Calcium-containing crystals enhance receptor activator of nuclear factor κB ligand/macrophage colony-stimulating factor–mediated osteoclastogenesis via extracellular-signal-regulated kinase and p38 pathways

Chi-Ching Chang1,2, Yu-Hui Tsai3,4, Yu Liu1, Shyr-Yi Lin2,5,6,* and Yu-Chih Liang7,8,*

Abstract

Objective. Diseases associated with calcium-containing crystal deposition can lead to local bone erosion. We aimed to determine whether calcium-containing crystal-hydroxyapatite, β-tricalcium phosphate and CPPD enhanced osteoclastogenesis and to define underlying mechanisms of action.

Methods. Osteoclastogenesis was studied by culturing murine RAW 264.7 osteoclast precursor cells with RANK ligand (RANKL)/M-CSF and/or calcium-containing crystals, and observing the tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells and TRAP activity. Resorption pit formation was used to evaluate osteoclast activity. Real-time RT-PCR analysis revealed osteoclast marker genes, including TRAP, cathepsin K and calcitonin receptor (CTR). Western blotting was used to analyse the phosphorylation levels of signal transduction molecules.

Results. Three kinds of calcium-containing crystal significantly enhanced RANKL/M-CSF-induced osteoclastogenesis in RAW 264.7 cells, as evidenced by the increased number of TRAP-positive multinucleated cells, TRAP activity and resorption pit formation in a dose-dependent manner. Hydroxyapatite, β-tricalcium phosphate and CPPD treatments significantly enhanced RANKL/M-CSF–induced mRNA expression of TRAP, cathepsin K and CTR. Moreover, the three kinds of calcium-containing crystal enhanced the phosphorylation of extracellular-signal-regulated kinase and p38 in RANKL/M-CSF–treated cells.

Conclusion. We concluded that calcium-containing crystals can promote osteoclastogenesis and bone resorption through the extracellular-signal-regulated kinase and p38 pathways. Together with synovial activation, this mechanism may be important in the pathogenesis of destructive arthropathies triggered by calcium-containing crystals.

Key words: calcium-containing crystals, osteoclastogenesis, receptor activator of nuclear factor κB ligand, extracellular-signal-regulated kinase, p38.

Rheumatology key messages

- Calcium-containing crystals deposition may play an important role in progression of bone erosion in vivo.
Introduction

Diseases associated with calcium-containing crystal deposition are a group of clinically heterogeneous arthritides including pseudogout, calcific periartthritis, Milwaukee shoulder syndrome and OA [1–3]. Calcium-containing crystals encompass CPPD and basic calcium phosphate (BCP) crystals. Furthermore, BCP crystals, including carbonate-substituted hydroxyapatite, β-tricalcium phosphate (β-TCP) and octacalcium phosphate, have heterogeneous structures, chemical compositions and biological properties.

Mechanisms of joint damage in diseases associated with calcium-containing crystal deposition remain unclear. Most studies have focused on the effects on synovial fibroblasts and chondrocytes. Calcium-containing crystals induce proliferation of fibroblasts, release of matrix-degrading molecules, production of nitric oxide, secretion of inflammatory mediators and production of cytokines [3–6]. Furthermore, calcium-containing crystals induce chondrocytes to produce nitric oxide and IL-1β [7] and to undergo apoptosis [8]. Calcium-containing crystals also enhance survival of and induce DNA synthesis in murine macrophages in vitro [9]. Enhanced local macrophage survival or proliferation in the synovium may also contribute to the synovial hypertrophy seen in association with BCP crystals. Bouchard et al. [10] reported that human osteoblasts stimulated with CPPD crystals and IL-1β exhibited reduced differentiation, indicating reduced bone formation and increased osteoblast-mediated osteoclastic activity, with possible repercussions on bone remodelling. Calcium-containing crystals are powerful activators of inflammatory cells such as monocytes and neutrophils, synovial fibroblasts and endothelial cells, all cells adjacent to microcrystal foci. However, whether direct contact between calcium-containing crystals and bone cells causes local bone remodelling such as bone erosion is not completely understood.

Bone erosion in joint disease may occur because of a number of factors, including alterations in the functions of osteoclasts, stromal cells, macrophages and T cells. Osteoclasts are multinucleated cells (MNCs) formed by fusion of monocyte/macrophage progenitors [11]. They are the principal resorptive cells of bone and play a central role in regulating the skeletal mass [12]. Bone-resorbing osteoclasts can be matured in vitro by culturing monocyte/macrophage precursors in the presence of M-CSF and the RANK ligand (RANKL) [13, 14] derived from osteoblasts and activated T cells. These cells also express proteins that represent an osteoclast lineage, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K and the calcitonin receptor (CTR). Osteoblasts also produce osteoprotegerin, a decoy receptor that prevents the binding of RANKL to its receptor, RANK, and thus inhibits osteoclastogenesis [14]. Calcium-containing crystals activate several intracellular signalling pathways that depend on cellular species and cellular effects [1]. Calcium-containing crystals may be internalized and dissolved intracellularly, with a subsequent increase in the intracellular calcium concentration and activation of protein kinase C and mitogen-activated protein kinase (MAPK) pathways, particularly p42/44 extracellular-signal-regulated kinase (ERK). A key signalling event induced by the binding of RANKL to RANK is activation of MAPK, NF-κB and Akt signalling [15].

The bone remodelling potential of calcium-containing crystals in human joints has rarely been studied. Therefore, we report the results of an in vitro study that examined whether calcium-containing crystals can stimulate osteoclastogenesis and osteoclastic bone resorption, thus highlighting new pathogenic mechanisms of diseases associated with calcium-containing crystal deposition.

Materials and methods

Crystals and reagents

Hydroxyapatite, β-TCP and CPPD crystals as well as 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma–Aldrich (St Louis, MO, USA). All crystals were prepared under sterile pyrogen-free conditions and suspended in deionized distilled water. Endotoxin levels of hydroxyapatite, β-TCP and CPPD crystal suspensions were determined using an E-toxate kit (Sigma, St Louis, MO, USA), and the endotoxin level was lower than detection level (0.05 EU). The mean diameters of hydroxyapatite, β-TCP and CPPD crystals were 20 nm, 10.57 μm and 2.39 μm, respectively, according to a laser scattering particle size distribution analysis. Kinase inhibitors (PD98059 and SB203580) were purchased from Tocris Bioscience (Bristol, UK). Anti-phospho-Akt, anti-phospho-p38, anti-phospho-c-Jun N-terminal kinase (JNK), anti-phospho-ERK, anti-phospho-inhibitor of NF-κB (IκB) and anti-IκB antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA); the anti-Akt, anti-ERK, anti-p38 and anti-JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and the anti-GAPDH antibody was purchased from Genetex International Corp. (Hsinchu city, Taiwan).

Cell culture and osteoclastogenesis

RAW 264.7 cells (BCRC 60,001) and U937 cells (BCRC 60,435) were obtained from the Food Industry Research and Development Institute (BCRC, Hsinchu, Taiwan) and maintained in z-minimal essential medium and RPMI 1640 medium (Life Technologies, Grand Island, NY, USA), respectively, with 10% fetal bovine serum and 1% antibiotic-antimycotic, and osteoclasts were generated by adding 10 ng/ml RANKL and 10 ng/ml M-CSF (Peprotech, London, UK), or 100 nM TPA.

Cell viability assay

RAW 264.7 cells were seeded in a 96-well plate (10³ cells/well) and then treated with crystals for 24 h. After treatment, the medium was removed, and the cells were incubated with 0.25 mg/ml 3-(4,5-dimethylthiazolyl-2)-2',5-diphenyltetrazolium bromide (MTT) reagent for 3 h. After removing the MTT reagent, dimethyl sulphoxide (DMSO) was added to dissolve the formazan. The absorbance was read on an ELISA reader at 570 nm, with a background wavelength of 630 nm.
Calcium-containing crystals enhance receptor

TRAP staining and TRAP activity
After osteoclastogenesis for 72 h, the medium was collected and incubated with the same volume of a TRAP activity agent (50 mM sodium acetate, 30 mM sodium tartrate and 2.5 mg/ml 4-nitrophenyl phosphate disodium salt hexahydrate) for 1 h at 37°C. Then, NaOH was added to stop the reaction, and the absorption was measured at 405 nm with a Synergy HT Microplate Reader (Winooski, VT, USA). To perform TRAP staining, cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde. Then, cells were incubated with the TRAP-staining solution (0.1 M sodium acetate, 30 mM sodium tartrate, 0.1% Triton X-100, 10 mg/ml naphthol AS-MX phosphate disodium salt and 0.3 mg/ml fast red violet LB salt) for 15 min at 37°C. Cells that contained three or more nuclei were counted as TRAP-positive MNCs under a light microscope (x>200) [16].

Resorption pit assay
RAW 264.7 cells (10³ cells/well) were seeded in a 96-well Corning Osteo Assay Surface plate (Corning Incorporated Life Science, Lowell, MA, USA) and treated with 10 ng/ml RANKL/M-CSF and/or calcium-containing crystals for 7 days. Cells were removed by adding a 6% (v/v) sodium hypochlorite solution, and wells were washed with deionized distilled water according to the manufacturer’s instructions. Resorption pits were visualized by light microscopy at x100 magnification, and areas of resorption pits were calculated using ImageJ software.

Western blot analysis
Equal amounts of total cellular proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose (NC) membranes (GE Healthcare Life Sciences, Taiwan, Taiwan). The NC membranes were incubated with various primary antibodies against Akt, phospho-Akt, ERK, phospho-ERK, JNK, phospho-JNK, p38, phospho-p38, IκB, phospho-IκB or GAPDH overnight and then with a secondary antibody conjugated with horseradish peroxidase. Immunocomplexes were visualized with ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Taipei, Taiwan).

Real-time PCR
Total RNA was isolated with an RNA extraction kit (Viogene BioTek Corp., New Taipei City, Taiwan) and cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Life Technologies). For the real-time PCR, a LightCycler System 480 (Roche Diagnostics Ltd, Taipei, Taiwan) was used according to the manufacturer’s instructions. Reactions were set up in a 96-well plate using 1 μl of cDNA with 10 μl of a KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, MA, USA) to which gene-specific upstream and downstream real-time PCR primers had been added. The final concentrations of the reaction components were 0.2 μM each primer: TRAP (NM 007388) forward 5’-CGACCATTGTTAGCCACATAC-3’, reverse 5’-TGCTCCTGAAGATCTGCAGGT-3’; cathepsin K (NM 007802) forward 5’-ATATGGGCGAGGATGAAAGTT-3’, reverse 5’-TGTTCCCCACAGGAATCTCT-3’; CTR (NM 001 042 725) forward 5’-AGTGCCCTTCTTAAGAGAAGGAGAAG-3’, reverse 5’-GGAGTGTCGCCAGACATG-3’; GAPDH (NM 002046) forward 5’-AAGTTTGGCATTTGGG-3’, reverse 5’-ACACATTGCGGTTAGGAAACA-3’. The conditions of the real-time PCR were: 95°C pre-incubation for 30 min; 45 cycles of 95°C for 10 s, 53°C for 20 s and 72°C for 1 s for amplification; and 95°C for 5 s, 65°C for 1 min and 97°C for a melting curve analysis. Expression levels of each mRNA were calculated using the comparative Ct method (2−ΔΔCt formula), and were normalized to the GAPDH mRNA level.

Statistical analysis
Experimental data were evaluated by SigmaStat (Systat Software Inc., Chicago, IL, USA). Student’s t-test was used to determine the significance of the difference. Data are presented as the mean (s.d.) of three independent experiments. P < 0.05 was considered significant.

Results
Hydroxyapatite, β-TCP and CPPD crystals enhanced RANKL/M-CSF- and TPA-induced osteoclastogenesis
To evaluate the roles of calcium-containing crystals in osteoclastogenesis, in this study we used the hydroxyapatite, β-TCP and CPPD crystals as well as RAW 264.7 murine osteoclast precursor cells. First, we found that treating cells with hydroxyapatite, β-TCP and CPPD crystals (0–2 mg/ml) had no effect on cell viability (supplementary Fig. S1, available at Rheumatology Online). In the presence of RANKL/M-CSF, RAW 264.7 cells were capable of differentiating into osteoclast-like cells, which are characterized by increased TRAP activity and the appearance of MNCs [17]. As shown in Fig. 1A–C, RANKL/M-CSF treatments indeed increased the number of TRAP-positive MNCs and TRAP activity in RAW 264.7 cells. In addition, hydroxyapatite, β-TCP and CPPD crystal treatments drastically enhanced RANKL/M-CSF-induced TRAP-positive MNCs and TRAP activity in a dose-dependent manner. β-TCP was better than hydroxyapatite and CPPD in inducing TRAP-positive MNCs. However, CPPD was a stronger inducer than β-TCP and hydroxyapatite for increasing TRAP activity. To further confirm that these crystals could enhance osteoclastogenesis in human macrophages, we used human myeloid lineage U937 cells and induced it to differentiate into macrophages by 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA alone not only differentiated U937 cells into macrophages but also further induced osteoclastogenesis, as evidenced by the increase in TRAP-positive cells and TRAP activity. In addition, these three crystals significantly enhanced TRAP-positive cells and TRAP activity in TPA-treated U937 cells (Fig. 1D and E). These results suggest that hydroxyapatite, β-TCP and CPPD crystals are potential promoters that can enhance the...
differentiation of macrophages into osteoclasts under appropriate conditions.

Osteoclastogenesis is associated with the upregulation of several specific genes in response to RANKL/M-CSF.

We therefore investigated the effects of hydroxyapatite, β-TCP and CPPD on expression of the three osteoclast-specific genes, TRAP, cathepsin K and CTR with a quantitative RT-PCR. RAW 264.7 cells were treated with

Fig. 1 Effects of calcium-containing crystals on RANKL/M-CSF- and TPA-induced osteoclastogenesis

(A–C) RAW 264.7 cells were incubated with various kinds of crystal, 10 ng/ml RANKL and 10 ng/ml M-CSF (R/M) for 72 h. (D and E) Human U937 cells were treated with 100 nM TPA for 2 days, and incubated with 62.50 μg/ml hydroxyapatite or β-TCP, or 31.25 μg/ml CPPD for another 4 days. (A) The numbers of TRAP-positive MNCs and (C and E) TRAP activity were determined, and (B and D) representative pictures of TRAP staining are shown. *P < 0.05 vs column 1; #P < 0.05 vs column 2. R/M: RANKL/M-CSF; HA: hydroxyapatite; β-TCP: β-tricalcium phosphate; TPA: 12-O-tetradecanoylphorbol-13-acetate.
Hydroxyapatite, β-TCP and CPPD crystal treatments drastically enhanced the mRNA expression of TRAP, cathepsin K (CathK) and calcitonin receptor (CTR) in RANKL/M-CSF/C150 treated cells (Fig. 2). Interestingly, hydroxyapatite and β-BCP also significantly increased the mRNA expression of cathepsin K and CTR in the absence of RANKL/M-CSF.

To further confirm the promotion of osteoclastogenesis by hydroxyapatite, β-TCP and CPPD crystals, a bone resorption pit assay was performed using a Coring Osteo Assay Surface plate. As shown in Fig. 3A, hydroxyapatite, β-TCP and CPPD crystals significantly increased areas of RANKL/M-CSF-induced resorption pits. A representative control plate and RANKL/M-CSF-induced resorption pits are shown in Fig. 3B. These results suggest that hydroxyapatite, β-TCP and CPPD crystals enhanced bone resorption activity in the presence of RANKL/M-CSF.

ERK and p38 were involved in osteoclastogenesis stimulated by hydroxyapatite, β-TCP and CPPD.

Three key signalling pathways of MAPKs, NF-κB and Akt are involved in osteoclastogenesis induced by RANKL in macrophages [15]. To investigate which signalling pathways are important for osteoblast differentiation enhanced by calcium-containing crystals, phosphorylation levels of Akt, IκB and MAPK members (ERK, JNK, p38) as well as IκB expression were determined. As shown in Fig. 4, RANKL/M-CSF alone significantly induced Akt phosphorylation, but had little effect on the phosphorylation of IκB, ERK, p38 and JNK or on IκB expression. Treatment with hydroxyapatite, β-TCP and CPPD crystals significantly enhanced the phosphorylation of ERK and p38, particularly increasing ERK phosphorylation in RANKL/M-CSF/C150-treated cells. The three kinds of calcium-containing crystal did not cause further change in the phosphorylation of IκB, Akt or JNK, or IκB expression in RANKL/M-CSF-treated cells. To further investigate the potential roles of ERK and p38 in calcium-containing crystal-enhanced osteoclastogenesis, pharmacological kinase inhibitors, including PD98059 (an ERK inhibitor) and SB203580 (a p38 inhibitor) were used. As shown in Fig. 5, hydroxyapatite, β-TCP and CPPD all significantly enhanced TRAP activity in RANKL/M-CSF-treated cells. However, these two kinase inhibitors significantly blocked...
To investigate whether calcium-containing crystals themselves can induce the phosphorylation of MAPKs and Akt, we used three kinds of crystal to treat RAW 264.7 cells in the presence of RANKL/M-CSF. As shown in Fig. 6, hydroxyapatite and \( \beta \)-TCP significantly induced the phosphorylation of Akt, ERK, JNK and p38 in dose- and time-dependent manner. However, CPPD only increased the phosphorylation of ERK and p38 but not that of Akt or JNK. These results suggest that each calcium-containing crystal alone can also activate several signal transductions, which might contribute to the enhancement of RANKL/M-CSF-induced osteoclastogenesis.

**Discussion**

In this study, we first demonstrated that calcium-containing crystals (hydroxyapatite, \( \beta \)-TCP and CPPD) can enhance RANKL/M-CSF-mediated osteoclastogenesis via ERK and p38 in RAW 264.7 osteoclast precursor cells. The results suggest that the enhancement of osteoclast formation by calcium-containing crystal deposition might play an important role in the progression of bone erosion *in vivo*.

Normal skeletal homeostasis relies on the coordination of osteoblast and osteoclast activities. Bouchard et al. [18] observed that MSU and CPPD crystals altered the normal phenotype of human osteoblasts, redirecting them towards reduced bone formation and amplified osteoblast-mediated bone resorption, abnormalities that could play a role in the bone destruction associated with chronic crystal-induced arthritis. Dalbeth et al. [19] also found that MSU crystals had profound inhibitory effects on osteoblast viability and differentiation, through altering physiological bone turnover then increasing osteoclast formation. However, MSU crystals at various time points and concentrations did not promote the formation of TRAP-positive MNCs in RAW 264.7 cells, either in the presence or absence of RANKL [20], indicating that MSU crystals did not directly promote osteoclast development at any point in osteoclastogenesis. On the contrary, our study showed that hydroxyapatite, \( \beta \)-TCP and CPPD promoted the formation of TRAP-positive MNCs in RAW 264.7 cells in the presence of RANKL/M-CSF. Indeed, the heterogeneity of crystals may lead to differences in their tolerance by human tissues, and may be one of the reasons why these crystals are identified in human tissues in very different clinical settings.

Crystal density, size and coating with various proteins are known to influence the inflammatory potential of crystal species [21]. Physicochemical heterogeneity may be an additional factor. Prudhommeaux et al. [22] showed that the inflammatory potential of BCP crystals appeared to vary according to the specific surface area and Ca:P ratio. We postulated that the bone remodelling effect of calcium-containing crystals depends on the calcium content and surface area. Therefore, further studies of the physicochemical properties of various crystals are needed to better understand differences in the behaviour of these crystals in bone remodelling.

A number of clinical observations have suggested that articular resident cells are primed by crystals to heightened responsiveness to local stimuli [23]. Cheung et al. [24] suggested that crystal-induced synovial fibroblast proliferation is a mechanism for increasing the number of synovial cells, which would then be capable of eliciting bone remodelling. The interaction of crystals with articular resident cells is believed to be a key factor in crystal-induced bone remodelling, and this may explain the lack of an inflammatory response to crystal deposits located in the ground substance of tendons or cartilages that could cause joint damage. Indeed, our studies suggest that crystal enhancement of osteoclastogenesis should be considered an active participant in tissue damage. In
support of this concept, clinical observations identified CPPD and BCP crystals within joint fluid in very diverse clinical settings, such as asymptomatic joints, acute arthritis and destructive arthropathies without overt inflammation [1]. The response of RAW 264.7 cells to calcium-containing crystals suggests that pathological calcification is not merely a passive consequence of chronic inflammatory disease but a possible contributing factor to osteoclastogenesis. Signalling events involved in calcium-containing crystal–induced joint damage remain unknown. Calcium-containing crystals activate multiple signalling transduction pathways and bone remodelling and differ depending on the crystal and cell type studied. Nair et al. [25] showed that BCP and CPPD crystals differentially activated members of the MAPK signal transduction cascade but not the p38 protein kinase cascade pathway in human fibroblasts. Previous studies also showed that BCP crystals activated synovial fibroblasts, inducing cellular proliferation and metalloproteinase secretion through a variety of intracellular signalling pathways, including protein kinase C, ERK, NF-κB and activator protein-1 [26–28]. In this study, the model of RANKL-induced osteoclastogenesis was used to examine the effects of the crystals. It has been known that at least

![Enhancement effects of calcium-containing crystals on the phosphorylation of ERK and p38 in RANKL/M-CSF-treated cells](https://academic.oup.com/rheumatology/article-abstract/54/10/1913/1790138/Calcium-containing-crystals-enhance-receptor)

**Fig. 4** Enhancement effects of calcium-containing crystals on the phosphorylation of ERK and p38 in RANKL/M-CSF-treated cells.

RAW 264.7 cells were treated with 10 ng/ml RANKL and 10 ng/ml M-CSF for 10–60 min, or pretreated with various kinds of crystal (1 mg/ml) for 10 min and then treated with 10 ng/ml RANKL and 10 ng/ml M-CSF for 10–60 min. Total cellular proteins were collected, and the protein and phosphorylated protein expression was detected by western blotting. R/M: RANKL/M-CSF; HA: hydroxyapatite; β-TCP: β-tricalcium phosphate; p-AKT: phospho-Akt; p-ERK: phosho-extracellular signal-regulated kinase; p-JNK: phospho-c-Jun N-terminal kinase; p-p38: phospho-p38; p-IκB: phospho-IκB.
Five distinct signalling pathways are involved in RANKL-dependent osteoclastogenesis, including Src/PI3K/Akt, IKK/NF-kB, ERK, JNK and p38 [29, 30]. We found that these crystals only enhanced the phosphorylation of ERK and p38, but they did not change the phosphorylation levels of Akt, IkB and JNK. Therefore, both ERK and p38 play an important role in crystal-enhanced osteoclastogenesis. c-Jun can bind c-fos to form a heterodimer.
Calcium-containing crystals promote osteoclastogenesis

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at Rheumatology Online.

References


Acknowledgements

We thank Dr Yu-Wei Wu, Dr Li-Hsuan Chiu, Ms. Zi-Hsuan Liu and Chen-Hung Wang for technical assistance. Conception and study design: L.S.Y., L.Y.C., C.C.C., T.Y.H. Technical support: T.Y.H. Data acquisition: C.C.C., L.Y. Data analysis and interpretation: C.C.C., L.S.Y., L.Y.C. Drafting of the article: C.C.C., L.Y.C. All other authors contributed to writing and revising the manuscript for scientific content and approved the final version before submission.

Funding: This study was supported by a grant (NSC101-2320-B-039-017-MY3) from the National Science Council, Taiwan and one (102TMU-TMUH-11) from Taipei Medical University Hospital.

(named activator protein-1) that is involved in the osteoclastogenesis. In RANKL-induced signalling pathways, c-Jun is a downstream molecule of JNK, and c-fos is activated by NF-κB [29, 30]. Since these crystals did not activate JNK and NF-κB signal pathways (Figs 4 and 6), and we also found that these crystals did not enhance the activation of c-Jun and c-fos in RANKL/M-CSF-treated cells (data not shown). In addition, the RANKL-independent osteoclastogenic regulatory molecules, such as NFAT2 and Myc, have not been examined in this study. In vitro experiments clearly demonstrated the deleterious role of calcium-containing crystals, which may stimulate cells through two mechanisms. These crystals can first activate cells after they are endocytosed or phagocytosed. The other mechanism of crystal activation involves a direct crystal–cell membrane interaction [31]. Binding to specific membrane receptors related to surface proteins, such as Toll-like receptor-2 and Toll-like receptor-4 [32, 33] or annexin V [34], has recently been shown. In the future, it remains for further studies to elucidate whether the mechanism by which crystals are activated is through being endocytosed or via surface-connected compartments. Previous studies have also demonstrated that inflammatory cytokines, such as IL-1, TNF and IL-17, promote osteoclastogenesis [35, 36]. Both BCP and CPPD regulate inflammatory responses by stimulation of cyclooxygenase-2, prostaglandin E2 and IL-1β expression [37, 38]. It is possible that the enhancement of osteoclastogenesis by calcium-containing crystals might be mediated through the induction of inflammatory cytokines.

In summary, the exact pathogenesis of calcium-containing crystals as a mediator of bone disease is unknown. Our studies demonstrated that calcium-containing crystals have the ability to increase RANKL/M-CSF-mediated osteoclastogenesis and resorption. Clearly, this study highlights new pathogenic mechanisms of diseases associated with calcium-containing crystal deposition, improves our knowledge of these common crystals and suggests new targets for drugs. Nevertheless, more work is required to further elucidate the relationship between the accumulation of calcium-containing crystals and the evolution of progressive tissue destruction.
Chi-Ching Chang et al.