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Down-regulation of mTOR leads to up-regulation of osteoprotegerin in bone marrow cells

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ABSTRACT

Osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor regulates bone mass by inhibiting osteoclastic bone resorption. mTOR, which is the mammalian target of rapamycin, is a kinase and central regulator of cell growth, proliferation, and survival. By using Rapamycin, we studied whether mTOR pathway is associated with OPG protein production in the mouse bone marrow-derived stromal cell line ST2. Rapamycin markedly increased the level of soluble OPG in ST2 cells. This antibiotic treatment resulted in the suppression of phosphorylation of mTOR. Rapamycin had no effects on the proliferation, differentiation, or apoptosis of the cells. Treatment with bone morphogenetic protein-4, which can induce OPG protein in ST2 cells, also resulted in a decrease in the density of the phospho-mTOR-band, suggesting that the suppression of the phospho-mTOR pathway is necessary for OPG production in ST2 cells. Thus, suitable suppression of mTOR phosphorylation is a necessary requirement for OPG production in bone marrow stromal cells.

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Introduction

Osteoprotegerin (OPG), which is known as osteoclastogenesis inhibitory factor [1], is a soluble decoy receptor for osteoclast differentiation factor as well as receptor activation of NF-κB ligand (RANKL). OPG inhibits osteoclastogenesis by interrupting cell-to-cell signaling between stromal ST2 cells and osteoclast progenitors; resulting in inhibition of the differentiation of osteoclasts [1]. Transgenic mice that overexpress OPG show osteopetrosis and lack osteoclasts [2]. Interestingly, OPG and RANKL in the alveolar bone stromal cells are associated with tooth eruption [3,4]. Since OPG mRNA is also present in gingival fibroblasts, periodontal ligament cells, and pulp cells [5], the alteration of OPG might be associated with the pathophysiology of bone-destructive diseases, such as periodontal disease and rheumatoid arthritis [6]. In earlier studies, we found an increase in the RANKL:OPG ratio in the synovial fluid and gingival crevicular fluid from patients with bone-destructive diseases, such as rheumatoid arthritis, periodontitis, and osteoarthritis [6–8]. Especially, since we found a common phenomenon, in these three inflammatory diseases, i.e., a decrease in the OPG level, a reduction in OPG production might trigger the pathophysiology and progression of bone-destructive diseases. Although OPG is expressed in a wide variety of tissues, it remains unclear how the expression of OPG is regulated. If the key signal cascade for OPG production can be elucidated, it can open the door for a new therapy for bone-destructive diseases by means of controlling OPG production.

The mammalian target of rapamycin (mTOR) is an evolutionally conserved serine/threonine protein kinase implicated in a wide array of cellular processes such as cell growth, proliferation, and survival [9,10]. The mTOR pathway has attracted broad scientific and clinical interest, particularly in light of the ongoing clinical cancer trials with mTOR inhibitors [11]. Rapamycin is a specific inhibitor of mTOR, and it exerts suppressive effects on proliferation, invasion, and metastasis and also induces apoptosis of tumor cells [12]. However, there has been no focus of attention on whether the mTOR signal cascade is involved in OPG production in osteoblastic cells.

To address these important issues described above, we presently studied whether the mTOR pathway is associated with OPG protein production in the mouse bone marrow-derived stromal cell line ST2. Interestingly, the present findings demonstrated that the inhibition of mTOR by rapamycin markedly induced OPG protein in the cells.

Materials and methods

Materials. Mouse bone marrow stromal cell line ST2 was obtained from RIKEN Cell Bank (Ibaragi, Japan). Rapamycin was purchased from Calbiochem (San Diego, CA, USA). α-Minimum essential medium (α-MEM) was from Gibco–BRL Laboratories (Grand Island, NY, USA), and fetal calf serum (FCS) from Cell Culture Laboratories (Cleveland, OH, USA). Anti-mTOR IgG and anti-

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phospho-mTOR IgG were obtained from Cell Signaling Technology Inc. (USA). Recombinant human bone morphogenetic protein (BMP)-4 was purchased from R&D systems (USA). All other chemicals used in this study were of analytical grade.

Cell culture. ST2 cells were cultured in plastic dishes containing α-MEM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in air with 5% CO_2, and were then subcultured until they were almost confluent. Incubation with rapamycin was done in medium containing 0.1% FCS. Conditioned medium was harvested from cultured cells and centrifuged to remove cell debris.

ELISA for mouse OPG. The amount of OPG secreted into the culture supernatant was measured by using an ELISA (R&D Systems, USA).

ELISA for mouse RANKL. The concentration of RANKL in the lysed cells was also determined by use of an ELISA (R&D Systems, USA), as described below. ST2 cells were lysed in RIPA buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, 0.1 mM phenylmethyl sulphonyl-fluoride, 0.5% deoxycholic acid, 0.1% SDS, 1% Nonidet P40, and phosphatase inhibitor cocktail-II (Sigma) and centrifuged at 14,000*g* for 10 min to remove insoluble materials. The content of total protein was measured by using a DC-Bio-Rad protein assay kit with bovine serum albumin as a standard.

Detection of mTOR and phospho-mTOR by Western blot analysis. ST2 cells were lysed in RIPA buffer and centrifuged at 14,000*g* for 10 min to remove insoluble materials. The lysates were dissolved in SDS electrophoresis buffer, and the proteins were separated on SDS–polyacrylamide gels (12%) and subsequently electrotransferred to polyvinylidene difluoride (PVDF) membranes. After having been blocked with PBS containing 0.05% Tween 20 and 5% nonfat dry milk, the membranes were incubated overnight at 4 °C with an antibody against either mTOR or phospho-mTOR; and the bound antibodies were detected with the corresponding secondary antibody conjugated to peroxidase (ECL Plus Western Blotting Reagent Pack, Amersham Int., Buckinghamshire, England). Blots were developed by using a chemiluminescence substrate, Lumingen PS-3, from Amersham Int. (ECL plus Western Blotting Detection System). Visualization of the bands in the PVDF membrane was performed with an Amersham Polaroid system (ECL mini-camera, Camera Luminometer for ECL).

Estimation of cell proliferation. Cell proliferation assays were performed by detecting 5-bromo-2′-deoxyuridine (BrdU)-labeled DNA with anti-BrdU monoclonal antibody (MoAb) labeled with peroxidase (Cell Proliferation ELISA, Roche, IN, USA). In short, ST2 cells grown in a 96-well microtiter plate (3×10^3cells/well) were incubated with different concentrations of Rapamycin (0–3000 nM) for 24 h at 37 °C in a humidified atmosphere (5% CO_2), and were then labeled with BrdU for 1 h. The amount of BrdU-labeled DNA was quantified by ELISA.

Estimation of cellular DNA fragmentation. Cellular DNA fragmentation was assayed by the detection of BrdU-labeled DNA fragments in the cytoplasm of cell lysates by use of solid phase-immobilized anti-DNA MoAb and anti-BrdU MoAb labeled with peroxidase (cellular DNA fragmentation ELISA, Roche, IN, USA). The cells were incubated in the presence of Rapamycin (0–3000 nM) for 8 h at 37 °C in a humidified atmosphere (5% CO_2). The amount of BrdU-labeled DNA was quantified by ELISA.

Cell-cycle analysis with a fluorescence-activated cell sorter (FACS). Cell-cycle analysis was made with a FACS and a cell-cycle analysis kit (Cycle Test plus, Becton–Dickinson, San Jose, CA, USA), as described previously [13].

Assessment of the osteoblastic phenotype. To assess the phenotype of the cultured cells, we examined alkaline phosphatase (ALP) activity and responsiveness to parathyroid hormone (PTH). ALP activity was determined by use of Blue-Phos substrate (microwell phosphatase substrate system: Kirkegaard & Perry Laboratory, MD, USA). The response to PTH was determined by measuring the amount of cAMP produced by the cells with or without exposure to 200 ng/ml human PTH (hPTH [1–34], Peptide Institute Inc., Japan) for 15 min. The concentration of cAMP in the cells was measured by use of an ELISA (Cayman Co., USA).

Statistical analysis. Data were presented as means ± SD of 4–6 cultures/group. Each experiment was repeated three times. Differences between control and experimental treatment groups were determined by using the paired Student's *t*-test. Differences were considered significant if P was <0.05.

Results

The treatment with rapamycin caused a marked concentration-dependent increase in the OPG level in the ST2 cells (maximum at 100 nM, Fig. 1A), with the maximum level being fivefold higher than the control. The elevation of OPG production occurred in a time-dependent manner, reaching a plateau at 18 h (Fig. 1B). Further, when we performed Western blotting of ST2 cell lysates by using anti-phosphorylated mTOR antibody, Rapamycin (100 or 3000 nM) caused a decrease in the density of the phosphorylated

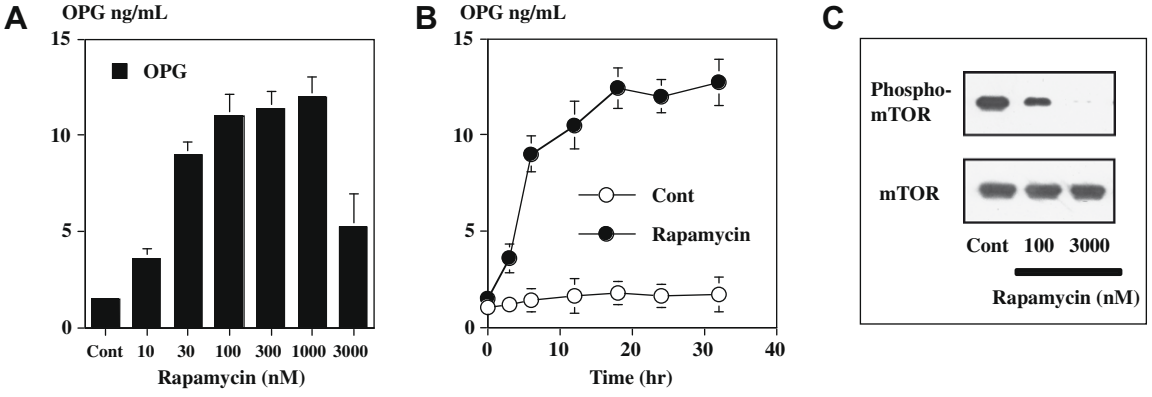

Fig. 1. Induction of OPG in ST2 cells by rapamycin. (A) Concentration-dependent production of OPG. ST2 cells were cultured for 24 h in the presence of rapamycin (0–3000 nM). Conditioned medium was harvested from the cultured cells, and the content of OPG in it was determined by ELISA. Values are expressed as mean ± SD of six wells (ng/ml). Each experiment was repeated three times, and the results shown are representative of these three independent experiments. (B) Time-dependent production of OPG. Cells were incubated for the indicated times in the presence or in the absence of rapamycin (100 nM). The content of OPG in the conditioned medium was determined by ELISA. Values are expressed as means ± SD of six wells. (C) Western blot analysis of mTOR and phospho-mTOR expression in rapamycin-treated ST2 cells. The data are shown for control ST2 cells (control, non-treated) and cells after rapamycin treatment (100 or 3000 nM) for 1 h. Equal amounts of the lysate protein were loaded in each well of the gel.

mTOR-band (Fig. 1C). In particular, rapamycin at 3000 nM completely inhibited the phosphorylation of mTOR.

Reciprocal gene expression of OPG and RANKL was demonstrated in 1α, 25$(OH)_2D_3$/dexamethasone-treated ST2 [14]. However, no significant difference in the RANKL protein level was found in ST2 cell lysates under a similar condition (Rapamycin: 100 nM, Fig. 2B).

It is widely known that the inhibition of mTOR has suppressive effects on cell proliferation and inductive effects on apoptosis in cells [12]. While rapamycin (3000 nM) caused substantial growth arrest in the cells ($P < 0.05$, Fig. 3A), relatively low concentrations of the antibiotic (30–300 nM) had no effect on the proliferation in the cells. In addition, rapamycin (30–1000 nM) except at 3000 nM had no effect on apoptosis as evaluated by DNA fragmentation ELISA (Fig. 3B).

Since osteoblast growth arrest is accompanied by differentiation, we measured ALP activity and PTH-responsive cAMP production in the ST2 cells to assess osteoblast differentiation. The control ST2 cells had very low levels of ALP activity and a low response to PTH in terms of producing cAMP. Exposure of the cells to rapamycin (100 nM) for 2 days had no effect on ALP activity and induced responsiveness for PTH-elicited cAMP (data not shown).

When a cell moves into a quiescent and/or terminally differentiated state from G1, it clearly enters a different metabolic state. In general, G0/G1 arrest in the cell cycle is induced by apoptotic stimulators and/or occurs in cells in the terminally differentiated state. To evaluate whether rapamycin induce differentiation of the cells, we performed cell-cycle analysis. FACS cell-cycle analysis demonstrated that treatment with rapamycin (100 nM) did not result in G0/G1 arrest in the ST2 cells (population rate: 61.8 ± 4.5% in control vs. 63.3 ± 4.9% in 100 nM Rapamycin). In contrast, a relatively high concentration of rapamycin (3000 nM) induced substantial G0/G1 arrest in the cells (82.2 ± 7.5%; $^{*}P < 0.05$ compared with the control). Taken together, rapamycin (100 nM, suitable for OPG production) had no effect on differentiation, proliferation, or apoptosis of ST2 cells.

Since we previously reported that BMP-4 induced OPG production in ST2 cells [15], we performed Western blot analysis to determine whether a common pathway is involved in OPG production. Treatment with BMP-4 (10 ng/ml) resulted in a potent decrease in the phosphorylation of mTOR, as shown in Fig. 4, suggesting that suppression of the phospho-mTOR pathway is necessary for OPG production in ST2 cells.

Discussion

The findings of the present study demonstrated that the expression of OPG is markedly induced by rapamycin in mouse bone marrow stromal cells. 1α,25-dihydroxyvitamin D_3, interleukin-1β, and tumor necrosis factor-α all increase OPG production in human

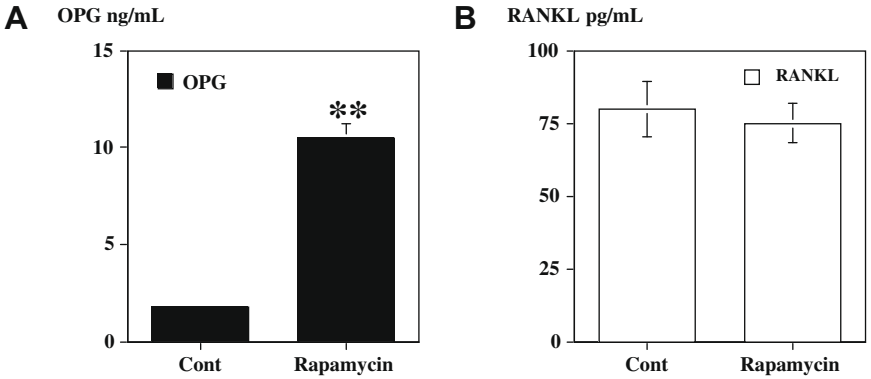

Fig. 2. Rapamycin-induced alteration of OPG level but not that of RANKL. (A) Cells were incubated for 24 h in the presence or in the absence of rapamycin (100 nM). The content of OPG in the conditioned medium was determined by ELISA. Values are expressed as means ± SD of six wells. $^{**}P < 0.005$ vs. control. (B) The RANKL content in the extracts was determined by a commercially available ELISA.

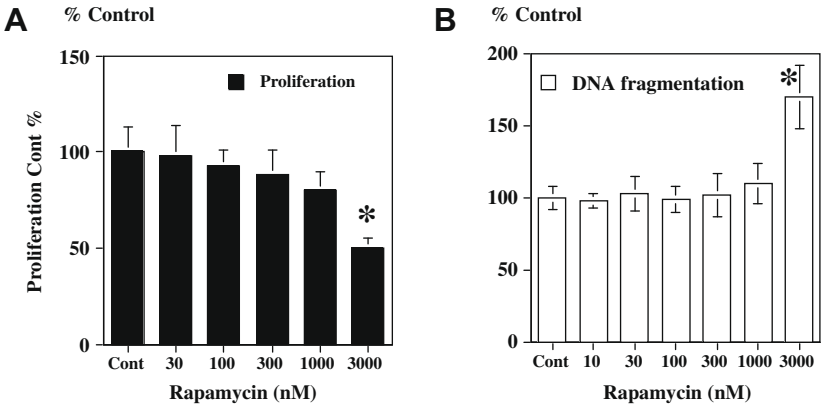

Fig. 3. (A) Effect of rapamycin on proliferation of ST2 cells. ST2 cells were incubated for 24 h in the presence of rapamycin (0–3000 nM). Cell proliferation was quantitatively determined by using a cell proliferation ELISA. Values represent means ± SD of four independent determinations performed. $^{*}P < 0.01$ vs. control (rapamycin: 0 nM). (B) Rapamycin-induced DNA fragmentation in ST2 cells ST2 cells were incubated for 8 h in the presence of rapamycin (0–3000 nM). Levels of apoptosis were quantitatively determined by using a cellular DNA fragmentation ELISA. Values represent means ± SD of four independent determinations performed. $^{*}P < 0.01$ vs. control (Rapamycin: 0 nM).

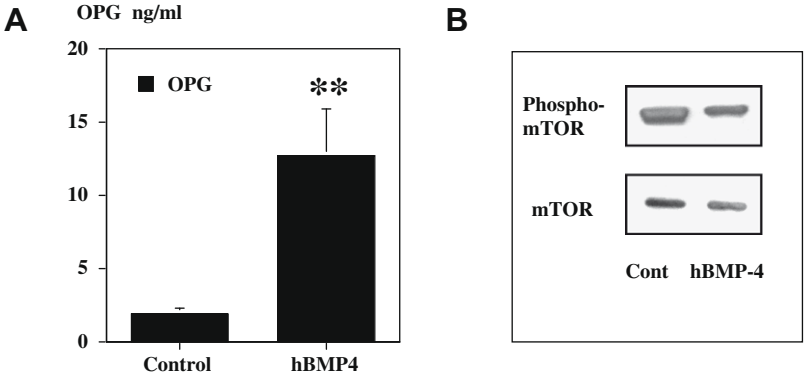

Fig. 4. (A) Induction of OPG in ST2 cells by hBMP-4. ST2 cells were cultured for 24 h in the presence of hBMP-4 (10 ng/ml). The conditioned medium was harvested from the cultured cells, and the content of OPG in it was determined by use of an ELISA. Values are expressed as means ± SD of six wells (ng/ml). Each experiment was repeated three times, and the results shown are representative of these three independent experiments. $^{**}P < 0.005$ vs. control. (B) Western blot analysis of mTOR and phospho-mTOR expression in hBMP-4-treated cells. The data are shown for control ST2 cells (control, non-treated) and cells after hBMP-4 treatment (10 ng/ml) for 1 h. Equal amounts of the protein had been loaded into each well of the gel.

osteoblastic cells [16]. While transforming growth factor-β1, BMP-2, and BMP-4 induce the expression of OPG mRNA and protein in osteoblastic cells [15–17], the present study is the first to show that a chemical compound, Rapamycin, caused a marked increase in the level of OPG protein in ST2 cells.

Although there is no report on protein induction caused by the suppression of phospho-mTOR, we demonstrated for the first time the significance of mTOR in the rapamycin-induced OPG production (Figs. 1 and 2). Although rapamycin (100 nM) induced OPG production, it had no effect on cell-cycle G0/G1 arrest or proliferation of ST2 cells under similar conditions. Since rapamycin also induced OPG protein in mouse osteoblastic cell line MC3T3-E1 (data not shown), this effect is not limited to ST2 cells. mTOR is a serine/threonine kinase central to a complex intracellular signaling pathway and is involved in diverse processes including cell growth and proliferation, survival, angiogenesis, autophagy, and metabolism. mTOR integrates input from multiple upstream signals, including growth factors and nutrients to regulate protein synthesis. In general, the suppression of phosphorylation of mTOR leads to the inhibition of protein synthesis associated with proliferation and/or growth. Therefore, the present pathway (the suppression of mTOR phosphorylation leading to OPG production) is very unique. As was shown in Figs. 1 and 2, a relatively high concentration of rapamycin (3000 nM) resulted in the potent suppression of phosphorylation of mTOR and reduced OPG production in the ST2 cells, suggesting that suitable and sensitive regulation of mTOR contributes to OPG production in the cells. There is no study focusing on the relationship between OPG and mTOR in osteoblastic cells except for one [18], which showed that while thrombin profoundly induced protein synthesis of OPG, rapamycin exerted an inhibitory effect on the thrombin-induced OPG synthesis.

In addition, our present data suggest a new risk to patients who are administered of rapamycin. Chronic administration of it might suppress the differentiation and activation of osteoclasts due to overexpression of OPG, and thus lead to osteopetrosis.

Imbalances in the RANKL/OPG system have been related to the pathogenesis of bone-destructive diseases [3,19,20]. Since we also reported a decreased level of OPG in the synovial fluid from patients with rheumatoid arthritis, periodontitis, and temporomandibular joint disorder [6–8], the locally produced OPG may play a functional role in the negative feedback regulation of osteoclastic bone resorption. OPG produced by osteoblasts is an important regulator of osteoclast development and function [21]. In addition, OPG plays an important potential protective or detrimental role in both vascular pathologies and tumorigenesis [22]. In light of these findings taken together with our current ones, the negative regulation of phosphorylation of mTOR enables control of bone formation through OPG production, and rapamycin shows therapeutic potential for bone-destructive diseases and other diseases.

In conclusion, the present results underline the importance of mTOR kinase acting as a regulator of OPG production in ST2 cells. The inhibition of mTOR kinase provides a crucial signaling transduction mechanism that positively regulates OPG production, and thus may positively affect bone formation as well.

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References

[1] H. Yasuda, N. Shima, N. Nakagawa, Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro, Endocrinology 139 (3) (1998) 1329–1337.
[2] W.S. Simonet, D.L. Lacey, C.R. Dunstan, M. Kelley, M.S. Chang, R. Lüthy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T.M. Hughes, D. Hill, W. Pattison, P. Campbell, S. Sander, G. Van, J. Tarpley, P. Derby, R. Lee, W.J. Boyle, Osteoprotegerin: a novel secreted protein involved in the regulation of bone density, Cell 89 (2) (1997) 309–319.
[3] Y.Y. Kong, U. Feige, I. Sarosi, B. Bolon, A. Tafuri, S. Morony, C. Capparelli, J. Li, R. Elliott, S. McCabe, T. Wong, G. Campagnuolo, E. Moran, E.R. Bogoch, G. Van, L.T. Nguyen, P.S. Ohashi, D.L. Lacey, E. Fish, W.J. Boyle, J.M. Penninger, Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand, Nature 402 (6759) (1999) 304–309.
[4] G.E. Wise, S.J. Lumpkin, H. Huang, Q. Zhang, Osteoprotegerin and osteoclast differentiation factor in tooth eruption, J. Dent. Res. 79 (12) (2000) 1937–1942.
[5] M. Sakata, H. Shiba, H. Komatsuzawa, T. Fujita, K. Ohta, M. Sugai, H. Suginaka, H. Kurihara, Expression of osteoprotegerin (osteoclastogenesis inhibitory factor) in cultures of human dental mesenchymal cells and epithelial cells, J. Bone Miner. Res. 14 (9) (1999) 1486–1492.
[6] S. Kotake, N. Udagawa, M. Hakoda, M. Mogi, K. Yano, E. Tsuda, K. Takahashi, T. Furuya, S. Ishiyama, K.J. Kim, S. Saito, T. Nishikawa, N. Takahashi, A. Togari, T. Tomatsu, T. Suda, N. Kamatani, Activated human T cells directly induce osteoclastogenesis from human monocytes, Arthritis Rheum. 44 (5) (2001) 1003–1012.
[7] M. Mogi, J. Otogoto, N. Ota, A. Togari, Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with Periodontitis, J. Dent. Res. 83 (2004) 166–169.
[8] T. Wakita, M. Mogi, K. Kurita, M. Kuzushima, A. Togari, Increase in RANKL:OPG ratio in synovial of patients with TMJ disorder, J. Dent. Res. 85 (7) (2006) 627–632.
[9] G.G. Chiang, R.T. Abraham, Targeting the mTOR signaling network in cancer, Trends Mol. Med. 13 (10) (2007) 433–442.
[10] Q. Yang, K.-L. Guan, Expanding mTOR signaling, Cell Res. 17 (8) (2007) 666–681.

[11] D.A. Guertin, D.M. Sabatini, Defining the role of mTOR in cancer, Cancer Cell 12 (1) (2007) 9–22.
[12] Y. Mamane, E. Petroulakis, O. LeBacquer, N. Sonenberg, MTOR, translation initiation and cancer, Oncogene 25 (48) (2006) 6416–6422.
[13] M. Mogi, A. Togari, Activation of caspases is required for osteoblastic differentiation, J. Biol. Chem. 278 (48) (2003) 47477–47482.
[14] T. Nakashima, Y. Kobayashi, S. Yamasaki, A. Kawakami, K. Eguchi, H. Sasaki, H. Sakai, Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines, Biochem. Biophys. Res. Commun. 275 (3) (2000) 768–775.
[15] M. Tazoe, M. Mogi, S. Goto, A. Togari, Involvement of p38MAP kinase on bone morphogenetic protein-4-induced osteoprotegerin in bone marrow-derived stromal cells, Arch. Oral Biol. 48 (8) (2003) 615–619.
[16] L.C. Hofbauer, C.R. Dunstan, T.C. Spelsberg, B.L. Riggs, S. Khosla, Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines, Biochem. Biophys. Res. Commun. 250 (3) (1998) 776–781.
[17] A. Yamaguchi, T. Ishizuya, N. Kintou, Y. Wada, T. Katagiri, J.M. Wozney, V. Rosen, S. Yoshiki, Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6, Biochem. Biophys. Res. Commun. 220 (1996) 366–371.
[18] U. Arayatrakoollikit, P. Pavasant, T. Yongchaitrakul, Thrombin induces osteoprotegerin synthesis via phosphatidylinositol 3′-kinase/mammalian target of rapamycin pathway in human periodontal ligament cells, J. Periodontal Res. 43 (5) (2008) 537–543.
[19] H. Takayanagi, K. Ogasawara, S. Hida, T. Chiba, S. Murata, K. Sato, A. Takaoka, T. Yokochi, H. Oda, K. Tanaka, K. Nakamura, T. Taniguchi, T-cell-mediated regulation of osteoclastogenesis by signaling cross-talk between RANKL and IFN-γ, Nature 408 (6812) (2000) 600–605.
[20] Y.T. Teng, H. Nguyen, X. Gao, Y.Y. Kong, R.M. Gorczynski, B. Singh, R.P. Ellen, J.M. Penninger, Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection, J. Clin. Invest. 106 (6) (2000) R59–R67.
[21] N. Udagawa, N. Takahashi, H. Yasuda, A. Mizuno, K. Itoh, Y. Ueno, T. Shinki, M.T. Gillespie, T.J. Martin, K. Higashio, T. Suda, Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function, Endocrinology 141 (9) (2000) 3478–3484.
[22] P. Reid, I. Holen, Pathophysiological roles of osteoprotegerin (OPG), Eur. J. Cell Biol. 88 (1) (2009) 1–17.