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Involvement of the G-protein-coupled receptor 4 in RANKL expression by osteoblasts in an acidic environment

Asuka Okito [a, b], Ken-ichi Nakahama [a, *], Masako Akiyama [a], Takashi Ono [b], Ikuo Morita [a]

[a] Department of Cellular Physiological Chemistry, Tokyo Medical and Dental University, Tokyo, Japan
[b] Department of Orthodontic Science, Tokyo Medical and Dental University, Tokyo, Japan

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ABSTRACT

Osteoclast activity is enhanced in acidic environments following systemic or local inflammation. However, the regulatory mechanism of receptor activator of NF-κB ligand (RANKL) expression in osteoblasts under acidic conditions is not fully understood. In the present paper, we detected the mRNA expression of the G-protein-coupled receptor (GPR) proton sensors GPR4 and GPR65 (T-cell death-associated gene 8, TDAG8), in osteoblasts. RANKL expression and the cyclic AMP (cAMP) level in osteoblasts were up-regulated under acidic culture conditions. Acidosis-induced up-regulation of RANKL was abolished by the protein kinase A inhibitor H89. To clarify the role of GPR4 in RANKL expression, GPR4 gain and loss of function experiments were performed. Gene knockdown and forced expression of GPR4 caused reduction and induction of RANKL expression, respectively. These results suggested that, at least in part, RANKL expression by osteoblasts in an acidic environment was mediated by cAMP/PKA signaling resulting from GPR4 activation. A comprehensive microarray analysis of gene expression of osteoblasts revealed that, under acidic conditions, the phenotype of osteoblasts was that of an osteoclast supporting cell rather than that of a mineralizing cell. These findings will contribute to a molecular understanding of bone disruption in an acidic environment.

1. Introduction

Bone remodeling is precisely regulated by the balance between bone formation and resorption. Osteoclasts, which are multinuclear cells, resorb old bone. On the other hand, osteoblasts synthesize the bone matrix on resorption pits to maintain bone integrity [1]. Excessive local bone resorption and inadequate bone formation result in bone diseases such as inflammatory arthritis, post-menopausal osteoporosis, and bone metastasis of tumors [2]. Receptor activator of NF-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) are essential for the differentiation of osteoclast precursors into functional osteoclasts since mice knocked out for these genes exhibited severe osteopetrosis owing to a defect in mature osteoclasts [3,4]. RANKL is mainly expressed by osteoblast lineage cells and its expression is regulated by the microenvironments that surround these cells such as inflammatory mediators, hypoxia, and acidosis [5]. Acidosis can arise locally as a result of hypoxia and inflammation. Chronic metabolic acidosis causes a decrease in bone mineral density and content in *in vivo* experiments [6,7]. It is therefore important to explore the mechanism by which osteoclast differentiation and activity are up-regulated in acidic conditions. Some papers have reported that acidic conditions promote osteoclast differentiation, activity, and survival [8–11]. Osteoblasts exhibit two phenotypes: one that produces bone matrix and another that supports osteoclast differentiation. Experimental evidences suggest that acidosis affects not only the function of osteoclasts but also the phenotype of osteoblasts. Differentiation and mineralization of osteoblasts have been reported to decrease in acidic conditions [12–15]. In addition to the effect of acidosis on this osteogenic phenotype, an effect of acidosis on the osteoclastogenic phenotype that supports osteoclast differentiation has also been reported [16,17].

In this study, we found up-regulation of RANKL expression in osteoblastic cells under acidic conditions. These cells expressed the G-protein-coupled proton sensors GRP4 and GPR65 (T-cell death-associated gene 8, TDAG8). We hypothesized that the up-

* Corresponding author. Department of Cellular Physiological Chemistry, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. Fax: +81 3 5803 0212.
E-mail address: nakacell@tmd.ac.jp (K.-i. Nakahama).

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regulation of RANKL under acidic conditions is mediated by GPR4. The cyclic AMP (cAMP) level of osteoblastic cells was increased under acidic conditions. Moreover, acidosis-induced RANKL expression was attenuated by the treatment of cells with the protein kinase A (PKA) inhibitor, H89. To confirm the involvement of GPR4 in acidosis-induced RANKL expression, knockdown and forced expression of GPR4 experiments were performed. RANKL expression was decreased in GPR4 knockdown cells and was increased in GPR4 overexpressing cells. These results suggested the possible involvement of GPR4 in bone loss under acidic conditions. Moreover, cDNA microarray data may suggest that osteoblasts tend to change their phenotype from osteogenic to osteoclastogenic under acidic conditions.

2. Materials and methods

2.1. Cell culture

Specific pathogen-free male ddY mice (6–8 weeks) were purchased from Japan SLC (Shizuoka, Japan). Mouse bone marrow cells were removed by flushing the femurs and tibias using a 27-gauge needle with phosphate buffered saline. The femurs and tibias without bone marrow were then cultured with alpha-modified Eagle's medium (αMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA) in 100 mm collagen-coated dishes at 37 °C, 95% humidity and 5% CO_2. Osteoblasts were isolated from the cells that had grown around the long bones at 2–4 weeks after initiation of the culture. The osteoblasts isolated from the long bones were confirmed to have osteoblast characteristics (mineralization and osteoclast supporting activity) and will be referred to as LBOB. TMS12 cells, which were previously characterized as murine osteoblasts, were isolated from bone marrow derived stromal cells [18]. MC3T3E1 cells (an ostoeblastic cell line) were purchased from the Riken BioResource Center (Ibaraki, Japan). These isolated osteoblasts and MC3T3E1 cells were cultured in alpha-modified Eagle's medium (αMEM, Invitrogen) supplemented with 10% FBS in 100 mm dishes at 37 °C, 95% humidity and 5% CO_2. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

2.2. Acidic culture conditions

Cells were seeded at a density of 10^6 cells/60 mm dish. Total RNA was extracted at 24 h after cultivation of the cells with 10% FBS containing αMEM (15 mM sodium bicarbonate) under a 5% or a 10% CO_2 condition at 37 °C, 95% humidity. The pH of the medium under 5% and 10% CO_2 conditions was confirmed as pH7.4 and pH 6.88 respectively. RANKL expression was evaluated by quantitative reverse transcription polymerase chain reaction (RT-PCR) (section 2.3) and the results are presented relative to GAPDH expression. To examine the effect of the PKA inhibitor, H89 (Sigma, USA), or the vehicle (dimethyl sulfoxide), the cells were pre-treated with these agents for 30 min under the 5% CO_2 condition before the 24 h of incubation under different CO_2 concentrations.

2.3. RT-PCR and real-time PCR

Cells were seeded at a density of 10^6 cells/60 mm dish. Total RNA was extracted from osteoblasts using the TRIzol reagent (Invitrogen). Five μg total RNA was subjected to reverse transcription. The cDNA samples were amplified using KAPA SYBR® FAST qPCR Kits (KAPA BIOSYSTEMS, Cape Town, South Africa). Quantitative analysis was performed using the comparative Ct Method (7500 Real-Time PCR System; Applied Biosystems, Foster City, CA, USA). Obtained PCR products were analyzed on a 2% agarose gel (Wako Chemicals, Osaka, Japan) to check the length of the PCR product. To detect and quantify the expression of mRNA in osteoblasts, the primer pairs used in this experiment were as follows: for mouse GAPDH (125 bp), 5′-CAACTCGGCCCCCAACACT-3' (forward) and 5′-TGGGTGCAGCGAACTTTAT-3' (reverse); for mouse GPR4 (218 bp), 5′-CTACCTGGCTGTGGCTCAT-3' (forward) and 5′-CAAAGACGCGGTACAGATTCA-3' (reverse); for mouse GPR65 (212 bp), 5′-GCTTCTGTGGAAAGATGAAACGA-3′ (forward) and 5′-TGTTTTCCGTGGCTTGGTTG-3′ (reverse); for mouse GPR68 (386 bp), 5′-TAAGAGCCTAGCCGGAAGGG-3′ (forward) and 5′-GCCAATCCCTCTCTTGCCAT-3′ (reverse); for mouse GPR132 (145 bp), 5′-CAGGACTGGCTTGGGTCATTT-3′ (forward) and 5′-GTGCTCTGAAGAACGGAGGT-3′ (reverse); for mouse RANKL (108 bp), 5′-CCTGAGGCCCAGCCATTT-3′ (forward) and 5′-CTTGGCCCAGCCTCGAT-3′ (reverse).

2.4. Plasmid construction

DNA fragments encoding full-length mouse GPR4 (GenBank ID: NM_175668) were amplified by a PCR-based technique using proofreading DNA polymerase (ExTaq polymerase, TAKARA, Japan). The primers used for cloning were 5′-TAGCCTGCCACAAAGCAAAC-3′ (forward) and 5′-CTGTCCTTATCTGCCAGAAACC-3′ (reverse). Obtained DNA fragments were cloned into pCR2.1-TOPO (Invitrogen). The GPR4 cDNA was then subcloned into the EcoRI site of the retrovirus expression vector, pQCXIP (Clontech, Mountain View, CA, USA). The constructs were verified by analysis of restriction enzyme digests and DNA sequencing.

2.5. cAMP assay

Osteoblastic cells were seeded on 96-well plates at a density of 2×10^4 cells/well and were cultured for 24 h in a 5% CO_2 condition. These cells were exposed to neutral or acidic medium that was equilibrated with 5% or 10% CO_2 respectively. These cells were incubated for 10 min under a 5% or 10% CO_2 condition at 37 °C. The concentration of cytosolic cAMP was measured using a cAMP EIA kit (Amersham PI, Little Chalfont, UK) according to the instruction manual.

2.6. Gene knockdown and over expression of GPR4

For GPR4 knockdown, short hairpin RNAs (clone ID; TRCN0000028167 and TRCN0000028164, Open Biosystems, Huntsville, AL, USA) for mouse GPR4 or empty vector (pLKO.1-pur, Sigma) were co-transfected with lentiviral packaging vectors (ViraPower™, Invitrogen) into HEK293FT cells (Invitrogen) using the Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after transfection, virus containing conditioned media was harvested. TMS12 cells were infected with the filtered viral supernatant and polybrene (10 μg/ml; Millipore, Billerica, MA, USA). GPR4 knockdown TMS12 cells and empty vector (EV) introduced cells were established by puromycin selection for 1 week and were named TMS12sh23 and control (EV) respectively. For GPR4 overexpression the GPR4 or mAG1 expression vector and pCL10A1 (a retroviral packaging vector, IMGENEX, San Diego, CA, USA) were co-transfected into HEK293FT cells using the Lipofectamine 2000 reagent. Forty-eight hours after transfection, virus containing conditioned media was harvested. TMS12 cells were infected with the filtered viral supernatant with polybrene (10 μg/ml). TMS12 cells overexpressing GPR4 or mAG1 were established by puromycin selection for 1 week, and were named mAG1 or GPR4OE.

2.7. Microarray analysis of osteoblast gene expression

TMS12 or GPR4OE cells were seeded on a 60 mm dish (10^6 cells/dish). Twenty-four hours after cultivation of the cells in a 5% or a 10% CO_2 condition, total RNA was extracted. The isolated RNA was amplified and labeled with Cy3 using the NimbleGen One-Color DNA Labeling Kit (Roche NimbleGen, Madison, WI, USA). The labeled cDNA samples were hybridized to the SurePrint G3 Mouse GE 8 × 60K Microarray (Agilent Technologies, Santa Clara, CA, USA). Hybridized arrays were scanned using an Axon GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA). Measurement of the microarray assay following mRNA extraction was outsourced to Takara Bio Inc. (Osaka, Japan). The microarray data analyses were performed with GeneSpring GX 12 (Agilent Technologies). Total detected entities were filtered by signal intensity value (lower cut-off 20th percentile) to remove very low signal entities. Normalization was performed with percentile shift in the analysis of signaling pathways.

2.8. Statistical analysis

JMP10 for Windows (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. Values are shown as means ± S.D. Comparisons were made using Student's *t*-test, Dunnett's multiple comparison test, or Tukey–Kramer's multiple comparison test. P values of less than 0.05 were considered significant.

3. Results

3.1. RANKL up-regulation under acidic conditions in osteoblasts

To confirm that RANKL expression is increased in osteoblasts in response to acidic stimulation, TMS12 and LBOB cells were cultured under 5% or 10% CO_2 conditions at 37 °C for 24 h. As shown in Fig. 1A, RANKL expression increased about 2–2.5-fold in both osteoblastic cell lines under acidic versus basic conditions. Previous reports suggested that these cells can sense pH using proton sensors. We therefore tested the expression of proton sensors in these osteoblastic cells. This experiment indicated that these osteoblasts expressed both of the proton sensors GPR4 and GPR65 (also known as TDAG8) (Fig. 1B).

3.2. Acidosis-induced RANKL up-regulation in osteoblasts via PKA

Proton sensing of both GPR4 and GPR65 has been reported to induce an increase in cytosolic cAMP in the cells. We therefore examined the cytosolic cAMP concentration in osteoblasts under 5% or 10% CO_2 conditions. As expected, the cAMP level in osteoblasts was increased under acidic conditions (Fig. 2A). Furthermore, RANKL expression under acidic conditions was abolished by treatment of the cells with H89, an inhibitor of the cAMP-dependent protein kinase PKA (Fig. 2B). This result suggested the involvement of the cAMP/PKA pathway in RANKL up-regulation under acidic conditions.

3.3. Involvement of GPR4 in RANKL up-regulation in acidosisa (gene knockdown and overexpression experiments)

Since we detected GPR4 and GPR65 expression in osteoblasts we aimed to clarify the role of GPR4 in RANKL expression under acidic conditions by performing GPR4 gene knockdown and overexpression experiments. As shown in Fig. 3A, GPR4 knockdown was established using an RNA interference technique (GPR4 mRNA expression was decreased by about 40% in comparison with parental cells, Fig. 3A). RANKL expression was significantly reduced in GPR knockdown TMS12 under neutral and acidic conditions (Fig. 3B). In contrast, forced expression of GPR4 caused an increase in GPR4 and RANKL expression under neutral and acidic conditions (Fig. 3C and D). These results strongly suggested that RANKL expression in an acidic condition is mediated, at least in part, by GPR4. Moreover, GPR4 can sense protons even in a neutral condition.

3.4. Comprehensive analysis

To understand the effect of proton sensing on gene expression in osteoblasts, a comprehensive analysis of gene expression by osteoblasts under acidic conditions was performed using a cDNA microarray. Among the expressed genes, we focused on adhesion molecules (Fig. 4A), growth factors (Fig. 4B), osteoclast differentiation factors (Fig. 4C), and markers of osteoblast mineralization (Fig. 4D) because these genes affect the phenotype of osteoblasts. Vascular cell adhesion molecule-1 (VCAM-1) expression tended to

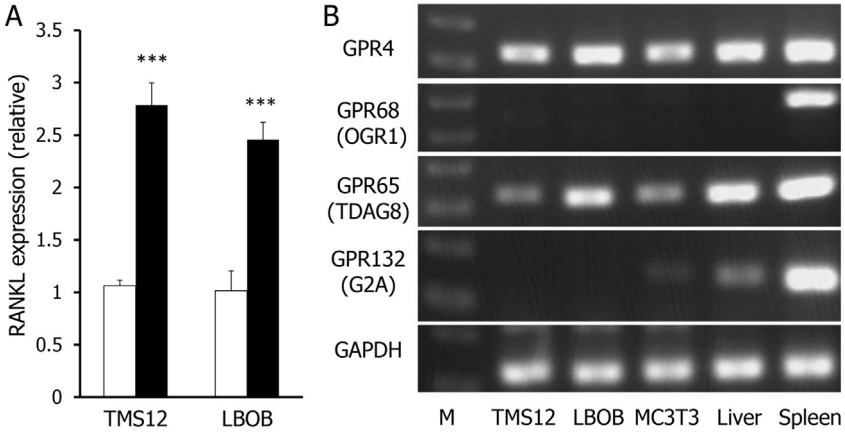

Fig. 1. Up-regulation of RANKL under acidic conditions and expression of proton sensors. (A) RANKL expression was increased in osteoblastic cells under acidic conditions. Twenty-four hours after cultivation of the cells in a 5% (open columns) or a 10% (closed columns) CO_2 condition. RANKL mRNA expression was evaluated using quantitative RT-PCR and the results are presented relative to GAPDH expression. Values are presented as means ± S.D., n = 3–4. Comparisons were made with Tukey–Kramer's multiple comparison test. ***p < 0.01 vs. the 5% CO_2 condition. (B) Expression of proton sensors. Total RNA was extracted from TMS12, LBOB, and MC3T3E1 cell lines and from mouse liver and spleen. G-protein-coupled receptor (GPR) mRNA expression was then analyzed using RT-PCR. The products of RT-PCR were separated by electrophoresis in a 2% agarose gel. Ethidium bromide stained gel images are presented. GAPDH mRNA expression was analyzed as a loading control. Lane M, molecular marker.

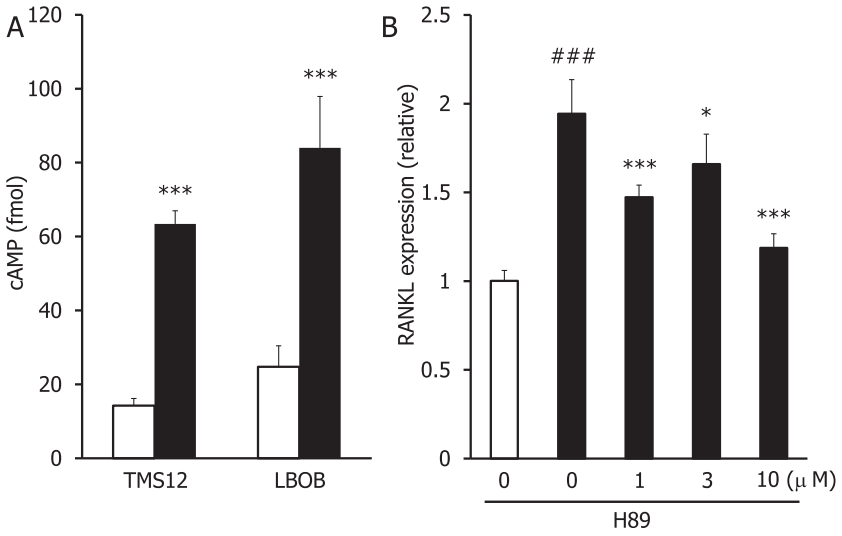

Fig. 2. Cytosolic cAMP elevation under acidic conditions and inhibitory effect of H89 on acid-induced RANKL expression. (A) Cytosolic cAMP levels were increased in osteoblastic cells under acidic conditions. Cells were exposed to a 5% CO_2 condition (open columns) or a 10% CO_2 condition (closed columns) for 10 min following which cAMP levels were measured using an EIA. Values are presented as means ± S.D. n = 9–10. Comparisons were made using Tukey–Kramer's multiple comparison test. ***p < 0.001 vs. control under the 5% CO_2 condition. (B) Effect of the PKA inhibitor H89 on acid-induced RANKL expression. TMS12 cells were pre-treated with H89 or the vehicle for 30 min under 5% CO_2 conditions followed by 24 h of cultivation of the cells under 5% (open columns) or 10% (closed columns) CO_2 conditions. RANKL mRNA expression was evaluated using quantitative RT-PCR and the results are presented relative to GAPDH mRNA expression. Values are presented as means ± S.D. n = 4. Comparisons were made using Dunnett's multiple comparison test. *p < 0.05, ***p < 0.001 vs. control under the 10% condition, ###p < 0.001 vs. control under the 5% condition.

increase in GPR4OE cells. Expression of transforming growth factor β-3 (TGF β-3) and RANKL depended on acidosis and GPR4 expression. Especially, expressions of alkaline phosphatase (ALP), osteopontin (OPN) and bone sialoprotein (BSP) were confirmed by real-time PCR (Suppl. Fig. 1). Interestingly, the expression of BSP was inversely correlated with that of RANKL. Collectively, these data might suggest that the osteoblast phenotype in an acidic condition was osteoclastogenic.

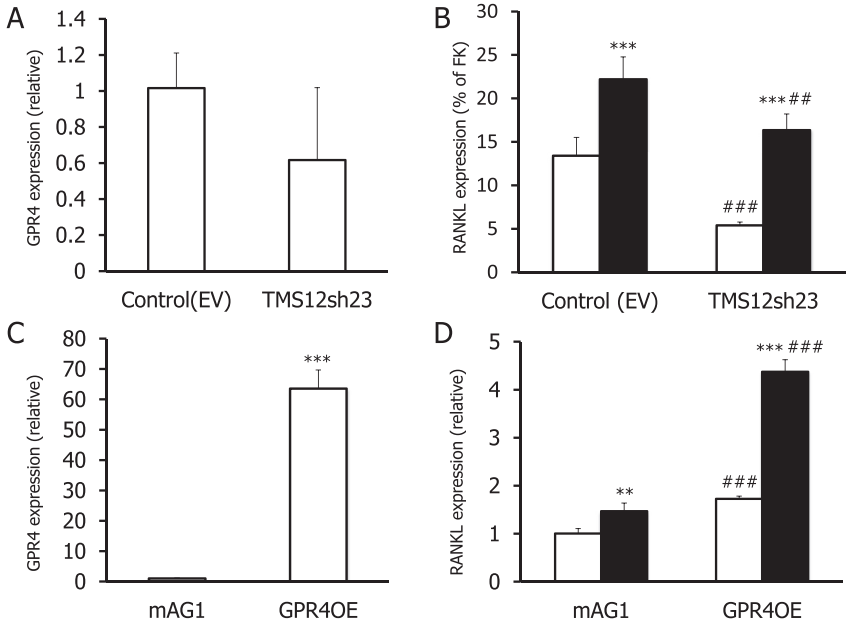

Fig. 3. Effect of GPR4 knockdown/overexpression on RANKL expression. (A) GPR4 knockdown in TMS12 cells (TMS12sh23 cells) tend to decrease in GPR4 expression. GPR4 mRNA expression was evaluated using quantitative RT-PCR and the results are presented relative to GAPDH mRNA expression. Values are presented as means ± S.D., n = 4. (B) RANKL mRNA expression was evaluated in Control (EV) and TMS12sh23 cells under 5% (open column) and 10% (closed column) CO_2 conditions using quantitative RT-PCR. To exclude cell reactivity against cAMP between Control (EV) and TMS12sh23, each value was represented as a % of RANKL expression in forskolin treated cells (relative to GAPDH mRNA expression). Values are presented as means ± S.D., n = 3. Comparisons were made using Tukey–Kramer's multiple comparison test. **p < 0.01, ***p < 0.001 vs. 10% the CO_2 condition, ###p < 0.001 vs. TMS12. (C) GPR4 overexpression in TMS12 cells (GPR4OE cells) caused a significant increase in GPR4 mRNA expression. GPR4 mRNA expression in GPR4OE or vector-expressing control cells (mAG1) was evaluated using quantitative RT-PCR and the results are presented relative to GAPDH mRNA expression. Values are presented as means ± S.D., n = 4. Comparisons were made using Student's *t*-test. ***p < 0.001 vs. mAG1. (D) RANKL expression in GPR4OE or mAG1 cells under 5% (open columns) or 10% (closed columns) CO_2 conditions was evaluated using quantitative RT-PCR. The results are presented relative to GAPDH mRNA expression. Values are presented as means ± S.D., n = 4. Comparisons were made using Tukey–Kramer's multiple comparison test. *p < 0.05, ***p < 0.001 vs. the 5% CO_2 condition, ###p < 0.001 vs. mAG1.

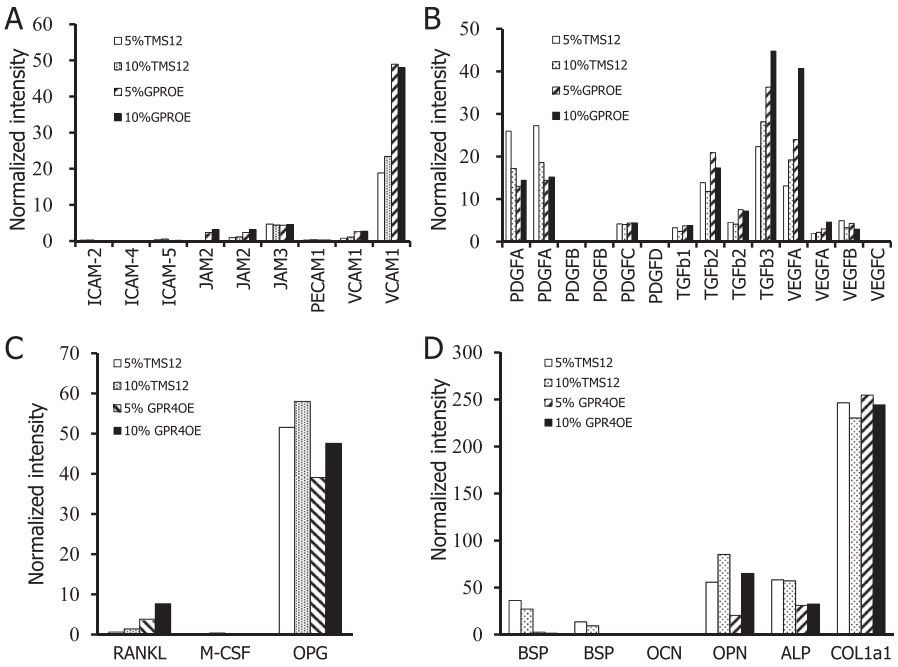

Fig. 4. Microarray analysis of osteoblast gene expression under acidic conditions. Twenty-four hours after cultivation of the TMS12 or GRPOE cells in 5% or 10% CO_2 conditions, total RNA was extracted and was then analyzed using a microarray. The microarray data were normalized and the expression of selected genes is presented as normalized intensity. (A) Expression of adhesion molecules. ICAM, intercellular adhesion molecule; JAM, junctional adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; VCAM, vascular cell adhesion molecule. (B) Expression of growth factors. PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor. (C) Expression of osteoclastogenic factors. RANKL, receptor activator of NF-κB ligand; M-CSF, macrophage-colony stimulating factor; OPG, osteoprotegerin. (D) Expression of osteogenic markers. BSP, bone sialoprotein; OCN, osteocalcin; OPN, osteopontin; ALP, alkaline phosphatase; COL1a1, alpha1(I) collagen. Some genes were detected by two different probes.

4. Discussion

Bone erosion is often observed in inflammatory disease and acidosis. To date it has been reported that osteoclast differentiation, activity, and survival are increased under acidic environments. However, there have only been a few papers that examined the effect of acidosis on osteoblast function, especially on osteoclast supporting activity. We report herein for the first time the possible role of GPR4 in RANKL expression in osteoblasts under acidic environments. We examined the expression of the proton-sensing G-protein coupled receptors, GPR4, GPR68 (OGR1), GPR65 (TDAG8), and GPR132 (G2A) in osteoblasts. These proton sensors sense extracellular protons and stimulate different intercellular signaling pathways [19]. Activation of GPR4 and TDAG8 cause an increase in cytosolic cAMP, while activation of GPR68 and 132 elicit cytosolic Ca^{2+} [20,21]. We found that the proton sensors GPR4 and GPR65 were expressed by cultured osteoblastic cells. A previous report showed the expression of GPR4, GPR65, and GPR68 in bone cells [22,23]. We could not detect GPR68 expression in the mouse osteoblastic cells used in our experiments. According to Hikiji et al., GPR65 mRNA and protein expression are localized in osteoclasts in bone tissue [24]. GPR4 was reported as a proton sensing G-protein-coupled receptor that elicits cAMP formation in 2003 [25]. Previous reports suggested that GPR4 plays important roles in the expression of adhesion molecules of endothelial cells and tumor malignancy under acidic conditions [26–28]. To the best of our knowledge, the role of GPR4 in bone cells has not yet been reported although its expression has been detected in osteoblastic cells. We therefore focused on the role of GPR4 in RANKL expression under an acidic condition. In our experiments, RANKL mRNA of osteoblasts was up-regulated in response to acidic environments via cAMP/PKA signaling. These results indicated the involvement of GPR4 in RANKL expression in osteoblasts under acidic environments. We next performed experiments to confirm the impact of GPR4 on RANKL expression using osteoblastic cells in which GPR4 was knocked down or over-expressed. RANKL expression was attenuated by shRNA knockdown of GPR4 whereas forced expression of GPR4 resulted in the up-regulation of RANKL under neutral and acidic conditions. These data strongly suggested that GPR4 mediated RANKL up-regulation in osteoblasts under acidic environments.

Promoter/enhancer analysis has revealed that the gene expression of RANKL is mainly regulated by cAMP/PKA signaling in osteoblastic cells [29]. This regulation was further confirmed by *in vivo* experiments [30]. These reports support our data that GPR4-mediated cAMP formation caused RANKL up-regulation.

Lastly, we showed the effect of acidosis on the gene expression of osteoblasts using a cDNA microarray. We focused on genes involved in adhesion, on growth factor genes, and on genes involved in osteoclast differentiation and in mineralization, because these genes are important for osteoblast functions (osteoclast differentiation/osteoblast mineralization). Chen et al. [27] reported that the expression of ICAM-1 and VCAM-1 is increased in GPR4 overexpressing endothelial cells under acidic conditions. Our results indicated that the expression of VCAM-1, TGFβ3, and VEGF-A is increased in response to both acidosis and GPR4 overexpression. These results may suggest that these genes are regulated by the same mechanism as RANKL. BSP and ALP were decreased by acidosis or by GPR4 overexpression, in contrast to osteoclast-inducible genes like RANKL and M-CSF. The combined data suggested that the phenotype of osteoblasts changes to support osteoclasts in acidic environments.

In this paper, we found that GPR4 was involved in RANKL expression under an acidic environment. The gene knockdown and overexpression of GPR4 experiments strongly suggested that, at least in part, GPR4 played an important role in RANKL expression

under acidic conditions, although we could not exclude the possibility that GPR65 (TDAG8) affected RANKL expression in osteoblasts.

Acidic environments are often observed at the site of active bone erosion such as that found in rheumatoid arthritis. Moreover, systemic acidosis with renal and respiratory disease is associated with bone loss. Our study provides insights into the mechanisms by which osteoclast differentiation may be promoted by GPR4-mediated RANKL expression of osteoblasts in an acidic environment. Thus GPR4 may be a candidate therapeutic target.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.142.

Appendix A. Supplementary data

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