Abstract. Thiazolidinediones are traditional anti-diabetic therapeutic agents that have been associated with bone loss and increased fracture risk. However, the underlying mechanisms of this side effect require further elucidation. The present study aimed to investigate the effect of pioglitazone (PIO), a thiazolidinedione, on osteoblastogenesis, osteoclastogenesis and the osteoprotegerin (OPG) / receptor activator of nuclear factor-κB ligand (RANKL) / RANK system. The MC3T3-E1 murine pre-osteoblastic cell line was treated with PIO and processed for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of OPG, RANKL, peroxisome proliferator-activated receptor γ (PPARγ), Runx-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP) and osteocalcin (OCN), and western blotting analysis of OPG and RANKL. The culture medium was collected for ELISA analysis of OPG and RANKL. Murine bone marrow monocytes (BMMCs) were treated with PIO in the presence of RANKL and macrophage-colony stimulating factor and subjected to tartrate-resistant acid phosphatase (TRAP) staining and activity measurement, and RT-qPCR analysis of cathepsin K, TRAP and RANK. Co-culture of MC3T3-E1 and BMMCs was performed in the presence of PIO, and TRAP staining was also conducted. PIO inhibited the osteoblastic differentiation of MC3T3-E1 cells, and promoted the osteoclastic differentiation of BMMCs with or without co-culturing with MC3T3-E1 cells. ELISA analysis indicated increased RANKL and decreased OPG expression levels in the medium of MC3T3-E1 cells treated with PIO.

PIO upregulated expression of RANKL and PPARγ and downregulated expression of OPG, RUNX2, ALP and OCN in MC3T3-E1 cells, while expression levels of RANK in BMMCs remained unchanged. These results suggest that PIO suppresses osteoblastogenesis and enhances osteoclastogenesis. In addition, PIO may also promote osteoclastogenesis by affecting the OPG-RANKL-RANK system.

Introduction

Thiazolidinediones (TZDs) are therapeutic agents commonly used to treat patients with type 2 diabetes, they have been demonstrated to affect bone metabolism (1). It has been reported that long-term usage of TZDs in diabetic patients induced bone loss and a higher risk of fracture (2,3). Bone homeostasis depends on the balance between bone formation by osteoblasts and bone resorption by osteoclasts (4). Bone loss and osteoporosis occur when bone resorption exceeds bone formation, and osteopetrosis may occur when bone formation predominates (5). The molecular osteoprotegerin (OPG) / receptor activator of nuclear factor-κB ligand (RANKL) / RANK axis is important in the regulation of homeostasis (6). OPG and RANKL are secreted by osteoblasts, RANKL mediates osteoclastogenesis by binding RANK expressed by osteoclasts, whereas OPG acts as a decoy receptor of RANKL to prevent osteoclastogenesis (6). Thus, osteoclastogenesis depends on the ratio of OPG to RANKL secreted by osteoblasts (6,7).

TZDs act via peroxisome proliferator-activated receptor γ (PPARγ), which is a member of the nuclear receptor superfamily of transcription factors (8), and the predominant factor involved in adipose metabolism. Activation of PPARγ results in adipogenic differentiation in various kinds of progenitor cells (9,10). A number of studies have demonstrated that TZDs inhibit osteoblastogenesis directly, but its effect on osteoclastogenesis remains to be determined. Few studies have investigated the effect of TZDs on the OPG/RANKL/RANK system and the paracrine regulation of osteoclastogenesis.

The present study investigated the direct effect of pioglitazone (PIO), a TZD, on osteoblastogenesis and osteoclastogenesis, and the paracrine mechanisms by which...
PIO affects osteoclastogenesis were also investigated by performing a co-culture system of a pre-osteoblastic cell line with bone marrow mononuclear cells.

Materials and methods

Reagents. Recombinant murine macrophage colony-stimulating factor (M-CSF) and RANKL were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The ALP Staining kit and TRAP Staining kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-RANKL (#4816; rabbit polyclonal; 1:1,000) antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and the anti-OPG (BA1475-1; rabbit polyclonal; 1:100-400) antibody, OPG and RANKL ELISA kits were obtained from Wuhan Boster Biological Technology, Co., Ltd. (Wuhan, China). PIO was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture. The MC3T3-E1 murine pre-osteoblastic cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in α-modified essential medium (α-MEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO₂. The media was changed every 3 days.

Isolation of monocytes. Bone marrow cells were isolated from the femora of C57BL/6 mice (male; age, 6–8 weeks; weight, 18-22 g; Beijing HFK Bioscience Co., Ltd., Beijing, China). The mice were housed separately in a temperature- and humidity-controlled (20–26°C and 40–70%, respectively) environment, with a 12/12 h light/dark cycle and free access to food and water. The mice were sacrificed by CO₂ inhalation and cervical dislocation. The cells were cultured in α-MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 15% FCS at 37°C in a humidified atmosphere of 5% CO₂. The media was changed every 3 days.

Osteoblast differentiation. For differentiation studies, MC3T3-E1 cells were cultured in 6-well plates (Corning Incorporated, Corning, NY, USA) at a density of 5x10⁵ cells/well. After 24 h of culture, the non-adherent BMMCs were collected. All procedures were approved by the Animal Care and Use Committee, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Osteoclast differentiation. BMMCs were cultured in 96-well plates (Corning Incorporated) at a density of 5,000 cells/well in conditioned medium containing 50 ng/ml M-CSF and 50 ng/ml RANKL and were treated with DMSO, 0.1 μM PIO or 1 μM PIO. After 4 days, the cells were harvested and TRAP staining and activity measurement, and western blotting were conducted.

OPG and RANKL ELISA. OPG and RANKL levels in the medium of osteoblast differentiation were analyzed using a commercially available ELISA kit according to the manufacturer's protocols. Each sample was assessed 3 times.

ALP activity and TRAP activity measurement. For measurement of ALP activity, the harvested cells were washed with phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.) twice prior to the addition of lysis buffer (1.5 M Tris-HCl (pH 9.2), 0.1 M ZnCl₂, 0.5 M MgCl₂-6H₂O, and Triton X-100; Wuhan Boster Biological Technology, Co., Ltd.), and the cells in each well were sonicated for 10 sec. The sonicated samples were added to a substrate solution containing 4-nitrophenyl phosphate (Sigma-Aldrich), 1.5 M alkaline buffer solution (Sigma-Aldrich) and H₂O₂ and incubated for 20 min at 37°C. Subsequently, 2 N NaOH was added to the samples to stop the reaction. Absorbance of the samples was measured at a wavelength of 405 nm using a microplate reader (Multiskan™ FC; Thermo Fisher Scientific, Inc.).

For measurement of TRAP activity, the harvested cells were fixed with 10% formalin (Sigma-Aldrich) for 10 min and 95% ethanol for 1 min, and then 100 μl of citrate buffer (50 mM; pH 4.6; Sigma-Aldrich) containing 10 mM sodium tartrate (Sigma-Aldrich) and 5 mM p-nitrophenolphosphate (Sigma-Aldrich) was added to the wells containing fixed cells in the plates. Following incubation for 1 h, enzyme reaction mixtures in the wells were transferred to new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at a wavelength of 405 nm using a Multiskan™ FC. Each experiment was performed in triplicate.

Co-culture of BMMCs with MC3T3-E1 cells. Using a 6-well Transwell plate (Corning Incorporated), MC3T3-E1 cells were cultured in the lower layer at a density of 5x10⁶ cells/well. After 24 h, the α-MEM medium was changed to α-MEM medium containing DMSO, 0.1 μM PIO or 1 μM PIO, and BMMCs were cultured in the upper layer of the Transwell plate at the density of 5,000 cells/well. The media was changed every 3 days. After 7 days, the cells in the upper layer were harvested to conduct TRAP staining.

RNA isolation and RT-qPCR analysis. Total RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). DNase I, RNase-free (#EN0521) and the RevertAid First Strand cDNA Synthesis kit (all from Thermo Fisher Scientific, Inc.) were used for reverse transcription. The reverse transcription reaction was conducted with total RNA, Oligo (dT)18 primers and nuclease-free water made up to a total volume of 12 μl. This mixture was then incubated at 65°C for 5 min, chilled on ice, spun down and placed back on ice. The 5X reaction buffer (4 μl), RiboLock RNase Inhibitor (1 μl), 10 mM dNTP Mix (2 μl), RevertAid M-MuLV RT (200 U/μl; 1 μl) were then added to a total volume of 20 μl, the mixture was mixed gently and
Table I. List of primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer F</th>
<th>Primer R</th>
<th>Product size</th>
<th>Accession no.</th>
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<td>18S</td>
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<td>ATGGTAGGACCGGAGCTA</td>
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<td>M35283.1</td>
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<tr>
<td>PPARγ</td>
<td>GGAAGACCACCTCGCATTCTTTT</td>
<td>GTAATCGAACCACCGACTGCA</td>
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<td>NM_001127330</td>
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<tr>
<td>RUNX2</td>
<td>GACTGTGATTCCCGTATGGCC</td>
<td>ACTTGGTTTTTCATAACAGGGA</td>
<td>84</td>
<td>NM_001146038</td>
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<tr>
<td>ALP</td>
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<td>GCTGCTGTCGCCAGTAATCG</td>
<td>61</td>
<td>NM_007431</td>
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<tr>
<td>OCN</td>
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<td>TGCTGCTGCACCGGACTG</td>
<td>187</td>
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<td>RANK</td>
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<td>ACTGTCGGAGGTAGGAGTGC</td>
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<tr>
<td>Cathepsin K</td>
<td>GAAGAAAGACTCACCAGGAAGCAG</td>
<td>CTGTATTCCCCGTTGTGTAGC</td>
<td>136</td>
<td>NM_007802</td>
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<tr>
<td>TRAP</td>
<td>CACTCCACCCCTGAGATTTGCT</td>
<td>CATCGTCTGCACGGGTCTG</td>
<td>118</td>
<td>NM_001102405</td>
</tr>
</tbody>
</table>

Primers are listed in the 5′-3′ direction, and product lengths are presented with the units ‘base pairs’. PPARγ, peroxisome proliferator-activated receptor γ; RUNX2, Runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin; RANK, receptor activator of nuclear factor-κB; TRAP, tartrate-resistant acid phosphatase; F, forward; R, reverse.

Figure 1. In vitro analysis of the effect of pioglitazone on osteoblasts. (A) ALP staining of osteoblasts at different pioglitazone concentrations. (B) ALP activities of osteoblast, as a quantitative index. (C) The effect of pioglitazone on gene expression in osteoblasts as analyzed by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. control; **P<0.01 vs. control. ALP, alkaline phosphatase; DMSO, dimethyl sulfoxide; PIO, pioglitazone; PPARγ, peroxisome proliferator-activated receptor γ; RUNX2, Runt-related transcription factor 2; OCN, osteocalcin.
centrifuged prior to incubation for 60 min at 42°C. For qPCR, Thermo Fisher Scientific, Inc. Maxima SYBR Green qPCR Master Mix (2X) Thermal cycling was conducted, which used a three-step cycling protocol: Pre-treatment at 50°C for 2 min, 1 cycle of 95°C for 10 min; 1 cycle of denaturation at 95°C for 15 sec; 40 cycles of annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The forward and reverse primers were obtained from Invitrogen (Thermo Fisher Scientific, Inc.) and are presented in Table I. Data are presented as the mean ± standard deviation for at least three independent experiments. Gene expression analysis was performed using RT-qPCR (iCycler iQ5 System; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to 18S RNA.

ALP and TRAP staining. Harvested cells were rinsed three times with PBS and fixed for 15 min in 4% paraformaldehyde (Wuhan Boster Biological Technology, Co., Ltd.) at 4°C. The cells were stained with ALP and TRAP using kits according to the manufacturer’s protocols. The staining was analyzed using an OsteoMeasure system (Osteometrics, Inc., Atlanta, GA, USA) connected to a Axioskop microscope (Zeiss, Oberkochen, Germany).

Western blotting. Total cell lysates were obtained by lysing cells in radioimmunoprecipitation assay buffer (Wuhan Boster Biological Technology, Co., Ltd.) containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium
deoxycholate, 2 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA and a protease inhibitor cocktail. Protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.). The proteins (20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blocked with bovine serum albumin (Wuhan Boster Biological Technology, Co., Ltd.) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature, and incubated with primary antibodies (anti-OPG and anti-RANKL) overnight at 4°C. The membrane was washed three times with TBST and incubated with the horseradish peroxidase-conjugated secondary antibodies (BA1039; goat anti-rabbit monoclonal; 1:1,000; Wuhan Boster Biological Technology, Co., Ltd.) for 1 h at room temperature, then washed three further times with TBST. Protein detection was conducted with a Pierce enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.). Densitometry was conducted using Quantity One software (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data were presented as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance with SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

PIO inhibits the differentiation of MC3T3-E1 cells into osteoblasts. To investigate the effect of PIO on osteoblast differentiation, MC3T3-E1 cells were cultured in osteoblastic differentiation media with or without PIO. ALP staining and ALP activity served as biomarkers of osteoblastic differentiation (Fig. 1). As presented in Fig. 1A and B, 1 µM PIO significantly reduced the number of ALP positive osteoblasts and ALP activity when compared with the control group (P<0.05). In addition, expression levels of osteoblastic genes, Runt-related transcription factor 2 (RUNX2) (P<0.01), ALP (P<0.05) and osteocalcin (OCN; P<0.05) were significantly decreased following treatment with 1 µM PIO (Fig. 1C). By contrast, PPARγ was significantly increased (P<0.05; Fig. 1C). No significant alterations were observed following 0.1 µM PIO treatment, apart from in RUNX2 expression.

Table II. The OPG and RANKL levels in osteoblast culture medium (detected by ELISA).

<table>
<thead>
<tr>
<th>Gene</th>
<th>DMSO (n=3)</th>
<th>PIO 0.1 µM (n=3)</th>
<th>PIO 1 µM (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPG (pg/ml)</td>
<td>476.5±26.8</td>
<td>431.6±26.18</td>
<td>326.1±34.4</td>
</tr>
<tr>
<td>RANKL (pg/ml)</td>
<td>792.5±43.1</td>
<td>815.5±33.2</td>
<td>1265.1±79.2</td>
</tr>
</tbody>
</table>

*aP<0.05; bP<0.01. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; DMSO, dimethyl sulfoxide; PIO, pioglitazone.
PIO promotes the differentiation of BMMCs into osteoclasts. The effect of pioglitazone on osteoclast differentiation was investigated in BMGCCs by culturing the cells in the presence of RANKL (50 ng/ml) and M-CSF (50 ng/ml) with or without PIO, and detecting the number of TRAP-positive cells and TRAP activity, which indicate the degree of osteoclast differentiation (Fig. 2). As presented in Fig. 2A, RANKL-mediated osteoclast differentiation was promoted by 1 µM PIO. The number of TRAP-positive cells (Fig. 2C and D) and TRAP activity (Fig. 2B) were significantly increased following treatment with 1 µM PIO. In addition, the gene expression levels of TRAP and cathepsin K, which indicate the function of osteoclasts, were significantly increased (P<0.05; Fig. 2E). Treatment with 0.1 µM PIO did not significantly affect the osteoclast differentiation of BMGCs.

PIO decreases the OPG/RANKL ratio in osteoblasts. The MC3T3-E1 cells were treated with PIO or DMSO for 7 days, and the mRNA and protein expression levels of OPG and RANKL were analyzed using RT-qPCR and western blotting, respectively. As presented in Fig. 3, the 1 µM PIO group exhibited a significant decrease of the ratio of OPG/RANKL mRNA expression levels (P<0.05) due to decreased OPG and increased RANKL expression levels compared with the control group. Furthermore, the protein expression levels of OPG, RANKL, and ratio of OPG to RANKL demonstrated the same trend of change as observed for the mRNA expression levels. The cells treated with 0.1 µM PIO demonstrated no significant difference compared with that of the control group.

PIO promotes osteoclastogenesis by modulating the osteoblast and osteoclast cross-talk. The OPG and RANKL expression levels in the osteoblast culture medium of MC3T3-E1 cells were detected by ELISA. Following PIO treatment, OPG expression levels decreased and RANKL expression levels increased, compared with DMSO-treated controls (Table II). Few TRAP positive cells were observed in BMGCs co-cultured with MC3T3-E1 cells without PIO treatment after 7 days (Fig. 4A). Following addition of PIO, an increased number of TRAP positive cells were observed (Fig. 4). Furthermore, the number of TRAP-positive cells among BMGCs in the PIO-treated co-culture system was significantly increased compared with BMGCs untreated with PIO (P<0.05; Fig. 4B). Notably, in these experiments, 0.1 µM PIO demonstrated the same effects as 1 µM pioglitazone; however, the difference was not statistically significant (Fig. 4A and B).

Discussion

Pioglitazone inhibited osteoblast differentiation and promoted osteoclast formation. As one of thiazolidinediones that are commonly prescribed for patients with diabetes, PIO is a PPARγ agonist, which may result in bone loss and an increased fracture rate in diabetic patients. The present study demonstrated that PIO inhibited osteoblast differentiation and promoted osteoclast formation directly, and enhanced osteoclastogenesis via the OPG/RANKL/RANK system, which is involved in paracrine regulation of osteoclastogenesis.

PIO negatively regulates osteoblast differentiation. Consistently with previous studies, the present study demonstrated that PIO inhibited osteoblastogenesis. Osteoblasts and adipocytes are commonly derived from mesenchymal stem cells (MSCs), and their differentiation is reliant on different stimulating signals. Activation of core binding factor-α1/RUNX2 results in osteoblast formation from MSCs, however, activation of PPARγ results in adipocyte formation. Competition exists between the differentiation of the two cell types, previous studies have shown that TZDs promote adipocytogenesis at expense of osteoblastogenesis (13-16). Furthermore, silencing PPARγ using synthetic small interfering RNA inhibited adipocyte differentiation and induced osteoblastic differentiation (17). However, the underlying mechanisms remain to be elucidated. In the present study, PIO was observed to upregulate PPARγ and downregulate RUNX2 in parallel with inhibition of osteoblastogenesis. Results from the current study support the findings of Jeon et al (18) that activation of PPARγ interfered with the transactivation ability of RUNX2 and suppressed its expression, thus resulting in the inhibition of OCN (an osteoblast-specific protein) expression. Furthermore, apoptosis of osteoblasts has been proven to be accelerated by TZDs. By contrast, Brudigam et al (19) demonstrated that rosiglitazone promoted osteoblastogenesis and produced a large quantity of reactive oxygen species and increased apoptosis, which resulted in attenuation of osteoblastogenesis.
**PIO increases osteoclastogenesis.** Osteoclasts are derived from bone marrow hematopoietic stem cells, which may be stimulated to differentiate by RANKL signaling and PPARγ activation (20). TZDs have been reported to affect osteoclast differentiation, however, results from various previous studies are conflicting. Chan et al (21) demonstrated that ciglitazone, a TZD, suppressed multinucleated osteoclast formation in a dose-dependent manner. Furthermore, Cho et al (15) demonstrated that rosiglitazone attenuated osteoclast formation and bone resorption by preventing RANK and enhancing PPARγ2 expression in osteoclasts. By contrast, Wan et al (22) demonstrated that activation of PPARγ stimulated osteoclastogenesis and bone resorption, and deletion of PPARγ prevented osteoclast formation and resulted in osteopetrosis. Wu et al (23) also demonstrated that rosiglitazone markedly increased the differentiation of mouse bone marrow cells into osteoclasts. In the present study, administration of exogenous RANKL and M-CSF, which are required for osteoclast differentiation, demonstrated that PIO exerts a direct effect on promoting differentiation of BMMCs into osteoclasts. Furthermore, contrary to results from Cho et al (15), the RANK expression levels in the cells remained unchanged, which indicated that this effect of PIO may involve PPARγ but not the RANKL-RANK response. Although c-Fos induction, TNF receptor-associated factor 6 and downstream extracellular signal-regulated kinase signaling, PPARγ coactivator 1β and estrogen-related receptor α have been reported to be associated with enhancement of PPARγ-mediated osteoclastogenesis (22), the underlying mechanisms remain to be elucidated.

**PIO decreases the OPG/RANKL ratio in osteoblasts.** The molecular OPG/RANKL/RANK axis participates in regulating bone metabolism, provides paracrine regulation for osteoclastogenesis (25). RANKL, expressed and secreted by osteoblasts, binds the extracellular RANK domain of pre-osteoclasts, and results in expression of specific genes involved in osteoclast differentiation and bone resorption (26). Furthermore, OPG, secreted by osteoblasts, acts as a soluble receptor antagonist for RANKL to prevent it binding to RANK and decreases osteoclastogenesis (6). Thus, the OPG/RANKL ratio and the expression of RANK in pre-osteoclasts are key in osteoclastogenesis (6,7). However, studies concerning the effect of TZDs on this paracrine regulation are rare.

In the present study, it was observed that the OPG/RANKL ratio decreased, due to a decreased OPG expression level and an elevated RANKL expression level, in osteoblasts in response to treatment with PIO. Furthermore, the expression levels of RANK remained unchanged following PIO treatment. Thus, the results of the present study suggest, in addition to the direct effect, PIO may positively regulate osteoclastogenesis via influencing the OPG/RANKL/RANK axis in the co-culture system mimicking the in vivo bone marrow microenvironment. Consistent with these findings, Lazarenko et al (27) demonstrated that PPARγ activation in osteoblasts promotes osteoclast differentiation by inducing the expression of RANKL. Previous in vitro and in vivo studies have demonstrated that TZD treatment lowers OPG levels (28-30). Contrary to results in the present study, Cho et al (15) demonstrated that rosiglitazone inhibited the RANK protein expression in monocytes induced by RANKL. This difference may be attributed to the higher dose of TZD used. In future experiments a larger range of doses of TZDs should be evaluated.

In conclusion, the present study demonstrates that PIO suppresses osteoclastogenesis and enhances osteoclastogenesis directly. It also decreases the OPG/RANKL ratio in osteoblasts, to promote osteoclast formation via a paracrine mechanism.

**Acknowledgements**

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**References**


