γ-Glutamyltranspeptidase Stimulates Receptor Activator of Nuclear Factor-κB Ligand Expression Independent of Its Enzymatic Activity and Serves as a Pathological Bone-resorbing Factor*\textsuperscript{a,g}

Shumpei Niida\textsuperscript{a,b}, Miyuki Kawahara\textsuperscript{c,d}, Yasuyuki Ishizuka\textsuperscript{a}, Yoshitaka Ikeda\textsuperscript{f,g}, Takako Kondo\textsuperscript{a,h}, Terumasa Hibi\textsuperscript{i}, Yu Suzuki\textsuperscript{j}, Kyoji Ikeda\textsuperscript{a}, and Naoyuki Taniguchi\textsuperscript{f}

From the \textsuperscript{a}Department of Geriatric Sciences, National Institute for Longevity Sciences, Aichi 474-8522, Japan, the \textsuperscript{b}Department of Orthodontics, Hiroshima University Faculty of Dentistry, Hiroshima 734-8553, Japan, the \textsuperscript{c}Department of Geriatric Sciences, National Institute for Longevity Sciences, Aichi 474-8522, Japan, the \textsuperscript{d}Department of Biochemistry, Osaka University Medical School, Osaka 565-0871, Japan, and the \textsuperscript{e}Sumitomo Pharmaceuticals Research Center, Osaka 554-0022, Japan

A novel bone-resorbing factor was cloned using an expression cloning technique, which involved a Xenopus oocyte expression system and an assay for osteoclast formation. A candidate clone was isolated from a BW5147 mouse T-lymphoma cell cDNA library. Sequencing analysis identified the factor as γ-glutamyltranspeptidase (GGT), which is an enzyme involved in glutathione metabolism. The addition of purified GGT protein to mouse bone marrow culture effectively induced formation of osteoclasts. An antibody against GGT inhibited osteoclast formation but not the enzymatic activity. We also demonstrated that an inactive form of GGT, the enzymatic activity of which had been blocked by chemical modification with a specific inhibitor, acivicin, supported osteoclast formation. These results indicate that GGT acts on osteoclast formation independent of its own enzymatic activity. Furthermore, both native GGT and inactive GGT stimulated the expression of the receptor activator of nuclear factor-κB ligand (RANKL) mRNA and protein from bone marrow stromal cells. This report is the first demonstration of a novel biological activity of GGT protein in a manner independent of its enzymatic activity.

Osteoclasts are potent bone resorbing cells that are derived from hematopoietic cells of the monocyte/macrophage lineage (1–3). Osteoclast differentiation is regulated by the simultaneous stimulation of colony stimulating factor-1 (CSF-1/M-CSF) and the receptor activator of nuclear factor-κB ligand (RANKL),\textsuperscript{3} which are produced by osteoblasts/stromal cells (3–8). The expression of these essential factors is stimulated by systemic bone-resorbing factors such as 1α,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}), parathyroid hormone, and interleukin (IL)-11 (9, 10). Increased osteoclast activity is responsible for progressive bone loss in postmenopausal osteoporosis and Paget disease (11, 12). Local bone destruction has also been observed in bone metastasis and rheumatoid arthritis (13, 14). Tumor cells that have metastasized to bone induce osteoclastogenesis via the secretion of bone-resorbing factors such as parathyroid hormone-related protein, IL-11, and prostaglandin E\textsubscript{2} (13).

Our previous study (15) demonstrated that the injection of BW5147 mouse T-lymphoma cells into AKR mice led to bone metastasis, accompanied by severe bone destruction. Conditioned medium from BW5147 cell cultures stimulated tartrate-resistant acid phosphatase-positive (TRAP\textsuperscript{3}) multinucleated osteoclast (MNC) formation in the mouse bone marrow culture. Because TRAP activity is expressed specifically in osteoclasts (16–18), it has been suggested that the BW5147 cells produce an osteoclast-forming factor that may play a role in pathological bone resorption such as metastatic lesions.

To identify and characterize the factor that promotes pathological bone resorption, we cloned a cDNA for the osteoclast-forming factor(s) from a BW5147 cell cDNA library by expression cloning using a Xenopus oocyte translation system. Consequently, we identified γ-glutamyltranspeptidase (GGT) as the bone-resorbing factor. GGT is an ectoenzyme that plays an important role in regulating glutathione metabolism (19) and is well known as a clinical marker for a number of diseases. In this study, we showed a possible mechanism by which GGT induces osteoclast formation using a mouse bone marrow culture system.

EXPERIMENTAL PROCEDURES

Cell Culture—A mouse T-lymphoma cell line, BW5147 (CRL-1588), (20) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Expression Cloning—Poly(A)\textsuperscript{+} RNA was prepared from BW5147 cells and size-fractionated by means of sucrose density gradient centrifugation.

Received for publication, October 30, 2003, and in revised form, November 21, 2003

\textsuperscript{1} The abbreviations used are: RANKL, receptor activator of nuclear factor-κB ligand; IL, interleukin; TRAP, tartrate-resistant acid phosphatase; MNC, multinucleated osteoclast; GGT, γ-glutamyltranspeptidase; 1α,25(OH)\textsubscript{2}D\textsubscript{3}, 1α,25-dihydroxyvitamin D\textsubscript{3}; I-GGT, inactive GGT; OPG, osteoprotegerin.
tion. The 2–4-kb fraction that had the greatest osteoclast-forming activity was used to construct a directional cDNA library using a ZAP-cDNA synthesis kit (Stratagene) and SuperScript II (Invitrogen). Then 50 ng of poly(A)+ RNAs or cRNAs, which were synthesized in vitro from the pools of clones, were injected into Xenopus oocytes. The conditioned media were assayed for osteoclast-forming activity, which was determined using the assay described below. Each positive pool was further subdivided and analyzed until a single clone was obtained.

Mouse Bone Marrow Culture—Bone marrow cells (2.5 x 10⁶ cells/ml) obtained from the tibiae and femurs of 5–12-week-old C3H/HeJ mice (Nippon Clea) were cultured in 96-well plates in 180 μl of α-minimal essential medium containing 10% fetal bovine serum and 10–5 M 1,25(OH)₂D₃. A 20-μl aliquot was added on day 1 of the culture. The culture medium was replaced with fresh medium containing a 20-μl aliquot at 3-day intervals. After 7 days of culture, the cells were fixed with 4% paraformaldehyde and stained for TRAP activity. The TRAP staining solution containing 50 mM sodium tartrate detects osteoclasts specifically (16, 17). The number of TRAP⁺ MNCs (>3 nuclei) was scored under a microscope. Bone marrow stromal ST2 cells, which support hematopoiesis, were also co-cultured with bone marrow hematopoietic cells (2.5 x 10⁶ cells/well) that were passed through a Sephadex G-10 column (Amersham Biosciences) under the same conditions.

Calcinocin Binding Assay—For autoradiography using 125I-salmon calcitonin, the cells were cultured on a chamber slide and incubated with 0.2 nM 125I-salmon calcitonin in α-minimal essential medium for 1 h at 37°C. After TRAP staining as described previously (18).

Pit Formation Assay—To determine the resorption activity of TRAP⁺ MNCs, the bone marrow cells were seeded on the dentine slices placed in 96-well plates or on calcium phosphate-coated slides (osteologic multistep slide, Millennium Biologic). The dentine slices and osteologic slides were stained for TRAP activity. After staining, the TRAP⁺ cells on the dentine or slides were removed with a scraper, and the number of pits was counted under a phase-contrast microscope.

GGT and Antibody—GGT was purified from rat kidney as described previously (21). When enzymatically inactive GGT (designated as I-GGT) was prepared, the purified enzyme was allowed to react with acivicin (a-αs,53)-a-amino-3-chloro-4,5-dihydro-5-isoazoleneacetic acid, which selectively inhibits the enzyme by each covalently binding to the active site (22, 23). After the residual activity was decreased to less than 0.1%, unreacted reagents were removed by gel filtration using Toyopearl HW40-F (Toyol) pre-equilibrated with phosphate-buffered saline. A goat IgG against rat GGT was prepared as described in the previous report (24). Non-immune goat IgG (Sigma) was used as a control antibody. Endotoxin was undetectable in these antibodies, as verified using an endospec kit (Seikagaku Co.).

GGT Assay—The enzymatic activity of GGT was measured using a GGT 419 assay kit (Sigma).

Ectrophoresis and Immunoblotting—The purified GGT was subjected to SDS-PAGE on 11% gels according to the Laemmli procedure (24). Non-immune goat IgG (Sigma) was used as a control antibody. A goat IgG against rat GGT was prepared as described in the previous report (21). The enzymatic activity of GGT was assayed using a GGT kit (Nippon Clea). The enzymatic activity of GGT was assayed using an enzymatic kit (Nippon Clea).

Expression Cloning of Osteoclast-forming Factor—The culture medium of Xenopus oocytes injected with poly(A)+ RNA from BW5147 cells induced the formation of a significant number of osteoclast-like cells in mouse bone marrow cultures, as indicated by an increased number of TRAP- and CT-receptor-positive MNCs (data not shown). After fractionation of the poly(A)+ RNA by sucrose density gradient centrifugation, to enrich the mRNA that encodes for this factor, the fraction from 2–4 kH, which contained the greatest amount of this factor, was used as a template to prepare a cDNA library. As a result, ~600,000 individual clones were obtained, which were then divided into 63 pools, each of which contained ~10,000 clones. These pools were grown separately, and cRNA was prepared from each pool by in vitro transcription. Following expression in the Xenopus oocytes as a result of cRNA injection, the resulting culture media were screened for osteoclast-inducing activity. One positive pool was found to contain high levels of activity, and this pool was further subdivided and screened. After several rounds of screening, several positive clones including colony stimulating factor-1, RANKL, IL-1, and IL-6 were obtained. One unique positive clone remained, and this cDNA clone was sequenced (GenBank™ accession number E15738). A database homology search indicated that the factor cloned was identical to mouse GGT (Ref. 27 and GenBank™ accession number U30509). We confirmed that the culture medium had 40 units/ml of ggt activity and that the BW5147 cells expressed GGT mRNA (Fig. 1A). The conditioned medium of oocytes injected with GGT mRNA induced many TRAP⁺ MNCs induced by GGT expressed the CT

![FIG. 1. Expression of GGT in BW5147 cells and the osteoclast-forming activity of GGT. RT-PCR analysis (A) indicates the expression of GGT mRNA in BW5147 cells. A sample of kidney was used as a control. Conditioned medium from Xenopus oocytes expressing GGT induced TRAP⁺ MNC formation in bone marrow cultures. The cultures were maintained for 7 days in the presence of a conditioned medium of oocytes injected with distilled water (B) and GGT cRNA (C). Most of the TRAP⁺ MNCs expressed calcitonin receptors (D). The black grains in the autoradiograph indicate the binding of 125I-salmon calcitonin. Resorption pit formation (stained red) was observed on a dentin slice (E).](image-url)
GGT Is a Novel Bone-resorbing Factor

receptor autoradiographically (Fig. 1D). Furthermore, resorption pits were observed microscopically on the dentine slices or osteologic slides after 7 days of culture (Fig. 1E).

Osteoclast-forming Activity of GGT—To verify the osteoclast-forming activity of GGT, the enzyme purified from rat kidney was subjected to an in vitro osteoclast-forming assay using bone marrow cultures without other bone-resorbing factors. The GGT used in this study produced only two protein bands, which corresponded to the large and small subunits of GGT (Fig. 2A). Treatment with a range of 5–625 ng/ml of GGT (equivalent to 0.08–10 nM) for 7 days in culture induced TRAP$^+$ MNC formation in a dose-dependent manner (Fig. 2B). Most of these cells expressed the CT receptor, and the resorption pit-forming activity was observed microscopically after 7 days of culture (data not shown). These results indicate that GGT serves as an inducer of TRAP$^+$ MNCs, which satisfies the major criteria for osteoclasts. No endogenous GGT activity was detected in bone marrow cultures. The same results were obtained in additional examinations using recombinant human GGT, which was produced in Spodoptera frugiperda SF21 cells with a baculovirus system as described previously (28) (data not shown).

Involvement of Expression RANKL in GGT-induced Osteoclast Formation—RANKL is the key regulator of osteoclastogenesis, and thus we examined whether RANKL is involved in the GGT-induced osteoclast formation. The osteoclast formation by GGT was inhibited dose dependently by OPG, a decoy receptor for RANKL (Fig. 3A). It was also found that GGT stimulated RANKL mRNA expression in bone marrow cultures (Fig. 3B). These results suggest that the induction of osteoclastogenesis by GGT involves RANKL expression.

Inhibition Studies of GGT-induced Osteoclast Formation—The osteoclast-forming activity of GGT in the bone marrow cultures was further supported by an inhibition study using an anti-GGT antibody. In bone marrow cultures containing 625 ng/ml purified GGT, TRAP$^+$ MNC formation was inhibited by the anti-GGT antibody in a dose-dependent manner (Fig. 4, solid bars), whereas substantial numbers of TRAP$^+$ MNCs developed with non-immune antibodies (data not shown). The antibodies per se had no adverse effects on cell viability. Interestingly, this antibody did not have a significant effect on neutralizing the enzyme activity of GGT in these examinations (Fig. 4, hatched bars), suggesting that the osteoclast-forming activity of GGT is not associated with its enzymatic activity.

Osteoclast-forming Activity of Enzymatically Inactive GGT—To further confirm that the osteoclast-forming activity of GGT does not require the enzyme activity, we prepared an inactive form of GGT (I-GGT), in which the active site was blocked by covalent binding of acivicin, and tested whether I-GGT supported TRAP$^+$ MNC formation in bone marrow cultures. TRAP$^+$ MNC-forming activity was also tested using the murine hematopoietic cell/ST2 cell coculture system that generates osteoclasts in vitro. In both systems, I-GGT also induced TRAP$^+$ MNC formation in a dose-dependent manner (Fig. 5). These results further support the suggestion that the osteoclastogenic activity of GGT does not require its own enzymatic activity.

Expression of RANKL in Stromal Cells—Most of the bone-resorbing factors act on the bone marrow stromal cells and stimulate RANKL production. To assess the induction of RANKL mRNA in response to GGT and I-GGT in stromal cells, we examined the expression of RANKL and OPG in bone marrow-adherent cells. Quantitative real time PCR analysis showed that both types of GGT induced a 4-fold increase in RANKL mRNA expression (Fig. 6A), whereas the level of OPG mRNA was slightly decreased (Fig. 6B). The same results were obtained in examinations using ST2 cells. In addition, the
immunoblot analysis showed that there was increased RANKL protein expression (Fig. 6C). These results suggest that GGT stimulates RANKL expression in bone marrow stromal cells and thereby serves as a bone-resorbing factor in osteolysis. In contrast, GGT had no effect on RANKL production from bone marrow hematopoietic cells (data not shown).

**DISCUSSION**

This study used expression cloning, based on in vitro osteoclast-forming activity in mouse bone marrow cultures, which led to the successful cloning of a novel bone-resorbing factor from a cDNA library of mouse T-lymphoma, BW5147 cells. This factor was identified as GGT. Many previous studies have focused on the enzymatic functions of GGT. These studies have revealed that GGT catalyzes the first step in the degradation of glutathione and plays an important role in glutathione metabolism (29). In addition, the expression of GGT is elevated under certain conditions, such as carcinogenesis (30–33), and it is used as a marker enzyme for many diseases. However, other biological activities of or significance of GGT have not yet been demonstrated.

We showed that the purified GGT induced osteoclast formation in bone marrow culture. This induction was inhibited by OPG, and RANKL mRNA was elevated in the bone marrow cells stimulated by GGT. Therefore, it is most likely that GGT induces osteoclasts via expression of RANKL. Because BW5147 mouse T-lymphoma cells expressed GGT mRNA, GGT appeared to contribute to osteoclast-mediated bone destruction in bone metastasis involving BW5147 cells.

As shown by experiments using anti-GGT antibody and chemically modified inactive GGT, the osteoclast-forming activity of GGT is not associated with its enzymatic activity, which catalyzes transpeptidation and hydrolysis of the γ-glutamyl moiety of glutathione and related compounds. In the induction of osteoclasts, therefore, it would be expected that GGT functions as a cytokine-like molecule, such as IL-1, via interaction with a presumable receptor. Furthermore, our results indicate that GGT acts on the bone marrow-adherent cells and the ST2 cells to express RANKL. Further investigation...
GGT Is a Novel Bone-resorbing Factor

Growth and development of osteoclasts was increased in GGT-deficient GGT\textsuperscript{−/−}/GGT\textsuperscript{−/−} mice (35). Although GGT\textsuperscript{−/−}/GGT\textsuperscript{−/−} mice have some bone abnormalities, including osteoclast, chondrocyte, and osteoblast, N-acetylcysteine treatment rescued most of these abnormalities. It was considered that the phenotypes observed in these mice are thought to result from impairment of cysteine metabolism (35). However, the osteoclast-forming activity of GGT is clearly independent of its enzymatic activity. GGT may act as an enhancer for RANKL expression under the pathological conditions, such as bone metastasis, and serve as a local bone-resorbing factor. Although the details remain to be clarified, this study has contributed to elucidating a distinct alternative mechanism or a mechanism that accelerates abnormal bone loss.

Expression of GGT is elevated in hepatic diseases and chronic alcoholism (33, 36, 37), which are frequently accompanied with osteopenia and osteoporosis (38, 39). Recently, it has been reported that OPG prevents an ethanol-mediated bone loss by inhibiting osteoclast formation (40). This is consistent with our inhibition study of GGT-induced osteoclast formation by OPG, and, thus, osteoclast formation by GGT may account for osteopenia induced by these hepatic diseases.

Acknowledgments—We thank M. Takatsuka, T. Yoshima, R. Mochizuki, I. Kodama, H. Tajina, and Y. Kondoh for their technical contributions. We also thank Drs. S.-I. Hayashi, T. Nakamura, and K. Watanabe for their valuable comments. M. Kawahara thanks Drs. N. Maeda and K. Tanne for their encouragement and help.

REFERENCES

1. Ash, P., Loutit, J. F., and Townsend, K. M. (1980) Nature 283, 669–670
2. Suda, T., Takahashi, N., and Martin, T. J. (1992) Endocr. Rev. 13, 66–80
3. Suda, T., Takahashi, N., Udagawa, N., Jimi, E, Gillespie, M. T., and Martin T. J. (1999) Endocr. Rev. 20, 345–357
4. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L. D., and Nishikawa, S. (1990) Nature 345, 442–444
5. Kodama, H., Yamasaki, A., Nose, M., Niida, S., Ohgame, Y., Abe, M., Kamegawa, M., and Suda, T. (1991) J. Exp. Med. 173, 269–272
6. Kodama, H., Nose, M., Niida, S., and Yamashita, A. (1991) J. Exp. Med. 173, 1291–1294
7. Yasuda, H., Shima, N., Kangawa, N., Yamaguchi, K., Kinoshita, M., Mochizuki, S., Tomoyasu, A., Yanai, K., Goto, M., Murakami, A., Tada, K., Morimaga, T., Higashino, K., Udagawa, N., Takahashi, N., and Suda, T. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 3597–3602
8. Takahashi, N., Udagawa, N., and Suda, T. (1999) Biochem. Biophys. Res. Commun. 256, 449–455
9. Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., Martin, T. J., and Suda, T. (1988) Endocrinology 123, 2600–2602
10. Udagawa, N., Takahashi, N., Akatsu, T., Sasaki, T., Yamaguchi, A., Kodama, H., Martin, T. J., and Suda, T. (1989) Endocrinology 125, 1805–1813
11. Roodman, G. D. (1996) Endocr. Rev. 17, 308–332
12. Roodman, G. D. (1996) Bone (N.Y.) 19, 209–212
13. Guise, T. A., and Mundy, G. R. (1998) Endocr. Rev. 19, 18–54
14. Feldmann, M., Brennan, F. M., and Maini, R. N. (1996) Annu. Rev. Immunol. 14, 397–440
15. Ishizuka, Y., Mochizuki, R., Yanai, K., Takatsuka, M., Nonomura, T., Niida, S., Horiuchi, H., Maeda, N., and Fukamizu, A. (1999) Biochem. Biophys. Acta 1450, 92–98
16. Minkin, C. (1992) Calcif. Tissue Int. 46, 285–290
17. Cole, A. A., and Walters L. M. (1987) J. Histochem. Cytochem. 35, 203–206
18. Suda, T., Jimi, E., Nakamura, I., and Takahashi, N. (1997) Methods Enzymol. 282, 223–235
19. Lieberman, M. W., Barrios, R., Carter, B. Z., Hubh, G. M., Lebovitz, R. M., Rajagopalan, S., Sepulveda, A. R., Shi, Z. Z., and Wao, D. F. (1995) Am. J. Pathol. 147, 1175–1185
20. Ross, E, La Riviere, G., Collard, J. G., Stukart, M. J., and De Baetselier, P. (1985) Cancer Res. 45, 6235–6243
21. Miester, A., Tate, S. S., and Griffith, O. W. (1981) Methods Enzymol. 77, 237–253
22. Ikeda, Y., Fujii, J., Anderson, M. E., Taniguchi, N., and Meister, A. (1995) J. Biol. Chem. 270, 22223–22228
23. Smith T. K., Ikeda Y., Fujii J., Taniguchi, N., and Meister, A. (1995) Proc. Natl. Acad. U.S.A. 92, 2360–2364
24. Takahashi, N., Udagawa, N., Yokosawa, N., Zhe, Z. N., Sako, F., Tsuchiya, Y., Sato, M., and Dempo, K. (1986) Ann. N. Y. Acad. Sci. 417, 203–212
25. Laemml U. K. (1970) Nature 225, 680–685
26. Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1982) Anal. Biochem. 105, 261–263
27. Shi, Z. Z., Habib, G. M., Lebovitz, R. M., and Lieberman, M. W. (1995) Gene (Amst.) 167, 233–237
28. Ikeda, Y., Fujii, J., Taniguchi, N., and Meister, A. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 126–130
29. Taniguchi, N., and Ikeda, Y. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72, 259–274
30. Hanigan, M. H., Frierson, F. H., Jr., Brown, J. E., Lovell, M. A., and Taylor, P. T. (1994) Cancer Res. 54, 286–290
31. Hanigan, M. H., Frierson, F. H., Jr., Swanson, P. E., and De-Young, B. R. (1999) Hum. Pathol. 30, 300–305
32. Taniguchi, N., House, S., Kuzumaki, N., Yokosawa, N., Yamagami, S., Iizuka, S., Makita, A., and Sekiya, C. (1985) J. Bone Miner. Res. 1, 129–136
33. Taniguchi, N., Iizuka, S., Zhe, Z. N., House, S., Yokosawa, N., Ono, M., Kinoshita, K., Makita, A., and Sekiya, C. (1985) Cancer Res. 45, 5835–5839
34. Lieberman, M. W., Wiseman, A. L., Shi, Z. Z., Carter, B. Z., Barrios, R., Ou, C.-N., Chevez-Barrion, F., Wang, Y., Habib, G. M., Goodman, J. C., Huang, S. L., Lebovitz, R. M., and Matzuk, M. M. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7923–7926
35. Levasseur, R., Barrios, R., Eletferiou, F., Glass, D. A., II, Lieberman, M. W., and Karsenty, G. (2003) Endocrinology 144, 2761–2764
36. Teschke, R., Neufeld, M., Nishimura, M., and Strubmeier, G. (1983) Gut 24, 625–630
37. Barouki, R., Chobert, M. N., Finidori, J., Aggerbeck, M., Nalpas, B., and Hanoune, J. (1985) Hepatology 3, 325–329
38. Heathcote J. (1999) Curr. Gastroenterol. Rep. 1, 455–458
39. Santolaria, F., Gonzalez-Reimers, E., Perez-Manzano, J. L., Milena, A., Gomez-Rodriguez, M. A., Gonzalez-Diaz, A., de la Vega, M. J., and Martinez-Riera, A. (2002) Alcohol 34, 147–157
40. Zhang, J., Dai, J., Lin, D. L., Habib, P., Smith, P., Murtha, J., Fu, Z., Yao, Z., Qi, Y., and Keller, E. T. (2002) J. Bone Miner. Res. 17, 1256–1263