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Bortezomib enhances the osteogenic differentiation capacity of human mesenchymal stromal cells derived from bone marrow and placental tissues

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ABSTRACT

Bortezomib (BZB) is a chemotherapeutic agent approved for treating multiple myeloma (MM) patients. In addition, there are several reports showing that bortezomib can induce murine mesenchymal stem cells (MSCs) to undergo osteogenic differentiation and increase bone formation *in vivo*. MSCs are the multipotent stem cells that have capacity to differentiate into several mesodermal derivatives including osteoblasts. Nowadays, MSCs mostly bone marrow derived have been considered as a valuable source of cell for tissue replacement therapy. In this study, the effect of bortezomib on the osteogenic differentiation of human MSCs derived from both bone marrow (BM-MSCs) and postnatal sources such as placenta (PL-MSCs) were investigated. The degree of osteogenic differentiation of BM-MSCs and PL-MSCs after bortezomib treatment was assessed by alkaline phosphatase (ALP) activity, matrix mineralization by Alizarin Red S staining and the expression profiles of osteogenic differentiation marker genes, *Osterix*, *RUNX2* and *BSP*. The results showed that 1 nM and 2 nM BZB can induce osteogenic differentiation of BM-MSCs and PL-MSCs as demonstrated by increased ALP activity, increased matrix mineralization and up-regulation of osteogenic differentiation marker genes, *Osterix*, *RUNX2* and *BSP* as compared to controls. The enhancement of osteogenic differentiation of MSCs by bortezomib may lead to the potential therapeutic applications in human diseases especially patients with osteopenia.

1. Introduction

Osteoporosis is the pathological condition of bone tissues which affects at least 20% of post-menopausal women [1]. Osteoporosis as characterized by the porous appearance of bone matrix could significantly reduce the strength of the affected bone and thereby more vulnerable to fracture [2]. The fractures caused by osteoporosis are difficult to treat due to the weakness of the porous bone surrounding the fracture site, and thus have been considered as one of the leading cause of morbidity in osteoporotic patients [1]. Hormonal therapy with estrogen can improve osteoporosis, however, its long term use causes serious side effects, especially increased risks of several cancers including breast, uterus and cerebrovascular diseases [3]. Apart from hormonal therapy, there are several anti-osteoporotic agents which have been used to treat osteoporotic patients, including Bisphosphonates, Denosumab and Teriparatide [4]. Although those agents could increase the bone density of osteoporotic patients by either inhibiting bone resorption (Bisphosphonates and Denosumab) or inducing bone formation (Teriparatide), their long term use could lead to some serious side effects, such as atypical fracture of femur,

Abbreviations: MSCs, mesenchymal stromal cells; PL, placenta; BM, bone marrow; ALP, alkaline phosphatase; BSP, bone sialoprotein; RUNX2, runt-related transcription factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MM, multiple myeloma.

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osteonecrosis of the jaw (in case of Bisphosphonates and Denosumab) [4–7] and osteosarcoma (in case of Teriparatide) [8]. Therefore, the novel therapeutic strategies are critically required to improve the clinical outcome of osteoporotic treatment.

Multipotent mesenchymal stromal cells (MSCs), which have a capacity to differentiate to osteoblasts are considered to be the potential source for treating several bone diseases by cell replacement therapy [9,10]. MSCs have been successfully isolated from various human tissues including bone marrow, adipose tissues, and postnatal tissues such as placenta [11–13]. However, the low number of functional osteocytes derived from MSCs has limited their use in clinical practice. Enhancement of osteogenic differentiation of MSCs is therefore necessary.

Bortezomib is the proteasome inhibitor used for treating several types of cancer especially multiple myeloma (MM) [14–16]. It has been observed that bortezomib could enhance bone formation both *in vitro* and *in vivo* by activating both β-catenin/TCF and BMP-2 signaling pathway [17–19]. Moreover, bortezomib could induce osteogenesis in several myeloma patients resulting in the improvement of osteolytic lesions [16,20–22]. Although, previous studies which investigated the effect of bortezomib on murine MSCs demonstrated that bortezomib induced osteoblastogenesis *in vitro* by showing the preferential differentiation of murine MSCs into osteoblasts in bortezomib-treated condition. The results showed higher ALP positive cells, increased osteoblast number and increased bone marrow-derived osteogenic CFU [18]. *In vivo* experiments in mice revealed that bortezomib increased bone formation in recipient mice. In addition, Bortezomib induced the expression of osteogenic differentiation marker genes, *bone sialoprotein* (*BSP*) and *Runx2* which are necessary for bone formation [18]. The present study aimed to investigate the effect of bortezomib on the osteogenic capacity of human MSCs derived from bone marrow and placental tissues as well as the underlying mechanisms.

2. Materials and methods

2.1. Isolation of human bone marrow-derived MSCs (BM-MSCs)

Bone marrow aspiration was performed in healthy volunteers after giving a written informed consent. Mononuclear cells (MNCs) were isolated using Ficoll–Paque density gradient centrifugation as previously described [23]. BM-MNCs were then cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 10% (v/v) Fetal Bovine Serum (FBS; LONZA), 100 U/ml Penicillin and 100ug/ml Streptomycin (M&H) at the density of 2×10^5 cells/cm^2. Cultures were maintained in humidified condition at 37 °C with 5% CO_2. After culture for 3 days, media were replaced and non-adherent cells were discarded. Adherent cells were cultured further under the same condition with media replacement every 3 days throughout the culture period.

2.2. Isolation of placental tissue-derived MSCs (PL-MSCs)

Placental tissues were collected from healthy full-term newborns after obtaining a written informed consent from their mothers. Placental tissues were cut into small pieces, 1–2 mm^3 in size, digested with 0.05% Trypsin–EDTA for 30 min at 37 °C, washed twice with 1X PBS and re-suspended in DMEM (GIBCO) supplemented with 10% (v/v) FBS (LONZA), 100 U/ml Penicillin and 100 μg/ml Streptomycin (M&H). After culture for 3 days, media were replaced and non-adherent cells were discarded. Adherent cells were cultured further under the same condition with media replacement every 3 days throughout the culture period.

2.3. Characterization of MSCs using flow cytometry analysis

5×10^5 BM-MSCs and PL-MSCs (passage 3) were incubated at 4 °C for 30 min in the dark with antibodies against human antigens, including fluorescein isothiocyanate (FITC) conjugated anti-CD45 antibody (BD Pharminogen), FITC-CD90 antibody (AbD-SeroTec), FITC-CD105 antibody (AbDSeroTec), Phycoerythrin (PE) conjugated anti-CD34 antibody (BD Pharminogen) and PE-CD73 antibody (BD Pharminogen). Stained cells were washed twice with 1X PBS and fixed with 1% paraformaldehyde before analyzed by FACS Calibur™ using Cell Quest® software (Becton Dickinson).

2.4. Osteogenic and adipogenic differentiation

For osteogenic differentiation, 1×10^5 MSCs were cultured in osteogenic medium [DMEM supplemented with 10% FBS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 0.1 μM Dexamethasone (SIGMA) and 50 μg/ml Ascorbic acid (SIGMA)]. On day 7 of culture, 10 mM β-glycerophosphate (MERCK) was added. On day 21 and 28 of culture, cells were harvested for Alizarin Red S staining (SIGMA) to determine bone matrix mineralization. For adipogenic differentiation, $1x10^5$ MSCs were cultured in HyClone Advance STEM Adipogenic Differentiation Kit (HyClone). On day 21 of culture, cells were harvested for Oil Red O staining (SIGMA) to detect lipid droplet accumulation.

2.5. Bortezomib treatment of MSCs

1×10^5 MSCs were cultured in osteogenic medium [DMEM supplemented with 10% FBS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 0.1 μM Dexamethasone (SIGMA) and 50 μg/ml Ascorbic acid (SIGMA) and various concentrations of bortezomib (1, 2 or 4 nM). On day 7 of culture, 10 mM β-glycerophosphate (MERCK) was added. Cells were harvested for ALP activity assay, Alizarin Red S staining and gene expression analysis. MSCs cultured in osteogenic medium without bortezomib supplementation served as controls.

2.6. Alkaline phosphatase (ALP) activity assay

After treating with 1, 2 or 4 nM bortezomib, MSCs were harvested on day 7 and day 10 of culture for colorimetric ALP activity assay (Sensolyte; Anaspec) according to the manufacturer's instruction. Briefly, cells were permeabilized with 0.2% Triton X-100 and incubated with *p*NPP substrate for 45 min at room temperature before measuring ALP activity using absorbance at 405 nm. The measured ALP activity was then compared to that of control and presented as the relative ALP activity.

2.7. Alizarin Red S staining

After treating with 1 nM or 2 nM bortezomib, BM-MSCs and PL-MSCs were harvested on day 21 and day 28 of culture for Alizarin Red S staining. Briefly, cells were fixed with 10% (v/v) formaldehyde before incubating with 40 mM Alizarin Red S dye for 20 min at room temperature. The stained cells were washed 3 times with water before examining by light microscopy.

2.8. Gene expression analysis by quantitative real-time PCR (qRT-PCR)

After treating with 1 nM or 2 nM bortezomib, BM-MSCs and PL-MSCs were harvested on day 3, day 7, day 14 and day 21 of culture for RNA isolation using TRIZOL® solution (INVITROGEN). Two micrograms of total RNA were reverse transcribed to synthesize cDNA using Superscript® III Reverse Transcriptase (INVITROGEN). The synthesized cDNAs were subjected to qRT-PCR analysis using

Table 1
Primer list.

Target gene	Forward primer	Reverse primer	Product size (bp)
Osterix	tgcttgaggaggaagttcac	ctgctttgcccagagttgtt	114
RUNX2	gacagccccaacttcctgt	ccggagctcagcagaataat	159
BSP	tgactcatccgaagaaaatgg	tcctctccatagcccagtgt	159
GAPDH	gtcaacggatttggtcgtattg	catgggtggaatcatattggaa	139

Abbreviations: RUNX2 = runt-related transcription factor 2, *BSP* = bone sialoprotein, *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase.

the ABI 7500 Real-time PCR System (Applied Biosystems). The PCR condition was the following: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 40 s. Finally, all PCR product quantifications were normalized with endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the 7500 software version 2.0.5 (Applied Biosystems, USA). Primers were specified in Table 1.

2.9. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Mann–Whitney *U* test was used to assess the significance of differences between the observed data. $p < 0.05$ was considered to be statistically significant. These tests were performed using SPSS Statistics v17.0.5.

3. Results

3.1. Characterization of BM-MSCs and PL-MSCs

BM-MSCs and PL-MSCs exhibited a similar morphology, being spindle-shaped cells with high nuclear/cytoplasmic ratio (data not shown). Both BM-MSCs and PL-MSCs expressed typical surface markers associated with MSCs, including positive for CD90, CD73 and CD105 and negative for hematopoietic cell markers, CD34 and CD45 (Fig. 1A and B). MSCs from both sources had a capacity to differentiate into osteocyte- and adipocyte-lineages, as shown by Alizarin Red S and Oil Red O staining, respectively (Fig. 1C and D).

3.2. The effect of bortezomib on the alkaline phosphatase activity and the quantities of osteocytes derived from cultured BM-MSCs and PL-MSCs

Bortezomib at the concentration of 4 nM could significantly increase the alkaline phosphatase activity of BM-MSCs in comparison to controls (BM-MSCs cultured without bortezomib) after culture in osteogenic inducing medium for 7 days (1.156 vs. 1.000, $p < 0.05$) (Fig. 2A) and 10 days (1.461 vs. 1.000, $p < 0.05$) (Fig. 2B). Although 1 nM and 2 nM of bortezomib failed to increase the alkaline phosphatase activity of BM-MSCs on day 7 of culture, both concentrations could significantly increase the alkaline phosphatase activity of BM-MSCs in comparison to controls on day 10 of culture (1.301 vs. 1.000, $p < 0.05$ for 1 nM bortezomib) and (1.567 vs. 1.000, $p < 0.05$ for 2 nM bortezomib) (Fig. 2B).

Compared with BM-MSCs, the effect of bortezomib on the alkaline phosphatase activity of cultured PL-MSCs was less pronounced. Only 4 nM of bortezomib could significantly increase the alkaline phosphatase activity of PL-MSCs on day 10 of culture in comparison to controls (1.176 vs. 1.000, $p < 0.05$) (Fig. 2D) while 1 nM and 2 nM of bortezomib failed to increase the alkaline phosphatase activity of cultured PL-MSCs under the conditions examined (Fig. 2D). Moreover, both 1 nM and 2 nM of bortezomib could increase the quantities of BM-MSCs- and PL-MSCs-derived osteogenic lineages on day 21 and day 28 of culture as qualitatively determined by Alizarin Red S staining (Fig. 3).

3.3. The effect of bortezomib on the expression levels of osteogenic lineage genes of cultured BM-MSCs and PL-MSCs

Both 1 nM and 2 nM of bortezomib significantly up-regulated the expression of osteogenic lineage genes, *Runt-related transcription factor 2* (*RUNX2*), *Osterix* and *Bone sialoprotein* (*BSP*) in cultured

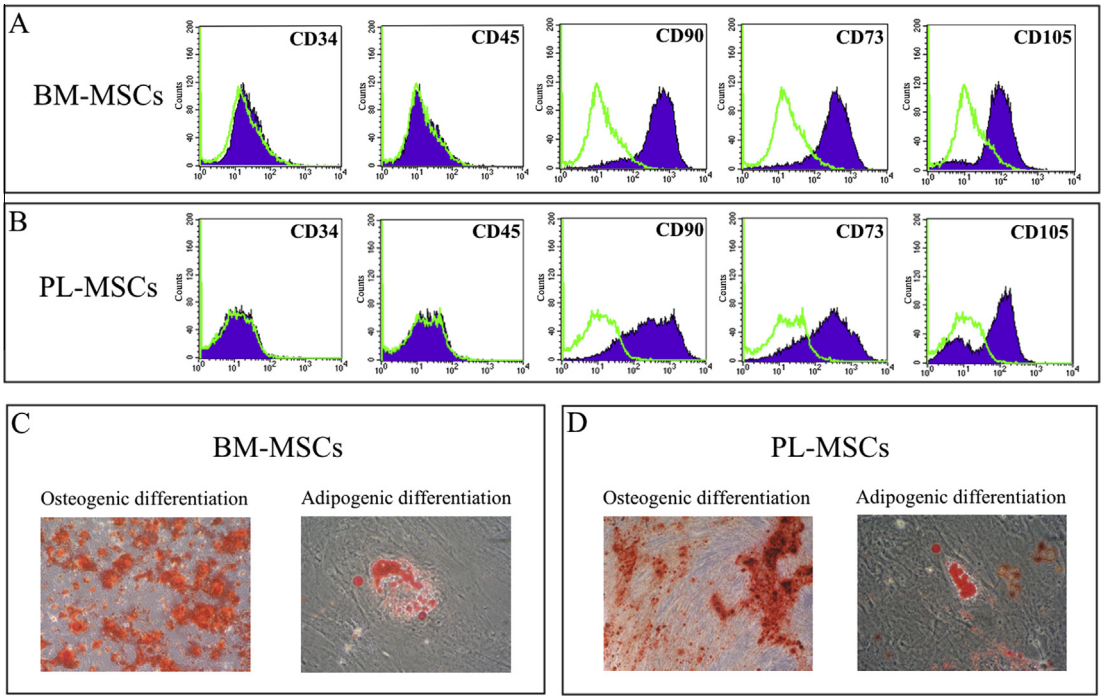

Fig. 1. Characterization of bone marrow-derived mesenchymal stromal cells (BM-MSCs) and placental tissue-derived mesenchymal stromal cells (PL-MSCs). (A) The cell surface marker expression profiles of BM-MSCs. (B) The cell surface marker expression profiles of PL-MSCs. (C) The osteogenic and adipogenic differentiation potential of BM-MSCs. (D) The osteogenic and adipogenic differentiation potential of PL-MSCs.

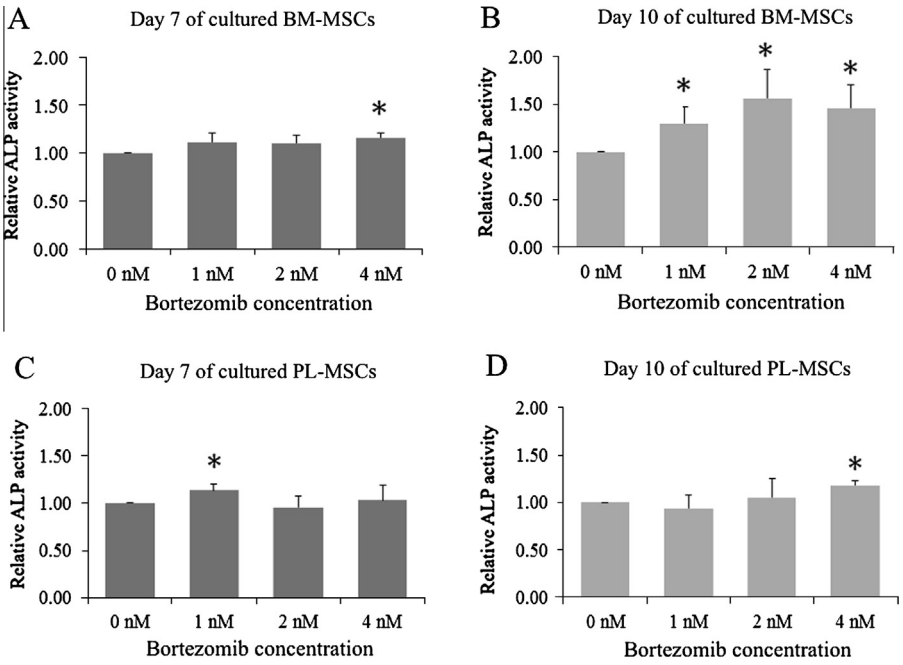

Fig. 2. The effect of bortezomib on the alkaline phosphatase (ALP) activity of cultured BM-MSCs and PL-MSCs. (A) Graph shows the relative ALP activity of BM-MSCs cultured in osteogenic medium supplemented with various concentrations of bortezomib at culture day 7. (B) The relative ALP activity of BM-MSCs cultured in osteogenic medium supplemented with various concentrations of bortezomib at culture day 10. (C) The relative ALP activity of PL-MSCs cultured in osteogenic medium supplemented with various concentrations of bortezomib at culture day 7. (D) The relative ALP activity of PL-MSCs cultured in osteogenic medium supplemented with various concentrations of bortezomib at culture day 10.

BM-MSCs on day 21 of culture compared with controls ($p < 0.05$) while there were no significantly changes in the expression levels of those osteogenic lineage genes during the earlier time points (days 3, 7 and 14 of culture) (Fig. 4A).

The effect of bortezomib on the expression levels of each osteogenic lineage gene of cultured PL-MSCs was varied. For *RUNX2* gene, 2 nM of bortezomib significantly upregulated the gene expression in cultured PL-MSCs on day 14 and day 21 of culture while the effect of 2 nM of bortezomib in up-regulating the gene expression was observed only on day 21 of culture (Fig. 4B).

For *Osterix* gene, 1 nM of bortezomib significantly up-regulated the gene expression in cultured PL-MSCs on days 7, 14 and 21 of culture while the effect of 2 nM bortezomib in up-regulating the gene expression were observed only on day 7 of culture but not on the later time points (Fig. 4B). In contrast to *RUNX2* and *Osterix* genes, both 1 nM and 2 nM of bortezomib had no effect on the expression level of *BSP* gene in cultured PL-MSCs under the conditions examined (Fig. 4B).

4. Discussion

At present, there are increasing numbers of patients suffering from degenerative bone diseases such as osteoporosis and osteolytic conditions associated with several cancers [24–26], however only few therapeutic options have been reported. Recently, transplantation of MSCs, which have the ability to generate osteoblasts both *in vitro* and *in vivo*, has been considered to be a potential treatment for several bone diseases [10]. The limited amount of functional MSC-derived osteocytes is the main obstacles of this therapeutic modality. Searching for the new agents that are able to enhance the osteogenic capacity of MSCs is thus required.

Our present study showed for the first time that *in vitro* bortezomib treatment could enhance the osteogenic differentiation of both human BM-MSCs and PL-MSCs as demonstrated by the increasing ALP activity, matrix mineralization and the expression of osteogenic differentiation marker genes. Previous studies in mice indicated that bortezomib could induce murine MSCs to undergo osteogenic differentiation as shown by higher expression

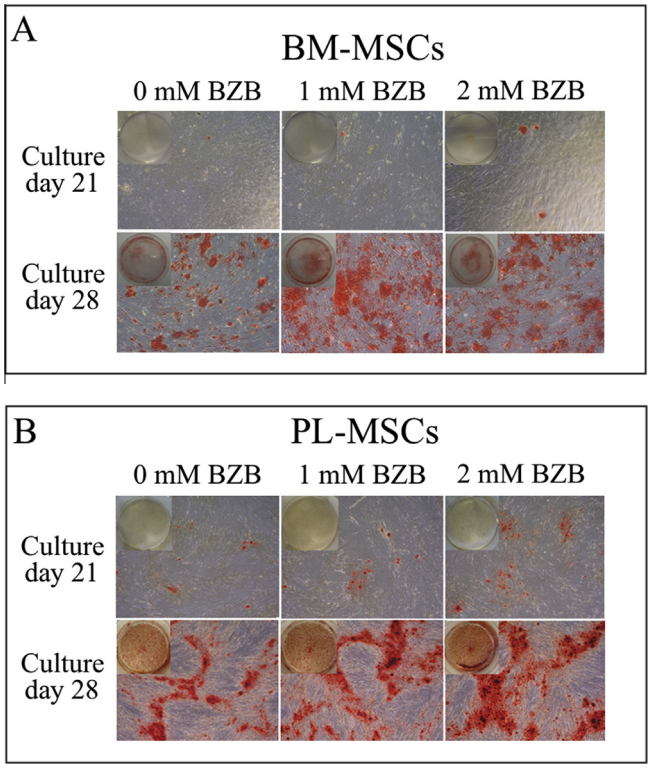

Fig. 3. The effect of bortezomib on the quantity of osteogenic lineage derived from cultured BM-MSCs and PL-MSCs. (A) The quantity of BM-MSCs-derived osteogenic lineage at culture day 21 and 28 as determined by Alizarin Red S staining. (B) The quantity of BM-MSCs-derived osteogenic lineage at culture day 21 and 28 as determined by Alizarin Red S staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

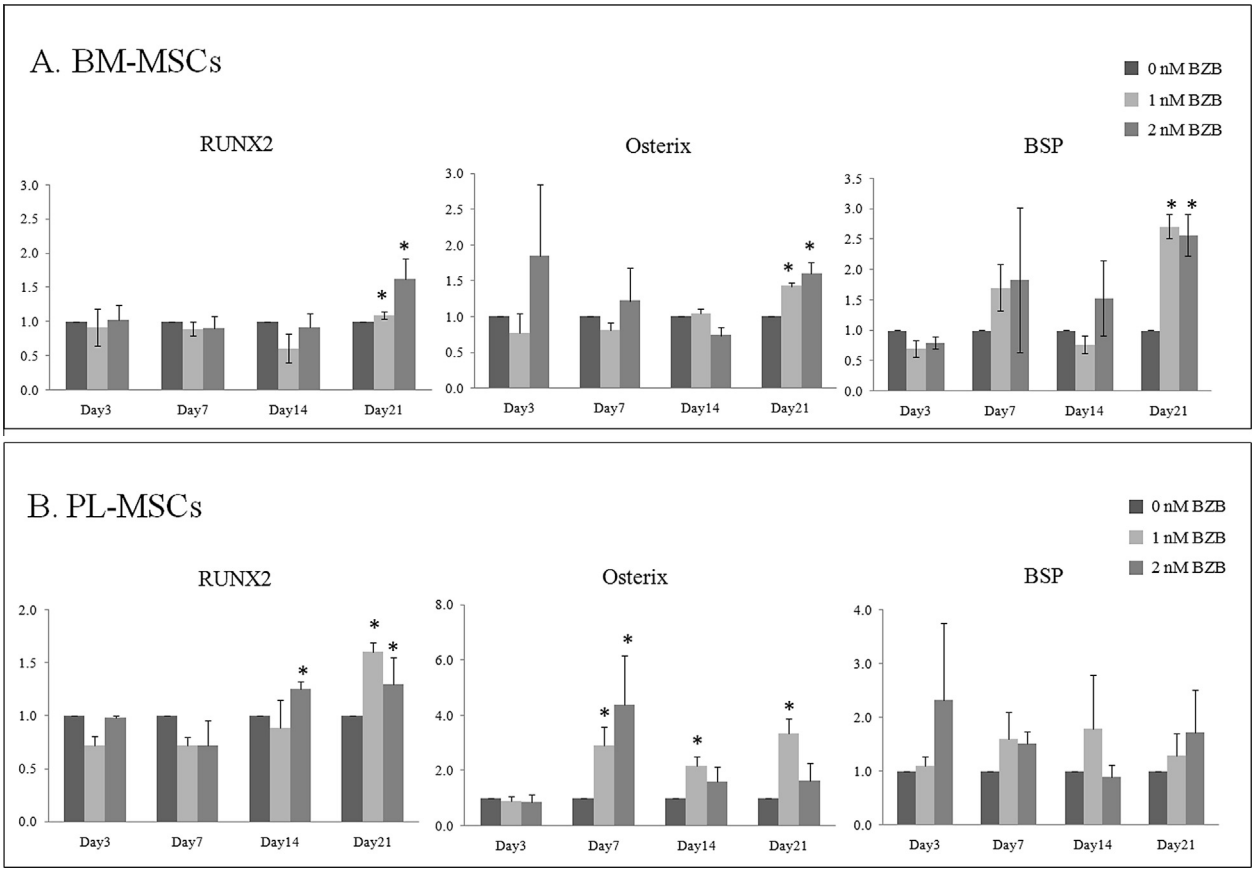

Fig. 4. The effect of bortezomib on the expression levels of osteogenic marker genes, *Runt-related transcription factor 2* (*RUNX2*), *Osterix* and *Bone sialoprotein* (*BSP*) in cultured BM-MSCs and PL-MSCs. (A) The expression levels of *RUNX2*, *Osterix* and *BSP* genes in BM-MSCs cultured in osteogenic medium supplemented with various concentrations of bortezomib. (B) The expression levels of *RUNX2*, *Osterix* and *BSP* genes in PL-MSCs cultured in osteogenic medium supplemented with various concentrations of bortezomib.

of *RUNX2* and *BSP* and higher ALP activity. Furthermore, bortezomib treatment could increase bone formation and rescued bone loss in a mouse model [18].

Inhibition of bone formation in myeloma arises from the suppression of Wnt/β-catenin signaling which is necessary for the differentiation of MSCs into osteoblasts [27–29]. Bortezomib has an ability to inhibit DKK-1 production, resulting in activation of Wnt/β-catenin signaling and bone formation [30,31]. In addition, bortezomib inhibits proteasome-mediated degradation of β-catenin [19].

We demonstrated in this study that apart from β-catenin/TCF signaling pathway, other critical osteogenic signaling molecules, including RUNX2 and Osterix were also up-regulated. This might arise from the possibility that bortezomib inhibits the destruction of intracellular proteins regulating the expression of those osteogenic transcription factors in BM-MSCs. Our results also demonstrated that bortezomib could exert the similar effect on the PL-MSCs, despite the effect is less pronounced compared to BM-MSCs. This might be due to the endogenous difference in osteogenic capacity between BM-MSCs and PL-MSCs as shown by the finding that the endogenous ALP activity of PL-MSCs was much lower than those of BM-MSCs cultured under the same condition (data not shown). Despite the difference, the suitable concentration of bortezomib could still significantly increase the osteogenic capacity of PL-MSCs.

Moreover, it was reported that bortezomib up to 500 nM can induce osteoblast differentiation *in vitro* [19]. However, the molecular mechanism of action of bortezomib on bone formation is not fully elucidated. Bortezomib can induce stabilization of β-catenin in cytoplasm and immigrate β-catenin into the nucleus, resulting in the stimulation of TCF/LEF transcription factor activities and ultimately increased bone formation [19].

Other than Wnt/β-catenin pathway, bortezomib could augment the bone formation of MSCs in mice through BMP-2 dependent pathway [18]. We demonstrated in this study that Osterix and RUNX2 which are the targets of BMP-2 signaling were up-regulated. We can then interpret that the augmentation of bone formation of MSC in human is mediated at least in part by BMP-2 dependent pathway.

Previous studies showed that bortezomib could inhibit proliferation and induce apoptosis of human myeloma cell lines. Bortezomib at the concentration of 10 nM initially had the effect on the cell lines and the optimal effect could be achieved with the 1,000 nM [15]. The standard dose of bortezomib in the treatment of multiple myeloma patients is 1.3 mg/m^2 which is equivalent to 232–312 nM [27]. Our studies indicated that the optimal concentration of bortezomib to be used for enhancing bone formation was only 1 nM, 200–300 folds lower than the concentration used in MM patients. It is therefore possible that bortezomib at lower dose can be effectively used to treat patients with osteoporosis. This will minimize the drug's side effects. However, clinical trials are badly needed to determine the appropriate dose and the safety of bortezomib for the treatment of osteoporosis.

In summary, this study demonstrated that low concentration of bortezomib could effectively enhance the osteogenic capacity of both BM-MSCs and PL-MSCs *in vitro* and could potentially be used as a therapeutic agent for treating patients with osteopenia such as osteoporosis in the future.

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