Original Paper

An Inhibitory Role of Osthole in Rat MSCs Osteogenic Differentiation and Proliferation via Wnt/ β -Catenin and Erk1/2-MAPK Pathways

Hongyang Hu^{a,b} Min Chen^c Guangzu Dai^b Guoqing Du^b Xuezhong Wang^b
Jie He^b Yongfang Zhao^b Dapeng Han^a Yuelong Cao^{a,b} Yuxin Zheng^{a,b}
Daofang Ding^{a,b}

^aInstitute of Arthritis Research, Shanghai Academy of Chinese Medical Sciences, Guanghua Integrative Medicine Hospital, Shanghai University of T.C.M, Shanghai, ^bShi's Center of Orthopedics and Traumatology, Shuguang Hospital Affiliated to Shanghai University of TCM, Shanghai, ^cReproductive medicine center, Shuguang Hospital Affiliated to Shanghai University of TCM, Shanghai, China

Key Words

Osthole • Mesenchymal stem cells • Proliferation • Osteogenic differentiation • Signaling pathway

Abstract

Background/Aims: Bone marrow-derived mesenchymal stem cells (MSCs) are responsible for new bone formation during adulthood. Accumulating evidences showed that Osthole promotes the osteogenic differentiation in primary osteoblasts. The aim of this study was to investigate whether Osthole exhibits a potential to stimulate the osteogenic differentiation of MSCs and the underlying mechanism. **Methods:** MSCs were treated with a gradient concentration of Osthole (6.25 μ M, 12.5 μ M, and 25 μ M). Cell proliferation was assessed by western blotting with the proliferating cell nuclear antigen (PCNA) and Cyclin D1 antibodies, fluorescence activated cell sorting (FACS), and cell counting kit 8 (CCK8). MSCs were cultured in osteogenesis-induced medium for one or two weeks. The osteogenic differentiation of MSCs was estimated by Alkaline Phosphatase (ALP) staining, Alizarin red staining, Calcium influx, and quantitative PCR (qPCR). The underlying mechanism of Osthole-induced osteogenesis was further evaluated by western blotting with antibodies in Wnt/ β -catenin, PI3K/Akt, BMPs/smad1/5/8, and MAPK signaling pathways. **Results:** Osthole inhibited proliferation of rat MSCs in a dose-dependent manner. Osthole suppressed osteogenic differentiation of rat MSCs by down-regulating the activities of Wnt/ β -catenin and Erk1/2-MAPK signaling. **Conclusions:** Osthole inhibits the proliferation and osteogenic differentiation of rat MSCs, which might be mediated through blocking the Wnt/ β -catenin and Erk1/2-MAPK signaling pathways.

© 2016 The Author(s)
Published by S. Karger AG, Basel

H. Hu, M. Chen and G. Dai contributed equally to this work.

Yuxin Zheng and Daofang Ding

Institute of Arthritis Research, Shanghai Academy of Chinese Medical Sciences,
Guanghua Integrative Medicine Hospital, Shanghai University of T.C.M,
Shanghai 200052 (China)
E-Mail sg_zyx1728@126.com / 051101049@fudan.edu.cn

Introduction

As the World Health Organization (WHO) experts defined, osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [1].

Mesenchymal stem cells (MSCs) originating from bone marrow are described as a population of progenitor cells capable of osteogenic, adipogenic and chondrogenic differentiation [2, 3]. Clinical relevance of MSCs has been highlighted by their capacity for *in vivo* differentiation and engraftment, and eventually by their efficacy in promoting wound healing and tissue regeneration [4-6]. A detailed study of osteogenic differentiation in 2015 indicated that both systemic and local injection of allogeneic MSCs promotes fracture healing by optimizing biomechanical properties, bone content, and callus sizes [7]. Ovariectomized (OVX) mice exhibited delayed osteoporotic fracture healing because of the lower bone marrow and peripheral blood MSCs after fracture [8].

Osthole, 7-methoxy-8-(3-methyl-2-butenyl) coumarin, is a coumarin derivative widely used in Traditional Chinese Medicine (TCM) [9]. It had been widely reported that Osthole exerts a significant positive effect on osteoporosis treatment by promoting osteoblast differentiation and inhibiting bone resorption [10-14]. Interestingly, a recent study on biomaterial suggested that Osthole stimulation may enhance extracellular matrix (ECM) production and positively affect cell behavior in cell sheet engineering of jaw bone marrow mesenchymal stem cells [15]. However, further studies investigating the impact of Osthole on the function of bone marrow-derived MSCs and the underlying mechanism remain quite limited.

Therefore, the purpose of the present study was to evaluate how Osthole affects the proliferation and osteogenic differentiation of rat bone marrow-derived MSCs. To the best of our knowledge, this investigation is the first to systematically elucidate the effect of Osthole on rat bone marrow-derived MSCs at the cellular and molecular levels.

Materials and Methods

Cell lines and culture

Rat primary MSCs were bought from Cyagen Company (Cyagen Biosciences Inc, Guangzhou, China), Basal medium for MSCs was supplemented with fetal bovine serum (FBS, RAWMX-05001-50, Cyagen) and penicillin-streptomycin (T150731G001, Cyagen). All media were changed every other day.

Osteogenic differentiation

Rat MSCs were seeded at 5×10^5 cells/well in 6-well plates in the maintenance medium. The medium was changed to osteogenic differentiation medium (RAWMX03021-175, Cyagen) the next day, which was supplemented with 10% FBS (RAWMX 05001-20, Cyagen), 10 mM β -glycerophosphate (T151112G001, Cyagen), 50 μ g/ml of L-ascorbic acid (T151013G001, Cyagen), L-glutamine (T151021g001, Cyagen), and 10^{-7} M dexamethasone (T151012G001, Cyagen). Rat MSCs were induced for one or two weeks for different purposes, and the medium was changed every other day.

Osthole-removal experiment. 12.5 μ M Osthole was removed from the medium after 7 days of induction, and maintained in osteogenic differentiation medium for another 14 days (Osthole-R group). MSCs were induced in osteogenic medium alone for 21 days (control group) or supplemented with 12.5 μ M Osthole for 21 days (Osthole group).

Cell density experiment. MSCs were seeded at the density of 5×10^4 /cm² (low, 70%-80% confluence), and induced in osteogenic differentiation medium alone or supplemented with 12.5 μ M Osthole (control-L group or Osthole-L group). MSCs were seeded at the density of 7.5×10^4 /cm² (high, 100% confluence), and induced in osteogenic differentiation medium alone or supplemented with 12.5 μ M Osthole (control-H group or Osthole-H group).

Western blotting

MSCs were treated with Osthole for 24 hours and lysed with lysis buffer (Beyotime, P0013B) containing PMSF. Lysates were centrifuged at 12,000 g at 4°C for 10 min. Protein concentrations were determined using BCA Protein Assay Kit (Cat.No 23227, Pierce, USA). Proteins were separated by 10% SDS-PAGE and transferred to PVDF membrane using a standard protocol. The membrane was incubated with primary antibodies and probed with the respective secondary antibodies. The membrane was exposed and scanned using Odyssey® Infrared Imaging system. The gray values of the resulting protein bands were quantified by using Image J software. The following antibodies were used: β -catenin (#9562, CST), TCF4 (#2569, CST), LEF1 (#2230, CST), GAPDH (#2118S, CST), PCNA (SC-25280, SantaCruz), CyclinD1 (#2978, CST). MSCs were treated with Osthole for 1 hour, 2 hours and 3 hours and lysed with Nuclear and Cytoplasmic Protein Extraction reagent (Beyotime, P0027). The cytoplasmic proteins were collected for detecting p-Erk1/2 (#9910, CST), Erk1/2 (#9102, CST), p-P38MARK (#9910, CST), P38MAPK (#9212, CST), Akt (#4691, CST), P-Akt-Ser473 (#4060, CST), P-Akt-Thr308 (#2965, CST), and P-Smad1/5/8 (#9511S, CST). The nuclear proteins were used to analyze Phospho- β -Catenin (#9561, CST).

Alkaline Phosphatase (ALP) and Alizarin red staining

Cells were fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 2 min, washed in PBS three times, incubated with 1-Step™ NBT/BCIP (Cat. No 34042, Pierce, USA) solution for 30 min at RT, and then rinsed with water. To detect mineralized nodules, cells were fixed in 95% ethanol and then stained with Alizarin red S (Cat. No A5533, Sigma-Aldrich, Switzerland) overnight.

ALP activity

Cells were induced for one week, and the Protein concentrations were determined using BCA Protein Assay Kit (Cat.No 23227, Pierce, USA). 20 μ g of total proteins were mixed with 100 μ L 1-step™ PNPP (Cat. No 37621, Pierce, USA), and then incubated at RT for 30 min. The reaction was stopped by addition of 50 μ L of 2M NaOH. As a result, yellow p-nitrophenol (pNP) compound is formed, which is measured by a maximum absorbance at 405 nm.

Cell cycle distribution

To assess the effect of Osthole on cell cycle distribution, flow cytometric analysis of nuclear DNA content was performed by staining with propidium iodide (P4170, Sigma). In brief, Rat MSCs were seeded in a 6cm dish and then allowed to attach overnight. The medium was replaced with osteogenic medium containing desired concentrations of Osthole or dimethyl sulfoxide (DMSO, control). Cells were incubated for 24 hours at 37°C, and harvested and washed with 1×PBS three times, and then fixed by 70% ethanol overnight at 4°C. The cells were then treated with 100 μ g/ml RNaseA and 100 μ g/ml propidium iodide for 30 min at 37°C. Stained cells were transferred to FACS tubes and detected using a flow cytometry (Beckman Coulter).

Cell Counting Kit (CCK-8) assay

Cell proliferation was measured using CCK-8 assay (Dojindo, Japan). Rat MSCs were plated at the density of 3000 cells/well in 96-well plates with osteogenic differentiation medium in the presence of gradient concentrations of Osthole (6.25 μ M, 12.5 μ M, and 25 μ M) for 24, 48, and 72 hours. After treatment, 10 μ L CCK-8 solution was added to each well and cells were incubated for 0.5 h at 37°C. The optical density of each well was measured using a microplate reader (SYNERGY4, USA) at a wavelength of 450 nm. At least three independent experiments were performed.

Intracellular calcium measurements

Cells were washed three times with HBSS solution (130 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 10 mM glucose), and then loaded with 1 μ M Ca^{2+} -sensitive dye fluo4-AM molecular Probes (Dojindo, Japan). Subsequently, the cells were incubated with probe for 60 min and then washed three times with HBSS for probe removal. Cells were investigated under inverted fluorescence microscope or measured with microplate Reader (excitation wavelength at 485 nm and emission wavelength at 528 nm). The percentages of absorbance values relative to control group were calculated according to this formula: $(\text{ControlOD}_{485, 528} - \text{experimental group OD}_{485, 528}) / \text{ControlOD}_{485, 528} \times 100\%$.

Table 1. Primer sequences for Real-time quantitative PCR. ALP= Alkaline phosphatase; ---OCN= Osteocalcin; RUNX2= runt-related transcription factor 2; Osterix= Sp7 transcription factor; OPN= osteopontin; OPG= osteoprotegerin; Col1a1= type I collagen, alpha 1 chain; Col1a2= type I collagen, alpha 2 chain; GAPDH= glyceraldehyde-3-phosphate dehydrogenase

Gene name	GeneBank accession no.	mRNA sequences (5'-3')
ALP-F	NM_013059.1	GCACAACATCAAGGACATCG
ALP-R		TCAGTTCTGTTCTTGGGGTACAT
OCN-F	M25490.1	CCCAATTGTGACGAGCTAGC
OCN-R		CTGTGCCGTCCATACTTTCG
Runx2-F	NM_001278483.1	AAGGAGCACAAACATGGCTG
Runx2-R		TCTTAGGGTCTCGGAGGGAA
Col1a1-F	NM_053304.1	CATGTTTCAGCTTTGTGGACCT
Col1a1-R		GCAGCTGACTTCAGGGATGT
Col1a2-F	NM_053356.1	CCTGGCTCTCGAGGTGAAC
Col1a2-R		CAATGCCAGAGGACCAG
Osterix-F	AY177399.1	CAATTGGTTAGGTGGTGGGC
Osterix-R		TCTTGGGGTAGGACATGCTG
OPN-F	M14656.1	CGGTGAAAGTGGCTGAGTTT
OPN-R		GGCTACAGCATCTGAGTGTG
OPG-F	U94330.1	TGAGGTTTCCAGAGGACCAC
OPG-R		GGAAAGGTTTCTGGGTTGT
GAPDH-F	NM_017008.4	TTCAACGGCACAGTCAAGG
GAPDH-R		CTCAGCACCAGCATCACC

Real-time quantitative PCR (qPCR)

After 7 days of culture, total RNAs were isolated with Trizol reagent (Cat. No 15596-026, Invitrogen). The first strand cDNA was synthesized with Reverse Transcription Reagent Kit (takara code DRR037A). qPCR was performed with SYBR® Premix Ex Taq™ (Cat.# RR420R). Delta-delta Ct method was used to analyze the data. Primer sequences are listed in Table 1. All the experiments were repeated independently three times.

Statistical analysis

The data are expressed as mean $\pm$ SD. Statistical correlation of data was checked for significance by One-Way ANOVA and Post Hoc Turkey HSD for multiple comparisons. Differences with $P < 0.05$ were considered significant. These analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

The effects of Osthole on the ALP activity in rat MSCs

Osteoblastic induction of rat MSCs was maintained for one or two weeks. ALP staining showed that the signal in Osthole-treated MSCs was significantly hindered compared with the negative control at day 7 and day 14 respectively (Fig. 1A, 1B). The ALP activities were further investigated, showing that the activities were also decreased in MSCs cultured with Osthole compared with the negative control and Osthole works here in a dose-dependent manner (Fig. 1C).

The influences of Osthole on the mineralized nodule formation and calcium influx in rat MSCs

To investigate the effects of Osthole on the mineralization potential of MSCs, calcified nodule formation was evaluated by Alizarin Red-S staining at day 21 after osteogenic culture. We observed that calcified nodules in Osthole groups appeared small in size compared

Fig. 1. Comparisons of ALP staining and activities between Osthole-treated group and control group. (A) Rat MSCs were stained for ALP levels after being induced with osteogenic differentiation medium for 7 days and 14 days respectively. (B) The details of ALP staining at day 7 and day 14. (C) the ALP activities were detected after cell induction for 7 days and 14 days. Data was expressed as mean $\pm$ SD from three separate experiments. * $p < 0.05$ versus control.

Fig. 2. Osthole affects the formation of calcified nodules and the intracellular calcium levels. (A) Mineralized nodules were stained after rat MSC induction with osteogenic differentiation medium for 21 days (upper lane), the details of the mineralized nodules (lower lane). (B) Fluorescent staining of MSCs by Fluo4-AM probe after treatment with differentiation medium for 6 hours. (C) The ratios of absorbance values in between Osthole groups and control group. Data was expressed as mean $\pm$ SD from three separate experiments. * $p < 0.05$ versus control.

with the control, and such effect was concentration dependent (Fig. 2A). The increase of intercellular calcium depends upon two different mechanisms [16]. Furthermore, a rise in

Fig. 3. Osteoblastic gene expression profiles in rat MSCs after treatment of Osthole. Rat MSCs were maintained in Osteoblast differentiation medium containing different concentrations of Osthole for one week. qPCR analysis of osteogenic gene expression in MSCs was performed, relative to internal control GAPDH and normalized to control cells; Data was expressed as mean $\pm$ SD from three separate experiments. * $p < 0.05$ versus control.

the intracellular Ca^{2+} levels caused by extracellular Ca^{2+} concentration serves as a signal in regulating the differentiation [17]. The cells were incubated with the induction medium for six hours, and the intracellular calcium was visualized by Fluo4-AM probe. We observed that the fluorescence intensity was decreased after stimulation with osteogenic medium containing increased concentrations of Osthole, confirming the negative stimulatory effect of Osthole on the mineralization (Fig. 2B, 2C).

Osthole treatment resulted in a dramatic reduction in osteoblastic gene expression

An increase in ALP activity and the mineralized nodule formation are indispensable to osteogenic differentiation. To further study the effect of Osthole on osteoblastic differentiation of rat MSCs, we analyzed the expression of osteoblastic genes by quantitative PCR. Our results revealed that Osthole treatment resulted in a dramatic reduction in the expression of osteoblastic genes including Col1a1, Col1a2, OCN, ALP, Runx2, Osterix, OPN, and OPG (Fig. 3), further suggesting a significant inhibitory role of Osthole in osteoblastic differentiation of MSCs.

Osthole treatment affected the osteogenesis-associated signaling pathways

To further understand the underlying mechanism of the inhibitory role of Osthole in osteoblastic differentiation, we screened the osteogenesis-associated signaling pathways: Wnt/ β -catenin, MAPK pathway, PI3K/Akt and BMPs/Smad1/5/8. The phosphorylation levels of these pathway downstream proteins were analyzed by western blot after Osthole treatment for 1 hour, 2 hours and 3 hours. Osthole treatment down-regulated the expressions of β -catenin, TCF4, and LEF, with a significant reduction in p-ERK1/2 expression (Fig. 4A, 4C). However, Osthole promoted the expression of P- β -catenin (Fig. 4B). In addition, Osthole treatment did not obviously influence the expression levels of p-Smad1/5/8, p-AKT(T308), p-AKT(S473), and p-P38MAPK (Fig. 4B, 4D).

Fig. 4. The influences of Osthole on the activities of Wnt/β-catenin, MAPK, PI3K-Akt and BMP/Smad1/5/8 signaling pathways. Rat MSCs were treated with different concentrations of Osthole (6.25 μM, 12.5 μM, and 25 μM) for 24 hours or 25 μM Osthole for 1 hour, 2 hours, and 3 hours. (A) The downstream molecules of Wnt/β-catenin including β-catenin, TCF4 and LEF1 were assessed. (B) The phosphorylation levels of β-catenin and smad1/5/8, which represented the activities of Wnts/β-catenin and BMPs/Smad1/5/8 pathways respectively, were analyzed. (C) The phosphorylation levels of the MAPK target proteins ERK1/2 and p38MAPK were detected at different time points. (D) The expression level of p-Akt (S473) and p-Akt (T308), which regulate the activities of PI3K-Akt signaling, were also analyzed after Osthole treatment. (E) Quantifications of P-β-catenin, P-Erk1/2, β-catenin, TCF4 and LEF1 protein were normalized to GAPDH using software Image J. Data was expressed as mean ±SD from three separate experiments. *p < 0.05 versus control. The Y axis indicates the values normalized to the control value.

The effect of Osthole on the proliferation of rat MSCs

It is well known that Osthole exhibits anti-cancer property partly by inhibiting the proliferation of tumor cells [18-20]. It was reported that Osthole did not display any cell toxicity in normal osteoblasts even at the concentration of 100 μM Osthole [12]. The effect of Osthole on rat MSCs is still unclear. To address this question, cell cycle profiles were analyzed by fluorescence-activated cell sorting (FACS) after treatment with different concentrations of Osthole. As shown in Fig. 5A, Osthole treatment resulted in an accumulation of cells at G0/G1 phase and a corresponding decrease at S phase, suggesting an anti-proliferative role of Osthole in rat MSCs. This was confirmed by a reduction in expression of proteins PCNA and CyclinD1 after Osthole treatment (Fig. 5B). Treatment of rat MSCs with Osthole from 24 to 72 hours further confirmed the inhibitory effect of Osthole on MSC proliferation (Fig. 5C).

The influence of Osthole removal on osteoblast differentiation

Osthole exhibited the inhibitory effect on osteoblast differentiation of rat MSCs. We further analyzed whether MSCs can recover the differentiation potential after Osthole removal from the induction medium. The results showed that the ALP activities in Osthole-R group or Osthole group were much lower than those in control group. However, no significant difference could be seen in between Osthole-R group and Osthole group (Fig. 6A). Mineralized nodule formation was inhibited evidently in Osthole groups (Fig. 6B), further confirmed by a reduction in expression of osteogenic genes after Osthole treatment. In addition to Runx2,

Fig. 5. The effect of Osthole on rat MSC proliferation evaluated by cell cycle, protein expression, and CCK-8 method. (A) Cells were incubated with Osthole for 24 hours, and cell cycles were analyzed by FACS. (B) The expression levels of PCNA and CyclinD1 were investigated by Western blot after treatment with Osthole for 24 hours. (C) MSCs were treated with gradient concentrations of Osthole for 24 hours, 48 hours, and 72 hours, the optical density of four groups at 450 nm were collected and analyzed. Data was expressed as mean $\pm$ SD from three separate experiments. * $p < 0.05$ versus control.

Osterix and OPN, there were no significant differences in the expression levels of those genes between Osthole-R group and Osthole group (Fig. 6C).

The effects of different MSC density on osteoblast differentiation in the presence of Osthole

Osthole effectively inhibited MSC proliferation, which may contribute to osteoblast differentiation. To reduce the influence of Osthole on cell proliferation, MSCs were seeded at low density and high density, respectively. We observed that the ALP activities in Osthole-L group (or Osthole-H group) were reduced compared to those in control-L group (or control-H group) respectively. Interestingly, we also noted the significant differences in ALP activities between control-L group and control-H group, whereas there was no statistical difference in ALP activities between Osthole-L group and Osthole-H group (Fig. 6A). MSCs were induced for 7 days, the condensation of MSCs more evident in control groups than in Osthole groups at different densities respectively (Fig. 7B, upper lane). The results of mineralized nodules staining were consistent with that of ALP activities (Fig. 7B, lower lane). Furthermore, the osteogenic expressions were investigated among these groups. The expression levels of these genes were down-regulated by Osthole compared to that in control cells, with a significant difference in mRNA expression between control-L group and control-H group.

Discussion

New bone formation involves a complex series of events, including the proliferation and differentiation of MSCs, and the terminal formation of mineralized ECM. ALP is highly

Fig. 6. The osteoblast differentiation of MSCs induced by Osthole at different time periods. Osthole was removed after 7 days of induction and cells were cultured in differentiation medium for another 14 days (Osthole-R group). MSCs were induced for 21 days (control group) or induced with 12.5 μ M Osthole for 21 days (Osthole group). (A) ALP activities were detected after 14 days of cell induction. (B) The mineralized nodules were stained after 21 days of cell induction (40 $\times$). (C) qPCR analysis of Osteogenic expression of MSCs after 14 days of cell induction, relative to internal control GAPDH and normalized to control cells; Data was expressed as mean $\pm$ SD from three separate experiments. * p < 0.05 versus control, # p < 0.05 versus Osthole-R.

expressed at early stage (day 7) of osteogenic differentiation to promote cell maturation and calcification, which is therefore considered as an important indicator for early osteogenesis [21-23]. In this study, Osthole significantly suppressed the rat MSCs ALP activities on day 7 and day 14 after cocultured in osteogenic medium, confirming its primary contribution to the early stages of osteogenic differentiation. Mineralized nodule is typically observed as a late phase marker for terminal differentiation. MSCs can be stained for mineral deposition by Alizarin red [24]. Here we noted the negative impact of Osthole on the development of mineralized nodules by detecting the calcium influx with Fluo4-AM probe.

Our further observation of Osthole-induced MSCs on day 7 indicates a clear reduction in mRNA expression of osteoblast-specific genes, such as transcription factors RUNX2 (one of the key factors to trigger the osteogenic differentiation cascade [25] and Osterix (a downstream protein of Runx2 and required for the differentiation of MSCs into osteoblasts [26]), Type I collagen (the main component of bone matrix and its degradation always results in bone loss [27, 28]), osteocalcin [29], and OPN [30], coinciding completely with our previous results of both ALP activities and calcified nodules.

The osteogenic differentiation of MSCs undergoes a complex process orchestrated by multiple signaling pathways. Recent studies in rabbit and human MSCs revealed the importance of PI3K/Akt pathway in promoting cell proliferation and osteoblast differentiation [31, 32]. A serine/threonine kinases family MAPK has an essential role in regulating cell proliferation and differentiation, and accumulating experimental data have uncovered that MAPK signaling pathway is closely associated with osteogenic differentiation. As a pre-requisite for MSC differentiation, inhibition of actin polymerization decreased osteogenic differentiation

Fig. 7. Comparisons of osteoblast differentiation potential after MSCs were seeded at low or high densities. MSCs were induced in osteogenic medium alone or supplemented with 12.5 μ M Osthole at low density (control-L group or Osthole-L group), and induced in osteogenic medium alone or supplemented with 12.5 μ M Osthole at high density (control-H group or Osthole-H group) (A) ALP activities were analyzed after 14 days of cell induction. (B) The phase contrast of MSCs after 7 days of cell induction (arrows indicate the confluence of MSCs), and the staining of mineralized nodules after 21 days of cell induction (40 $\times$). (C) qPCR analysis of osteogenic gene expression after MSC induction with Osthole for 7 days at different densities, relative to internal control GAPDH and normalized to control cells; Data was expressed as mean $\pm$ SD from three separate experiments. * $p < 0.05$ versus control, # $p < 0.05$ versus Osthole-L.

of MSC by targeting p38 MAPK [33]. miR-21 modulation of Erk1/2-MAPK signaling pathway regulated the process of MSC differentiation [34]. Ghrelin-mediated activation of Erk1/2 pathway accelerated the growth of rabbit MSCs [35]. Quercetin was also demonstrated to promote the osteogenic differentiation of rat MSCs by activating MAPK cascade [36]. Thus, these findings implicate the Erk1/2-MAPK signaling to be an essential controller of bone formation. Bone morphogenetic proteins (BMPs) are master regulators of osteoblast differentiation [14, 37]. Secreted BMPs bind receptors to activate Smad transcription factors, which in turn control the expression of target genes [38, 39]. Additionally, altered expression of Wnt signaling pathway components was observed in osteogenic differentiation of MSCs isolated from osteoarthritis patients. Further studies implicate some natural components

extracted from plants to have potential for the promotion of the MSC differentiation towards osteoblasts [40-42].

In general, the activity of a signaling pathway can be reflected by detecting the expression and/or activities of its downstream target proteins. From data presented in this study, we observed that the Osthole in osteogenic differentiation of MSCs dramatically inhibits the Wnt/ β -catenin and Erk1/2-MAPK signaling pathways rather than the p38 MAPK, BMPs/Smad1/5/8 and PI3K/Akt mediated signaling pathways.

Despite advances in MSCs-mediated tissue engineering, the challenge remains for the high survival and functional ability. Enhancement of MSC survival and functional ability would be the key for potential applications of MSCs in tissue regeneration [43]. High-densities MSCs were essential to promote the establishment of cell-cell contacts with formation of multicellular clusters stabilized by endogenous ECM, and also to stimulate osteogenic differentiation [44]. Gelatin microsphere-mediated aggregation of MSCs enhanced their osteogenic differentiation [45]. Aged human MSCs displayed the reduced proliferation and differentiation potential when compared with those from young donors [46]. Therefore, MSC proliferation was largely believed to be critical for osteogenic differentiation. In this study, Osthole evidently inhibited the proliferation of rat MSCs by down-regulating the expression of cell proliferation markers PCNA and CyclinD1, which is consistent with our previous data evaluated by FACS and CCK8 [47]. Thus, the results of our studies indicate that Osthole suppressed the osteogenic differentiation of MSCs, possibly in part by the Osthole-induced reduction in cell proliferation.

To further confirm whether Osthole prevents MSCs from osteogenic differentiation through inhibiting MSC proliferation, two additional experiments were conducted in this study. First of all, Osthole was removed from the medium on day 7 during the induction process, and MSCs continued to be cultured for 14 days. We observed that the ALP activities and the expression of osteogenic genes were down-regulated consistently in between Osthole-R group and Osthole group, suggesting that Osthole removal, to some degree, alleviate the inhibition extent of osteoblast differentiation especially the expression of osteogenic transcription factors Runx2 and Osterix compared with the continuous effect of Osthole on MSCs. Calcified nodules, as the mature marker for osteogenesis, was not formed efficiently in Osthole-removal (Osthole-R) group and Osthole group, providing an indication that Osthole removal on day 7 failed to induce the terminal differentiation of MSCs. Thus, these observations suggest that MSC proliferation play a vital role in the subsequent initiation of differentiation. Secondly, MSCs at the beginning of induction were seeded at high density reaching 100% confluence, and maintained in induction medium for 7 days or 21 days. Both Osthole-L group and Osthole-H group exhibited the decreased ALP activities and calcified nodule formation, and the reduction in expressions of osteogenic genes, indicating that the inhibition of osteoblast differentiation by Osthole is not dependent on MSC proliferation alone. We noticed that MSCs converged when cells reached 100% confluence, and calcified nodules were formed from the confluence site during the process of cell differentiation. However, we did not investigate the evident converge of MSCs in the presence of Osthole even after 100% confluence reached. Therefore, we made a preliminary conclusion that the failed confluence might be the second factor for osteogenic differentiation. Additionally, we observed that an increase in MSC density alone significantly accelerated cell differentiation (control-H versus control-L), confirming an essential role of cell proliferation for osteoblast differentiation.

It has been reported that Osthole promoted the differentiation and maturation of osteoblasts [11-14]. However, our observations in this study seem contradictory to other reported findings, which are as follows: Firstly, the MSC proliferation was inhibited by Osthole. Secondly, the activity of Erk1/2-MAPK pathway was down-regulated efficiently by Osthole. Thirdly, the differentiation and maturation of MSCs towards osteoblasts were disrupted by Osthole. The possible explanation for the distinction described above is that MSCs share the similar biological properties with cancer cells [48]. It is not difficult to explain that Osthole interrupts the proliferation of MSCs which conforms to the inhibitory effect

of growth on various types of cancer cells, and MSC proliferation initiates the osteogenic differentiation [22]. Moreover, the Erk1/2-MAPK signaling was highly activated in tumor cells but inactivated by Osthole [18, 49]. In this study, we noted that Osthole inhibits the proliferation and osteogenic differentiation of MSCs in part by blocking this pathway. In addition, Osthole also inhibits the activity of Wnt/ β -catenin signaling pathway, which is different from the previous report [12].

Bone marrow-derived MSCs are a population of non-hematopoietic multipotent stem cells capable of differentiating into adipocytes and osteoblasts. Osteoblasts belonging to somatic cells can only differentiate into osteocytes. MSCs are more sensitive to external factors than osteoblasts. Taken together, Osthole inhibited the osteogenic differentiation of rat MSCs, and this is likely to be the way Osthole affects osteogenesis in human ESCs. Future experimental analysis of Osthole will need to take into account the potential involvement of human ESCs in osteogenic differentiation.

Acknowledgements

This study was supported by National Natural Science Foundation of China (81373665), Shanghai Municipal Science and Technology Commission (13411950500) and Shanghai three years plan of TCM (ZYSNXD-CC-YJXY-JS08), Shanghai young teacher training program (ZZszy12017).

Disclosure Statement

The authors declare that they have no competing interests.

References

- 1 Kanis JA: Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. *Osteoporos Int* 1994;4:368-381.
- 2 Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY: The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013;19:35-42.
- 3 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143-147.
- 4 English K, French A, Wood KJ: Mesenchymal stromal cells: facilitators of successful transplantation. *Cell Stem Cell* 2010;7:431-442.
- 5 Murphy MB, Moncivais K, Caplan AI: Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp Mol Med* 2013;45:e54.
- 6 Xu X, Kratz K, Wang W, Li Z, Roch T, Jung F, Lendlein A, Ma N: Cultivation and spontaneous differentiation of rat bone marrow-derived mesenchymal stem cells on polymeric surfaces. *Clin Hemorheol Microcirc* 2013;55:143-156.
- 7 Huang S, Xu L, Sun Y, Zhang Y, Li G: The fate of systemically administrated allogeneic mesenchymal stem cells in mouse femoral fracture healing. *Stem Cell Res Ther* 2015;6:206.
- 8 Pang J, Guo HL, Ding DF, Wu YY, Zhao YF, Gu XF, Zheng YX: Changes of mesenchymal stromal cells mobilization and bone turnover in an experimental bone fracture model in ovariectomized mice. *Int J Clin Exp Pathol* 2015;8:1028-1038.
- 9 You L, Feng S, An R, Wang XH: Osthole: a promising lead compound for drug discovery from a traditional Chinese medicine (TCM). *Nat Prod Commun* 2009;4:297-302.
- 10 Zhang W, Ma D, Zhao Q, Ishida T: The effect of the major components of *Fructus Cnidii* on osteoblasts *in vitro*. *J Acupunct Meridian Stud* 2010;3:32-37.
- 11 Zhang Q, Qin L, He W, Van Puyvelde L, Maes D, Adams A, Zheng H, De Kimpe N: Coumarins from *Cnidium monnieri* and their antiosteoporotic activity. *Planta Med* 2007;73:13-19.

- 12 Tang DZ, Hou W, Zhou Q, Zhang M, Holz J, Sheu TJ, Li TF, Cheng SD, Shi Q, Harris SE, Chen D, Wang YJ: Osthole stimulates osteoblast differentiation and bone formation by activation of beta-catenin-BMP signaling. *J Bone Miner Res* 2010;25:1234-1245.
- 13 Ming LG, Zhou J, Cheng GZ, Ma HP, Chen KM: Osthole, a coumarin isolated from common cnidium fruit, enhances the differentiation and maturation of osteoblasts *in vitro*. *Pharmacology* 2011;88:33-43.
- 14 Kuo PL, Hsu YL, Chang CH, Chang JK: Osthole-mediated cell differentiation through bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase 1/2 pathway in human osteoblast cells. *J Pharmacol Exp Ther* 2005;314:1290-1299.
- 15 Gao LN, An Y, Lei M, Li B, Yang H, Lu H, Chen FM, Jin Y: The effect of the coumarin-like derivative osthole on the osteogenic properties of human periodontal ligament and jaw bone marrow mesenchymal stem cell sheets. *Biomaterials* 2013;34:9937-9951.
- 16 Jørgensen NR: Short-range intercellular calcium signaling in bone. *APMIS Suppl* 2005;5:36.
- 17 An S, Gao Y, Huang Y, Jiang X, Ma K, Ling J: Short-term effects of calcium ions on the apoptosis and onset of mineralization of human dental pulp cells *in vitro* and *in vivo*. *Int J Mol Med* 2015;36:215-221.
- 18 Xu X, Zhang Y, Qu D, Jiang T, Li S: Osthole induces G2/M arrest and apoptosis in lung cancer A549 cells by modulating PI3K/Akt pathway. *J Exp Clin Cancer Res* 2011;30:33.
- 19 Lintao Wang, Yanyan Peng, Kaikai Shi, Haixiao Wang, Jianlei Lu, Yanli Li, Changyan Ma: Osthole inhibits proliferation of human breast cancer cells by inducing cell cycle arrest and apoptosis. *J Biomed Res* 2015;29:132-138.
- 20 Daofang Ding, Songpu Wei, Yi Song, Linghui Li, Guoqing Du, Hongsheng Zhan, Yuelong Cao: Osthole Exhibits Anti-Cancer Property in Rat Glioma Cells Through Inhibiting PI3K/Akt and MAPK Signaling Pathways. *Cell Physiol Biochem* 2013;32:1751-1760.
- 21 Karsenty G, Wagner EF: Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* 2002;2:389-406.
- 22 Hu Z, Peel SA, Ho SK, Sándor GK, Clokie CM: Role of bovine bone morphogenetic proteins in bone matrix protein and osteoblast-related gene expression during rat bone marrow stromal cell differentiation. *J Craniofac Surg* 2005;16:1006-1014.
- 23 Beck GR Jr, Zerler B, Moran E: Phosphate is a specific signal for induction of osteopontin gene expression. *Proc Natl Acad Sci USA* 2000;97:8352-8357.
- 24 Yun HM, Park KR, Quang TH, Oh H, Hong JT, Kim YC, Kim EC: 2, 4, 5 - Trimethoxyl dalbergiquinol promotes osteoblastic differentiation and mineralization via the BMP and Wnt/ β -catenin pathway. *Cell Death Dis* 2015;6:e1819.
- 25 Schaffler A, Buchler C: Concise review: adipose tissue-derived stromal cells—basic and clinical implications for novel cell-based therapies. *Stem Cells* 2007;25:818-827.
- 26 Choung HW, Lee DS, Lee HK, Shon WJ, Park JC: Preameloblast-Derived Factors Mediate Osteoblast Differentiation of Human Bone Marrow Mesenchymal Stem Cells by Runx2-Osterix-BSP Signaling. *Tissue Eng Part A* 2016;22:93-102.
- 27 Huang CY, Reuben PM, Cheung HS: Temporal expression patterns and corresponding protein inductions of early responsive genes in rabbit bone marrow-derived mesenchymal stem cells under cyclic compressive loading. *Stem Cells* 2005;23:1113-1121.
- 28 Kurt-Sirin O, Yilmaz-Aydogan H, Uyar M, Seyhan MF, Isbir T, Can: Combined effects of collagen type I $\alpha 1$ (COL1A1) Sp1 polymorphism and osteoporosis riskfactors on bone mineral density in Turkish postmenopausal women. *Gene* 2014;540:226-231.
- 29 Duan X, Xu H, Wang Y, Wang H, Li G, Jing L: Expression of core-binding factor $\alpha 1$ and osteocalcin in fluoride-treated fibroblasts and osteoblasts. *J Trace Elem Med Biol* 2014;28:278-283.
- 30 Chen Q, Shou P, Zhang L, Xu C, Zheng C, Han Y, Li W, Huang Y, Zhang X, Shao C, Roberts AI, Rabson AB, Ren G, Zhang Y, Wang Y, Denhardt DT, Shi Y: Anosteopontin-integrin interaction plays a critical role in directing adipogenesis and osteogenesis by mesenchymal stem cells. *Stem Cells* 2014;32:327-337.
- 31 Tong Y, Feng W, Wu Y, Lv H, Jia Y, Jiang D: Mechano-growth factor accelerates the proliferation and osteogenic differentiation of rabbit mesenchymal stem cells through the PI3K/AKT pathway. *BMC Biochem* 2015;16:1.
- 32 Baker N, Sohn J, Tuan RS: Promotion of human mesenchymal stem cell osteogenesis by PI3-kinase/Akt signaling, and the influence of caveolin-1/cholesterol homeostasis. *Stem Cell Res Ther* 2015;6:238.

- 33 Sonowal H, Kumar A, Bhattacharyya J, Gogoi PK, Jaganathan BG: Inhibition of actin polymerization decreases osteogenic differentiation of mesenchymal stem cells through p38 MAPK pathway. *J Biomed Sci* 2013;20:71.
- 34 Mei Y, Bian C, Li J, Du Z, Zhou H, Yang Z, Zhao RC: miR-21 modulates the ERK-MAPK signaling pathway by regulating SPRY2 expression during human mesenchymal stem cell differentiation. *J Cell Biochem* 2013;114:1374-1384.
- 35 Ye N, Jiang D: Ghrelin accelerates the growth and osteogenic differentiation of rabbit mesenchymal stem cells through the ERK1/2 pathway. *BMC Biotechnol* 2015;15:51.
- 36 Li Y, Wang J, Chen G, Feng S, Wang P, Zhu X, Zhang R: Quercetin promotes the osteogenic differentiation of rat mesenchymal stem cells via mitogen-activated protein kinase signaling. *Exp Ther Med* 2015;9:2072-2080.
- 37 Lo YC, Chang YH, Wei BL, Huang YL, Chiou WF: Betulinic acid stimulates the differentiation and mineralization of osteoblastic MC3T3-E1 cells: involvement of BMP/Runx2 and beta-catenin signals. *J Agric Food Chem* 2010;58:6643-6649.
- 38 Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR, Chen D: Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. *J Cell Biol* 2002;157:1049-1060.
- 39 Kishi Y, Fujihara H, Kawaguchi K, Yamada H, Nakayama R, Yamamoto N, Fujihara Y, Hamada Y, Satomura K, Masutani M: PARP Inhibitor PJ34 Suppresses Osteogenic Differentiation in Mouse Mesenchymal Stem Cells by Modulating BMP-2 Signaling Pathway. *Int J Mol Sci* 2015;16:24820-24838.
- 40 Tornero-Esteban P, Peralta-Sastre A, Herranz E, Rodríguez-Rodríguez L, Mucientes A, Abásolo L, Marco F, Fernández-Gutiérrez B, Lamas JR: Altered Expression of Wnt Signaling Pathway Components in Osteogenesis of Mesenchymal Stem Cells in Osteoarthritis Patients. *PLoS One* 2015;10:e0137170.
- 41 Gu Q, Chen C, Zhang Z, Wu Z, Fan X, Zhang Z, Di W, Shi L: Ginkgo biloba extract promotes osteogenic differentiation of human bone marrow mesenchymal stem cells in a pathway involving Wnt/ β -catenin signaling. *Pharmacol Res* 2015;97:70-78.
- 42 Tao K, Xiao D, Weng J, Xiong A, Kang B, Zeng H: Berberine promotes bone marrow-derived mesenchymal stem cells osteogenic differentiation via canonical Wnt/ β -catenin signaling pathway. *Toxicol Lett* 2016;240:68-80.
- 43 Chen J, Crawford R, Chen C, Xiao Y: The key regulatory roles of the PI3K/Akt signaling pathway in the functionalities of mesenchymal stem cells and applications in tissue regeneration. *Tissue Eng Part B Rev* 2013;19:516-528.
- 44 Maia FR, Lourenço AH, Granja PL, Gonçalves RM, Barrias CC: Effect of cell density on mesenchymal stem cells aggregation in RGD-alginate 3D matrices under osteoinductive conditions. *Macromol Biosci* 2014;14:759-771.
- 45 Hayashi K, Tabata Y: Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions. *Acta Biomater* 2011;7:2797-2803.
- 46 Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT: Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. *J Transl Med* 2014;12:8.
- 47 Du G, Song Y, Wei L, Li L, Wang X, Xu Q, Zhan H, Cao Y, Zheng Y, Ding D: Osthole Inhibits Proliferation and Induces Catabolism in Rat Chondrocytes and Cartilage Tissue. *Cell Physiol Biochem* 2015;36:2480-2493.
- 48 Berger L, Shamaï Y, Skorecki K, Tzukerman M: Tumor Specific Recruitment and Reprogramming of Mesenchymal Stem Cells in Tumorigenesis. *Stem Cells* 2016;34:1011-1026.
- 49 Chen TJ, Zhou YF, Ning JJ, Yang T, Ren H, Li Y, Zhang S, Chen MW: NBM-T-BMX-OS01, an Osthole Derivative, Sensitizes Human Lung Cancer A549 Cells to Cisplatin through AMPK-Dependent Inhibition of ERK and Akt Pathway. *Cell Physiol Biochem* 2015;36:893-906.