Regular Article

Osteoclast Differentiation Is Suppressed by Increased *O*-GlcNAcylation Due to Thiamet G Treatment

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Received March 10, 2020; accepted July 5, 2020

Osteoclasts are the only bone-resorbing cells in organisms and understanding their differentiation mechanism is crucial for the treatment of osteoporosis. In the present study, we investigated the effect of Thiamet G, an *O*-GlcNAcase specific inhibitor, on osteoclastogenic differentiation. Thiamet G treatment increased global *O*-GlcNAcylation in murine RAW264 cells and suppressed receptor activator of nuclear factor-*κ*B ligand (RANKL)-dependent formation in tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells, thereby suppressing the upregulation of osteoclast specific genes. Meanwhile, knockdown of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase promoted the formation TRAP-positive multinuclear cells. Thiamet G treatment also suppressed RANKL and macrophage colony-stimulating factor (M-CSF) dependent osteoclast formation and bone-resorbing activity in mouse primary bone marrow cells and human peripheral blood mononuclear cells. These results indicate that the promotion of *O*-GlcNAc modification specifically suppresses osteoclast formation and its activity and suggest that chemicals affecting *O*-GlcNAc modification might potentially be useful in the prevention or treatment of osteoporosis in future.

Key words osteoclast; *O*-linked *N*-acetylglucosamine modification; differentiation; bone; Thiamet G

INTRODUCTION

Osteoclasts are tartrate-resistant acid phosphatase (TRAP)-positive multinuclear giant cells that differentiate from monocyte/macrophage lineage cells in response to macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-*κ*B ligand (RANKL).[1–3] Osteoclasts are specialized in bone resorption and their excessive activity is associated with detrimental bone diseases such as osteoporosis and neoplastic bone metastasis. Molecules that inhibit osteoclast differentiation and/or function may have potential application in the treatment of such diseases.[4,5]

O-GlcNAcylation is a post-translational modification of serine (Ser)/threonine (Thr) residue in intracellular proteins with *N*-acetylglucosamine (GlcNAc).[6,7] This modification is regulated mainly by three factors: *O*-GlcNAc transferase (OGT), which modifies substrate protein with GlcNAc; *O*-GlcNAcase, which removes GlcNAc from the substrate; uridine 5'-diphosphate (UDP)-GlcNAc, which is used for the modification and is synthesized intracellularly from glucose, glucosamine (GlcN), and *N*-acetylglucosamine (GlcNAc). *O*-GlcNAc modification occurs in many proteins including kinases and transcription factors and functions in various biological phenomena, such as signaling, cancer, and cell differentiation.[7]

Reportedly, *O*-GlcNAcylation is involved in the differentiation of bone-related cells such as chondrocytes and osteoblasts.[8–10] Moreover, it has been reported that GlcNAc, GlcN, and an *O*-GlcNAcase inhibitor PUGNAc, promote *O*-GlcNAcylation and suppress osteoclast differentiation.[11,12] However, GlcNAc and GlcN incorporation into cells affect sugar metabolism and PUGNAc inhibits not only *O*-GlcNAcase but also GH20 human *β*-hexosaminidases.[6,7] Therefore, it is uncertain whether the suppression of osteoclast differentiation by these chemicals occurs exclusively by the promotion of *O*-GlcNAcylation. In the present study, we investigated the effect of Thiamet G, an *O*-GlcNAcase specific inhibitor,[13] on osteoclastogenesis to elucidate the mechanism underlying suppression of osteoclast differentiation.

MATERIALS AND METHODS

Cell Culture The murine RAW264 cell line established from male BAB/14 mice[14] was obtained from the RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in modified Eagle's medium alpha (MEM*α*) medium purchased from Wako (Osaka, Japan) with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and antibiotics (1×penicillin/streptomycin purchased from Wako) at 37°C under 5% CO_2. Uncharacterized cryopreserved human male peripheral blood mononuclear cells (PBMCs) were purchased from Cellular Technology, Ltd. (Shaker Heights, OH, U.S.A.).

Osteoclast Differentiation and TRAP Staining of RAW264 Cells and Human PBMCs RAW264 cells were seeded into a 96-well cell culture plate at a density of 1000 cells/well. After 1 d, the cells were stimulated with 250 ng/mL soluble RANKL (sRANKL) (Oriental Yeast, Tokyo, Japan) in the presence of 10 μM or indicated concentrations of Thiamet G (Sigma-Aldrich, St. Louis, MO, U.S.A.) or PUGNAc (Wako), both of which inhibit *N*-acetyl-D-glucosaminidase (*O*-GlcNAcase). The cells were further cultured for 4 d to allow differentiation. Thiamet G and PUGNAc were dissolved in dimethyl sulfoxide (DMSO).

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Differentiated cells were subjected to TRAP staining and TRAP-positive cells containing 3 or more nuclei were counted as described previously.[11]

Human male PBMCs were seeded into a 96-well cell culture plate at a density of 1×10^5 cells/well. After 1 d, the cells were stimulated with 25 ng/mL human M-CSF (PeproTech, Rocky Hill, NJ, U.S.A.) and 50 ng/mL sRANKL in the presence of 10 μM Thiamet G. Then the cells were further cultured for 8 d to allow differentiation. Cultivation medium were replenished every 3 or 4 d. Differentiated cells were subjected to TRAP staining, as described above.

Isolation and Osteoclastic Differentiation of Mouse Primary Bone Marrow Cells Four-week-old female ddY mice were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). The isolation of the primary bone marrow cells from the mice and the cultivation of the cells was done according to a previous study.[15] After culturing for 24 h, floating cells were collected as hematopoietic stem cells. The cells were seeded into a 96-well cell culture pate at a density of 4.0×10^4 cells/well and maintained in the presence of 10 μM of Thiamet G or DMSO with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 5 d. For TRAP staining, the cells were fixed using Mildform (Wako) and stained using an Acid phosphatase, leukocyte (TRAP) kit (Sigma-Aldrich).

The study was approved by the Josai University Animal Use Committee and the mice were maintained in accordance with the university guidelines for the care and use of laboratory animals.

Resorption Assay To assess resorption, mouse primary bone marrow cells were seeded into an Osteo Assay Surface 24-well plate (Corning, Cambridge, MA, U.S.A.) at a density of 2.5×10^5 cells/well or human PBMCs were seeded on an Osteo Assay Surface Stripwell Microplate (Corning) at 1×10^5 cells/well and allowed to differentiate. Differentiation protocols were as described in "Osteoclast Differentiation and TRAP Staining of RAW264 Cells and Human PBMCs" and "Isolation and Osteoclastic Differentiation of Mouse Primary Bone Marrow Cells," except that human PBMCs were allowed to differentiate for 14 d. After that, to remove the cells, the plate was incubated with 10% bleach. Then, the plate was washed with distilled water followed by air drying. The image of dried plate was taken by a digital camera, and the area of resorbed surface was measured using NIH ImageJ software.[16]

Real-Time PCR RAW264 cells were stimulated with sRANKL and allowed to differentiate for 4 d, as described in "Osteoclast Differentiation and TRAP Staining of RAW264 Cells and Human PBMCs." Total RNA was extracted from the cells and cDNA was generated using the Power SYBR® Green Cells-to-CT™ Kit (Life Technologies, Carlsbad, CA, U.S.A.). Real-time PCR was performed using the Power SYBR® Green PCR Master Mix (Life Technologies) and the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) as described previously.[12] The primers used for PCR were also described previously,[12] except those for Ogt, 5′-TGG ACA CAC CAC AGG GAT GG-3′ and 5′-GGC AAG AGT CTC TCC TGG CAT-3′.

RNA Interference RAW264 cells were seeded into a 96-well cell culture plate at a density of 1000 cells/well. Then small interfering RNA (siRNA) was transfected by using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instruction. The siRNAs used were as follows: Ogt siRNA #1, 5′-CAU UUA UGA UAA UCG GAU Att-3′ and 5′-UAU CCG AUU AUC AUA AAU Gtg-3′; Ogt siRNA #2, 5′-GAU UGU CUG UGA UUG GAC Att-3′ and 5′-UGU CCA AUC ACA GAC AAU Ctg-3′; and negative control siRNA (Silencer select negative control siRNA #1; Applied Biosystems). After 1 d, the media were replaced and the cells were stimulated with 250 ng/mL sRANKL (Oriental Yeast, Tokyo, Japan). The cells were further cultured for 4 d to allow differentiation, then subjected to real-time PCR or TRAP staining.

Western Blotting RAW264 cells were seeded into a 6-well plate at a density of 20000 cells/well. After 1 d, cells were stimulated with RANKL in the presence of 10 μM Thiamet G, PUGNAc or DMSO for 24 h. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously[12] except that using horseradish peroxidase (HRP)-conjugated anti-*O*-GlcNAc (RL2) mouse monoclonal antibody (Abcam, Cambridge, U.K.). Equal protein loading was confirmed by Coomassie brilliant blue staining.

For the Western blotting of mouse bone marrow cells, cells were seeded at 1.2×10^6 cells/well (9.5 cm^2) and maintained in the presence of 10 μM Thiamet G or DMSO with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 5 d. Then the cells were harvested for Western blotting as described above.

Statistics StatMate 3 software (ATOMS Inc., Tokyo, Japan) was used for statistical analysis. Data were expressed as mean ± standard deviation (S.D.) and analyzed by Tukey's multiple comparison test. p-Values <0.01 were considered statistically significant. We repeated each experiment at least two times and obtained similar results.

RESULTS AND DISCUSSION

Thiamet G Suppresses the Osteoclastic Differentiation of RAW264 Cells We first investigated the effect of Thiamet G on the osteoclastic differentiation of murine RAW264 cells, which differentiate to osteoclast-like cells upon RANKL stimulation and is known to well reflect the physiological process.[17] RAW264 cells were treated with RANKL in the presence of Thiamet G or PUGNAc for 24 h, then extracted and subjected to Western blotting using an anti-*O*-GlcNAc antibody (Fig. 1A). Both GlcNAcase inhibitors led to a global increase in *O*-GlcNAcylated proteins. However, Thiamet G treatment promoted more *O*-GlcNAcylation as compared with PUGNAc. This might reflect the difference in the inhibition activity of these inhibitors: the *K*i value of Thiamet G (21 nM) is lower than that of PUGNAc (50 nM).[6]

To clarify the effect of these inhibitors on osteoclastic differentiation, we treated RAW264 cells with RANKL in the presence of Thiamet G or PUGNAc for 4 d to allow differentiation, then subjected the cells to TRAP staining and real-time PCR of osteoclast marker genes (Figs. 1B–D). We found that both Thiamet G and PUGNAc suppressed the RANKL-dependent formation of TRAP-positive multinuclear cells and the induction of cathepsin K and MMP-9. We also found that the treatment of lower concentrations of Thiamet G (0.1 and 1 μM) significantly suppressed the differentiation (Fig. 1C). These results indicated that specific inhibition of the *O*-GlcNAcase suppresses osteoclastogenesis of the murine osteoclast differentiation model. It should be noted that results

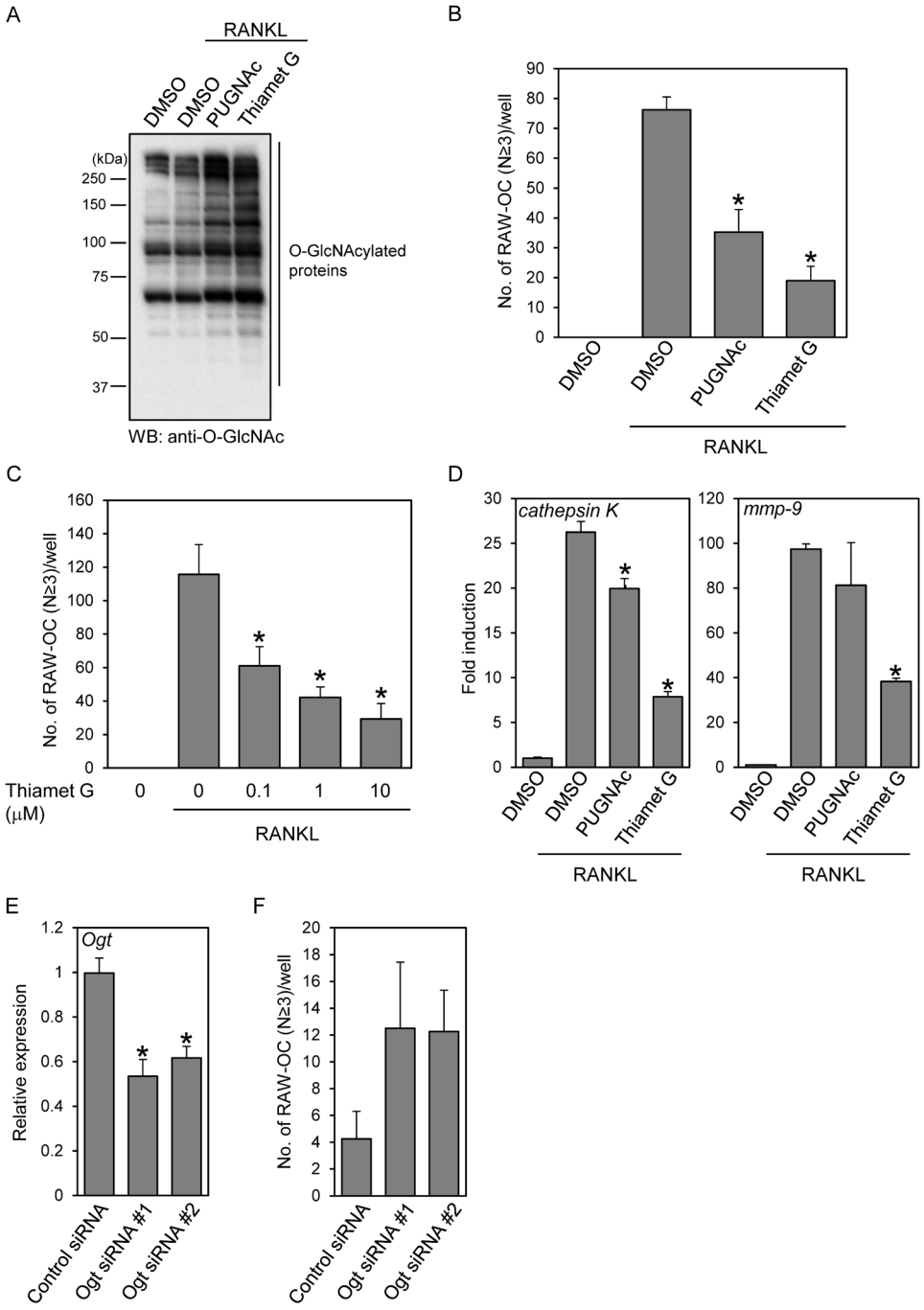

Fig. 1. Effects of *O*-GlcNAcase Inhibitors on Osteoclastic Differentiation of Murine RAW264 Cells

(A) RAW264 cells were treated with RANKL in the presence of 10 μM of PUGNAc, Thiamet G or DMSO for 24 h, then the cells were lysed and subjected to Western blotting with the anti-*O*-GlcNAc antibody. (B–D) RAW264 cells were treated with RANKL in the presence of 10 μM or indicated concentrations of PUGNAc, Thiamet G or DMSO and incubated for 4 d to allow differentiation. Then the differentiated cells were subjected to (B, C) TRAP-staining or (D) real-time PCR analysis of osteoclast marker genes, cathepsin K and matrix metallopeptidase 9 (MMP-9). (E and F) RAW264 cells were transfected with indicated siRNAs, and treated with RANKL. After incubation for 4 d, the cells were subjected to (E) real-time PCR or (F) TRAP-staining. Data are expressed as mean ± S.D. $*p < 0.01$ *vs.* control (DMSO with RANKL or Negative control siRNA).

with Thiamet G are not sufficient to conclude the definitive role of *O*-GlcNAcase, even the specificity of Thiamet G is high.

In addition, the effect of knockdown of Ogt, which is speculated to suppress *O*-GlcNAcylation, was examined. RAW264 cells were transfected with two different sequences of Ogt siRNAs (Ogt siRNA #1 and #2) or Negative control siRNA, then the effects of these siRNAs on the expression Ogt mRNA and the formation of TRAP-positive multinuclear cells were verified (Figs. 1E, F). Transfection of each Ogt siRNA suppressed the amount of Ogt mRNA and tended to promote the formation of osteoclast-like cells. These results also indicated the importance of *O*-GlcNAcylation on osteoclastogenesis and further supported the effect of *O*-GlcNAc inhibitors on osteoclastogenesis.

Thiamet G Suppresses the Osteoclast Differentiation and Bone Resorption Activity of Murine Bone Marrow-Derived Cells Next, we investigated the effect of Thiamet G using

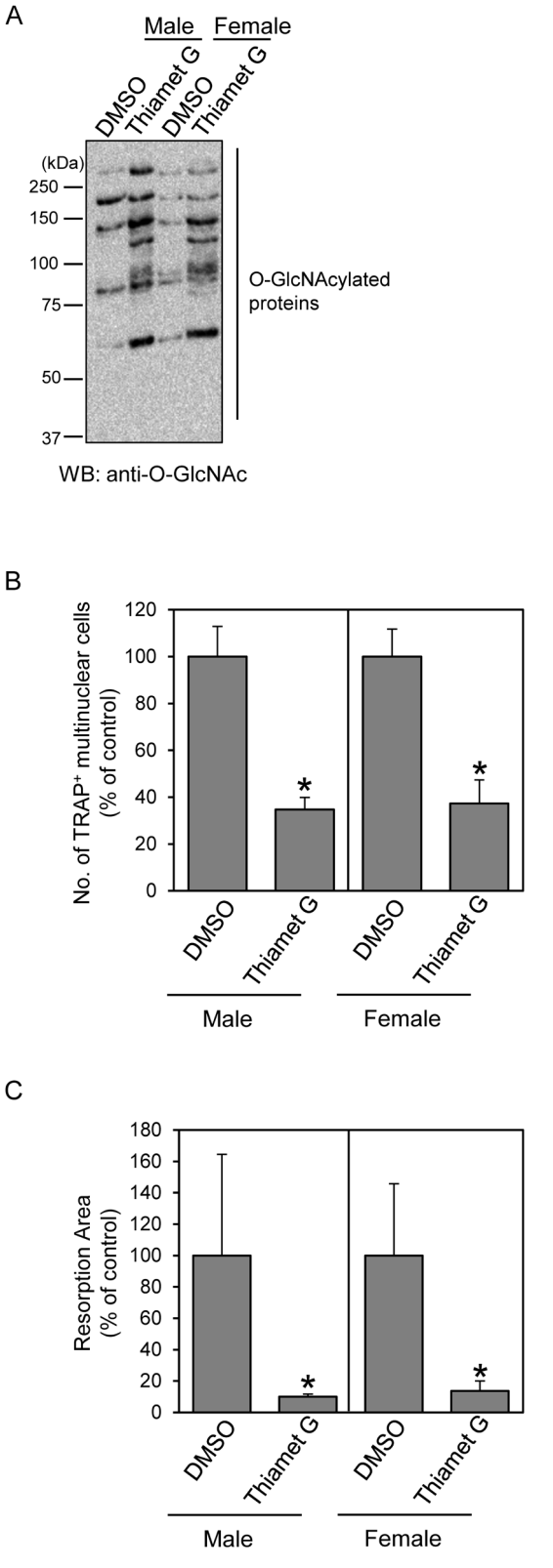

Fig. 2. The Effect of Thiamet G on Osteoclastic Differentiation of Mouse Bone Marrow-Derived Cells

(A, B) Mouse bone marrow-derived cells from male and female ddY mice were treated with M-CSF and RANKL in the presence of Thiamet G for 5 d. The cells were then subjected to (A) Western blotting with anti-*O*-GlcNAc antibody, or (B) subjected to TRAP-staining. (C) For bone resorption assay, mouse bone marrow-derived cells were allowed to differentiate as described above, except that the cells were seeded on an Osteo Assay Surface Plate. Data are expressed as mean ± S.D. $*p < 0.01$ *vs.* control (DMSO with RANKL).

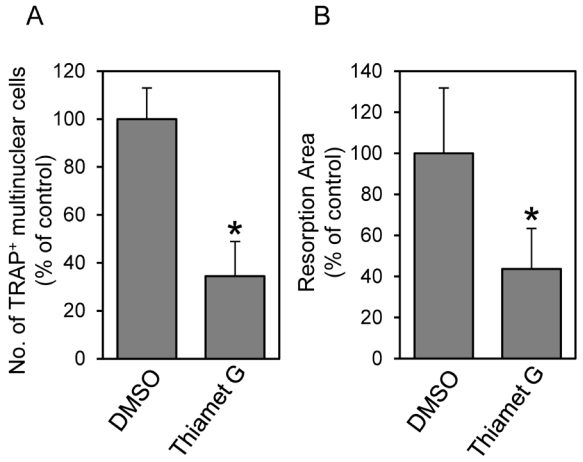

Fig. 3. The Effect of Thiamet G on Osteoclastic Differentiation of Human PBMCs

(A) Human male PBMCs were treated with M-CSF and RANKL in the presence of Thiamet G and incubated for 14 d to allow differentiation. Afterwards, the differentiated cells were subjected to TRAP-staining. (B) For bone resorption assay, PBMCs were allowed to differentiate as described above, except that the cells were seeded on an Osteo Assay Surface Plate. After 14 d, the resorption areas on the plates were measured using NIH ImageJ software. Data are expressed as mean ± S.D. $*p < 0.01$ *vs.* control (DMSO with RANKL).

mouse primary bone marrow cells under physiological conditions. We isolated the primary bone marrow cells from male and female mice, and treated them with M-CSF and RANKL in the presence of Thiamet G or DMSO. Western blotting with an anti-*O*-GlcNAc antibody revealed that Thiamet G treatment increased global *O*-GlcNAcylated proteins in cells derived from both male and female mice (Fig. 2A). Meanwhile, Thiamet G treatment suppressed the formation of TRAP-positive multinuclear cells and bone resorption activity (Figs. 2B, C). These results were consistent with those using RAW264 cells and indicated that the promotion of *O*-GlcNAcylation suppresses osteoclast differentiation and activity of murine cells regardless of their gender.

Thiamet G Suppresses Osteoclast Differentiation and Bone Resorptive Activity of Human PBMCs We then investigated whether Thiamet G treatment also suppresses the osteoclastogenesis of human cells. We treated human male PBMCs with M-CSF and RANKL in the presence of Thiamet G or DMSO. TRAP staining and resorption assay revealed that Thiamet G treatment reduced the number of TRAP-positive multinuclear cells and bone resorption activity (Figs. 3A, B). These results are consistent with the results obtained using RAW264 cells and mouse primary bone marrow-derived cells, indicating that the promotion of *O*-GlcNAcylation using Thiamet G suppresses osteoclast differentiation.

In the present study, we mainly investigated the effect of the promotion of global *O*-GlcNAcyaltion on osteoclast differentiation using Thiamet G and observed suppression of osteoclastogenesis. Although the underlying molecular mechanism remains undetermined, it has been indicated in previous studies that PUGNAc treatment suppresses nuclear factor-kappaB (NF-κB) signaling pathway, which is important for osteoclast differentiation.[4,11] In addition, we identified vimentin as a potential *O*-GlcNAcylated protein in RAW264 cells (data not shown). Since *O*-GlcNAc modification of vimentin affects its function and antibody against citrullinated vimentin induces osteoclastogenesis,[18,19] it is possible that *O*-GlcNAcylation af-

fects osteoclastogenesis through the modification of vimentin; this should be investigated in the future.

It has been reported that GlcN supplementation might increase global *O*-GlcNAcylation, attenuating bone loss in ovariectomized mice,[15] and that the promotion of *O*-GlcNAcylation accelerates osteoblast differentiation.[10] In the present study, we found that promotion of *O*-GlcNAcylation suppresses osteoclast differentiation. The results of this study suggest that chemicals promoting global *O*-GlcNAcylation might have a positive effect on the prevention or treatment of osteoporosis; however, further careful investigations are needed to elucidate this.

Acknowledgments We are grateful to Mr. Hidehiko Senba, Ms. Kaho Takahashi, and Ms. Mana Yanagidaira (Josai University, Faculty of Pharmacy and Pharmaceutical Sciences) for technical assistance.

Conflict of Interest The authors declare no conflict of interest.

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