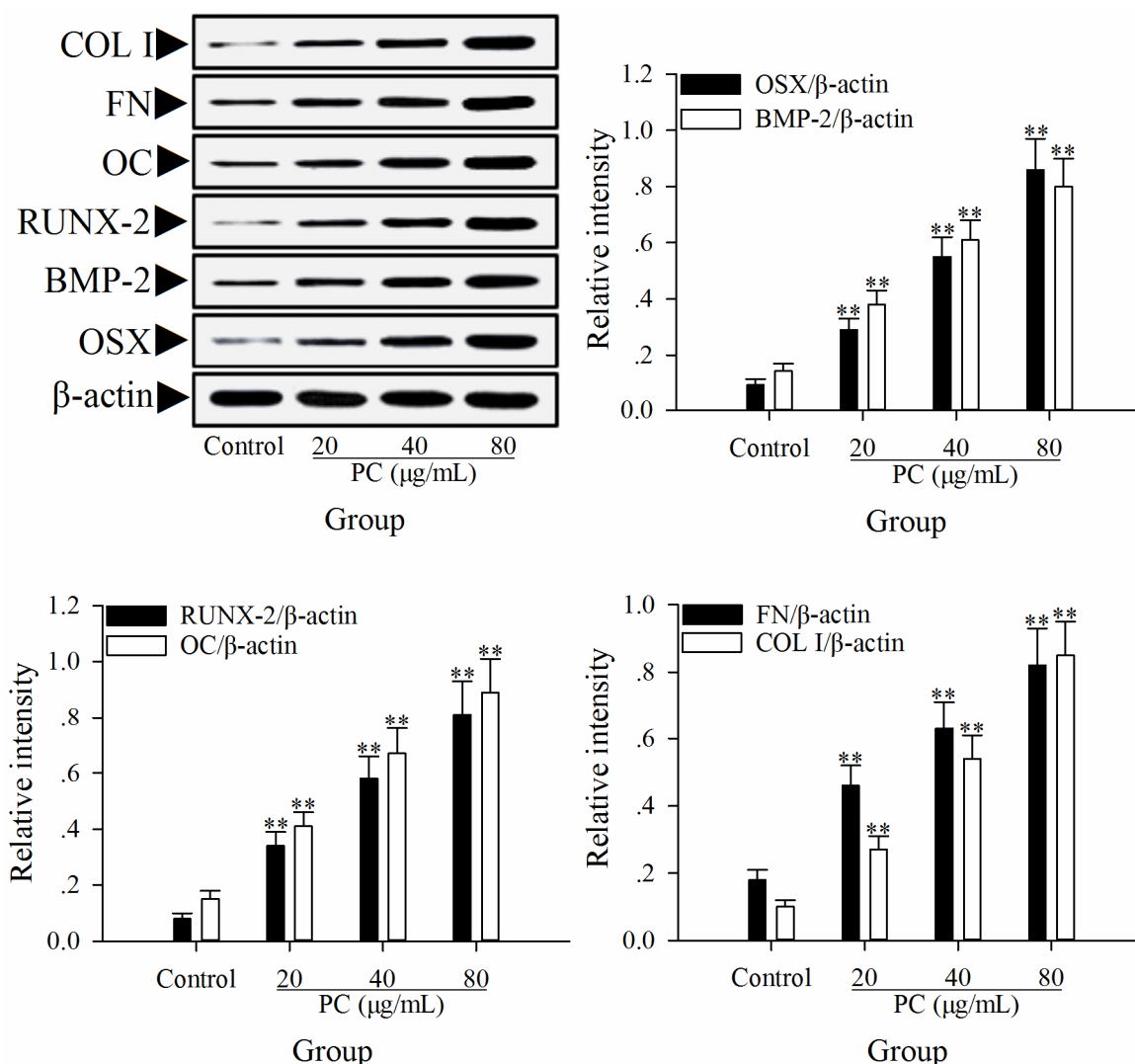


Figure 3: Up-regulative effect of PC on expressions of bone formation-related proteins (OSX, BMP-2, RUNX-2, OC, FN and COL I) in MG63 cells; ** $p < 0.01$, compared with those in the control group

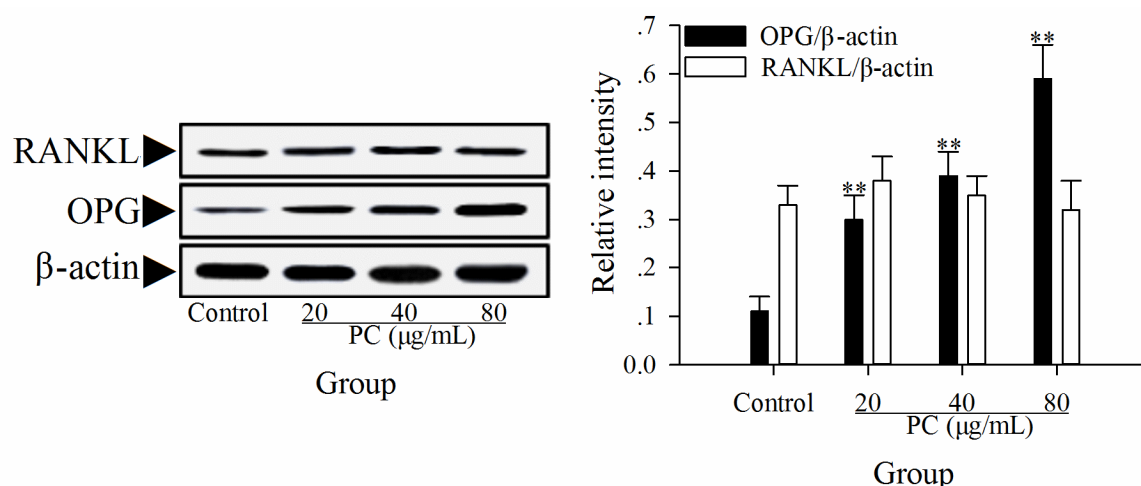


Figure 4: Effect of PC on expressions of bone resorption-related proteins (OPG and RANKL) in MG63 cells; ** $p < 0.01$, compared with that in the control group

The differentiation of pre-osteoblasts into mature osteoblasts is enhanced by BMP-2 through up-regulation of the expression of OSX [20]. OC, a key protein involved in osteoblasts differentiation and maturity, is positively related to the rate of bone formation and exhibits a regulative effect on extracellular matrix mineralization [21]. FN, an important cell adhesion factor, has a positive influence on osteoblasts differentiation and expression of COL I [22,23]. ALP and COL I are the main protein of reflecting osteoblasts functions [15]. Thus, ALP activity and the expressions of OSX, BMP-2, RUNX-2, OC, FN and COL I have positive influences on the differentiation of pre-osteoblasts into mature osteoblasts and on the osteoblasts functions. These indicate that ALP activity and the expression levels of these proteins are positively related to bone formation. In this study, PC significantly increased ALP activity and the expressions of OSX, BMP-2, RUNX-2, OC, FN and COL I in MG63 cells relative to those in the control group, suggesting that PC promoted the bone formation function of MG63 cells.

Osteoclasts differentiation and maturity are enhanced by the combination of RANKL produced by osteoblasts and RANK [24]. The OPG produced by osteoblasts is a decoy receptor for RANKL and inhibits combination of RANKL and RANK [25]. Briefly, RANKL promotes osteoclasts differentiation and maturity, and OPG inhibits RANKL-induced osteoclasts differentiation and maturity. Thus, RANKL is positively related to bone resorption, and OPG is negative related to bone resorption. In this study, PC significantly increased the expression of OPG protein in MG63 cells without any effect on the expression of RANKL protein in MG63 cells relative to those in the control group, indicating

that PC inhibited the bone resorption function of MG63 cells.

CONCLUSION

The findings of this work indicate that PC promotes the bone formation function of MG63 cells by up-regulating ALP activity and the expressions of bone formation-related proteins (OSX, BMP-2, RUNX-2, OC, FN and COL I). In addition, PC inhibits bone resorption function of MG63 cells by up-regulating the expression of bone resorption-related protein (OPG) without significant effect on the expression of bone resorption-related protein (RANKL). Thus, PC may have a potential application in the treatment of OP.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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REFERENCES

- Svedbom A, Hernlund E, Ivergard M, Compston J, Cooper C, Stenmark J, McCloskey EV, Jönsson B, Kanis JA, the EU review panel of the IOF. Osteoporosis in the European Union: a compendium of country-specific reports. *Arch Osteoporos* 2013; 8(1-2): 137.
- Svedbom A, Ivergard M, Hernlund E, Rizzoli R, Kanis JA. Epidemiology and economic burden of osteoporosis in Switzerland. *Arch Osteoporos* 2014; 9: 187.
- Yang J, Wu N, Peng J, Yang X, Guo J, Yin S, Wang J. Prevention of retinoic acid-induced osteoporosis in mice by isoflavone-enriched soy protein. *J Sci Food Agric* 2016; 96(1): 331-338.
- Simpson AH, Murray IR. Main differences in osteoporotic fracture models: which should I use? *Injury* 2016; 47(Suppl 1): S15-S20.
- Luan T, Wei SL. The present treatment situation for idiopathic adolescent or adult osteoporosis. *J Pract Tradit Chin Inter Med* 2013; 27(6x): 162-163.
- Charles JF, Aliprantis AO. Osteoclasts: more than 'bone eaters'. *Trends Mol Med* 2014; 20(8): 449-459.
- Weinstein RS. Glucocorticoid-induced osteoporosis and osteonecrosis. *Endocrinol Metab Clin North Am* 2012; 41(3): 595-611.
- Kim MJ, Seo BI, Shin SS, Park JH. Effect of *Polygoni multiflori radix* and *Cynanchi wilfordii radix* on prevention of osteoporosis in ovariectomized rats. *Korea J Herbol* 2004; 19(1): 23-23.
- Seo BI, Park JH, Lee ES, Choi DJ, Choi HS, Kim HK, Seo YB, Kim IR, Park JH, Seo GT. Effect of *Polygoni Multiflori Radix* and *Polygoni Multiflori Radix Preparat* on prevention of osteoporosis in ovariectomized rats. *Korea J Herbol* 2005; 20(2): 137-148.
- Chen SR, Zou LY, Wu T. Research progress of *Polygonum multiflorum* for preventing and treating osteoporosis. *J Mod Med Health* 2011; 27(22): 3428-3431.
- Hu XQ, Zhuo J, Liu GZ, Xie RM. Effect of stilbene glucoside on bone mineral density and bone strength of rats. *China J Chin Med* 2011; 26(157): 696-698.
- Zhou LX, Lin M, Li JB, Li SZ. Chemical studies on the ethyl acetate insoluble fraction of the roots of *Polygonum multiflorum* Thunb. *Acta Pharmaceutica Sinica* 1994; 29(2): 107-110.
- Zhan YC, Zhang LP, Mao GX, He XB, Si SY, Wang Z. Effects of salidroside on osteoblast differentiation and related signaling factors in human osteosarcoma cell line MG63. *Chin Med Biotechnol* 2013; 8(6): 414-419.
- Liu M, Liao EY, Zhou HD, Hu PA. Effect of 17- β estradiol (E2) on Cbfa 1 expression in MG63 cells. *Chin J Osteoporos* 2008; 14(3): 166-169.
- Li MQ, Wang H, Cheng ZY, Li ML, Wu J. Effects of alendronate on the function of osteoblasts. *J Biomed Eng* 2012; 29(5): 908-912.
- Komori T. Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem* 2006; 99(5): 1233-1239.
- Nishio Y, Dong Y, Paris M, O'Keefe RJ, Schwarz EM, Drissi H. Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. *Gene* 2006; 372: 62-70.
- Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, Aburatani H, Nishimura R, Yoneda T. BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* 2008; 283(43): 29119-29125.
- Nakashima K, de Crombrughe B. Transcriptional mechanisms in osteoblast differentiation and bone formation. *Trends Genet* 2003; 19(8): 458-446.
- Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM. BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem Biophys Res Commun* 2003; 309(3): 689-694.
- Wang Q, Zhang H, Geng LX, Li QJ, Gan HQ, Wang H, Bi C, Wang ZQ. Expressions of osteogenesis related factors in MG63 cells co-cultured with domestic. *Chin J Repara Reconstr Surg* 2014; 28(11): 1422-1427.
- Moursi AM, Damsky CH, Lull J, Zimmerman D, Doty SB, Aota S, Globus RK. Fibronectin regulates calvarial osteoblast differentiation. *J Cell Sci* 1996; 109(Pt 6): 1369-1380.
- Sottile J, Shi F, Rublyevska I, Chiang HY, Lust J, Chandler J. Fibronectin-dependent collagen I deposition modulates the cell response to fibronectin. *Am J Physiol Cell Physiol* 2007; 293(6): 1934-1946.
- Tanaka S. Regulation of bone destruction in rheumatoid arthritis through RANKL-RANK pathways. *World J Orthop* 2013; 4(1): 1-6.
- Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, Gouin F, Redini F, Heymann D. Receptor activator of nuclear factor κ B ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. *Am J Pathol* 2003; 163(5): 2021-2031.