

—Original—

# Osthole improves therapy for osteoporosis through increasing autophagy of mesenchymal stem cells

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**Abstract:** Osteoporosis is a common skeletal disorder resulting in elevated fracture risk. Improvement of osteogenic differentiation is thought to be the top priority in osteoporosis treatment projects. Significant characteristics of bone marrow mesenchymal stem cells (BMMSCs), especially attractive ability to differentiate into osteoblasts, have made them alternatives for osteoporosis treatment. However, therapeutic effect with BMMSCs remains to be improved. Here, osthole, a bioactive simple coumarin derivative extracted from many medicinal plants, was introduced to pre-stimulate BMMSCs and then applied in osteoporosis therapy. The results showed that osthole-treated-BMMSCs (OBMMSCs) brought a better outcome than BMMSCs alone in estrogen deficiency-induced osteoporosis model. And elevated autophagy level was suggested to be the underlying mechanism of the ability of osthole to promote osteoblast differentiation, which is indicated by the upregulation of protein and mRNA expression level of autophagy-associated genes, *Beclin1* and *LC3*. We concluded from these experiments that OBMMSCs are more effective than BMMSCs in osteoporosis treatment maybe through upregulation level of autophagy level induced by osthole.

**Key words:** autophagy, mesenchymal stem cells, osteoblast differentiation, osteoporosis, osthole.

## Introduction

Osteoporosis (OP) is a physical status characterized by increased risk of fracture resulting from loss of bone mass and bone strength [1]. It's predictable that incidence of OP will keep increasing among the elderly as life expectancy keeps going up, especially among women [32]. Nowadays, osteoanabolic drugs focusing on stimulating bone formation like parathyroid hormone [34] and antiresorptive reagents paying attention to in-

hibit bone resorption such as zoledronic acid [22], lasofoxifene [8], denosumab [7], and odanacatib [4] are the two major pharmacologic approaches to the therapy of OP. Increase in bone mineral density (BMD) and reduction in fractures are confirmed to happen after taking these medications. Although the symptoms and complications are the main targets of the drug [41] and long-term adverse effects are existed [18, 38]; so, a new treatment aiming at the imbalance of bone formation and resorption is expected to further advance the treatment

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of OP. Mesenchymal stem cells come into our sight for their advantages of multipotent differentiation potential [6] and easy access; therefore, they have been widely used for tissue repair [36]. Bone marrow mesenchymal stem cells (BMMSCs) derived from bone marrow stromal cells has drawn much attention for its ability to serve as a good therapy for skeletal disease [15, 29, 42]. It's well believed that promoting osteoblasts differentiation and inhibiting adipocytes differentiation of BMMSCs can alleviate development of OP [3, 27]. Although BMMSCs have been much explored as a therapeutic agent for osteoporosis [15, 47], how to enhance the osteogenic ability of BMMSCs in vivo is still an issue waiting to be solved.

It's well documented that autophagy plays an important role in bone metabolism [30]. Conditional knockout of autophagy-related gene *Atg7* in osteoblasts results in decrease of trabecular bone mass, decline in numbers of osteoblasts, and reduction of bone formation [31], which figures out that autophagy is involved in maintaining skeletal homeostasis and the mineralization process [26]. Recent studies have also pointed out that autophagy dysfunction is related with OP [9] and autophagy can be a target of the treatment for glucocorticoid-induced osteoporosis treatment [11, 35]. In BMMSCs of estrogen deficiency-induced osteoporotic mice, autophagy level was reduced and ability of osteogenic differentiation was decreased, while rapamycin could rescue osteogenic differentiation of BMMSCs accompanying with elevation of autophagy level and attenuation of osteoporotic phenotype [33].

Osthole is a fundamental component with pharmacological activity extracted from Chinese Medicine *cnidium monnieri* [45]. Osthole has been reported to stimulate osteoblast differentiation and bone formation [17, 25, 39] which suggests that it may be an effective alternative of therapy for OP. In the present study, we investigated the therapeutic effect of BMMSCs pre-stimulated by osthole and demonstrated that osthole indeed promoted OP treatment by BMMSCs. It was further elucidated that autophagy level has been elevated in BMMSCs after osthole stimulation. Through this research, we proposed that osthole may advance autophagy in BMMSCs and promote BMMSCs to differentiate into osteoblasts, which can further improve the treatment for OP.

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## Materials and Methods

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### *Animals*

C57BL/6J mice of eight weeks old were purchased from the animal center of Chongqing Medical University in Chongqing, China. Performances of all animal experiments were under the experimental animal guidelines of the National Institutes of Health and approved by the Animal Care and Use Committee of Chongqing Medical University. All mice were housed under specific pathogen-free conditions (22°C, 12-h light/12-h dark cycles, and 50%–55% humidity) with free access to food pellets and tap water.

### *BMMSCs isolation*

The mouse femurs and tibias were harvested from 8-week-old mice, and then the bone marrow stromal cells were flushed out from the bone marrow cavity with a syringe to culture medium containing  $\alpha$ -MEM (Invitrogen, Calsbad, CA, USA), 20% fetal bovine serum (FBS) (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA), 10 U/ml penicillin (Invitrogen), 10 g/ml streptomycin (Introvigen) and 2 mM L-glutamine (Hyclone, GE Healthcare Life Sciences). The resultant medium was centrifuged at 1,000 revolutions per min (rpm) for 5 min and then the deposits were suspended with basal medium and cultured for 3 days. Next, the floating cells were removed from the suspension and the attached cells were cultured until they reached confluence. Afterwards, the limiting-dilution technique was used to obtain single-cell suspensions of primary cells, and passage 0 (P0) cells were cultured. BMMSCs were acquired after expansion. Cells at passage 3–5 (P3) were put to application in this study.

### *BMMSCs pre-stimulated by osthole*

It was reported before that confluent BMMSCs cultured with medium containing  $10^{-5}$  M osthole for 3 days could acquire their most significant osteogenic capability as ALP activities were promoted and expression of osteogenic genes was increased [10]. So in our study, confluent cells were incubated with basal medium containing  $10^{-5}$  M osthole for 3 days, then the resultant osthole-treated-BMMSCs (OBMMSCs) were analyzed and injected into mice as described below.

### *Flow-cytometric analysis*

*In vitro*-expanded BMMSCs at early passages (P3)

were examined by flow-cytometric analysis to characterize their immunophenotype, and mesenchymal and non-mesenchymal stem cell-associated surface markers were measured. 2 ml of 0.05% trypsin (Sigma-Aldrich, Darmstadt, Germany) was added to liberate adherent cells after being washed twice with PBS. The single-cell suspension was washed with PBS containing 3% FBS twice and re-suspended in PBS containing 3% FBS. With BMMSCs prepared, identification of the BMMSCs phenotype was performed. Antibodies combined with phycoerythrin (PE) or fluorescein isothiocyanate (FITC) against CD90, CD34, CD11b (BioLegend, San Diego, CA, USA), CD73-PE (eBioscience, San Diego, CA, USA), and Sca-1, CD14 [46] (Abcam, Cambridge, UK) were added into each tube loaded with nearly  $5 \times 10^5$  BMMSCs/200  $\mu$ l of PBS, and then the BMMSCs were incubated for 1 h at 4°C in the dark. BMMSCs which were incubated without any antibodies were used as negative control. Whereafter, BMMSCs were washed with 1 ml wash buffer twice and analyzed by a flow cytometer (Beckman Coulter, Fullerton, CA, USA). OBMMSCs had also been examined as described above.

#### *Osteogenic differentiation*

For osteogenic differentiation assay,  $1 \times 10^5$ /well BMMSCs (P3) reaching 80% confluence were incubated in 12-well plates with osteogenic medium containing 5 mM  $\beta$ -glycerophosphate (Sigma-Aldrich), 50  $\mu$ g/ml ascorbic acid (Sigma-Aldrich) and 10 nM dexamethasone (Sigma-Aldrich). The osteogenic medium was refreshed every 3 days. And 21 days later, cells were stained with 2% Alizarin Red (Sigma-Aldrich). The same performance was done to OBMMSCs to confirm their osteogenic differentiation. The stain was eluted with 2% cetylpyridinium chloride (Sigma-Aldrich) for 30 min, and the absorbance was quantitatively measured at 520 nm.

#### *Adipogenic differentiation*

For adipogenic differentiation assay,  $1 \times 10^5$ /well BMMSCs (P3) reaching 100% confluence were incubated in 12-well plates with adipogenic medium,  $\alpha$ -MEM added with 20% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 5  $\mu$ g/ml insulin (Sigma-Aldrich), 50  $\mu$ M indomethacin (Sigma-Aldrich),  $1 \times 10^{-6}$  M dexamethasone, and 0.5  $\mu$ M 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich). The adipogenic medium was refreshed every 2–3 days. And 7 days

later, 4% formaldehyde was applied to fix cells at room temperature (RT) for 30 min and then 0.5% oil red (Sigma-Aldrich) in methanol was added to stain cells for 20 min at RT. The same performance was done to OBMMSCs to confirm their adipogenic differentiation. The stain was eluted with methanol for 30 min, and the absorbance was quantitatively measured at 520 nm.

#### *Cell proliferation assay*

Cell proliferation of BMMSCs and OBMMSCs was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which was carried out every day for the 7-day culture period. BMMSCs and OBMMSCs, which were incubated with basal medium containing with or without osthole for 3 days respectively, were plated at a density of  $2 \times 10^3$  cells/well in 96-well plates, and incubated with 20  $\mu$ l of 5 mg/ml MTT solution (Sigma-Aldrich, 5 mg/ml) was for 4 h. Afterwards, the medium was replaced by formazan salts dissolved in 150  $\mu$ l of dimethylsulfoxide (DMSO, Sigma-Aldrich), and the absorbance was quantitatively measured at 490 nm.

#### *Western blot*

Lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) was employed to lyse harvested cells, and the concentration of total protein isolated from cells was assessed with BCA protein assay reagent (Bio-Rad, Hercules, CA, USA). In SDS-polyacrylamide gels, 30 micrograms of each sample was loaded and then after separation, proteins were transferred to immunoblot PVD membranes (Millipore, Billerica, MA, USA). The membranes were blocked with blocking buffer containing 5% non-fat milk and 2 mg/ml BSA in PBST for 1 h and then were incubated overnight with the primary antibodies against Beclin1 (1:1,000; Abcam), LC3 (1:1,000; Abcam) and  $\beta$ -actin (1:2,000; Abcam). The membranes were incubated with secondary antibody (Boster, Pleasanton, NJ, USA) in blocking solution for 2 h at RT after washing in PBST for 10 min. The membranes were washed again with PBST and then be visualized by an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA) and the results were quantified by ImageJ.

#### *RNA isolation and real-time polymerase chain reaction*

Total RNA was extracted with Trizol (Sigma-Aldrich) reagent according to manufacture's protocol. Then, 1,000

ng mRNA was reverse-transcribed to cDNA using a PrimeScript RT reagent kit (Takara Bio Inc., Kusatsu, Japan). SYBR Premix Ex Taq II kit (Takara) and a CFX96™ Real-time RT-PCR System (Bio-Rad) were used to perform the real-time RT-PCR analysis respectively. The primer sequences for real-time RT-PCR are listed below:

*Beclin1* (F:CAGTACCAGCGGGAGTATAGTGA; R:TGTGGAAGGTGGCATTGAAGA),

*LC3* (F:CCTGTCCTGGATAAGACCAAGTT; R:CTCCTGTTTCATAGATGTCAGCGAT),

*β-actin* (F:CTGGCACCACACCTTCTACA; R:GGTACGACCAGAGGCATACA).

#### *ELISA for serological markers*

To detect biochemical markers of bone turnover, blood samples were acquired from the aorta ventralis upon euthanasia. Serum was isolated and stored at  $-80^{\circ}\text{C}$ . Serum levels of procollagen 1 N-terminal peptide (P1NP), bone alkaline phosphatase (ALP), and tartrate-resistant acid phosphatase (TRAP) were measured using the relevant enzymelinked immunoassay (ELISA) kits (IDS, Frankfurt, Germany). Serum levels of calcium were measured using a Plasma Emission Spectrometer (iCAP 6000; Thermo Fisher Scientific, Waltham, MA, USA).

#### *Induction of OP and study design*

Thirty female C57BL/6J mice (8-weeks-old) were randomly divided into two groups and underwent either sham surgery or bilateral ovariectomy (OVX) under general anesthesia by the dorsal approach. Before injection,  $1 \times 10^6$  BMMSCs or  $1.0 \times 10^6$  OBMMSCs were suspended in 200 ml PBS. Mice in the OVX group were injected intravenously with  $1.0 \times 10^6$  mouse BMMSCs,  $1.0 \times 10^6$  OBMMSCs, or equivalent PBS alone at 8 weeks after surgery. Mice were sacrificed one month later, and all femurs were isolated intactly and scanned by micro CT (Siemens Inveon Micro CT, Munich, Germany).

#### *Micro-computed tomography analysis*

Femurs were scanned with the Inveon micro-CT (Siemens Inveon Micro CT). Setting of X-ray source were 80 kV and 500  $\mu\text{A}$  microfocus. Interested region was selected manually in the marrow cavity. Bone morphometric parameters, including bone mineral density (BMD, mg/cc), bone volume relative to total volume

(BV/TV, %), trabecular number (Tb. N,  $\text{mm}^{-1}$ ), trabecular thickness (Tb. Th, mm) and trabecular spacing (Tb. Sp, mm) were assessed.

#### *Statistical analysis*

Data are exhibited as mean  $\pm$  SD. Comparisons were performed by Student's *t*-test or one-way ANOVA. All analyses were performed using the SPSS statistical package (version 16.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (version 7.05).  $P < 0.05$  was considered statistically significant.

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## Results

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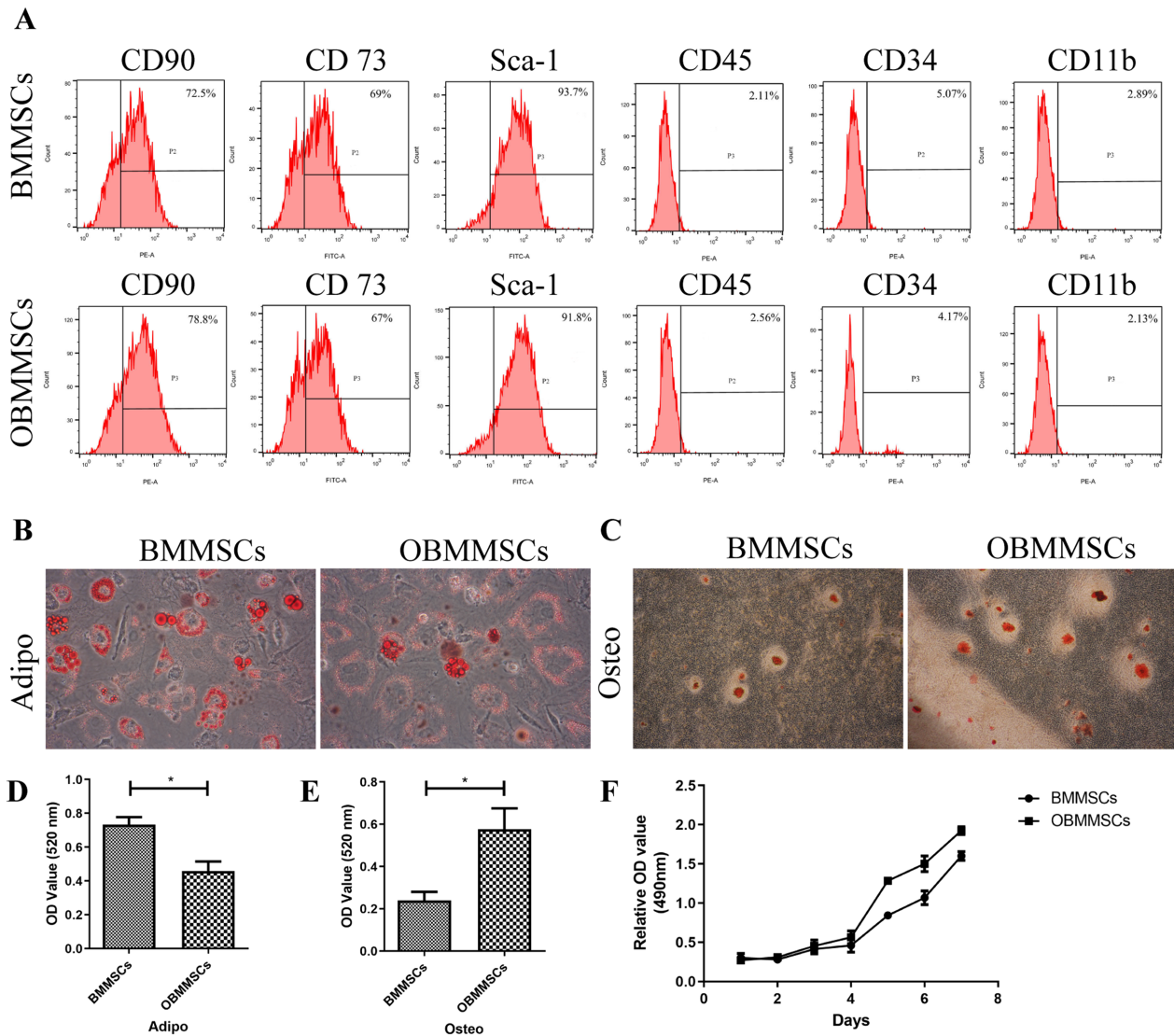
#### *Characterization of BMMSCs and OBMMSCs*

To characterize BMMSCs and OBMMSCs pre-stimulated with osthole for 3 days as documented [10], specific cell surface markers were tested. Results demonstrated that the obtained BMMSCs and osthole-treated-BMMSCs (OBMMSCs) were positive for CD73, CD90, and Sca-1 but negative for the CD45, CD34, and CD11b (Fig. 1A). Besides, BMMSCs and OBMMSCs still maintained their abilities to differentiate into osteoblasts and adipocytes (Figs. 1B and C). Quantitative analysis showed that OBMMSCs decreased adipogenic differentiation (Fig. 1D) and increased osteogenic differentiation (Fig. 1E), compared with BMMSCs. In addition, OBMMSCs proliferation activity was higher than that of BMMSCs after 7 days of incubation, though the proliferation rate was similar in the first 3 days (Fig. 1F).

Together these data revealed that OBMMSCs satisfied BMMSCs criteria and OBMMSCs exhibited a higher potential for osteogenic differentiation and cell proliferation rate than BMMSCs.

#### *Therapeutic outcome of osteoporosis with BMMSCs and OBMMSCs*

To test effects of OBMMSCs on therapy of osteoporosis, estrogen deficiency induced osteoporosis mice model was constructed by ovariectomy (OVX), and sham surgery was simultaneously performed in another group of mice as control. All mice were assessed by micro CT (Fig. 2A), which showed that trabecular bone value/total value (BV/TV) (Fig. 2C), bone mineral density (BMD) (Fig. 2D), trabecular thickness (Tb. Th) (Fig. 2E) and trabecular number (Tb. N) (Fig. 2F) were decreased, while trabecular spacing (Tb. Sp) (Fig. 2B) was signifi-



**Fig. 1.** Characterization of bone marrow mesenchymal stem cells (BMMSCs) and osthole-treated-BMMSCs (OBMMSCs). (A) The analysis of surface antigens of BMMSCs and OBMMSCs by flow cytometry. Adipogenic (B, D) and osteogenic (C, E) differentiation of BMMSCs and OBMMSCs were determined by Oil Red O staining and Alizarin Red S staining respectively. (F) Proliferation activity of BMMSCs and OBMMSCs. Data were analyzed by Student's *t*-test. Results are presented as means  $\pm$  SD, \**P*<0.05.

cantly increased in the OVX model compared with the sham group.

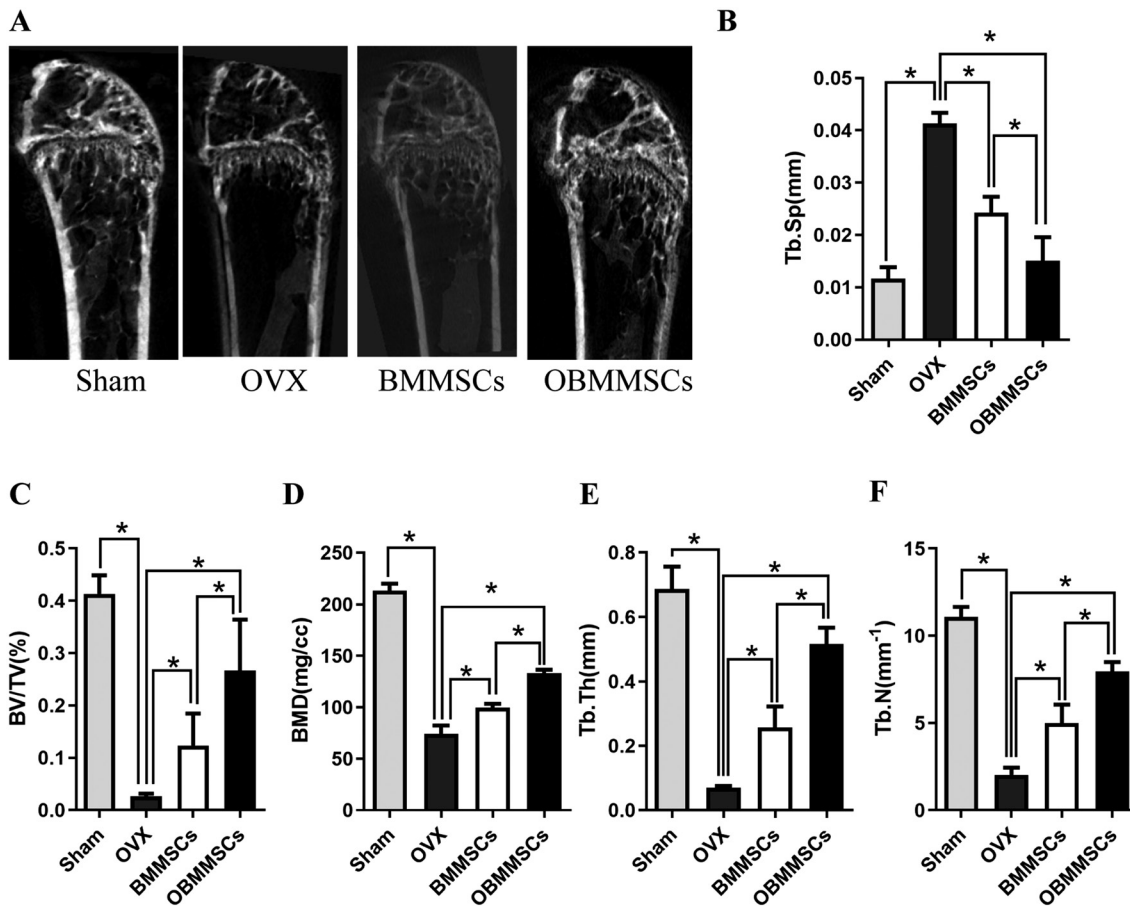
With the OVX model well established, we intravenously injected OBMMSCs or BMMSCs into the OVX mice 8 weeks after surgical operation as previously described. The femur bone mass was analyzed by micro CT one month later. Newly formed bone tissues were observed, and it was markedly more in OBMMSC-treated mice than in BMMSCs-treated mice (Fig. 2A). In addition, the distal femoral trabecular BV/TV, BMD, Tb. Th, Tb. N of OBMMSCs-treated mice also showed

remarkably higher than those of BMMSCs-treated mice (Figs. 2C–F), whereas, Tb. Sp was lower in OBMMSCs-treated mice than that in BMMSCs-treated mice (Fig. 2B).

Results mentioned above consistently indicate that OBMMSCs are more effective in the prevention of osteoporosis than BMMSCs.

#### *Osteoblastic and osteoclastic activity of BMMSCs and OBMMSCs*

Knowing OBMMSCs can attenuate development of



**Fig. 2.** Therapeutic outcome of osteoporosis with bone marrow mesenchymal stem cells (BMMSCs) and osthole-treated-BMMSCs (OBMMSCs). (A) Micro-CT analysis of trabecular bone mass in the femora of mice in each group one month later after injection. Quantitative analysis was performed including (B) trabecular spacing (Tb. Sp), (C) trabecular bone volume (BV/TV), (D) bone mineral density (BMD), (E) trabecular thickness (Tb. Th), and trabecular number (Tb. N). Data were analyzed by one-way ANOVA. Results are presented as means  $\pm$  SD, \* $P$ <0.05.

osteoporosis, we were interested in the problem that how OBMMSCs affect bone formation and resorption. So serological assessment was carried out in sham group, OVX group (PBS injected), OBMMSCs group, and BMMSCs group one month after injection. ELISA of osteogenic markers, P1NP and ALP, was performed in the OVX and sham group first. Serum levels of P1NP and ALP in the OVX group were considerably lower than those in the sham group (Figs. 3A and B). In addition, serum level of calcium in the OVX group was also decreased than that in the sham group (Fig. 3D). Whereafter, ELISA of osteoclastic marker, TRAP, was also conducted. Serum level of TRAP in the OVX group was significantly higher than in the sham group as expected (Fig. 3C).

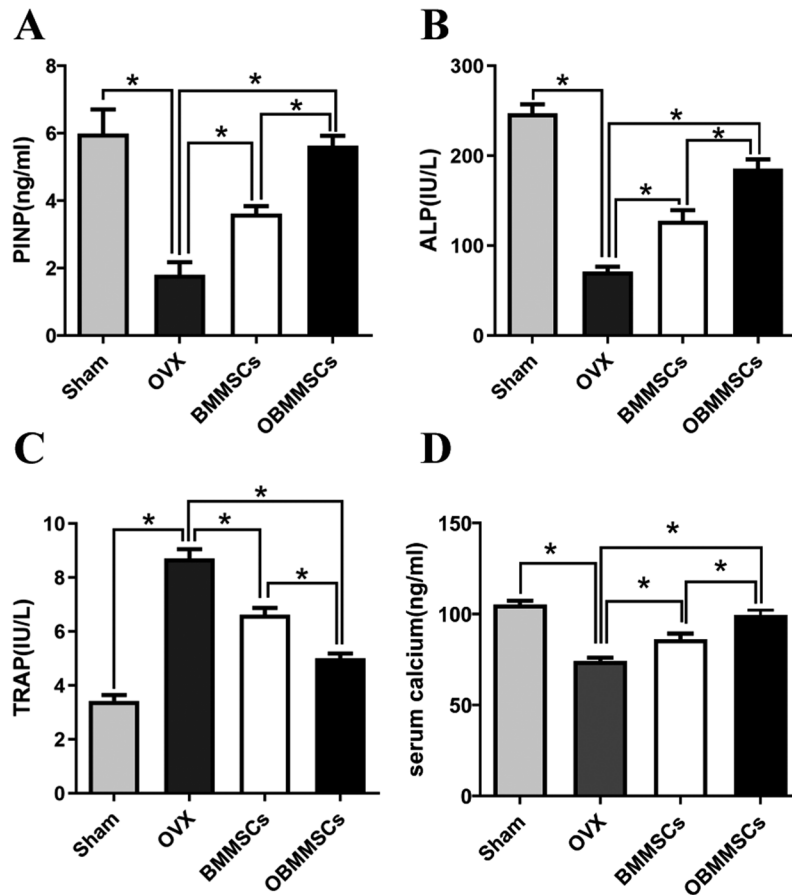
Serum levels of P1NP and ALP were increased in

OBMMSCs and BMMSCs group when compared with those in the OVX group (Fig. 3A). Importantly, serum levels of P1NP and ALP in the OBMMSCs group were dramatically higher than those in the BMMSCs group. Serum level of calcium was also elevated after OBMMSCs and BMMSCs treatments, and OBMMSCs improved calcium level more than BMMSCs. On the contrary, serum level of TRAP was reduced in the OBMMSCs and the BMMSCs group, and its level in the OBMMSCs group was much lower than in the BMMSCs group (Fig. 3C).

We concluded from those data that osthole had a great impact on osteoblastic and osteoclastic activities.

#### *Osthole promotes autophagy in BMMSCs*

Based on previous reports that osteogenic differentia-



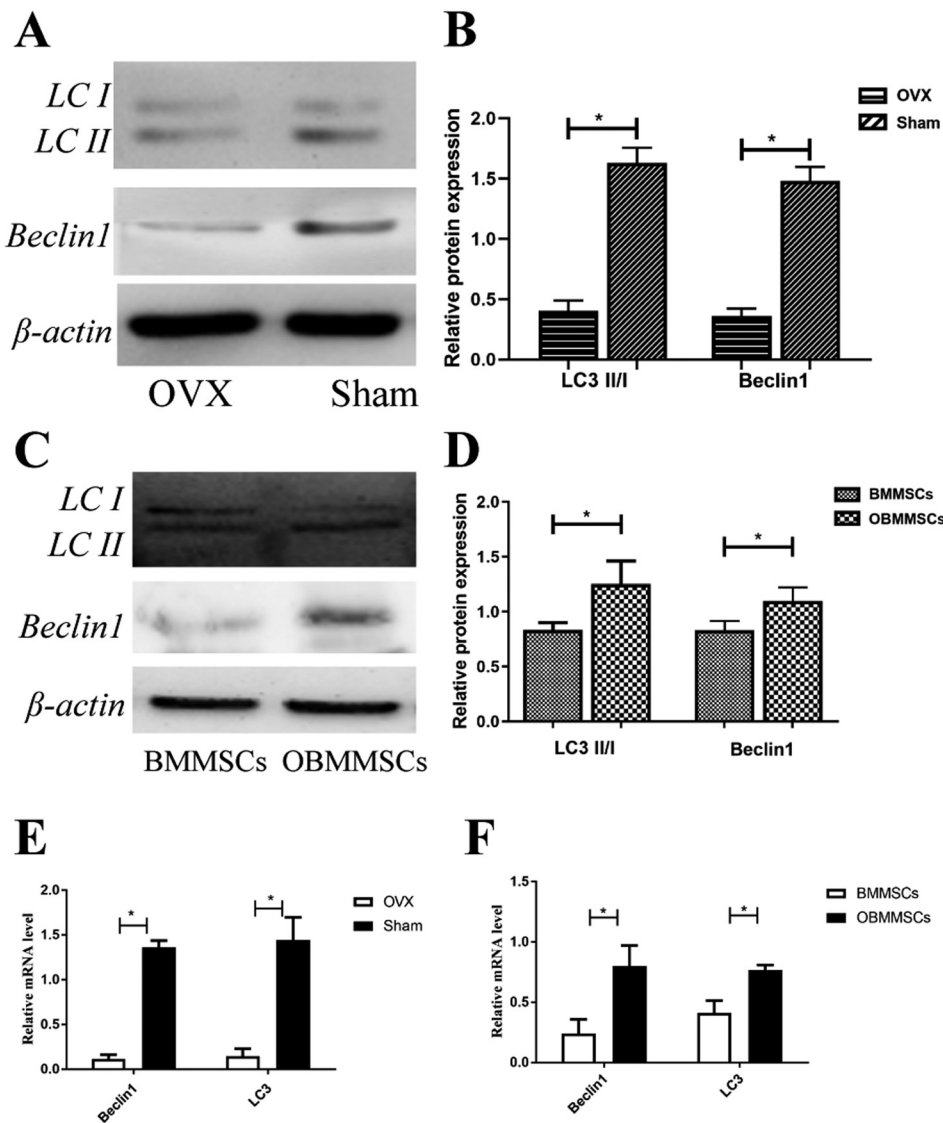
**Fig. 3.** Osteoblastic and osteoclastic activity of bone marrow mesenchymal stem cells (BMMSCs) and osthole-treated-BMMSCs (OBMMSCs). Serum procollagen 1 N-terminal peptide (PINP) (A), bone alkaline phosphatase (ALP) (B), and tartrate-resistant acid phosphatase (TRAP) (C), calcium (D) concentrations in mice from each group. Data were analyzed by one-way ANOVA. Results are presented as means  $\pm$  SD, \* $P$ <0.05.

tion of BMMSCs in OVX mice can be rescued through elevating autophagy level [33], we were wondering whether there was relationship between autophagy and osthole-prestimulation. So we examined expression levels of autophagy-associated genes, *LC3* and *Beclin1*. First of all, both protein and mRNA expressions of *LC3* and *Beclin1* of BMMSCs isolated from the OVX mice were significantly reduced compared with those of the Sham mice, indicating that autophagy level declined in the OVX mice (Figs. 4A, B and E). Further study testified that osthole treatment resulted in upregulation of *LC3* and *Beclin1* protein and mRNA production in BMMSCs (Figs. 4C, D and F).

From the above data, we suggest that osthole promotes autophagy in BMMSCs, leading to a better treatment effect for OP.

## Discussion

Osteoporosis is a common metabolic skeletal disease which makes the trabecular bone be prone to fracture and leads to high incidence of fractures, mortality and morbidity, which seriously influences the quality of aging individuals [41, 44]. It's a worldwide concern waiting for more safe and effective therapeutic methods. BMMSCs are an option embracing cell supply and gene therapy. Gene modified stem cell transplantation has been studied to enhance the osteogenic differentiation ability of BMMSCs and offers broad prospects for osteoporosis treatment [2, 16, 20, 22]. However, growth factors added to culture medium cannot display its complete function for its short half-lives. As a consequence, its effects on promoting differentiation and proliferation



**Fig. 4.** Autophagy level in bone marrow mesenchymal stem cells (BMMSCs) and osthole-treated-BMMSCs (OBMMSCs). (A, B) Western blot was performed to examine expression of Beclin1 and LC3 in the ovariectomy (OVX) and Sham group at protein level. (E) Real-time PCR was performed to detect the mRNA expression of *Beclin1* and *LC3*. (C, D, F) Expression of Beclin1 and LC3 in BMMSCs and OBMMSCs at protein and mRNA level was also observed. Data were analyzed by Student's *t*-test. Results are presented as means  $\pm$  SD, \* $P$ <0.05.

couldn't come up to our expectations. And in recent several decades, many Chinese herbal medicines attract much interest for their regulation in stem cell differentiation and function in tissue regeneration [43]. It has been well established that application of the Chinese Medicine (CM) facilitates osteoblast differentiation which benefits in therapy for bone fracture. These facts point out that CM can be important alternatives of growth factors [21].

Osthole is a coumarin derivative possessing efficiency

of anti-inflammatory, anti-apoptosis, anti-antitumor, anti-bacterial, anti-allergic, anti-osteoporotic [5, 24, 25, 28, 52]. It also plays protective roles in cardiovascular system, reproductive system and skeletal diseases [25, 48]. It's approved that osthole exhibits estrogen-like effects, promoting osteoblast proliferation and differentiation [17]. Osthole has been demonstrated to have relationship with several signaling pathways involved in osteoblast differentiation. BMP-2/p38 signaling pathway has been proved to regulate the early differentiation stage



while ERK1/2 plays a role in late differentiation stage of osteoblast differentiation mediated by osthole [17, 25]. Wnt/ $\beta$ -catenin-BMP pathway has also been suggested to take part in promotion of osteoblast differentiation resulted from osthole treatment [39]. And importantly, osthole promotes osteoblast differentiation in a strictly dose-dependent manner, as Hu *et al.* [14] reported that osthole suppressed osteogenic differentiation of MSCs with concentrations 6.25, 12.5, and 25  $\mu$ M. In contrast,  $10^{-5}$  M osthole is widely reported to enhance osteoblast differentiation [10, 17, 25, 49]. Additionally, the tissue where MSCs derived from can also influence the role of osthole in osteoblast differentiation [10, 14]. So in our study, we combined BMMSCs and  $10^{-5}$  M osthole to treat OP and the results indicate that BMMSCs pre-stimulated by osthole can produce a better amelioration of development of OP.

Autophagy is a cellular degradation tool for large molecules and also involves in the removal of dysfunctional organelles to maintain cellular metabolism [13]. It's reported that upregulation of autophagy can extend the lifespan of aged mice and restore the self-renewal capacity of stem cells [12, 37]. Autophagy level is reduced in aged BMMSCs according to mainstream view [23, 51], and it's reported that autophagy activation can repair degenerative properties of aged BMMSCs [23]. We take an observation on the correlation of autophagy and osthole-mediated osteoblast differentiation. Autophagy level in the OVX mice was lower than that in the Sham mice, while the level in OBMMSCs is higher than that in BMMSCs. We speculate that OBMMSCs have a better therapeutic effect than BMMSCs due to the elevated autophagy level induced by osthole. In vitro study proposed that upregulated autophagy could protect osteoblast cells from tumor necrosis- $\alpha$ -induced [50] and oxidative stress-mediated apoptosis [19]. Induction of osteoblast autophagic activity by  $\beta$ -ecdysterone inactivating mTOR is reported to be the mechanism for treating glucocorticoid-induced osteoporosis [40]. So in our study, the exact molecular targets for osthole to induce autophagy activity and the mechanisms for osthole-induced autophagy to promote osteoblast differentiation of BMMSCs still needs and is worthy of further investigations. Autophagy level of BMMSCs from OVX mice, which are either treated by BMMSCs stimulated or not stimulated by osthole, is needed to be explored to strengthen our conclusion. In addition, experiments on proliferation and apoptosis of osteoblasts could give

further understandings of the mechanisms on the improvement of OP by osthole treatment.

In summary, the therapy combining BMMSCs and osthole remarkably ameliorated the condition of osteoporosis due to low estrogen level. And autophagy is associated with promotion of osteoblast differentiation and osthole application.

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### Conflicts of Interest

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The authors declare no conflict of interest.

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