Int J Clin Exp Med 2017;10(1):224-241
www.ijcem.com /ISSN:1940-5901/IJCEM0039328

Original Article

Different functions of the two isoforms of osteopontin (iOPN and sOPN) in the proliferation and differentiation of osteoblast and osteoclast: iOPN inhibits osteoclasts' proliferation while sOPN stimulates osteoblasts' differentiation and osteoclasts' resorption

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Received September 2, 2016; Accepted November 12, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Osteopontin (OPN) plays an important role in maintaining bone homeostasis. However, functions of its two isoforms (iOPN and sOPN) in bone are poorly understood. This paper aimed to identify which isoform of OPN could be beneficial to bone regeneration. The CCK-8 assays showed that cell proliferation was significantly inhibited in iOPN-over expressed RAW264.7 cells (Adv-iOPN) compared to wild RAW264.7 cells (Adv-Control) via increasing cells at G0 phase. Osteogenic differentiation was dramatically enhanced after treatment with sOPN through AKT and p38 pathway. ALP activity, collagen expression, calcium mineralization and the expression of osteogenic-related gene Runx2 was significantly increased by sOPN. In addition, osteoclastogenesis was dramatically enhanced after treatment with sOPN through AKT and NF-KB pathway. TRAP positive cell count, bone resorption pit formation and expression of osteoclastic marker genes (TRAP, RANK and Cathpesin-K) were significantly increased in RAW264.7 cells. The present results also indicated that iOPN has an inhibitory role in bone loss by preventing osteoclast proliferation, while sOPN plays positive roles in bone formation of osteoblasts and osteoclast resorption.

Keywords: Osteopontin isoforms, osteoclast, osteoblast, proliferation and differentiation, osteoclast resorption

Introduction

Chronic periodontitis is a major public health problem in China that causes damage to the soft tissues and bones supporting the teeth. It's reported that over 80% of the Chinese adults suffer from mild to moderate periodontitis [1]. It is a major cause of anodontia in adults and has also been implicated as a risk factor for various systemic inflammatory disorders including diabetes mellitus (DM), rheumatoid Arthritis (RA), and cardiovascular disease (CVD) [2-4]. However, to date, no diagnostic and prognostic biomarkers or therapeutic targets for periodontitis have been identified.

Osteopontin (OPN), one of the major non-collagenous bone proteins may be considered as a potential biomarker and therapeutic target for periodontitis. It has been detected in gingival crevicular fluid and plasm, associated with the progression and prognostic of periodontal diseases in patients [5-7]. However, the related detail molecular mechanism remains unclear. We believe that the functions of OPN are worthy of attention. On the one hand, OPN regulates host immune function via regulating macrophages and other immune cells [8-10], influencing the development and progression of periodontal disease. On the other hand, OPN regulates the proliferation & differentiation of osteoblasts and osteoclasts [11-13], affecting the resorption and reconstruction of alveolar bone in periodontal disease. Importantly, the latter effect of OPN arouses our attentions. In addition, OPN has two types of isoform [14-16]: one is the full length OPN with the signal peptide that targets OPN for secretion (sOPN), whereas

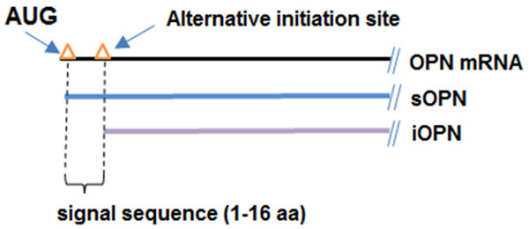


Figure 1. Alternative translation of osteopontin (OPN) generates intracellular and secreted isoforms. The two isoforms of OPN are translated from a single OPN mRNA species with different translation initiation sites. sOPN is translated from the beginning site and is targeted to secretory vesicles, while iOPN is translated from the alterative initiation site and stays in the cell. The first 16-aa is a signal sequence in mouse OPN protein which has 294-aa (Summarized from [15, 16]).

another is the intracellular form of OPN (iOPN) lacking the OPN signal sequences by alternative translation. The two isforms of OPN are translated from a single OPN mRNA species with different translation initiation site (**Figure 1**). When translation starts from the beginning site, the peptide product (sOPN) includes a signal sequence and is targeted to secretory vesicles; while alternative translation generates iOPN which lacks a signal sequence and stays in the cell [15, 16]. So far, OPN has always been studied as an extracellular protein (sOPN), and current understanding regarding iOPN is rather limited. Thus, the role of iOPN is much less characterized compared with that of sOPN. Thus, it would be necessary to delineate which isoform of OPN is responsible for pathophysiological events in bone.

Bone is a dynamic tissue that is constantly remodeled, shaped and repaired through its whole lifetime. The cell populations (osteoblasts and osteoclasts) that participate in this process are derived from different progenitor pools that are under distinct molecular control mechanisms [17, 18]. These two cell types are necessary for the maintenance of bone mass as well as bone mineral density. Excessive bone resorption by osteoclasts relative to bone formation by osteoblasts causes imbalances of bone remodeling and consequently leads to diseases such as periodontitis, osteoporosis and arthritis [19-21].

This study aimed to compare the effects of two isoforms of OPN on the proliferation & differentiation of osteoblast and osteoclast, and ascertain how the OPN subtype mediates the signaling molecules in bone-remodeling process. We evaluated effects of sOPN and iOPN on cell viability and differentiation in mouse pre-osteoblast MC3T3-E1 and monocyte-macrophage RAW264.7 cell lines. In addition, we also explored the related potential mechanisms.

Materials and methods

Preparation of MC3T3-E1 cells and RAW264.7 cells

Mouse pre-osteoblast cell line MC3T3-E1 subclone 4 cells and mouse mononuclear macrophage RAW264.7 cells (ATCC, Bethesda, MD, USA) were obtained from the Chinese Medicine Academy of Sciences Cell Bank (Beijing, China). MC3T3-E1 cells were cultured in an osteogenic induction medium containing α-MEM (α-MEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 U penicillin/ml and 100 U streptomycin/ml antibiotics, 50 mg/ml ascorbic acid, 10 mM β-glycerophosphate (P/S, AA, βGP; Sigma Chemicals, St. Louis, MO, USA). RAW264.7 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/mL streptomycin and 50 ng/mL RANKL (PeproTech Rocky Hill, NJ, USA). Both the cell lines were routinely incubated at 37°C with 5% CO_2 and saturated humidity. Cells were sub-cultured before reaching confluence using PBS and 0.25% trypsin/EDTA (Sigma Chemicals, St. Louis, MO, USA).

Preparation of osteopontin isoforms

Recombinant mouse OPN (as sOPN) had been purchased from R&D Systems (Minneapolis, MN, USA). After 24 h seeding, cells were treated with different concentrations (ranging from 0 to 400 ng/ml) of the sOPN. Media was changed every other day with the appropriate treatment addition. In order to examine the function of iOPN in cells, we use an adenoviral vector carrying an iOPN cDNA (Adv-iOPN) to infect cells. The construction of the overexpression recombinant adenovirus containing iOPN was carried out with the Adeno-X Expression System (Clontech, Inc, Shanghai, China).The gene of iOPN was chemically synthesized by JIKA company (Shanghai, China) which deleted

the codons from 1 to 15 from gene sOPN (Spp1, NM_009263) as well as plus codons of 3 flag to the N end. Then iOPN expression plasmid was ligated into linearized pDC315-EGFP vector (JIKAI, shanghai, China). The corresponding amplicon was cloned in the pShuttle plasmid (Clontech, Inc, Shanghai, China) using the Age I and NheI restriction sites. The recombinant adenoviruses (Adv-iOPN) were generated by transfection into HEK293 cells with Lipofectamine Transfection Reagent (Invitrogen, Grand Island, NY, USA). Expression of iOPN protein was detected using western blotting assay. An adenoviral vector without the OPN insert (Adv-control) was used as another control. For the infection of MC3T3-E1 or RAW264.7 with the recombinant Adenoviruses, cells were seeded in normal cultures during 24 hrs. The cell cultures were then incubated with 100-800 MOI (multiplicity of infection) of either Adv-iOPN or Adv-control during 8 hrs in normal medium, in order to allow the virus adsorption and choose the best MOI. After the infection, media was changed for cell differentiation.

Cell proliferation assessment by the CCK8 assay

Cell proliferation was measured using a CCK-8 Kit (Dojindo CK04-11, Tokyo, Japan). MC3T3-E1 cells of the forth passage and RAW264.7 cells of the fifth passage were seeded in 96-well plates at a density of 3×10^3 and 2×10^4 cells/well. MC3T3-E1 cells were treated with different concentrations of sOPN (0, 40, 400 ng/ml) for 1, 3, 5, 7 days, or infected with adv-iOPN and adv-control for 8 hrs and then incubated in an induction medium for the same time. RAW264.7 cells were treated by similar way for 0, 12, 24, 36, 48 hours. After treatment, 10 μl of CCK-8 buffer was added to each well, and the cells were incubated at 37°C for 2 h, after which the absorbance was measured at 450 nm on an absorbance microplate reader (ThermoMultiskan MK3, USA). Cell viability was calculated relative to that of the control cells from the formula: cell viability = (OD experimental group-OD zeroing)/(OD control group-OD zeroing).

Cell cycle analysis by flow cytometry

RAW264.7 cells were infected with adv-iOPN and adv-control for 8hrs and then incubated in an induction medium for 24 and 48 hours (the time point based on the results of CCK 8 assay). Cells were harvested and washed in cold PBS, then incubated in 50 ug/ml of propidium iodide (PI) solution (containing 0.03% TritonX-100) (Sigma-Aldrich) at room temperature for 25 min. For each sample, at least 2×10 cells/ml were analyzed by BD FACSAria Cell Sorter (Becton Dickinson, San Jose, CA, USA). Cell cycle profiles were calculated by Modfit LT software.

Alkaline phosphatase (ALP) staining for alkaline phosphatase (ALP) activity assay

Alkaline phosphatase (ALP) activity was evaluated by staining method and quantitative measurement. MC3T3-E1 cells were cultured in 24-well cell culture dish under the osteogenic induction culture conditions as well as the treatment of two OPN isoforms. At day 4 and day 7, cells were digested by trypsin (0.25%) and seeded in 96-well plates. After 24 h, re-seeded cells were stained using a BCIP/NBT alkaline phosphatase color development kit (Beyotime, China) according to the manufacturing protocol. Stained cells were visualized with inverted light microscope. For ALP activity measurement, cells were washed with PBS and finally lysed in RIPA cell lysis buffer (Beyotime, Shanghai, China). ALP activity in the lysate was measured using an ALP testing kit (Jiancheng Biotechnology, Nanjing, China). The ALP activity was normalized by the total protein concentration for each sample using a BCA protein assay (Beyotime, Shanghai, China).

Collagen staining

The collagen secretion was carried out at day 7 after fixing samples in 4% PFA for 15 min. For the degree of collagen secretion, the material-cell constructs were stained in a 0.1% solution of Sirius Red (Beyotime, Shanghai, China), in saturated picric acid overnight. After rinsing with 0.1 M acetic acid until no more color appeared, optical images for each sample were acquired. Quantitatively, the stain was eluted in 400 mL of the destain solution (0.2 M NaOH/methanol 1:1) before optical density at 570 nm was measured using a microplate reader. The experiments were carried out in quadruplicate.

Alizarinred-staining

To measure extracellular matrix mineralization deposits for bone nodule formation, the cellular

Table 1. Primers used for real time RT-PCR

Genes		
Runx-2	Forward	5'-TTTAGGGCGCATTCCTCATC-3'
	Reverse	5'-TGTCCTTGTGGATTAAAAGGACTTG-3'
BMP2	Forward	5'-CCAACCATGGATTCGTGGTG-3'
	Reverse	5'-GGTACAGCATCGAGATAGCA-3'
TRAP	Forward	5'-AGCAGCCAAGGAGGACTACGTT-3'
	Reverse	5'-TCGTTGATGTCGCACAGAGG-3'
RANK	Forward	5'-ACCTCCAGTCAGCAAGAAGT-3'
	Reverse	5'-TCACAGCCCTCAGAATCCAC-3'
Cathepsinn-K	Forward	5'-TTAATTTGGGAGAAAAACCT-3'
	Reverse	5'-AGCCGCCTCCACAGCCATAAT-3'
GAPDH	Forward	5'-GGGCTCTCCAGAACATCATC-3'
	Reverse	5'-CAAAGTGGTCGTTGAGGGCA-3'

matrix was stained using alizarin red dye that combines with Ca^{2+} in the matrix [18]. Cells were cultured in a 12-well cell culture dish with an osteogenic induction medium as well as the treatment of two OPN isoforms. At day 14, cultures were washed twice with PBS, fixed in 4% Paraformaldehyde for 30 min, and rinsed with deionized wate. Finally, the cells were stained with 40 mmol/l alizarin red solution (pH 4.4) for 5 minutes at room temperature. Stained cells were visualized with inverted light microscope. Quantitative analyses of the mineralization indicated by alizarin red staining were performed using Image Software Pro Plus 6.0.

TRAP staining

To confirm the generation of multinucleated osteoclast-like cells, RAW 264.7 cells were seeded in 48 well plates at a density of 6×10^4 cells/well with an osteogenic induction medium as well as the treatment of two OPN isoforms. At day 5, were then fixed and stained for TRAP activity using the TRAP-staining kit (Sigma-Aldrich, Shanghai, China) according to the manufacturer's instructions. TRAP-positive multinucleated (3 or more nuclei) osteoclasts were visualized by light microscopy and photographed. Each osteoclast formation assay was performed at least 3 times.

Resorption pit assay

RAW 264.7 cells were seeded in Corning Osteo Assay Surface 24 well plates (Corning Inc, USA) at a density of 10,000 cells/cm^2 and treated during the entire experiment for 6 days. Cells were removed by incubating with 5% sodium hypochlorite for 5 min. Then the wells were stained by modified Von Kossa method as described [15]. Briefly, the plates were treated with 300 μl/well 5% (w/v) silver nitrate solution for 30 min in darkness at room temperature. After washing 5 times with distilled water, wells were treated with 300 μl/well 5% (w/v) sodium carbonate in 10% commercial balanced formalin for 5 min at room temperature. Then, the wells were washed 5 times with distilled water. The plates were aspirated and air dried prior to imaging. The count of resorption pits were quantified using Image J software (NIH, USA). Two wells were assessed per treatment in three different experiments.

RNA isolation and real-time RT-PCR analysis

RAW264.7 and MC3T3-E1 cells were seeded in 6-well plates with induction medium as well as the treatment of two OPN isoforms. The RNA levels were analyzed by real-time PCR at day 7 or day 14 for MC3T3-E1 cells, day 3 or day 5 for RAW264.7 cells. Total RNA was isolated using RNA extraction kit (Biomed, Beijing, China) according to the manufacturer's protocol. The purified total RNA was used for cDNA synthesis with a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas K1622, China) and oligo (dT) primers. Quantitative real-time PCR (qRT-PCR) was performed using a ReverTra Ace® qPCR RT kit (Toyobo, Osaka, Japan) in an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA). Primers synthesized from Sangong Biotech (Shanghai, China) were listed in **Table 1**. For MC3T3-E1 cells, real-time RT-PCR was performed for Runt-related transcription factors 2 (Runx2) and bone morphogenetic protein 2 (BMP2). For RAW264.7 cells, real-time RT-PCR was performed for tartrate resistant acid phosphatase (Trap), cathepsin K (CtsK), Receptor activator of nuclear factor kappa-B (Rank). The transcript levels were normalized by the GAPDH transcript levels. Thermal cycling parameters were 94°C for 5 min, followed by 35 cycles of amplifications at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 5 min as the final extension step.

Western blotting

The cytoplasmic and nuclear proteins were prepared using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China)

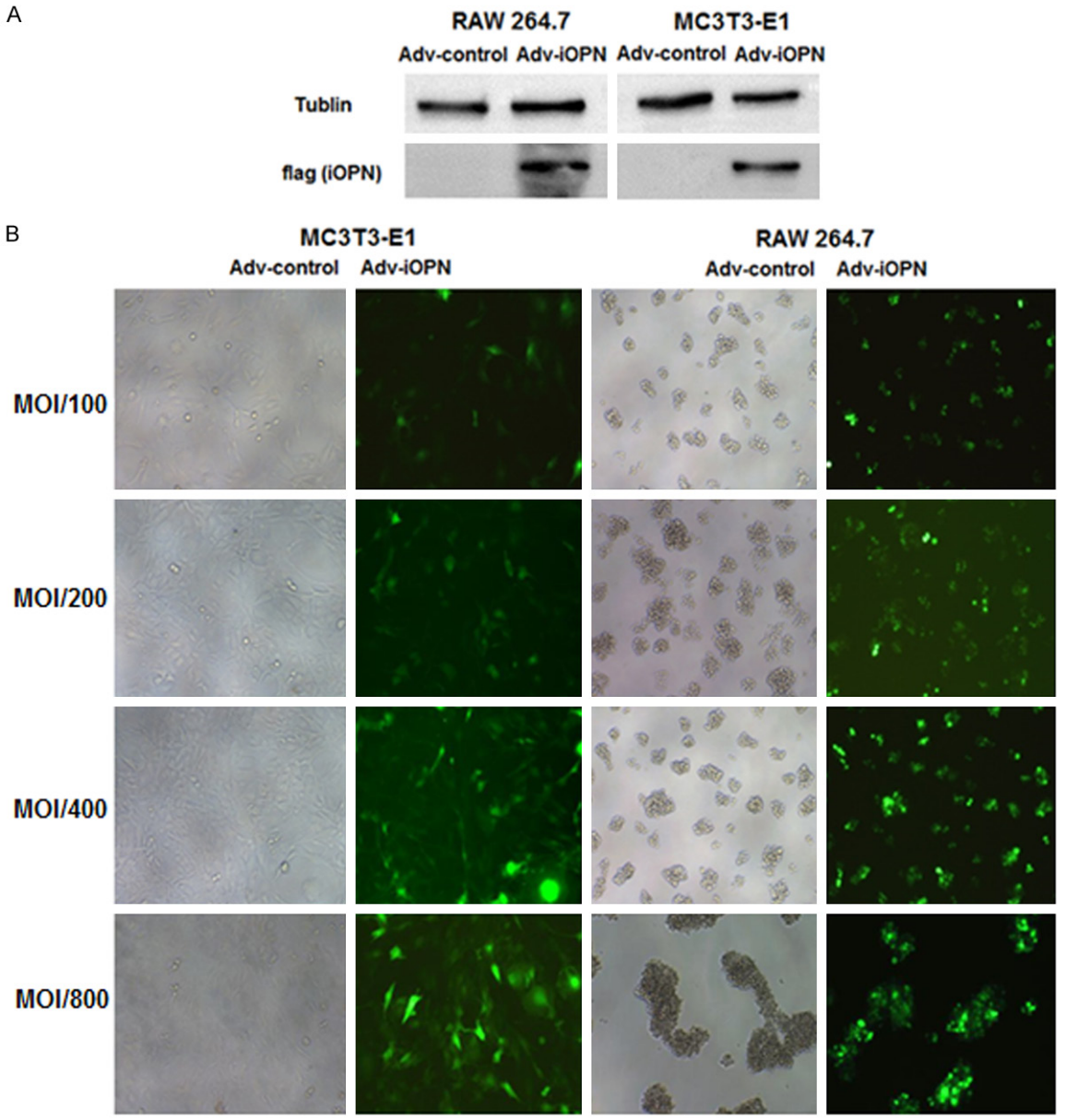


Figure 2. Up-regulation of iOPN in MC3T3-E1 and RAW264.7 cells. A: Flag-iOPN protein expression. Cells were cultured with standard medium before confluence, and protein expression levels were measured by western blot. Tublin was used as an internal control. B: MC3T3-E1 and RAW264.7 cells were infected by adv-iOPN (MOI = 100, 200, 400, 800). Cells were observed by phase-contrast microscopy (×100).

according to manufacturer's instruction. The protein concentration was determined using BCA assay kit (Beyotime, Shanghai, China). Equal amounts of protein (50 μg) were separated by SDS-PAGE and electro-transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked for 1 h at room temperature with blocking buffer (3% BSA in TBST). Then, the membrane was incubated overnight at 4°C with the primary antibody diluted in blocking buffer. After three times washes in TBST, the membrane was incubated with an appropriate HRP conjugated secondary antibody diluted in blocking buffer. Images were visualized and captured using Micro chemi Chemiluminescence system 4.2 (DNR, Jerusalem, Israel).

Statistics

All data are presented as means ± S.D. Differences between groups were assessed

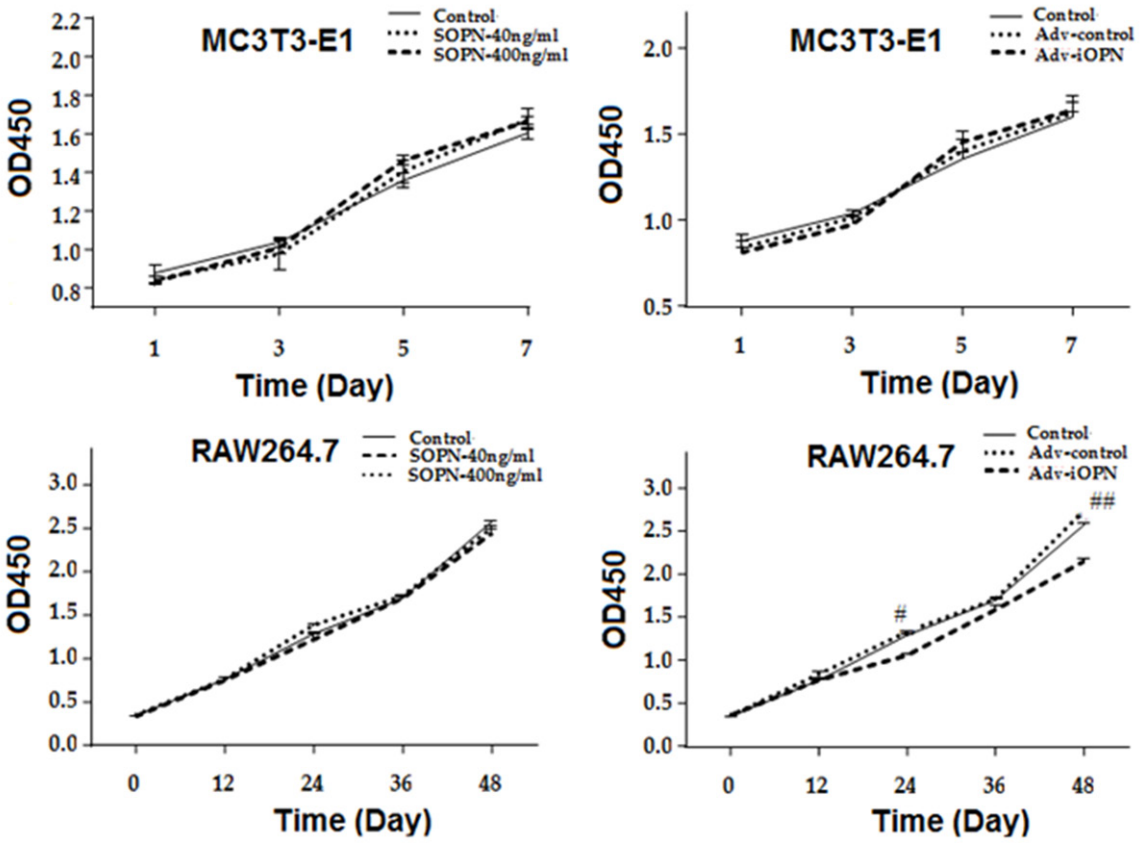


Figure 3. Results of CCK-8 assay. Data were expressed as means ± standard deviation (SD) (n = 4). #P<0.05 and ##P<0.01 vs. adv-control.

Multiple comparisons of the data were performed by ANOVA with Dunnett's test. *P* values less than 0.05 were regarded as significant.

Results

Establishment of iOPN-up-regulated MC3T3-E1 and RAW264.7 cell lines

To confirm that the iOPN was up-regulated in MC3T3-E1 and RAW264.7 cells, the western blotting assays were carried out using a flag antibody since flag and GFP were fused to iOPN gene. Cells infected with Adv-control (adenoviral control without iOPN insert), or uninfected (control) were also generated. Adv-iOPN-infected cells showed significant up-regulation of iOPN, while no obvious iOPN expressions could be seen in adv-control-infected cells (**Figure 2A**), confirming that the adv-iOPN works in the primary osteoblasts and osteoclasts. **Figure 2B** shows the multiplicity of infection (MOI) dependence of fluorescence signal in adv-iOPN infected cells, appearing saturated at MOI 400 (MC3T3-E1 cells) and 200 (RAW264.7 cells).

Cell proliferation and cell cycle progression

Effects of sOPN and iOPN on proliferations of MC3T3-E1 and RAW264.7 cells were measured by Cell Counting Kit-8 (CCK-8) assay. As can be seen from the **Figure 3**, no significant differences were observed between the optical density (OD) values of cells after sOPN treatment (0, 40, 400 ng/ml) in MC3T3-E1 and RAW264.7 cells. It also showed that when RAW264.7 cells were treated with adv-iOPN for 24 hours, the OD values decreased significantly compared with adv-control treated cells in 24 and 48 hours (P<0.01), while no significant differences were observed in MC3T3-E1 cells. The OD values of cells infected with adv-control or uninfected (control) had no significant differences.

We further investigated whether iOPN affected the cell cycle of RAW264.7 cells or not. Following PI staining, cell cycle distribution analyses of

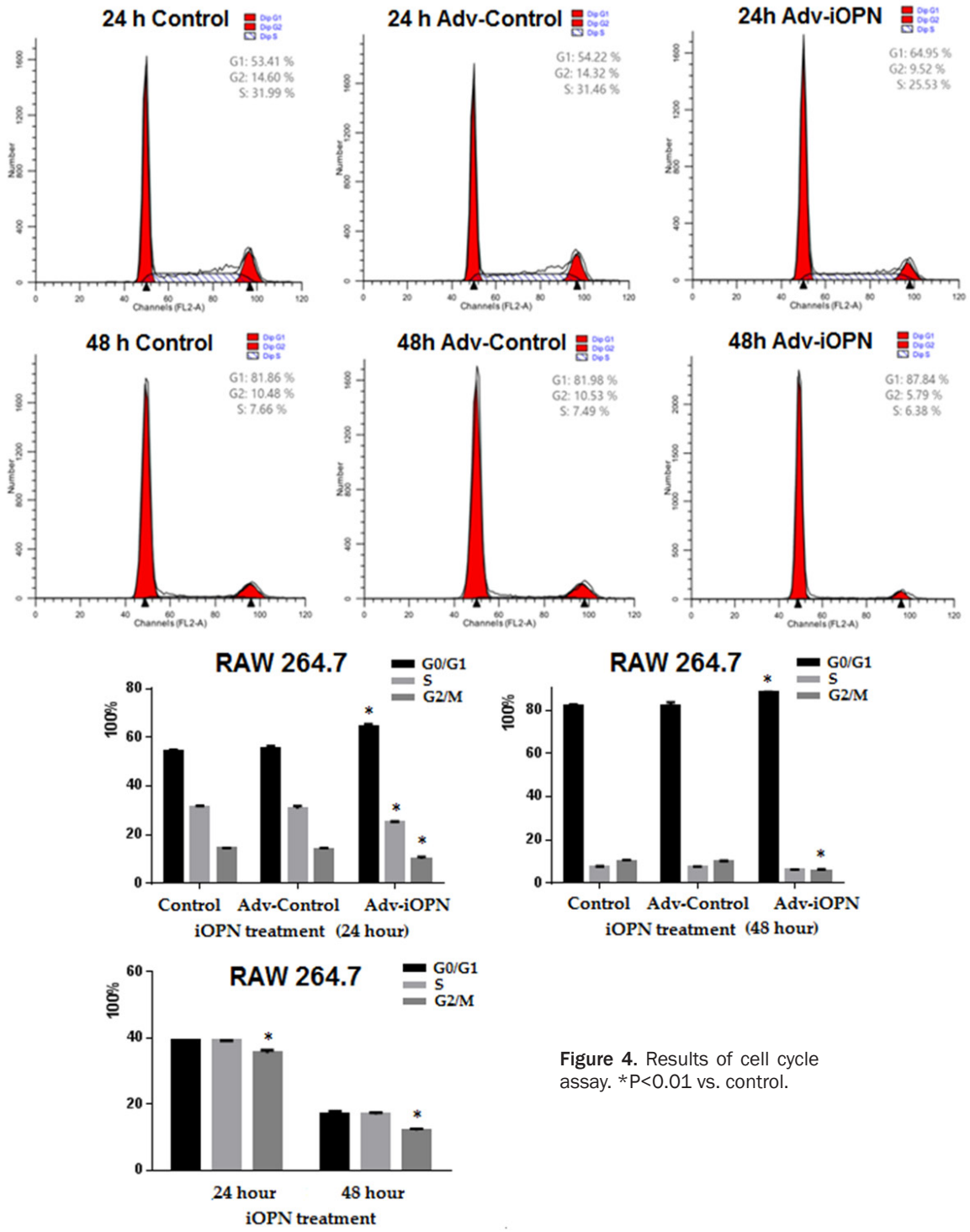


Figure 4. Results of cell cycle assay. *P<0.01 vs. control.

RAW264.7 cells were conducted by FCM scanning. As shown in **Figure 4**, Infection with adv-iOPN lead to increased cell cycle arrest in G0/G1 phase at 24 or 48 days (P<0.05), and G2/M phase as well as PI index were significantly reduced compared with the adv-control-infected cells (P<0.05).

Formation of the mineralized nodules and expression of osteogenic marker genes

Next, we examined effects of iOPN and sOPN on MC3T3-E1 osteogenic differentiation via determining alkaline phosphatase (ALP) activity, collagen I secretion, calcium mineralization,

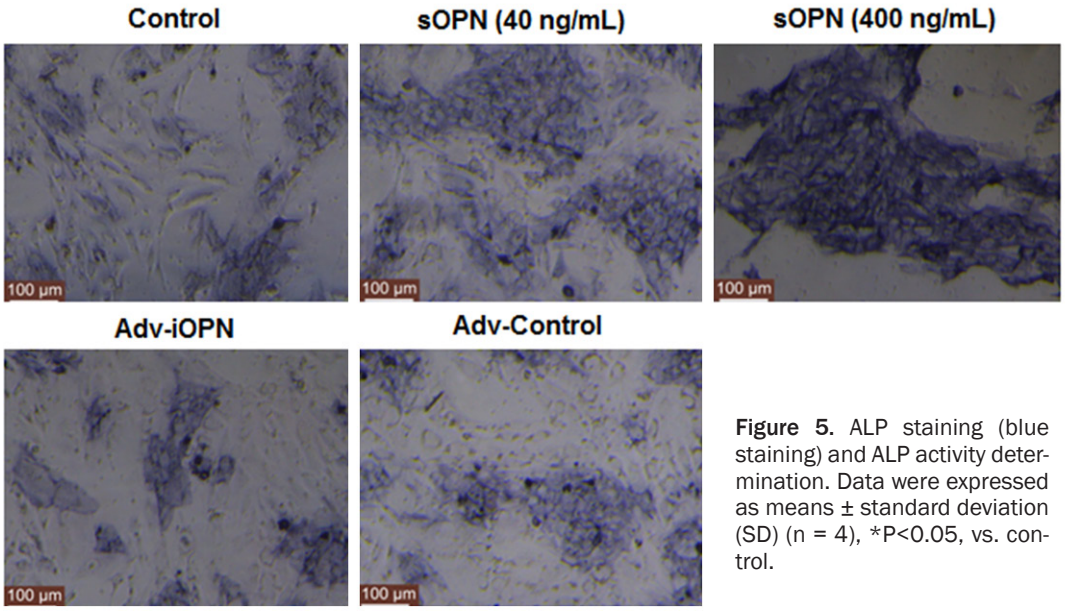
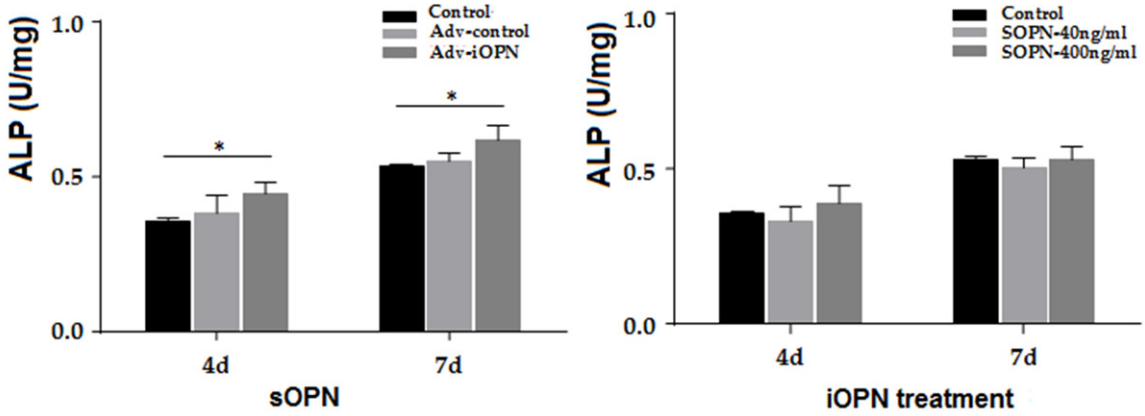


Figure 5. ALP staining (blue staining) and ALP activity determination. Data were expressed as means ± standard deviation (SD) (n = 4), *P<0.05, vs. control.



and mRNA expressions of osteogenic genes (Runx2 and BMP2). ALP activity was evalu-ated by staining method and quantitative measurement. In contrast to the control group, MC3T3-E1 cells treated with sOPN (400 ng/ml) showed more intense ALP staining at 7 days. The further results of ALP activity by quantitative measurement were consistent with the result of staining method described above (**Figure 5**). The collagen secretion significantly increased after sOPN (400 ng/ml) treatment compared to control after 7 days of culture by both staining and quantitative assays (P<0.01) (**Figure 6**). Mineralization is important for bone formation. Quantitative assays of mineralization were carried out using alizarin red staining. The presence of calcium deposits showed that treatment with 40 and 400 ng/mL sOPN significantly increased the mineralization in MC3T3-E1 cells at 14 days compared with the control cells (P<0.05, P<0.01), while high dose (400 ng/ml) of sOPN had greater effects compared to low dose (400 ng/ml) sOPN (P<0.05) (**Figure 7**).

To study whether osteoblast marker gene expression was affected by iOPN or sOPN overexpression during osteoblastic differentiation, cells were cultured with osteogenic medium for the indicated number of days and then mRNA were analyzed by real-time RT-PCR (**Figure 8**). The results showed that high doses of sOPN (400 ng/ml) induced higher mRNA expression levels of Runx2 compared to control at both 7days and 14 days.

No significant difference was observed among the adv-iOPN infected, adv-control infected and control cells regarding any osteogenic differentiation markers, such as ALP activity, the colla-

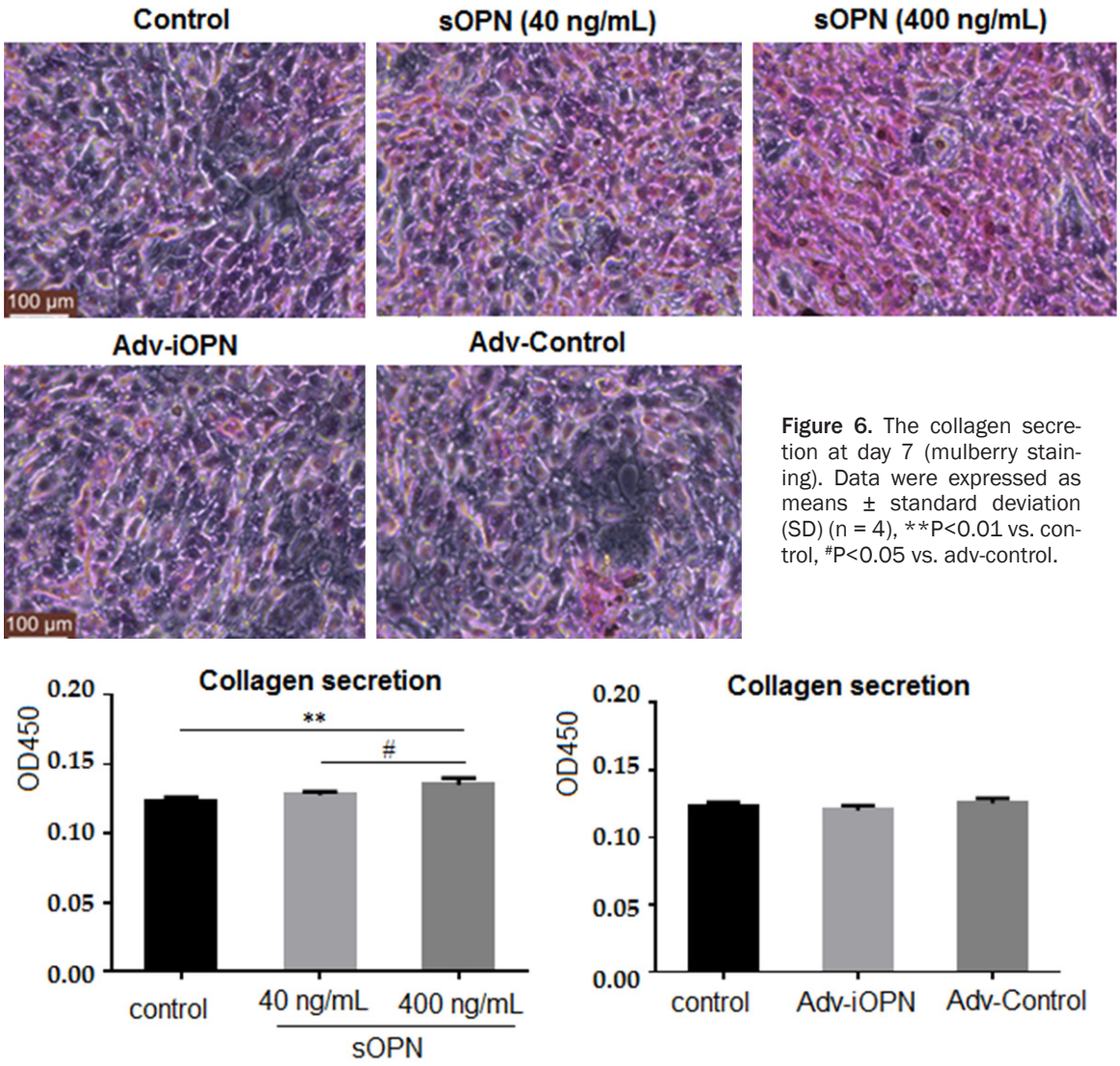


Figure 6. The collagen secretion at day 7 (mulberry staining). Data were expressed as means ± standard deviation (SD) (n = 4), **P<0.01 vs. control, #P<0.05 vs. adv-control.

gen I secretion, calcium deposition, and osteogenic gene expressions (**Figures 5**-**8**).

Effect of the OPN isoforms on osteoclastogenesis

Furthermore, we investigated the effect of iOPN and sOPN on RAW264.7 osteogenic differentiation by mature multinucleated osteoclasts formation, resorption activity and the mRNA expression of osteoclastic genes (Trap, RANK and CathepsinK) (**Figures 9**-**11**). RAW264.7 cells were treated with different concentrations of sOPN or infected with adv-iOPN and adv-control in RANKL-induced culture medium. Mature TRAP positive multinucleated osteoclasts (with 3 or more nuclei) were seen in each treated group after the fifth day of incubation. However, sOPN (400 ng/ml) significantly increased number of multinucleated trap-positive cells and modified their morphology, with greater osteoclast (OCL) and more nuclei than control cells (**Figure 9**) (P<0.05). No significant difference was observed in sOPN (40 ng/ml) or adv-iOPN groups compared to the control. To further investigate the effects of OPN isoforms on RANKL mediated bone resorption, cells were cultured above Corning OsteoAssay Surface. sOPN treatment increased bone resorption pit formation at 400 ug/ml compared with the control (**Figure 10**) (P<0.05). No significance was found for treatment with sOPN (40 ng/ml) or adv-iOPN in contrast to the control group. Finally, we investigated the effects on the mRNA expressions of osteoclastic genes. Cells treated with sOPN (400 ng/ml) showed incr-

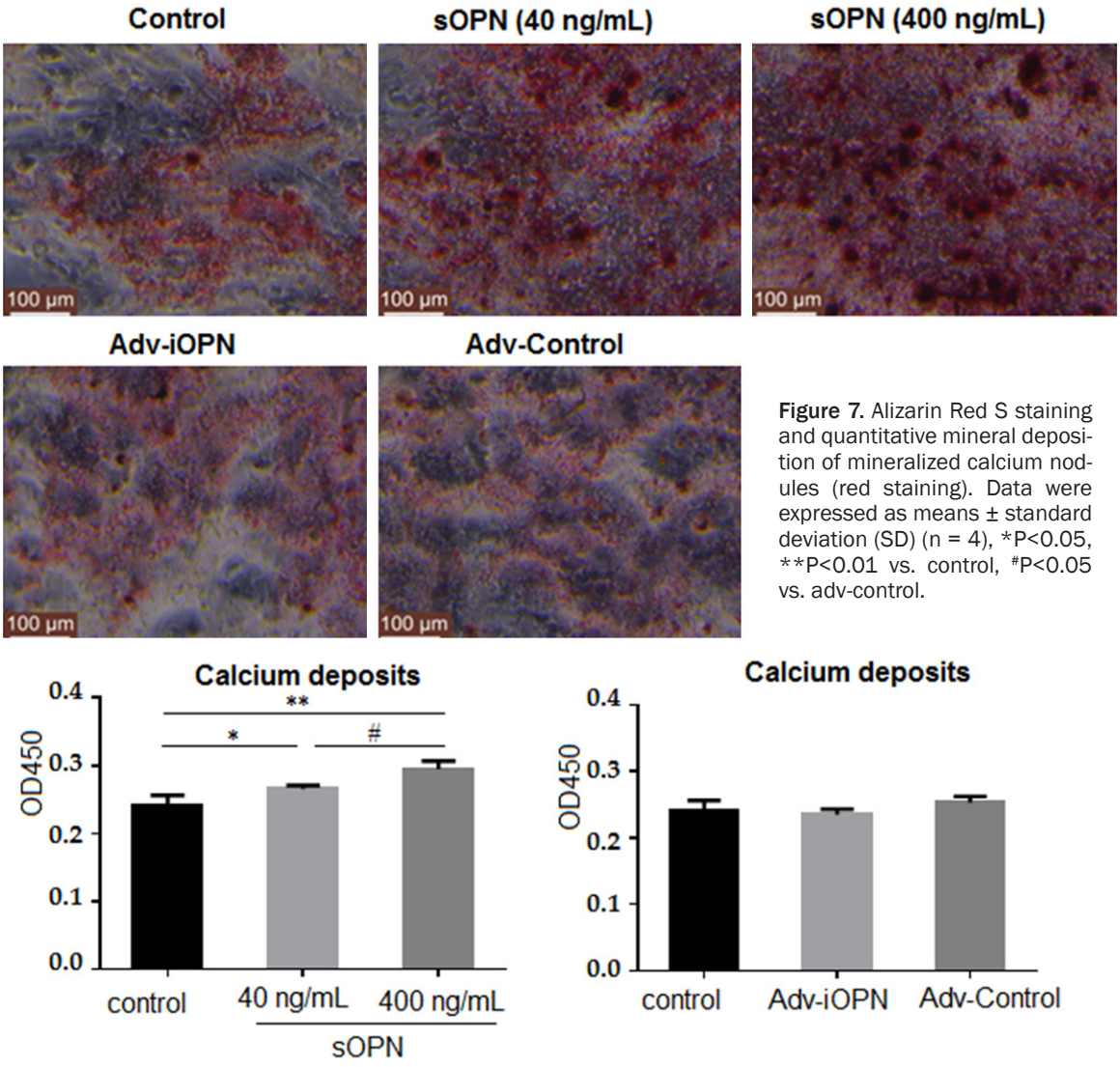


Figure 7. Alizarin Red S staining and quantitative mineral deposition of mineralized calcium nodules (red staining). Data were expressed as means ± standard deviation (SD) (n = 4), *P<0.05, **P<0.01 vs. control, #P<0.05 vs. adv-control.

eased Trap, RANK, and Cathepsin K mRNA expression at the early phase of induction (day3), although no significance was found for sOPN treatment (40 ng/ml) compared with control (**Figure 11**). Additionally, no difference was observed in mRNA expression of TRAP, RANK, and Cathepsin K between adv-iOPN and adv-control infected cells. Collectively, these findings demonstrateds OPN (400 ng/ml) promote bone resorption by osteoclasts.

Signaling pathway in the sOPN-induced osteogenic and osteoclastic differentiation

Since sOPN plays a crucial role in osteogenic and osteoclastic differentiation, it would be necessary to delineate which signaling cascades are responsible for the pathophysiological events in bone. Osteopontin can activate the phosphorylation of several kinases (P13K, NIK, MAPK) by interacting with integrin avl33 and CD44 receptors. To investigate the molecular mechanism, we examined the phosphorylation of AKT, NF-KB, p38, JNK and ERK by western blotting. As shown in **Figure 12**, sOPN significantly inhibited the phosphorylation of p38 and AKT, but did not alter the total p38 and AKT in MC3T3-E1 cells. In contrary, sOPN significantly inhibited the phosphorylation of AKT and p65, but did not alter the expression of total AKT and p65 in RAW264.7 cells. These results demonstrated that pAKT and p-p38 were activated in the sOPN-mediated osteogenic differentiation; while in the sOPN mediated osteoclast differentiation, the expression of pAKT and pNF-kB was activated.

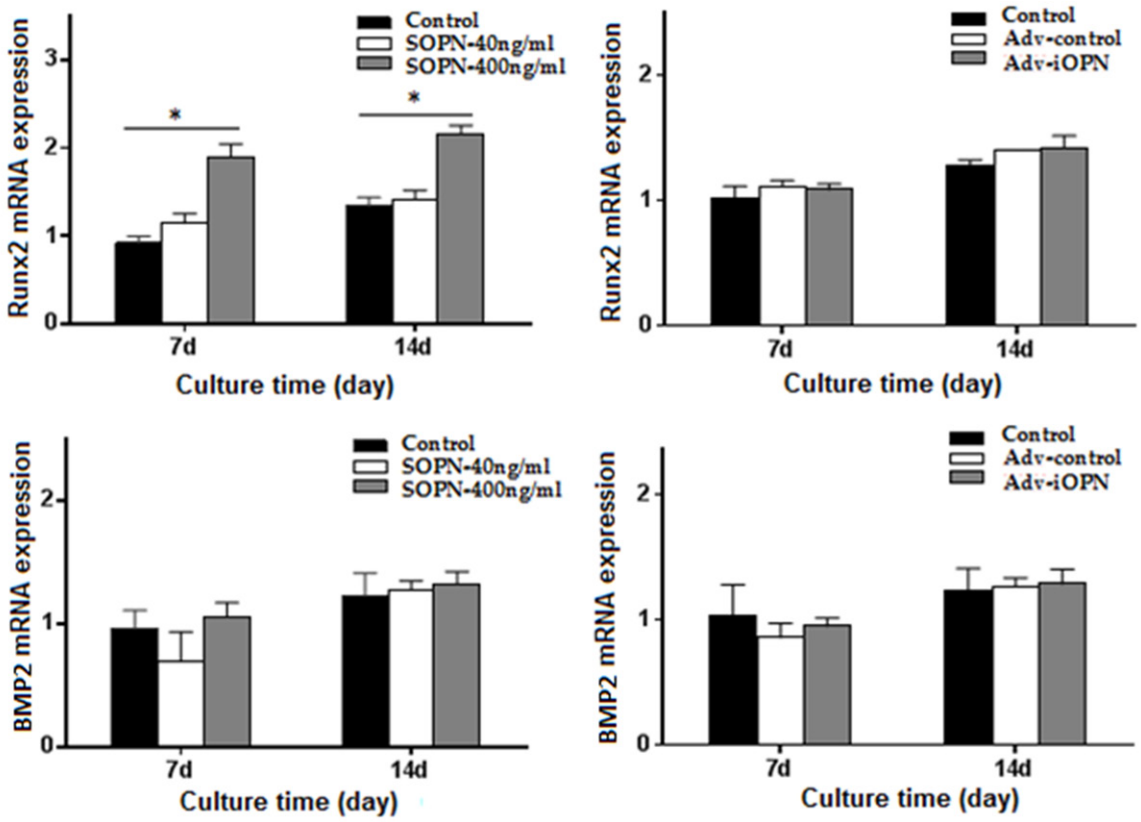


Figure 8. The expression of osteogenic marker genes (Runx-2 and BMP2). Data were expressed as means ± standard deviation (SD) (n = 4), *P<0.05, vs. control.

Discussion

The role of osteopontin (OPN) in bone metabolism has been reported previously [11-13], and it is considered as a kind of secreted cytokine that plays the dual-directional regulation effects in bone balance. OPN can not only affect the mineralization osteogenesis, but also participates in regulating the bone resorption of the osteoclast during bone resorption process. It is the potential target for treating bone destructive diseases such as chronic periodontitis and osteoporosis. To date, the role of OPN in the dynamic balance between bone resorption and reconstruction are still controversial, and the precise mechanism of which remains unclear. The discovery of the intracellular osteopontin (iOPN) subtype may give a reasonable explanation [14-16]. This research was the first to identify the different roles of two subtypes of OPN in bone metabolism. The results indicated that the secreted osteopontin (sOPN) could promote the differentiation and maturity of the MC3T3-E1 cells, as well as the ability to form the mineralized nodules. In addition, it could also promote the differentiation and bone resorption ability of RAW264.7 cells. In contray, the iOPN could remarkably inhibit the proliferation level of RAW264.7 cells, but it showed no notable effects on the proliferation of MC3T3-E1 cells as well as the differentiation of the two kinds of cells. Thus, it could be seen that the effects of the two subtypes of OPN were greatly different, and the roles of OPN in bone metabolism could not be generalized, which had also complicated its mechanism.

In normal bone tissue, the osteoclast (OC) and osteoblast (OB) are maintained at certain quantities, and their interaction in mediating bone resorption and osteogenesis is also in a dynamic balance [18]. The effects of the two OPN subtypes on the proliferation of OB and OC are what we are interested in. CCK-8 assay was conducted in this research, the results of which demonstrated that various concentrations (0, 40, 400 ng/ml) of sOPN had no effect on the proliferation of MC3T3-E1 and RAW264.7 cells,

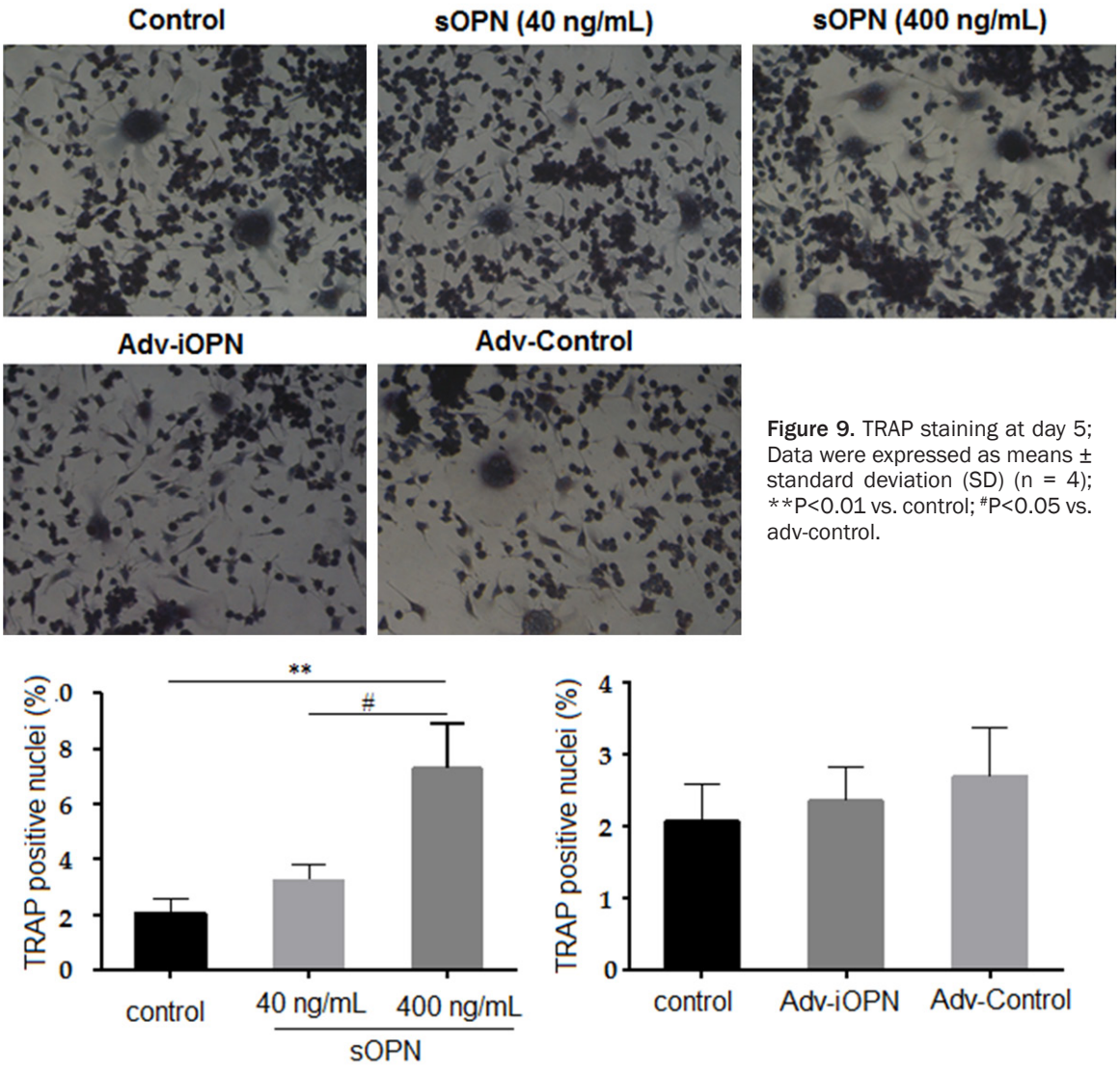


Figure 9. TRAP staining at day 5; Data were expressed as means ± standard deviation (SD) (n = 4); **P<0.01 vs. control; #P<0.05 vs. adv-control.

while iOPN could markedly inhibit the proliferation of RAW264.7 cells, but it did not affect the growth curve of MC3T3-E1 cells. As the proliferation was precisely regulated by the cell cycle, we further investigated in combination with the cell cycle PI staining for the inhibiting effect of iOPN on growth, the results of which indicated that iOPN inhibited cell proliferation mainly through extending the G0 phase and shortening the G2/M phase. There were scholars investigating the effects of OPN on the proliferation of OC previously. Koyama et al [22] discovered in the experiment that the number of OC was distinctly lower in OPN knockout mice than in wild type mice, suggesting that OPN might play an important role in stimulating the number of OC, but the two subtypes were not distinguished in the research. It was suggested in our research that iOPN was the major subtype that inhibited the proliferation process of OC, which was mainly achieved through extending the G0 phase. Such discovery is of great significance, since the balance between bone resorption and osteogenesis is the key factor for maintaining the normal bone mass; theoretically speaking, inhibiting the proliferation of osteoclast without influencing the growth of osteoblast may tilt the balance in the osteogenesis' favor, thus it may become the target for treating bone destructive diseases.

Certainly, apart from the cell proliferation, the differentiation, maturity and activation of OB and OC can affect the balance between bone resorption and bone reconstruction in a more

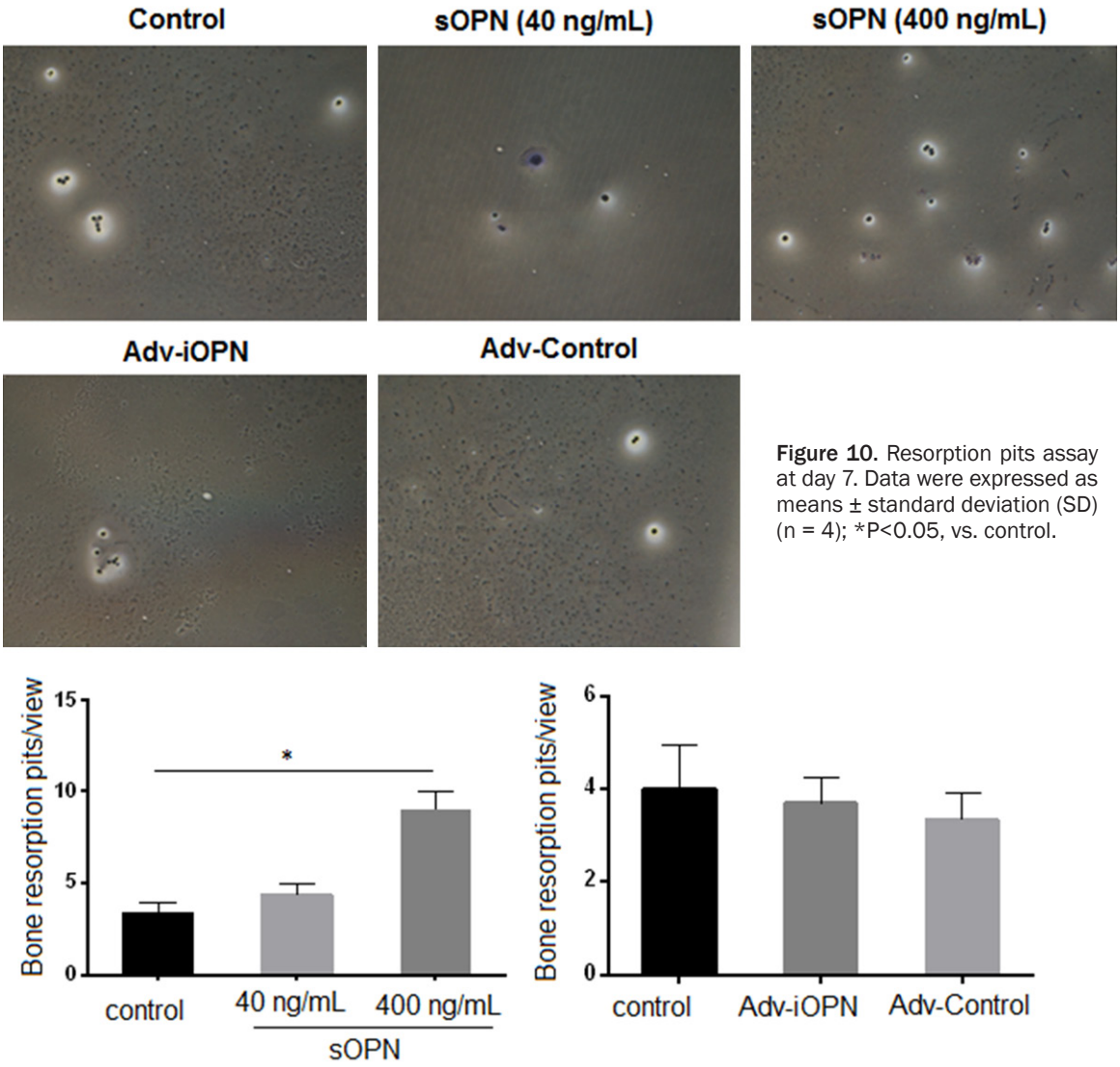


Figure 10. Resorption pits assay at day 7. Data were expressed as means ± standard deviation (SD) (n = 4); *P<0.05, vs. control.

direct way. We further investigated the roles of the two OPN subtypes during this process. In this research, the effects of sOPN and iOPN on the differentiation of the osteoblastic progenitor cells and the mineralization and osteogenesis abilities were determined systemically through the expression of the alkaline phosphatase (ALP), collagen (COL), mineralized nodules and osteogenic differentiation marker genes. During the osteogenesis process, the most obvious feature in the early stage of differentiation is the sharply increased ALP; therefore, ALP is the early marker of mature extracellular matrix [23, 24]. ALP promotes the increase in the content of local phosphoric acid, lowered PH, and matrix mineralization. COL (mainly the type I-COL) is secreted by OB in the osteogenesis and forms the skeleton structure. Some non-collagenous proteins are secreted to the extracellular matrix, which bind with the calcium and phosphorus to form the hydroxyapatite crystal, and the calcium phosphate deposites eventually and forms the mineralized nodules [24-27]. Therefore, the three indicators, namely, ALP, COL and mineralized nodules represent the features in the early, middle and late stages of osteogenic differentiation, respectively. This research promoted notably up-regulated ALP expression on the 4th and the 7th days through inducing the differentiation of MC3T3-E1 cells into OB as well as high concentration of sOPN (400 ng/ml); while the low concentration of sOPN (40 ng/ml) would prompt an increasing trend of the ALP expression, but it was of no statistically significant difference, and iOPN would not affect the ALP expression.

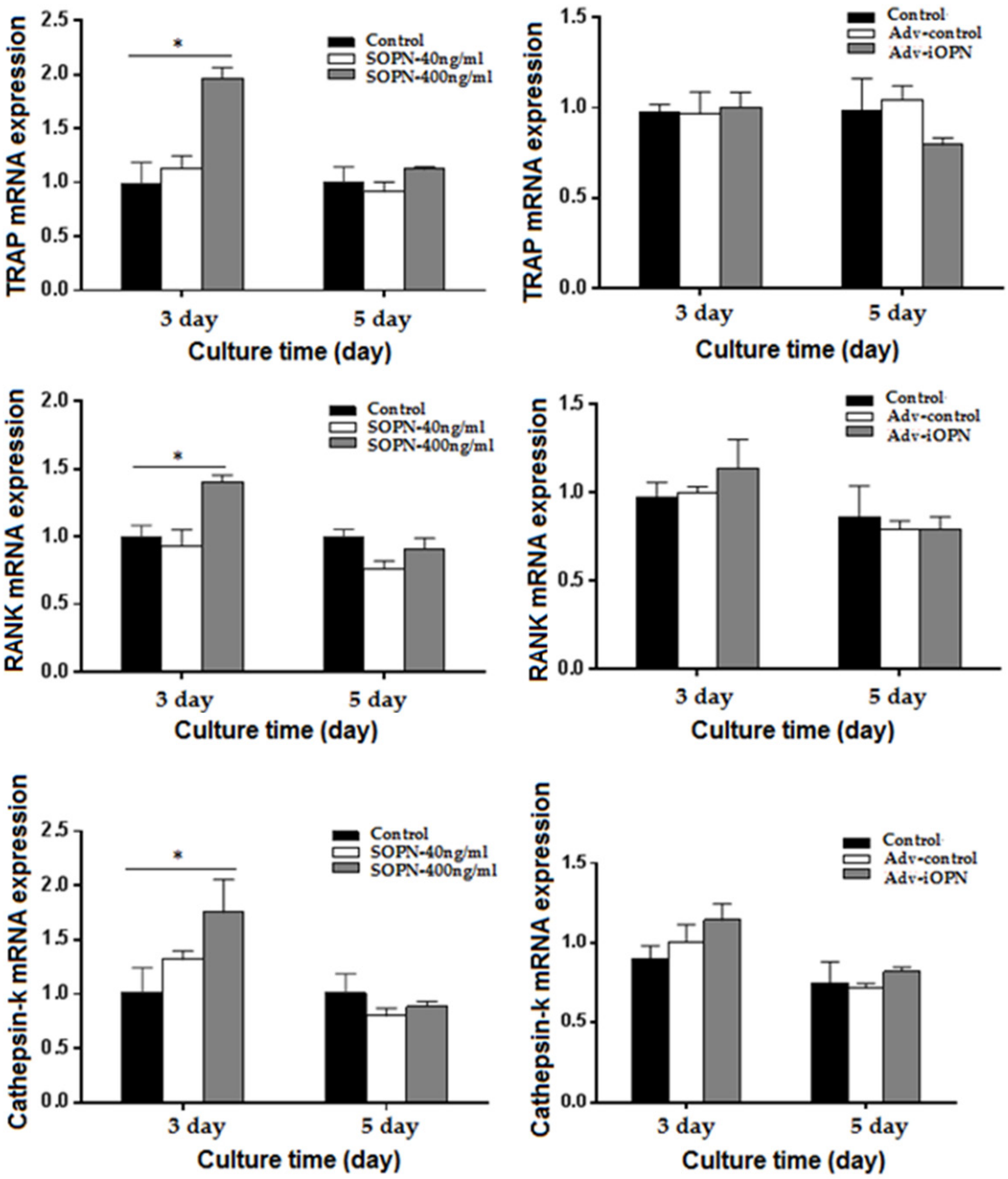


Figure 11. The expressions of osteogenic marker genes (TRAP, RANK and Cathepsin-K). Data were expressed as means ± standard deviation (SD) (n = 4); *P<0.05, vs. control.

sOPN (400 ng/ml and 40 ng/ml) would give rise to remarkably increased secretion of I-COL on the 7th day, as well as markedly up-regulated mineralized nodule expression on the 14th day, and the expression of both COL and mineralized nodules would be notably up-regulated with the increase in sOPN concentration in a dose-dependent manner, but iOPN showed no effect. The whole differentiation process was accompanied with the expression of the differentiation characteristic genes, and the expression of Runx2 mRNA was up-regulated after being treated with 400 ng/ml of sOPN, while iOPN did not affect the expression of these genes. Runx2 is an important cytokines in osteogenic differentiation [28-29], and it can directly stimulate the transcription of genes like osteocalcin (OCN) and type I collagen during

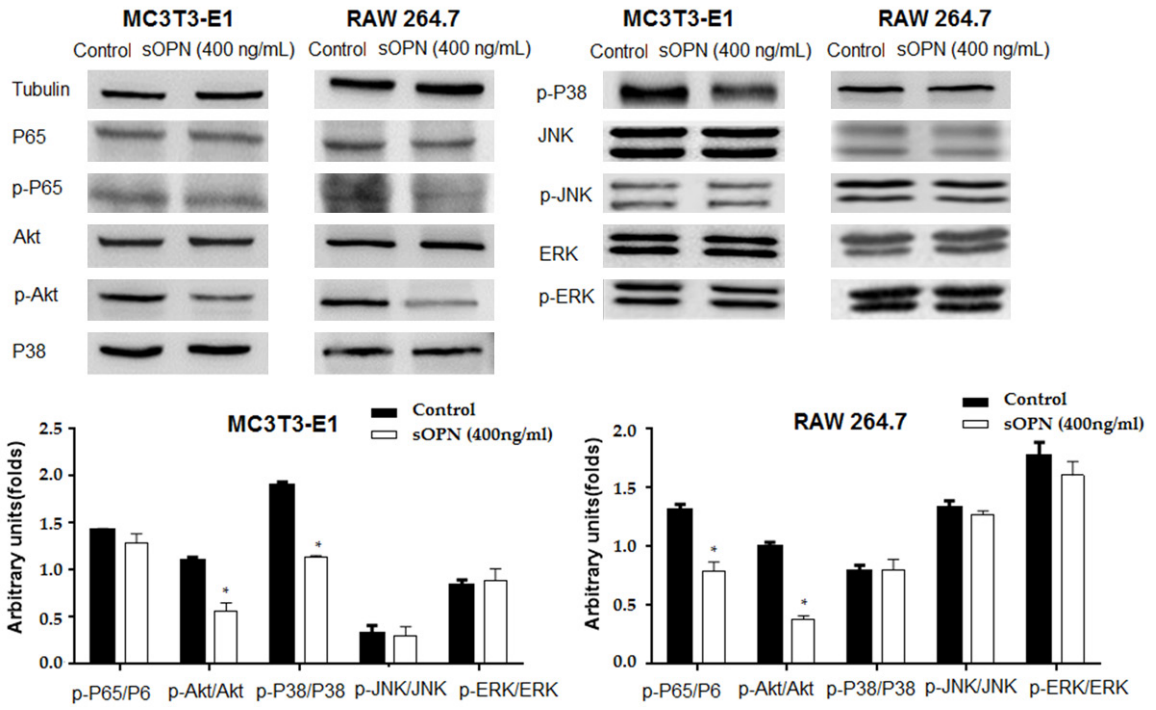


Figure 12. Effects of sOPN on P65, Akt, P38 and ERK. Data were expressed as means ± standard deviation (SD) (n = 4); *P<0.05, **P<0.01 vs. control.

the differentiation of bone marrow-derived mesenchymal stem cell into OB; while the latter has strong affinity of the mineralized component hydroxyapatite in bone and binds with it, in addition, it can recruit OC and OB through signal transduction so as to correspond to bone resorption and deposition, respectively. To sum up, a lot of evidences in our research proved that sOPN played a major role in the differentiation and osteogenic ability of MC3T3-E1, and it showed a concentration-dependent trend, while iOPN did not affect this process.

On the other hand, we conducted research to distinguish the effects of the two OPN subtypes on the differentiation and bone resorption of RAW264.7 cells. Tartrate-resistant acid phosphatase (TRAP) is recognized as the characteristic enzyme of OC, the activity of which can be treated as the symbol of bone resorption function and activity of OC [30]. Bone resorptive pit is the direct result of the bone resorption of OC, the number and size of which directly reflect the bone resorption ability of OC [31]. In this research, both the results of the number of the positive TRAP staining cells and the determination of the number of the bone resorptive pits demonstrated that 400 ng/ml of sOPN could significantly promote the differentiation and bone resorption ability of OC, while the mRNA expression of the characteristic genes cathepsin K, TRAP and RANK was increased during the differentiation process. Cathepsin K was the major enzyme that participated in the degradation of ossein, which was also the manifestation of the bone resorption ability [32]. RANK was the surface receptor of OC, which could promote the differentiation of OC when binding with RANKL, and participate in the regulation of the RANK/RANKL/OPG system [33]. To sum up, our research proved from various aspects that 400 ng/ml of sOPN could enhance the osteoclast differentiation and bone resorption ability, while iOPN did not affect the process.

Our research revealed that sOPN could mediate the differentiation and maturity of the osteoblastic progenitor cell as well as the osteoclast precursors at the same time, and such process involved multiple cytokines, which directly or indirectly regulated the expression of the key nuclear genes through the complicated signal transduction pathways. It was found in this research that the relevant factors in these transduction pathways were of great theoretical and practical significance. sOPN contains

the RGD sequence to bind with the integrin, as well as the sequences to bind with CD44V6/7 or interact with other adhesion receptors, and they bind with each other to initiate a series of signal pathways. It is known that the differentiation-associated downstream effectors of OPN include at least the following: Akt, JNK, p38, ERK and NF-kB [34-38]. It was found in this research that pAKT and p-p38 were activated in the sOPN-mediated osteogenic differentiation; while in the sOPN-mediated osteoclast differentiation, the expression of pAKT and pNF-kB was activated. AKT took part in regulating the differentiation of the two kinds of cells induced by sOPN at the same time, and it was suggested in recent research that, PI3K/Akt signal pathway participated in the proliferation and differentiation of OB and OC, as well as the regulation of apoptosis, which played a key regulating role in bone metabolism. Akt had three kinds of subtypes, namely, Aktl/PKBα, Akt2/PKBβ and Akt3/PKB; and it was suggested in research that AKT1 and AKT2 had stronger association with osteogenesis [39, 40]. The activation of the AKT downstream mTOR signal pathway was the important pathway to regulate the activity of OC and the proliferation of osteoclast precursors. JNK, p38 and ERK were all the members of the MAPKs family, and there was research reporting that JNK, p38 and ERK were associated with the differentiation markers of OB with regard to participating in osteogenic differentiation [41]. It was indicated in this research that P38 might be the major regulating factor for sOPN to induce the differentiation and mineralization process of the osteoblastic progenitor cells. NF-kB played an important role in the recruitment and maturity of macrophages as well as the production of some inflammatory factors (such as TNF-α, IL-1β, IL-6), and it was revealed in this research that sOPN might promote the differentiation and functional expression of OC through up-regulating NF-kB.

Conclusions

The results of this research demonstrated that various OPN subtypes played distinct roles in bone metabolism; to be more specific, the intracellular subtype inhibited the proliferation of the osteoclast precursors, while the extracellular subtype promoted the differentiation and function of both the osteoblastic progenitor cells and the osteoclast precursors. Besides, the effects of sOPN on the differentiation of the two kinds of cells were a regulation process that involved multiple factors. The research results had provided novel target and thinking for treating bone destructive diseases in clinic, such as chronic periodontitis and osteoporosis; however, these results were obtained in vitro, which remained to be further verified in vivo and to investigate its precise regulating mechanism.

Acknowledgements

This paper was supported by the Zhejiang Provincial Natural Science Foundation of China (Grant No. LY14H140007).

Disclosure of conflict of interest

None.

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