Omentin-1 attenuates arterial calcification and bone loss in osteoprotegerin-deficient mice by inhibition of RANKL expression

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Aims
Omentin-1 (also known as intelectin-1) is a recently identified visceral adipose tissue-derived cytokine that is inversely related to obesity. Our previous study showed that omentin-1 inhibits osteoblastic differentiation of calcifying vascular smooth muscle cells (CVSMCs) in vitro. This study was undertaken to investigate the effects of omentin-1 on arterial calcification and bone metabolism in vivo.

Methods and results
In vitro, omentin-1 stimulated production of osteoprotegerin (OPG) and inhibited production of receptor activator for nuclear factor ΚB ligand (RANKL) in both CVSMCs and osteoblasts. In vivo, adenovirus-mediated over-expression of omentin-1 attenuated arterial calcification and bone loss in OPG−/− mice. All these in vitro and in vivo actions were abrogated by blockade of the PI3K–Akt signalling pathway. Furthermore, omentin-1 reduced serum levels of RANKL, tartrate-resistant acid phosphatase-5b and osteocalcin, all of which are increased dramatically in OPG−/− mice.

Conclusion
These data suggest that omentin-1 ameliorates arterial calcification and bone loss in vivo through the regulation of the RANK signalling pathway.

Keywords
Omentin-1 • Arterial calcification • Bone • Osteoprotegerin • Receptor activator for nuclear factor ΚB ligand

1. Introduction
Patients with osteoporosis display a high prevalence of arterial calcification. Bone loss and arterial calcification frequently occur simultaneously and share many of the same risk factors, such as ageing, estrogen deficiency, inflammatory diseases, chronic renal failure, or the use of glucocorticoids. The osteoprotegerin–receptor activator for nuclear factor ΚB ligand (OPG–RANK–RANKL) autocrine/paracrine axis represents one of the most promising targets for mediating arterial calcification and bone homeostasis.

Adipose tissue has been recognized as a highly active endocrine organ that, in addition to the uptake, storage, and synthesis of lipids, secretes a variety of adipokines (e.g. leptin, resistin, and adiponectin) controlling insulin sensitivity, neuroendocrine activity, food and water intake, breeding, inflammatory response, cardiovascular function, and bone metabolism. Omentin-1, a novel adipokine, is primarily expressed in visceral adipose tissue and is highly abundant in plasma. This protein, also called intelectin-1, is expressed in intestinal Paneth cells as well. Analysis of the 313 amino acid full-length human omentin-1 protein sequence suggests that the amino-terminal portion of omentin-1 contains a highly hydrophobic region that is a typical signal sequence for protein secretion. The circulating concentration of omentin-1 is decreased in patients with obesity or diabetes and increased after weight loss, correlating positively with serum adiponectin and high-density lipoprotein levels, and negatively with body mass index, waist circumference, insulin resistance, and serum leptin levels. Although the biological function of omentin-1 is largely unknown, recent reports have demonstrated that omentin-1...
promotes insulin-mediated glucose transport in adipocytes,9 induces
temol in isolated blood vessels,14 and inhibits tumour necro-
sis factor-induced vascular inflammation in human endothelial cells.15
We recently demonstrated that omentin-1 inhibits osteoblastic
differentiation of human calcifying vascular smooth muscle cells (CVSMCs) in vitro.16
Osteoprotegerin knock-out (OPG-/-) mice exhibit progressive
massive arterial calcification and intensive high-turnover bone loss,
and display characteristics of normal multiplication and growth;16 therefore, this animal model is ideal for investigating whether
omentin-1 regulates arterial and bone metabolism depending on the
RANK pathway in vivo. Thus, we aimed to test the effects of
omentin-1 on arterial calcification and bone metabolism in OPG-/-
mice.

2. Methods

More detailed information on methods is in the Supplementary material
online.

2.1 Ethics statement

The investigation conforms to the Guide for the Care and Use of Laboratory
Animals published by the United States National Institutes of Health (NIH
publication no. 85-23, revised 1996). Approval was granted by the Second
Xiangya Hospital of Central South University Ethics Review Board.

2.2 Reagents

Recombinant human omentin-1 was purchased from Cell Sciences Inc.
(Canton, MA, USA). Pentobarbital sodium, dimethyl sulfoxide,
1,25-dihydroxyvitamin D3 [1,25(OH)2D3], phenol red-free α-minimal
essential medium (α-MEM), and Dulbecco’s modified Eagle’s medium
were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum
was purchased from Gibco-BRL Corp. (Grand Island, NY, USA).
LY294002 and 1,6-hydroxyethyl-chiro-inositol 2-(R)-2-O-methyl-3-
0-octadecylcarbonate (HIMO) were purchased from Calbiochem Corp.
(San Diego, CA, USA). Recombinant mouse RANKL, mouse OPG,
and human adiponectin were purchased from R&D Systems (Minneapolis,
MN, USA). Antibodies for OPG, RANKL, and β-actin were purchased
from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies
for Akt and phosphorylated Akt (p-Akt) were purchased from Cell Signal-
ing Technology Inc. (Danvers, MA, USA).

2.3 Cell culture

To isolate primary cells, mice received a 150 mg/kg intraperitoneal dose of
pentobarbital sodium prior to death, which was confirmed by the absence
of a heartbeat.

Primary mouse calvarial osteoblasts and bone marrow cells were iso-
lated from 8-week-old female wild-type (WT) mice or OPG-/-
mice.17–19 vascular smooth muscle cells (VSMCs) and CVSMCs were iso-
lated from 8-week-old female WT mice.20,21

2.4 Assessment of proliferation, osteocalcin
secretion and matrix mineralization in primary
mouse osteoblasts

Osteoblast proliferation was measured using the [3H]thymidine incorpor-
ation assay.22 Osteocalcin secretion and matrix mineralization were also
measured.18

2.5 Genetic suppression of Akt by small
interfering RNA

To suppress Akt, the osteoblasts were cultured and transfected with either
Akt small interfering RNA (siRNA) or control siRNA (Santa
Cruz Biotechnology Inc.) using Lipofectamine 2000 (Invitrogen, Carlsbad,
CA, USA). The levels of Akt expression were assessed by western blotting
as described below.

2.6 Analysis of OPG and RANKL mRNA
expression by quantitative real-time PCR
(qRT-PCR)

Total RNA was isolated using TriZol (Invitrogen). Reverse transcription
and qRT-PCR methods and primer sequences are available in Supplementary
material online. The qRT-PCR was performed using a Roche Molecular
Light Cycler (Roche Molecular Biochemicals, Indianapolis, IN, USA).

2.7 Analysis of Runx2, OPG, RANKL, Akt, and
p-Akt expression by western blotting

Equal amounts of protein (40 μg/lane) were analysed for Runx2, OPG,
RANKL, p-Akt, and Akt by western blotting.16,18

2.8 Measurements of cellular OPG and RANKL
protein secretion by ELISA

Cell supernatants were collected for OPG and RANKL protein pro-
duction assay using commercial ELISA kits according to the manufacturers’
instructions. Cells were also harvested and counted. The RANKL and
OPG levels were normalized to cell numbers in each well. The mouse
OPG and RANKL ELISA kits were obtained from R&D Systems.

2.9 Mice

Female OPG-/- and WT mice (C57BL/6J) were provided by the Shanghai
Research Center for Biomodel Organisms. An OPG knockout construct
was prepared as described by Bucay et al.23 The animals were genotyped
by PCR analysis as previously described.23,24

Adenovirus producing the full-length human omentin-1 was prepared by
using the Adenovirus Expression Vector Kit (Takara, Kyoto, Japan).
Adenovirus β-galactosidase (Ad-Gal; control) or adenovirus omentin-1
(Ad-Ome) at a dose of 1 × 10⁸ plaque-forming units, or a comparable
volume of vehicle (phosphate-buffered saline), was injected into the tail
vein of 4-week-old OPG-/- or WT mice once every 2 weeks for a
total of 8 weeks.

In a separate experiment, the phosphoinositide 3-kinase (PI3K) inhibitor
LY2940029 (20 mg/kg; dissolved in dimethyl sulfoxide) or vehicle
(dimethyl sulfoxide) was intraperitoneally injected into the abdomen of
4-week-old OPG-/- mice 1 day before first injection of Ad-Ome or
Ad-Gal and every other day for 6 weeks.

At the end of administration, blood was collected with a capillary tube
from the retro-orbital venous plexus of 8 fasted mice under general
anaesthesia (a tactile stimulus failed to induce a response, and the forelimb
or hindlimb pedal withdrawal reflex response was absent or delayed) with
pentobarbital sodium (50 mg/kg intraperitoneal) which did not kill the
mice. The serum was separated from the blood and stored at −70°C
until analysis. Bones and arteries were also dissected for further analysis.

2.10 Measurements of serum omentin-1,
adiponectin, RANKL, tartrate-resistant acid
phosphatase-5b, and osteocalcin

The levels of serum omentin-1, adiponectin, RANKL, tartrate-resistant acid
phosphatase-5b (TRAP-5b), and osteocalcin were detected by performing
an ELISA or an RIA using commercial kits according to the manufacturers’ instructions.
2.11 Measurement of arterial calcification in mice
The thoracic aorta was dissected, rinsed in phosphate-buffered saline, and fixed in 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C. Haematoxylin and eosin staining was used to detect calcification, and the images were captured with a digital microscope (Leica Microsystems, Wetzlar, Germany). To measure calcium content, aortic segments were dissected and dried at 55°C, and calcium was extracted with 10% formic acid overnight at 4°C. The colorimetric quantification of calcium was achieved through a reaction with o-cresolphthalein, and total protein was determined using the Bradford protein assay.

2.12 Bone mineral density measurement
The whole left femur and lumbar vertebral segment (L2–L4) of each mouse were scanned by dual energy X-ray absorptiometry (DXA) using a PIXIImus densitometer (GE Lunar Corp., Madison, WI, USA) to determine the bone mineral density (BMD).

2.13 Tartrate-resistant acid phosphatase staining
Deparaffinized bone sections or formalin-fixed cells were stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts) using a TRAP staining kit (Sigma).

2.14 Statistical analysis
All calculations were performed using SPSS version 13.0 for Windows (SPSS, Chicago, IL, USA). Data are presented as means ± SD. Comparisons were made using a one-way ANOVA followed by the LSD post hoc test for multiple comparisons, and differences were considered significant at \( P < 0.05 \).

3. Results

3.1 Omentin-1 stimulates proliferation and inhibits differentiation in primary mouse osteoblasts
As shown in Supplementary material online, Figure S1A–C, omentin-1 (25–200 ng/mL) stimulated \([^{3}H]\)thymidine incorporation and suppressed osteocalcin secretion and Runx2 protein expression in osteoblasts in a dose-dependent manner. Omentin-1 (200 ng/mL) also inhibited matrix mineralization in osteoblasts (see Supplementary material online, Figure S1D).

3.2 Omentin-1 stimulates OPG and inhibits RANKL production in primary mouse osteoblasts and CVSMCs
As shown in Figure 1A, in osteoblasts, the levels of OPG mRNA were dramatically increased and the levels of RANKL mRNA dramatically decreased after treatment with omentin-1 starting at 25 ng/mL, indicating that omentin-1 up-regulates OPG expression and down-regulates RANKL expression at transcriptional level. In OPG−/− osteoblasts, the levels of RANKL mRNA were also dramatically reduced after the treatment with omentin-1 at 200 ng/mL (Figure 1A). Omentin-1 (25–200 ng/mL) was also observed to stimulate OPG protein production in culture media of osteoblasts, CVSMCs, and VSMCs, and to inhibit RANKL protein production in culture media of osteoblasts and CVSMCs in a dose-dependent manner (Figure 1B); however, RANKL protein was not detected in culture media of VSMCs by ELISA.

3.3 Omentin-1 regulates OPG and RANKL production in osteoblasts and CVSMCs through the PI3K–Akt signalling pathway
Omentin-1 stimulated the activity of Akt in osteoblasts and CVSMCs after 5, 15, 30, or 60 min of incubation, as demonstrated by an increase in the levels of phosphorylated Akt; the peak activation of Akt occurred at 30 min (Figure 2A). Transfection of osteoblasts with Akt siRNA inhibited Akt protein expression (Figure 2B).

Pre-treatment of osteoblasts or CVSMCs with both LY294002 (a selective PI3K inhibitor) and HIMO (a selective Akt inhibitor) had no effect on basal OPG and RANKL mRNA expression or protein secretion, but abolished the effects of omentin-1 on OPG and RANKL production (Figure 2C and D). The findings with Akt siRNA corroborated the findings with LY294002 and HIMO. Treatment of osteoblasts with Akt siRNA suppressed the effects of omentin-1 on OPG and RANKL mRNA expression (Figure 2C).

3.4 Omentin-1 has no effect on the circulating endogenous adiponectin levels in WT and OPG−/− mice
At the end of the animal experimental protocol, measurements for serum omentin-1 and adiponectin levels were undertaken, as shown in Supplementary material online, Table S1. Circulating endogenous omentin-1 and adiponectin levels in all WT mice were equal to those in OPG−/− mice. No difference in serum endogenous omentin-1 or adiponectin was observed between each of the WT and OPG−/− mice. Treatment of WT or OPG−/− mice with Ad-Ome resulted in an approximately 1.5-fold increase in the serum omentin-1 levels compared with those in vehicle- or Ad-Gal-treated WT or OPG−/− mice.

3.5 Omentin-1 inhibits vascular calcification in OPG−/− mice
As shown in Figure 3A, microscopic examination of haematoxylin and eosin stained sections revealed massive calcification in the media of thoracic aortas from OPG−/− mice compared with those from WT mice; however, calcification was significantly attenuated in the arteries of Ad-Ome-treated OPG−/− mice. The calcium content in the aortas from OPG−/− mice also increased relative to WT mice (Figure 3B). Treatment with Ad-Ome significantly reduced the aortic calcium content in OPG−/− mice (Figure 3B). Treatment of OPG−/− mice with LY294002, a selective PI3K inhibitor, aggravated arterial calcification by itself, and abolished the beneficial effect of omentin-1 on attenuation of arterial calcification in these mice (Figure 3C).

3.6 Omentin-1 partly restores BMD in OPG−/− mice
TRAP-stained sections of the metaphyseal region of the distal femur of each mouse showed increased numbers of osteoclasts in OPG−/− mice (treated with either vehicle or Ad-Gal) compared with those in WT mice; however, calcification was significantly attenuated in the arteries of Ad-Ome-treated OPG−/− mice.

DXA scanning and analyses of the left femur and lumbar vertebral segment (L2–L4) of each mouse demonstrated that the femurs and lumbar vertebrae of vehicle- or Ad-Gal-treated OPG−/− mice
Omentin-1 regulated OPG and RANKL production in osteoblasts and CVSMCs. Osteoblasts were cultured in the serum-free α-MEM and treated with vehicle or omentin-1 (25–200 ng/mL) for 48 h. (A) Total RNA was extracted for OPG and RANKL mRNA expression assay by qRT-PCR. Results are expressed as fold of the vehicle-treated group. (B) Cell culture media were collected for OPG and RANKL protein production assay using ELISA kits. The bars represent the means ± SD, n = 5, *p < 0.05.
Figure 2. Omentin-1 regulated OPG and RANKL production in osteoblasts and CVSMCs through the PI3K–Akt signalling pathway. (A) Western blot analysis of Akt activation. The osteoblasts and CVSMCs were cultured in serum-free α-MEM for 6 h and then treated with omentin-1 (200 ng/mL) for 5–60 min. The cell lysates were analysed by western blotting and incubated with antibodies against p-Akt and Akt. Representative results are shown. (B) Either control siRNA or Akt siRNA was transfected into osteoblasts. Expression of Akt was determined by western blot analysis using an Akt antibody. Representative results are shown. (C) The osteoblasts were pre-treated with vehicle, PI3K inhibitor LY294002 (10 μM), or Akt inhibitor HIMO (10 μM) for 3 h prior to treatment with omentin-1 (200 ng/mL) for 48 h. The cells were also transfected with control siRNA or Akt siRNA before treatment with omentin-1 (200 ng/mL) for 48 h. The total RNA was extracted for OPG and RANKL mRNA expression assay by qRT-PCR. Results are expressed as fold of the vehicle-treated group. (D) The osteoblasts and CVSMCs were pre-treated with vehicle, PI3K inhibitor LY294002 (10 μM), or Akt inhibitor HIMO (10 μM) for 3 h prior to treatment with 200 ng/mL omentin-1 for 48 h. The cell culture media were collected for OPG and RANKL protein production assay using ELISA kits. The bars represent the means + SD, n = 5, *P < 0.05.
Figure 3 Omentin-1 inhibited arterial calcification in OPG−/− mice. (A) The thoracic aorta sections were stained with haematoxylin and eosin to detect calcification. Representative microscopic views are shown. Scale bar represents 500 µm. The area of the calcified lesion is stained dark blue and indicated by arrows. (B and C) Calcium content in the thoracic aorta. The bars represent means + SD. *P < 0.05. For B, n = 7; for C, n = 6.

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had lower BMD than did those of treatment-matched WT mice (Figure 4B). Administration of Ad-Ome partly restored BMD in OPG−/− mice (Figure 4B). In WT mice treated with Ad-Ome, BMD tended to be slightly decreased, but the reductions relative to the baseline controls did not achieve statistical significance (Figure 4B).

OPG−/− mice treated with LY294002 alone displayed more severe bone loss than vehicle-treated OPG−/− mice (Figure 4C). LY294002 also abolished the bone-sparing effect of omentin-1 in OPG−/− mice (Figure 4C).

3.7 Omentin-1 decreases serum levels of RANKL and bone turnover markers in OPG−/− mice

Serum levels of RANKL, TRAP-5b, and osteocalcin were assessed at the end of treatment. Serum RANKL, osteocalcin, and TRAP-5b concentrations were significantly increased in either vehicle- or Ad-Gal-treated OPG−/− mice compared with treatment-matched WT mice (Figure 5). After Ad-Ome administration, serum RANKL, osteocalcin, and TRAP-5b levels were significantly reduced in both OPG−/− and WT mice; changes were more obvious for the OPG−/− animals (Figure 5).

3.8 Omentin-1 and adiponectin counteract each other’s effects on regulation of OPG/RANKL expression in osteoblasts and on osteoclast formation in co-cultures of osteoblasts and bone marrow cells

Omentin-1 stimulated OPG and suppressed RANKL expression in osteoblasts; on the contrary, adiponectin inhibited OPG and enhanced RANKL expression in osteoblasts (Figure 6A). Accordingly, omentin-1 reduced osteoclast formation in co-culture systems of osteoblasts and bone marrow cells, and adiponectin promoted osteoclast formation in the same co-culture systems (Figure 6B). The effects of omentin-1 and adiponectin on regulation of OPG/RANKL expression and osteoclast formation were counteracted by each other (Figure 6A and B). As expected, the stimulatory effect of RANKL on osteoclast formation was markedly stronger than that of adiponectin, thus, RANKL exerted more vigorous counteraction on the suppressive effect of omentin-1 on osteoclast formation (Figure 6B).

3.9 Omentin-1 and adiponectin synergistically inhibit CVSMC calcification

CVSMCs treated with omentin-1 or adiponectin alone showed less matrix mineralization than vehicle-treated CVSMCs (Figure 6C). A combination treatment of omentin-1 and adiponectin achieved a synergistic suppression of matrix mineralization in CVSMCs (Figure 6C).

4. Discussion

This study demonstrated that recombinant human omentin-1 could induce OPG and inhibit RANKL production in primary mouse osteoblasts and CVSMCs in vitro, and adenovirus-mediated over-expression of human omentin-1 in OPG−/− mice could ameliorate bone loss and arterial calcification in vivo. All these actions were dependent on the PI3K–Akt signalling pathway.

Levels of both omentin-1 mRNA in adipose tissue and serum omentin-1 are inversely related to obesity. Serum omentin-1 level is reported to be decreased among diabetic subjects as well. Circulating levels of omentin-1 correlate negatively with body mass index, waist circumference, leptin, fasting insulin, and insulin resistance, and positively with high-density lipoprotein. Omentin-1 promotes insulin-mediated glucose transport in adipocytes, induces vasodilatation in isolated blood vessels, and inhibits tumour necrosis factor-induced vascular inflammation in endothelial cells. It also inhibits osteoblastic differentiation of CVSMCs. Interestingly, it has been noted that this pattern of results for omentin-1 is similar to adiponectin, an insulin sensitizer and cardiovascular protective adipokine. In fact, circulating adiponectin levels are positively correlated with serum omentin-1 levels. Importantly, up-to-date reports show that adiponectin and omentin-1 are the only two adipokines whose levels are decreased in human obesity. This indicates that both adiponectin and omentin-1 are markers of
leanness, and may exert beneficial actions to oppose obesity and its negative effects.

Our previous studies showed that adiponectin prevents arterial calcification but exerts a negative effect on bone mass.20,28-31 The arterial calcification in adiponectin−/− mice is attributed to loss of the inhibitory effect of adiponectin on osteoblastic differentiation of CVSMCs.20 Adiponectin induces human osteoblast proliferation and differentiation, and increases osteoclast formation indirectly via stimulating RANKL and inhibiting OPG production in osteoblasts.28,29 Serum adiponectin levels correlate positively with bone turnover biochemical markers and negatively with BMD in men and postmenopausal women.30,31 These findings reveal that adiponectin promotes more bone resorption than bone formation, and eventually decreases bone mass. Thus, we postulated that omentin-1 must also play a pivotal role in the regulation of arterial and bone metabolism.

Our previous study has shown that omentin-1 inhibits osteoblastic differentiation of CVSMCs.15 In the present study, we found that omentin-1 also inhibited differentiation of osteoblasts, as evidenced by the suppressive effects of omentin-1 on Runx2 and osteocalcin expression, and matrix mineralization. Furthermore, we found that omentin-1 stimulated proliferation of osteoblasts.

RANKL, known as a member of the tumour necrosis factor superfamily,32 binds to its receptor, RANK, expressed on the surface of osteoclasts, osteoclast precursors, and VSMCs. This promotes

Figure 4 Adenovirus-mediated over-expression of omentin-1 suppressed osteoclast formation and partly restored BMD in OPG−/− mice. (A) Representative microscopic views of TRAP-stained sections of the metaphyseal region of the distal femurs. Scale bar represents 20 μm. Osteoclasts are indicated by arrows. (B and C) The whole left femur and lumbar vertebral segment (L2–L4) of each mouse were scanned by DXA to determine the BMD. The bars represent means ± SD, *P < 0.05. For B, n = 7; for C, n = 6.
found arterial calcification. RANKL-infused animal models exhibit RANKL, can directly block all the RANKL-mediated actions. 34,35

high-turnover bone loss, 36,37 while
underlie not only the pathology of osteoporosis but also that of arterial calcification.2– 4

Therefore, imbalances in the RANKL/OPG ratio or RANK signalling may contribute to the spontaneous calcification in VSMCs. Furthermore, we demonstrated that omentin-1 stimulated OPG and inhibited RANKL production in both CVSMCs and osteoblasts,29 suggesting that omentin-1 has protective effects on both bone and arterial tissue. Notably, omentin-1 could inhibit RANKL expression in OPG−/− osteoblasts, indicating that the suppressive effect of omentin-1 on RANKL expression is independent of its stimulatory action on OPG.

Our previous study showed that omentin-1 inhibits osteoblastic differentiation of CVSMCs via the PI3K–Akt pathway.16 Thus, we evaluated whether the PI3K–Akt pathway was also involved in the regulation of OPG and RANKL production by omentin-1 in VSMCs and osteoblasts. In relation to this, Zhang et al.42 reported that platelet-derived growth factor induces OPG expression in VSMCs through the PI3K–Akt signal pathway. In the present study, treatment with either of the chemical inhibitors of PI3K–Akt, or the Akt siRNA, abolished the effects of omentin-1 on OPG and RANKL production in osteoblasts or CVSMCs, revealing that omentin-1 stimulated OPG and inhibited RANKL production in CVSMCs and osteoblasts via the PI3K–Akt signalling pathway.

As omentin-1 regulated OPG and RANKL expression in CVSMCs and osteoblasts directly in vitro, it may play an important role in bone remodelling and arterial metabolism in vivo. In the present study, OPG−/− mice were infected with an adenovirus vector encoding omentin-1 or LacZ. The results showed that OPG−/− mice developed arterial calcification and osteoporosis, and adenovirus-mediated over-expression of omentin-1 attenuated arterial calcification and bone loss in these mice. Furthermore, we found that the ability of omentin-1 to enhance beneficial vascular and skeletal actions was completely abrogated by LY294002 in vivo. Taken together, these findings suggested that omentin-1 activated the PI3K–Akt signalling pathway, which in turn decreased RANKL expression, resulting in alleviation of arterial calcification and bone loss in the OPG−/− mice. Notably, LY294002 aggravated arterial calcification and bone loss in OPG−/− mice by itself, in agreement with the reported protective effects of PI3K–Akt on VSMC calcification,43 and the decreased BMD in Akt1 knock-out mice with PI3K–Akt signalling deficiency.44

We went further to examine the effects of omentin-1 on systemic levels of RANKL in OPG−/− mice. As expected, we derived observations of a profound increase in serum RANKL level in OPG−/− mice, not in WT mice. This is in good agreement with our previously reported findings and others’ reports that serum RANKL level is dramatically increased in OPG−/− mice.23,45 Indeed, we demonstrated here that administration of omentin-1 resulted in a lower RANKL level in OPG−/− mice. These results further supported our data on the inhibitory effect of omentin-1 on RANKL production in

Figure 5 Adenovirus-mediated over-expression of omentin-1 reduced the serum levels of RANKL, TRACP-5b, and osteocalcin in OPG−/− and WT mice. The bars represent means ± SD; n = 7, *P < 0.05.

osteoclast formation and activation, suppresses osteoclast apoptosis, and induces VSMC calcification.32–34 OPG, a decoy receptor for RANKL, can directly block all the RANKL-mediated actions.34,35 Therefore, imbalances in the RANKL/OPG ratio or RANK signalling underline not only the pathology of osteoporosis but also that of arterial calcification.2–4 OPG−/− mice develop severe bone loss and profound arterial calcification.3 RANKL-infused animal models exhibit high-turnover bone loss,36,37 while RANKL knock-in mice display increased aortic calcium deposition, compared with wild-type control animals.38 Recently, the RANKL–OPG pathway has emerged as a promising target to reduce bone loss and arterial calcification.39

VSMCs, osteoblasts, and their immature precursors express and secrete OPG and RANKL;2,34 however, RANKL expression in VSMCs is so weak that it can only be detected by immunohistochemical staining or RT-PCR, and small amounts of RANKL in the conditioned medium of VSMCs are undetectable by ELISA.40 The expression level of RANKL in the conditioned medium of CVSMCs (a subpopulation of aortic smooth muscle cells possessing the ability of spontaneous calcification, and putatively involved in arterial calcification41) remains unclear. In this study, by using ELISA kits, we detected OPG in conditioned medium of VSMCs, CVSMCs, and osteoblasts, and we also detected RANKL in conditioned medium of CVSMCs and osteoblasts, but not VSMCs. Given that RANKL is able to induce VSMC calcification in vitro,34 a high level of RANKL may contribute to the spontaneous calcification in CVSMCs. Furthermore, we demonstrated that omentin-1 stimulated OPG and inhibited RANKL production in both CVSMCs and osteoblasts,29 suggesting that omentin-1 has protective effects on both bone and arterial tissue.
Figure 6  Effects of the combination treatment of omentin-1 and adiponectin on OPG and RANKL expression in osteoblasts, osteoclast formation, and CVSMC calcification. (A) Omentin-1 and adiponectin counteracted each other’s effects on regulation of OPG and RANKL expression in osteoblasts. Osteoblasts were cultured in serum-free α-MEM and treated with vehicle, omentin-1 (200 ng/mL), adiponectin (30 μg/mL), or omentin-1 + adiponectin for 48 h. The cells were lysed for OPG and RANKL protein expression analysis by western blotting. On the left, representative results are shown. On the right, the data are expressed as densitometric ratios of OPG/β-actin or RANKL/β-actin. (B) Omentin-1 and adiponectin counteracted each other’s effects on regulation of osteoclast formation in co-culture systems of osteoblasts and bone marrow cells. The osteoblasts were seeded into 24-well plates and cultured to subconfluence. Subsequently, the bone marrow cells were added to the osteoblasts, and co-cultured in α-MEM containing 10% fetal bovine serum in the presence of 1,25(OH)2D3 (10−7 M), and treated with vehicle, omentin-1 (200 ng/mL), adiponectin (30 μg/mL), omentin-1 + adiponectin, RANKL (50 ng/mL), or omentin-1 + RANKL for 9 days, with replacement of the medium every 2–3 days. The cultures were stained for TRAP. Top panels show representative microscopic views (scale bar represents 40 μm). Osteoclasts are indicated by the arrows. Bottom panel shows quantification of osteoclast numbers by counting TRAP+ multinucleated cells containing more than three nuclei. (C) Omentin-1 and adiponectin synergistically inhibited CVSMC calcification. The CVSMCs were seeded into 24-well plates and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and β-glycerophosphate (10 mM), in the presence of vehicle, omentin-1 (200 ng/mL), adiponectin (30 μg/mL), or omentin-1 + adiponectin for 16 days, with replacement of the medium every 2–3 days. Matrix mineralization was determined by Alizarin red S staining. Representative microscopic views (scale bar represents 150 μm) are shown (top panels), and the Alizarin Red S staining was quantified via extraction with cetylpyridinium chloride (bottom panel). The amount of released dye was quantified by spectrophotometry at 540 nm. In A–C, the bars represent means ± SD, n = 3, */p < 0.05.
OPG−/− osteoblasts in vitro. These findings also further indicated that the suppressive effects of omentin-1 on bone loss and arterial calcification in OPG−/− mice must be mediated by its systemic inhibitory action on RANKL production.

Moreover, the increased numbers of femoral osteoclasts, and the elevated levels of the bone resorption marker TRACP-5b and the bone formation marker osteocalcin in OPG−/− mice found here are consistent with the fact that OPG−/− mice exhibit high-turnover bone loss. After administration of omentin-1, the number of osteoclasts and the serum levels of TRACP-5b and osteocalcin of the OPG−/− mice were decreased. These changes may have been caused by decreased RANK signalling.

Of note, in this study, even though omentin-1 reduced serum levels of RANKL, TRACP-5b, and osteocalcin in WT mice, it could not enhance BMD in these mice; in contrast, omentin-1 slightly reduced BMD in WT mice, although the reductions were not significant. These results could be explained by the hypothesis that the 4-week-old WT mice used here need a positive balance of bone turnover during the bone growth phase, and that the suppression of both bone formation and resorption by omentin-1 in these mice would not be beneficial for bone growth.

Furthermore, it is interesting to see that although both omentin-1 and adiponectin are decreased in human obesity, they have opposite roles in regulation of OPG and RANKL expression. In this study, we found that the effects of omentin-1 and adiponectin on OPG and RANKL expression in osteoblasts could be counteracted by each other. In co-cultures of osteoblasts and osteoclast precursors, we demonstrated that omentin-1 and adiponectin exerted opposite effects on osteoclast formation, and that they counteracted each other’s effects on regulation of osteoclastogenesis. Thus, we hypothesized that the decreased levels of both omentin-1 and adiponectin in obesity might result in negligible effects on bone, even though many existing data indicate that obesity is associated with high BMD. The protective effect of obesity on bone may be mainly due to increased mechanical loading, and effects of estrogen and insulin. Moreover, even though omentin-1 and adiponectin counteracted each other’s effects on regulation of osteoclast formation, they had direct synergistic inhibitory effects on CVSMC calcification in vitro. Considering the high incidence of arterial calcification in obese subjects, and the inhibitory actions of both omentin-1 and adiponectin on arterial calcification, the decreased levels of omentin-1 and adiponectin in obesity may be responsible for the development of arterial calcification in obesity.

In conclusion, we demonstrated that omentin-1 stimulated OPG and inhibited RANKL production in CVSMCs and osteoblasts via the PI3K–Akt signalling pathway in vitro. Omentin-1 also reduced systemic RANKL levels in OPG−/− mice. This effect contributed to suppression of both arterial calcification and bone loss. Our present findings suggest that increasing concentrations of omentin-1 may be beneficial for protecting bone and artery in patients with high-turnover osteoporosis and arterial calcification.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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