Omentin-1 induces osteoblast viability and differentiation via the TGF-β/Smad signaling pathway in osteoporosis

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Abstract. Osteoporosis is a bone-related disease that results from impaired bone formation and excessive bone resorption. The potential value of adipokines has been investigated previously, due to their influence on osteogenesis. However, the osteogenic effects induced by omentin-1 remain unclear. The aim of the present study was to determine the regulatory effects of omentin-1 on osteoblast viability and differentiation, as well as to explore the underlying molecular mechanism. The present study investigated the effects of omentin-1 on the viability and differentiation of mouse pre-osteoblast cells (MC3T3-E1) using quantitative and qualitative measures. A Cell Counting Kit-8 assay was used to assess the viability of MC3T3-E1 cells following treatment with different doses of omentin-1. Omentin-1 and bone morphogenetic protein (BMP) inhibitor were added to osteogenic induction mediums in different ways to assess their effect. The alkaline phosphatase (ALP) activity and Alizarin Red S (ARS) staining of MC3T3-E1 cells treated with omentin-1 and/or BMP inhibitor were used to examine the effects of omentin-1 on differentiation and mineralization. Western blotting was used to further explore its potential mechanism, and to study the role of omentin-1 on the viability and differentiation of osteoblasts. The results showed that omentin-1 altered the viability of MC3T3-E1 cells in a dose-dependent manner. Omentin-1 treatment significantly increased the expression of members of the TGF-β/Smad signaling pathway. In the omentin-1 group, the ALP activity of the MC3T3-E1 cells was increased, and the ARS staining area was also increased. The mRNA and protein expression levels of BMP2, Runt-related transcription factor 2, collagen1, osteopontin, osteocalcin and osterix in the omentin-1 group were also significantly upregulated. All these effects were reversed following treatment with SIS3 HCl. These results demonstrated that omentin-1 can significantly promote osteoblast viability and differentiation via the TGF-β/Smad signaling pathway, thereby promoting bone formation and preventing osteoporosis.

Introduction

Osteoporosis is a debilitating disease that predominantly affects elderly individuals worldwide (1). At present, >200 million patients suffer from osteoporosis worldwide (2). According to research statistics, >1.6 million fractures are caused by osteoporosis (3,4). For women aged >50 years, the risk of suffering from fractures due to osteoporosis is 40-50% (5). Osteoporosis is a systemic skeletal disease that is characterized by a decrease in mass and deterioration of the bone microstructure (6). The consequences brought on by osteoporosis are bone fragility and susceptibility to fractures, and this can cause brittle fractures (7). At the cellular level, the pathogenesis of osteoporosis is primarily related to the disruption of bone remodeling (8). Bone remodeling primarily depends on osteoblast osteogenesis and osteoclast bone absorption, so as to maintain a certain balance (9). When a disease or aberrant inflammation disrupt this balance, osteoporosis may occur (10).

In bone tissues, osteoblasts originate from mesenchymal stem cells (MSCs), whereas osteoclasts are derived from hematopoietic stem cells (11). The differentiation of osteoblasts is achieved through the initial organic phase, which is production of collagen matrix, and the final inorganic phase, which is mineralization by hydroxyapatite crystals on the collagen scaffold (12). After the completion of matrix secretion and mineralization, the osteoblasts, which are embedded in the matrix, are known as bone cells, whereas the osteoclasts remaining on the bone surface turn into flattened lining cells or gradually undergo apoptosis. Importantly, bone marrow MSCs (BMSCs) are pluripotent. Apart from osteoblasts, BMSCs can differentiate into several different cell types, including adipocytes, myocytes, chondrocytes, endothelial
cells and vascular smooth muscle cells (13). Amongst these cell lineages, adipogenic differentiation is particularly relevant to the homeostasis of bone, as cells undergoing osteoblast formation can be diverted to adipocytes, leading to the loss of these cells from the osteoblast pool and the reduction of overall osteogenic potential (13).

The imbalance of adipocyte differentiation and regulation of osteoblasts is commonly seen in aging and diabetic individuals, which can lead to the impairment of fat bone marrow, impairment of osteoblast renewal and an increase in the incidence of chronic bone loss (13-15). Beyond that, adipocytes secrete a series of biologically active signaling molecules, which can also influence bone homeostasis. It has been reported that several of these molecules, including chemerin, resistin, visfatin, leptin and adiponectin, affect the development and function of osteoblasts and osteoclasts (16-21).

Omentin-1, a 34 kDa adipokine selectively expressed in omental adipose tissue, is abundant in the plasma (22,23). Omentin-1 has been demonstrated to serve as a significant factor in multiple physiological processes, including insulin action, cardiovascular function and the inflammatory response (23-25). A previous study demonstrated that omentin-1 plays an important role in protecting against vascular calcification (26). A clinical study has also shown that omentin-1 levels are inversely correlated with obesity and insulin resistance (27). Regarding its effects on bone, a previous study reported that circulating omentin-1 levels are inversely correlated with bone mineral density (BMD) in the lumbar region of the spine in Iranian postmenopausal women (28).

The levels of circulating omentin-1 correlates positively with adiponectin and negatively with body mass index and the leptin levels (29). In a co-culture system of osteoblasts and osteoclast precursors, omentin-1 can reduce osteoclast formation by stimulating osteoprotegerin (OPG) (30). To date, only a few studies have investigated the association between omentin-1 and osteoblast proliferation and differentiation. Moreover, the osteogenic effect induced by omentin-1 remains unclear. The TGF-β/Smad signaling pathway is involved in the regulation of cell differentiation and it is a key pathway related to osteogenesis (31-33). To understand the underlying mechanism driving the effects of omentin-1 on osteogenic differentiation in MC3T3-E1, the role of the TGF-β/Smad pathway in this process was investigated. The aim of the present study was to determine the regulatory effects of omentin-1 on osteoblast viability and differentiation, as well as to explore the underlying molecular mechanism.

Materials and methods

Cell culture and treatment. Mouse embryo osteoblast precursor cell line MC3T3-E1 was obtained from American Type Culture Collection (ATCC, CRL-2593™). The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ humidified incubator. In order to stimulate osteogenic induction, the MC3T3-E1 cells were seeded in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 10 mM β-glycerophosphate, 50 µg/ml ascorbic acid and 10 nM dexamethasone (all Sigma-Aldrich; Merck KGaA) (34). Subsequently, cells were treated with different doses of omentin-1 (100, 500 and 1,000 ng/ml; Cell Science, Inc.) for 24 h at 37°C and/or 3 µM SIS3 HCl (Selleck Chemicals) for 6 h at 37°C for the following experiments.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). iScript™ Reverse Transcription SuperMix kit (Bio-Rad Laboratories, Inc.) was used to reverse transcribe 2 µg RNA into cDNA according to the manufacturer’s protocol. qPCR was performed using SYBR-Green MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR amplification program was as follows: 94°C for 60 sec, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. The primer sequences used for PCR were as follows: Bone morphogenetic protein 2 (BMP2) forward, 5'-AGGCTCTGCTATTGCCATGTCG-3' and reverse, 5'-ATTCTCTGTCGTAACGCCCA-3'; Runt-related transcription factor 2 (Runx2) forward, 5'-CCCAATCTTCTGTCCTC-3' and reverse, 5'-AGTGAATACTTGCCTGTCG-3'; Collagen1 forward, 5'-TAAGGGTGACAGAGGGCATG-3' and reverse, 5'-GGAGCGCTAGGACAGGCTT-3'; osteopontin (Opn) forward, 5'-TCAAAAGTCAGCAGGAATTC-3' and reverse, 5'-CGGGTTGCTGTCCTTCTA-3'; osteocalcin (Ocn) forward, 5'-CTCCTTCATTCCGCGTTCCG-3' and reverse, 5'-GTAAGGCGGCTGTAAGGCCTG-3'; Osterix forward, 5'-TCCTCGGATATGACTCATCCTC-3' and reverse, 5'-CCAAGGAGGTGTTGTTGTCGCC-3' and GAPDH forward, 5'-GGGAACTCCTGGCGTCTGAT-3' and reverse, 5'-GAGTGGCTGGTGTGCGTCA-3'. Gene expression analysis was performed using the 2-ΔΔCt method (35) and normalized to GAPDH.

Cell viability. The cells were cultured in 96-well culture plates at a density of 2x10⁴ cells/well. After 3 days, a Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology) was performed to evaluate cell viability. The CCK-8 solution was added to each group, and the cells were incubated at 37°C for 1 h. The absorbance of each well was measured at a wavelength of 450 nm using a microplate spectrophotometer.

Western blot assay. Cells in the different treatment groups were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentrations were measured using Bradford reagent (Bio-Rad Laboratories, Inc.). Equal quantities of proteins (40 µg/lane) were separated via 10% SDS-PAGE (Bio-Rad Laboratories, Inc.) and transferred to a PVDF membrane (MilliporeSigma). After being blocked with 5% non-fat milk in 0.1% tris-buffered saline with Tween-20 for 1 h at room temperature, the membranes were incubated with primary antibodies against BMP2 (1:1,000; cat. no. ab214821; Abcam), phosphorylated (p)-Smad1 (1:1,000; cat. no. ab226821; Abcam), p-Smad5 (1:2,000; cat. no. ab92698; Abcam), Smad1 (1:1,000; cat. no. ab33902; Abcam), Smad5 (1:1,000; cat. no. ab40771; Abcam), Runx2 (1:1,000; cat. no. ab236639; Abcam), collagen1 (1:1,000; cat. no. ab138492; Abcam), Opn (1:1,000; cat. no. ab63856; Abcam), Ocn (1:1,000; cat. no. ab133612; Abcam) and osterix (1:1,000; cat. no. ab209484; Abcam) and GAPDH (1:2,500;
cat. no. ab9485; Abcam) overnight at 4°C, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. #7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. The blots were then visualized using an enhanced chemiluminescence system (Beyotime Institute of Biotechnology). Densitometry analysis was performed using ImageJ (Version 1.49; National Institutes of Health).

Cell differentiation analysis. Alkaline phosphatase (ALP) activity, an early biochemical marker widely used to assess osteogenic activity, was measured. Briefly, the cells were seeded in 12-well culture plates at a density of 4x10^4 cells per ml for 7 days. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature, followed by treatment with nitroblue tetrazolium (Sigma-Aldrich; Merck KGaA) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. Cells were washed with deionized water and observed under an inverted light microscope (Nikon Corporation; x200 magnification).

Cell mineralization analysis. Mineralization was evaluated by quantifying the formation of calcium phosphate in cells using an Alizarin Red Staining (ARS) kit (Sigma-Aldrich; Merck KGaA). Briefly, the cells were cultured in 12-well culture plates at a density of 4x10^4 cells per ml. After 21 days, the cultured cells were fixed with 95% ethanol for 10 min at room temperature. To stain the calcium deposits, 2% ARS solution (Sigma-Aldrich; Merck KGaA) was applied for 15 min at room temperature. To measure the degree of mineralization, the ARS released from the cell matrix was incubated in cetyl pyridinium chloride for 15 min at room temperature and quantified by spectrophotometry at 540 nm.

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. Differences between groups were determined using an unpaired two-tailed Student's t-test for comparisons between two groups, and one-way ANOVA with a post hoc Tukey's test was used for comparisons between multiple groups. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Omentin-1 stimulates viability of osteoblasts. The effect of omentin-1 on MC3T3-E1 cell viability was assessed using a CCK-8 assay. As shown in Fig. 1, the viability of osteoblasts was altered in a dose-dependent manner following treatment with omentin-1, and the effect was significant when treated with 1,000 ng/ml omentin-1 after 3 days of treatment.

Omentin-1 activates the Smad signaling pathway and increases BMP2 expression in osteoblasts. To explore the potential mechanisms by which omentin-1 exerted its effects on osteoblasts, western blotting was performed. As shown in Fig. 2, omentin-1 at a dose of 1,000 ng/ml increased the protein expression levels of BMP2, p-Smad1 and p-Smad5 (P<0.05). However, 1,000 ng/ml omentin-1 did not exert a notable effect on the expression levels of Smad1 and Smad5.

Figure 1. Effects of different concentrations of omentin-1 on the viability of MC3T3-E1 cells. Cell Counting Kit-8 assay was performed to assess cell viability. Results are expressed as the mean ± SD. ***P<0.001 vs. control.

Omentin-1 promotes the viability of osteoblasts through the TGF-β/Smad signaling pathway. As shown in Fig. 3A and B, SIS3 HCl, a Smad inhibitor, significantly inhibited BMP2 protein levels and phosphorylation of Smad1 and Smad5 compared with the control group, while 1,000 ng/ml omentin-1 significantly increased the expression levels of the three proteins. Meanwhile, SIS3 HCl attenuated the effects of omentin-1 on the expression of BMP2, p-Smad1 and p-Smad5 in MC3T3-E1 cells. In addition, CCK-8 analysis showed a significant increase in cell viability in omentin-1-treated MC3T3-E1 cells, and this increase in viability was significantly attenuated by SIS3 HCl (Fig. 3C).

Omentin-1 regulates differentiation and mineralization of osteoblasts via the TGF-β/Smad signaling pathway. Next, the effects of omentin-1 on osteoblasts were assessed. As shown in Fig. 4A and B, ARS staining in the omentin-1-treated cells showed an increased mineralization rate compared with the control group, and SIS3 HCl significantly attenuated the omentin-1-induced mineralization. In addition, ALP activity was significantly increased in the omentin-1 group, whereas SIS3 HCl significantly weakened the activity of ALP in MC3T3-E1 cells (Fig. 4C).

Analysis of cellular Runx2, collagen1, Opn, Ocn and osterix expression. Data from RT-qPCR and western blot assays showed that the expression levels of osteogenesis-related proteins, including BMP2, Runx2, collagen1, Opn, Ocn and osterix, were significantly upregulated in the omentin-1 group. However, the expression levels of these proteins in MC3T3-E1 cells treated with SIS3 HCl were significantly reduced (Fig. 5A-C).

Discussion

Bone remodeling and skeletal homeostasis are dynamic life-long processes that rely on the balance and integrated actions between osteoblastic bone formation and osteoclastic bone resorption (36-38). Osteoblastic lineage cells interact with osteoclastic lineage cells, which is necessary for the formation of functional osteoclasts (39). Certain bone-related diseases, such as osteoporosis, rheumatoid arthritis and
osteoporosis, are associated with abnormal bone metabolism and accelerated bone loss (40). Bone formation and repair are intrinsically associated with the balanced activity of osteoblasts (41). Osteoblasts are responsible for bone matrix secretion and mineralization, and they tightly regulate osteoclast activation and differentiation (42). Dysfunctional behaviors of osteoblasts lead to improper bone matrix deposition and mineralization, affecting the size, shape and integrity of the skeletal structures (43).

MC3T3-E1, a popular osteoblast cell line that represents a pre-osteoblastic phenotype, is a mouse calvaria clonal cell line that has given rise to several subclones (44). Decreased proliferative activities have been observed at passage numbers above 36, whereas their replicative senescence has been displayed when these cells reach a high passage number of 60, which is similar to that of human cells, at which point they show inconsistent cell cycling (45). This important feature also makes these cells suitable as novel models...
for in vitro bone research, including bone remodeling and formation (46).

Omentin-1, which was discovered from a human omental fat cDNA library, is primarily expressed in visceral adipose tissue (23). Omentin-1 can promote osteogenic differentiation, although this has been contested (47-52). Therefore, the present study investigated the effects of omentin-1 on MC3T3-E1 cells by observing proliferation patterns, with the aim of determining the regulatory effect of omentin-1 and identifying the underlying molecular mechanisms. When MC3T3-E1 cells were treated with different concentrations of omentin-1 (100-1,000 ng/ml) for 3 days, the viability of MC3T3-E1 cells increased significantly. Additionally, the effect of omentin-1 was weakened after adding a BMP inhibitor. This suggested that omentin-1 could stimulate the viability of osteoblasts. Based on these experiments, 1,000 ng/ml omentin-1 was used for subsequent experiments, and 0 ng/ml omentin-1 was used as the control group to further study the effects of omentin-1 on osteoblasts.

Regulation of gene expression by TGF-β is achieved primarily through the initiation of intracellular signal transduction and the activation of the Smad signaling pathway (53,54). TGF-β promotes osteogenic differentiation, and at the same time, it also inhibits adipogenic differentiation of human MSCs (55). The TGF-β/Smad pathway is also key to adipogenesis (56). TGF-β inhibits adipogenic differentiation in human preadipocytes, which is mediated primarily by the Smad family members (57,58). Smad3 inhibits adipogenic conversion, whereas interfering with Smad3 function confers resistance to inhibition of adipogenesis by TGF-β (56). The present results showed that SIS3 HCL treatment significantly inhibited the protein expression levels of BMP2, p-SMAD1 and p-SMAD5, while 1,000 ng/ml omentin-1 increased the protein expression levels of BMP2 and p-Smad1/5. However, 1,000 ng/ml omentin-1 exerted no effect on Smad1 and Smad5, which are also members of the TGF-β/Smad pathway.

To confirm the effects of the omentin-1/TGF-β/Smad pathway on MC3T3-E1 cells, cells were pretreated with a selective BMP TGF-β/Smad signaling inhibitor, SIS3 HCl (3 µM per day), 3 days prior to treatment with 1,000 ng/ml omentin-1. Cell viability was promoted in the 1,000 ng/ml omentin-1 group, and SIS3 HCl attenuated the increase in viability mediated by omentin-1. The results from the CCK-8 assays revealed that omentin-1 significantly promoted the viability ability of the MC3T3-E1 cells via the TGF-β/Smad pathway.

In previous years, several studies have investigated the relationship between omentin-1 and bone metabolism. On the one hand, adiponectin has been shown to induce osteoblast proliferation and differentiation (50), as well as to increase bone mass via osteoclastogenesis suppression and osteoblastogenesis activation (51), and the results of the present study were in agreement with these previous findings. Conversely, adiponectin has been verified to induce osteoclast formation via stimulation of RANKL and inhibition of OPG production (48,49). This suggests that adipokines also serve an important role in osteoclastic balance; thus the effects of adipokines on osteoclasts should form the basis of future studies. A previous study showed that there was an inverse correlation between omentin-1 and broadband ultrasound
attenuation levels in postmenopausal women. This association was not mediated by OPG levels (52). The results from in vitro and in vivo studies have shown contradictory results. Results from a meta-analysis of observational studies demonstrated an inverse relationship between adiponectin levels and BMD, with five studies showing a negative association, and another five showing no association (47). The reasons for such discrepant findings are unclear, but it is conceivable that the microenvironment in human bodies can be affected by various factors. The ability of cell culture and animal models to emulate the complex processes that take place in the human body is limited, such as possible adiponectin-dependent counter-regulatory mechanisms.

Osteoporosis, a type of bone-related disease, results from impaired bone formation and excessive bone resorption. Current strategies to augment bone formation, such as the use of growth factors (59), have shown promising results. However, the osteoblast lineage specification and bone regeneration remain unclear. An improved understanding of these may considerably improve the therapeutic strategies available.
In order to determine how omentin-1 guides osteoblast bone formation, the effects of several potential mediators involved in the pro-osteogenic pathway were assessed using western blotting in the current study. Osteoblasts are derived from osteoprogenitors that differentiate by progressively expressing maturation markers (60). Runx2 is a transcription factor that serves a role in osteoblast differentiation. Previous studies have shown that silencing of Runx2 can block the differentiation of osteoblasts in mice (61,62). Patients with cleidocranial dysplasia, which is caused by heterozygous mutations in the Runx2 gene, are characterized by an underdeveloped collarbone, short stature, excess teeth, fontanelle patency and other bone growth-related defects (63). Osterix is a zinc finger transcription factor present in osteoblasts and serves a leading role in the osteoblast differentiation process (63). A previous study showed that Runx2 can regulate the levels of osterix (64). Collagen-1 is a primary structural protein present in the extracellular space in the bone, making up 25-35% of the entire-body protein content (65). Depending upon the degree of mineralization, collagen tissues may be rigid (bone), compliant (tendon) or have a gradient from rigid to compliant (cartilage) (66). As the skeleton is the main structural component of the body, collagen-1 is responsible for maintaining the strength of these structures (67). Opn is also known as bone sialoprotein 1, a protein in humans encoded by the SPP1 gene (68). Ocn is a vitamin K-dependent calcium-binding protein (69). Opn and Ocn are two typical biomarkers of osteoblasts, and are involved in osteogenic differentiation and the mineralization of extracellular matrix during bone formation and repair (70,71). Previous studies have shown that Runx2 stimulates the differentiation of MSCs into osteoblasts by regulating Opn and Ocn activity (61,72). In the present study, it was shown that omentin-1 significantly upregulated the expression of Runx2, Collagen-1, Opn, Ocn and osterix, suggesting that it accelerated bone formation.

Based on the aforementioned results, it was noticed in the present study that SIS3 HCL treatment alone only affected the TGF-β/Smad pathway, but had no significant effects on the viability, differentiation and mineralization of osteoblasts and the expression of osteogenesis-related proteins. However, SIS3 HCL exerted obvious roles when the cells were treated with omentin-1. Hence, we speculated that under normal conditions, osteoblasts were only partially affected by SIS3 HCL except for the expression of TGF-β/Smad pathway, but omentin-1 had significant promoting effects on cell differentiation and mineralization via regulating the TGF-β/Smad pathway. Thus, there is a noticeable effect of SIS3 HCL on biological function and signaling and omentin-1-mediated osteoblasts.

In conclusion, the present study examined the potential effects of omentin-1 on the viability and differentiation of osteoblasts and the signaling pathways involved (Fig. 6). Omentin-1 promoted osteoblast viability in a dose-dependent manner. Western blotting revealed that omentin-1 induced the activation of BMP2 and p-Smad1/5. Furthermore, omentin-1 promoted osteoblast viability, differentiation and mineralization, and these effects were impeded by a TGF-β/Smad inhibitor. These findings indicated that omentin-1 promoted osteoblast viability and differentiation via the TGF-β/Smad signaling pathway, suggesting that omentin-1 may be a potential target in the treatment of osteoporosis.
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