Inhibitory effect and mechanism of 1,25-dihydroxy vitamin D_3 on RANKL expression in fibroblast-like synoviocytes and osteoclast-like cell formation induced by IL-22 in rheumatoid arthritis

H. Wen, Y. Liu, J. Li, D. Wei, D. Liu, F. Zhao

Department of Rheumatology, Shanxi Medical University, the Second Hospital of Shanxi Medical University, Taiyuan, Shanxi Province, China.

Abstract

Objective

To explore the inhibitory effect and mechanism of 1,25-dihydroxy vitamin D_3 (l,25(OH)$_2D_3$) on receptor activator of nuclear factor-κB ligand (RANKL) expression in fibroblast-like synoviocytes (FLSs) and osteoclastogenesis induced by interleukin (IL)-22 in patients with rheumatoid arthritis (RA).

Methods

Fibroblast-like synoviocytes from patients with rheumatoid arthritis (RA-FLSs) were cultured and stimulated for RANKL expression with IL-22 in the absence or presence of various concentrations of l,25(OH)$_2D_3$, and JAK-2 inhibitor or p38 MAPK inhibitor at the optimised time point of IL-22 treatment. The level of RANKL messenger RNA (mRNA) or protein was measured using real-time polymerase chain reaction (RT-PCR) or western blot method. To assess the impact of l,25(OH)$_2D_3$ on osteoclastogenesis, isolated monocytes were activated by M-CSF and RANKL or cocultured with FLSs stimulated by IL-22 in the presence or absence of l,25(OH)$_2D_3$ and those inhibitors. TRAP-positive cells as differentiated osteoclasts were stained for alkaline phosphatase.

Results

FLSs stimulated with IL-22 for 72 hours were used in further experiment because of the highest expression of RANKL at this time point. The expression of RANKL mRNA and protein in IL-22-stimulated FLSs were significantly inhibited by 1 nM of 1,25(OH)$_2D_3$ ($p<0.05$). Interestingly, this inhibition was reversed by inhibitor of JAK-2/STAT-3 or p38 MAPK/NF-κB signalling. In monocytes cocultured with IL-22-stimulated FLSs in the presence of exogenous RANKL and M-CSF, 1,25(OH)$_2D_3$ could block the process of osteoclastogenesis by JAK-2/STAT-3 or p38 MAPK/NF-κB signalling.

Conclusion

1,25(OH)$_2D_3$ may exert inhibitory effect on osteoclastogenesis of RA-FLSs by down-regulating RANKL expression, which could be mediated by IL-22 through JAK-2/STAT-3 and p38 MAPK/NF-κB signalling.

Key words

rheumatoid arthritis (RA), 1,25(OH)$_2$D$_3$, fibroblast-like synoviocytes (FLS), RANKL, IL-22

*Hongyan Wen, MD, PhD**
*Yang Liu, MD**
Jinchan Li, MD
Dandan Wei, MD
Dandan Liu, MD
Fangfang Zhao, MD

**These authors contributed equally to this work.*

Please address correspondence to:
Dr Hongyan Wen,
Department of Rheumatology,
Shanxi Medical University,
the Second Hospital of Shanxi Medical University,
382 Wu Yi Road, Taiyuan,
Shanxi Province 030001, China.
E-mail: wenhongyan0509@aliyun.com

Received on September 19, 2017; accepted in revised form on January 8, 2018.

Funding: this study was supported by research grants from the National Nature Science Foundation of China (no.81301532/H0603), Fund Program for the Scientific Activities of Selected Returned Overseas Professionals in Shanxi Province-2017, the Shanxi Science and Technology Research Projects of China (no. 201603D321074), and the Shanxi Scholarship Council of China (2017-119).

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, mainly characterised by persistent synovitis induced by extensive synovial cell proliferation, synovial tissue hyperplasia, and pannus formation. Activated synoviocytes attacking cartilage and bone cause arthritis and joint deformity, and, even worse, may lead to high risk of morbidity and mortality (1). It is reported that about 75% of RA patients would have joint erosions after 2 years of disease onset (2). Therefore, early diagnosis and early reversal of bone destruction could be assumed enormous importance. However, the exact aetiology and pathogenesis of RA bone erosions are still unknown, while genetic and environmental factors seem to play a key role (3). One of the crucial environmental factors related to RA is vitamin D deficiency (4-7).

Traditional vitamin D was reported to act as a hormone precursor to exert its anti-inflammatory and immune-regulation role (8-10), including the release of related chemokines and cytokines (11-13). Among cytokines, IL-22 can be produced by many cells including activated NK and T cells and suggested to play crucial roles in innate immune responses, autoimmunity and tissue regeneration (14). However, the roles of IL-22 and vitamin D in rheumatoid arthritis are still unclear.

Fibroblast-like synoviocytes (FLSs) existing in articular cavity, are characterised by excessive proliferation in inflammatory environment and play a crucial role in bone destruction. A variety of inflammatory cytokines and matrix proteins can stimulate their proliferation and differentiation (15). Cytokines such as IL-1, IL-6, IL-17, and tumor necrosis factor-α (TNF-α), especially IL-22 can activate FLSs. IL-22 was also highly expressed in FLSs and could promote the proliferation of FLS (16, 17). The activation of FLSs induces the expression of receptor activator of nuclear factor-κB ligand (RANKL), which is necessary to promote monocyte/macrophage to differentiate into osteoclasts (18), eventually leading to the destruction of cartilage and bone. Interestingly, 1,25-dihydroxy vitamin D$_3$ (1,25(OH)$_2$D$_3$) can significantly reduce the expression of cytokines including IL-17, IL-22, IL-6 and TNF-α in patients with RA and may have a plausible link with NF-κB (19).

Our previous study found that the levels of 1,25(OH)$_2$D$_3$ in patients with RA were significantly lower than those of healthy volunteers (20). The negative correlation between serum 1,25 (OH)$_2$D$_3$ levels and several markers of RA disease activity suggests the involvement of vitamin D in the pathogenesis of RA (20). Furthermore, 1,25(OH)$_2$D$_3$ could reduce the production of RANKL and the secretion of TNF-α, IL-17, and IL-6 in peripheral blood mononuclear cells (PBMCs) of RA patients (21). These findings have reminded us that the bone-preserving effects of 1,25(OH)$_2$D$_3$ in RA patients could be at least partly ascribed to regulate the expression of RANKL signalling pathway. Here, we provided new insights into the stimulus-specific roles of 1,25(OH)$_2$D$_3$ in RA. Our findings demonstrated that the presence of 1,25(OH)$_2$D$_3$ could down-regulate RANKL expression in RA-FLS and inhibit osteoclastogenesis mediated by JAK-2/STAT-3 and p38 MAPK/NF-κB signalling, delaying the process of bone destruction of RA. Therefore, 1,25(OH)$_2$D$_3$ may be of value in preventing bone erosions in patients with RA.

Patients and methods

Patients and tissue specimens

PBMCs were obtained from 18 RA patients (6 men and 12 women: age, mean±SD: 44±5 years) and 18 age-, sex-matched healthy volunteers. Synovial samples were isolated from another 3 patients with RA (1 men and 2 women: age, mean±SD: 43±6 years) through blind type needle synovial biopsy surgery. All patients fulfilled the American College of Rheumatology (ACR) 1987 revised criteria for RA (22). Informed consents were obtained from all patients and healthy volunteers, and the experimental protocol was approved by the Medical Ethics Committee of the Second Hospital of Shanxi Medical University, which complied with the Declaration of Helsinki (approval no.: 2013ky007).

Reagents

1,25(OH)2D3 was supplied by Sigma Company (St. Louis, USA). IL-22 and

macrophage colony-stimulating factor (M-CSF) were purchased from PeproTech Company (New Jersey, USA). AG490 (JAK-2/STAT pathway inhibitor) and SB203580 (p38 MAPK pathway inhibitor) were obtained from R&D Company (Santa Monica, USA). All the other chemical agents used were of analytical grade.

T lymphocyte activation and IL-22 expression assay

To assay down-regulated effect of 1,25$(OH)_2D_3$ on IL-22 expression of PBMCs activated by anti-CD3/anti-CD28, these cells were isolated from RA patients as reported previously (16). The PBMCs were suspended in phenol red-free Iscove's modified Dulbecco's medium (IMDM) (Gibco, Breda, The Netherlands) supplemented with 10% charcoal-treated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker, Walkersville, MD) and cultured for 72 hours in 96-well plates (200μl/well). Different concentrations (0.1 nM, 1 nM, and 100 nM) of 1,25$(OH)_2D_3$ were used to stimulate the cells with the presence of anti-CD3 (300 ng/ml, 1XE, Sanquin, Amsterdam, The Netherlands) and anti-CD28 (400 ng/ml, 15E8, Sanquin, Amsterdam, The Netherlands). At 72 hours after treatment, the culture supernatant was collected for further examination of IL-22 by a sandwich Enzyme-linked immunosorbent kit (ELISA) (16).

RANKL expression of FLSs stimulated by IL-22

FLSs, synoviocytes type B, from RA were isolated from the synovial tissue using a process of enzymatic digestion (23). The isolated FLSs were suspended in DMEM/F12 (Gibco) supplemented with 10% FCS, 100 units/ml penicillin, and 100g/ml streptomycin (Gibco) and inoculated into 6-well plates (2 ml/well). The FLSs were stimulated with IL-22 at 10ng/ml for 48 hours, 72 hours or 96 hours, and then selected one optimal time point. At the optimal time point (72 hours after treatment), the FLSs were incubated in the presence or absence of different concentrations (0.1 nM,1 nM, and 100 nM) of 1,25$(OH)_2D_3$ and/or different pathway inhibitors (50 μM) (AG490 for JAK-2/STAT-3, SB203580 for p38 MAPK/NF-κB). After the inhibitor treatment for 1 hour and the addition of IL-22 again, FLSs were incubated with different concentrations (0.1 nM, 1 nM, and 100 nM) of 1,25$(OH)_2D_3$ for 72 hours. Expression of RANKL messenger RNA (mRNA) and protein was examined by real-time polymerase chain reaction (PCR) and western blot.

Measurement of RANKL messenger RNA (mRNA) expression by real-time polymerase chain reaction (PCR)

mRNA was extracted using RNAzol B (Biotex Laboratories), according to the manufacturer's instructions. Inner reference (β-actin) cDNA was amplified with the following oligonucleotides: 5'-GGA CTT CGA GCA AGA GAT GG-3' as the forward primer sequence, and 5'-TGT GTT GGC GTA CAG GTC TTT G-3'as the reverse primer sequence. Target gene (RANKL) cDNA was amplified with the following oligonucleotides: 5'-ACC AGC ATC AAA ATC CCA AG-3' as the forward primer sequence, and 5'-CCC CAA AGT ATG TTG CAT CC-3' as the reverse primer sequence. Reverse transcription of 2 μg total RNA was carried out at 37°C for 15 minutes using the SuperScript reverse transcription system (Takara). Quantitative PCR was performed in a 20μl final volume in capillary tubes in a LightCycler instrument (Roche Diagnostics). The reaction mixture contained 2 μl of LightCycler FastStart DNA MasterMix for SYBR Green I (Roche Diagnostics), 0.5 μM of each primer, 4 mM MgCl2, and 2 μl of template DNA. All of the capillaries were amplified with the predenaturing of polymerase at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The temperature transition rate was 20°C/second for all steps. The double-stranded PCR product was measured during the 72°C extension step by the detection of fluorescence associated with the binding of SYBR Green I to the product. Melting curve analysis was performed immediately after the amplification protocol, under the following conditions: 0 seconds (hold time) at 95°C, 15 seconds at 71°C, and 0 seconds (hold time) at 95°C. Negative controls were also included and contained all elements of the reaction mixture, except for the template DNA. All samples were processed in triplicate.

The fluorescence curves were analysed using LightCycler software (v. 3.0; Roche Diagnostics). The relative expression level of mRNA in each sample was calculated as the level of RANKL normalised to the endogenously expressed housekeeping gene (β-actin).

Western blot analysis of RANKL expression

The IL-22-stimulated FLSs were incubated with various doses of 1,25$(OH)_2D_3$, either AG490 (50 μM) or SB203580 (50 μM), or 1,25(OH)2D3 plus either AG490 or SB203580. After incubation for 72 hours, whole cell lysates of FLS were prepared by homogenisation in the lysis buffer (RIPA lysate, Thermo Bio, USA). The protein concentration in the supernatant was measured using the Bicinchonininc acid (BCA) method. The protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (BOSTER Bio) and transferred to a nitrocellulose (NC) membrane. For Western hybridisation, 2μl of anti-RANKL antibody (Abcam Bio.) was added and incubated overnight at 4°C. After wash and addition of 2μl horseradish peroxidase- conjugated goat anti-rabbit antibody (BBI life sciences Bio), the hybridised bands were detected using an enhanced chemiluminescence detection kit and Hyperfilm-ECL reagents (BOSTER Bio).

Effect of 1,25$(OH)_2D_3$ on monocytes

PBMCs obtained from healthy volunteers were separated from the buffy coats by Ficoll-Hypaque (Sigma) density gradient centrifugation. Monocytes were purified using magnetic bead separation (Miltenyi Biotec, Cologne, Germany) from PBMCs and stained with a monocyte-specific anti-$CD14^+$ monoclonal antibody to assess the purity of the preparation, which revealed that 90% of the isolated cells expressed $CD14^+$. Osteoclast precursors were prepared using the monocyte-enriched fraction from the peripheral blood. The

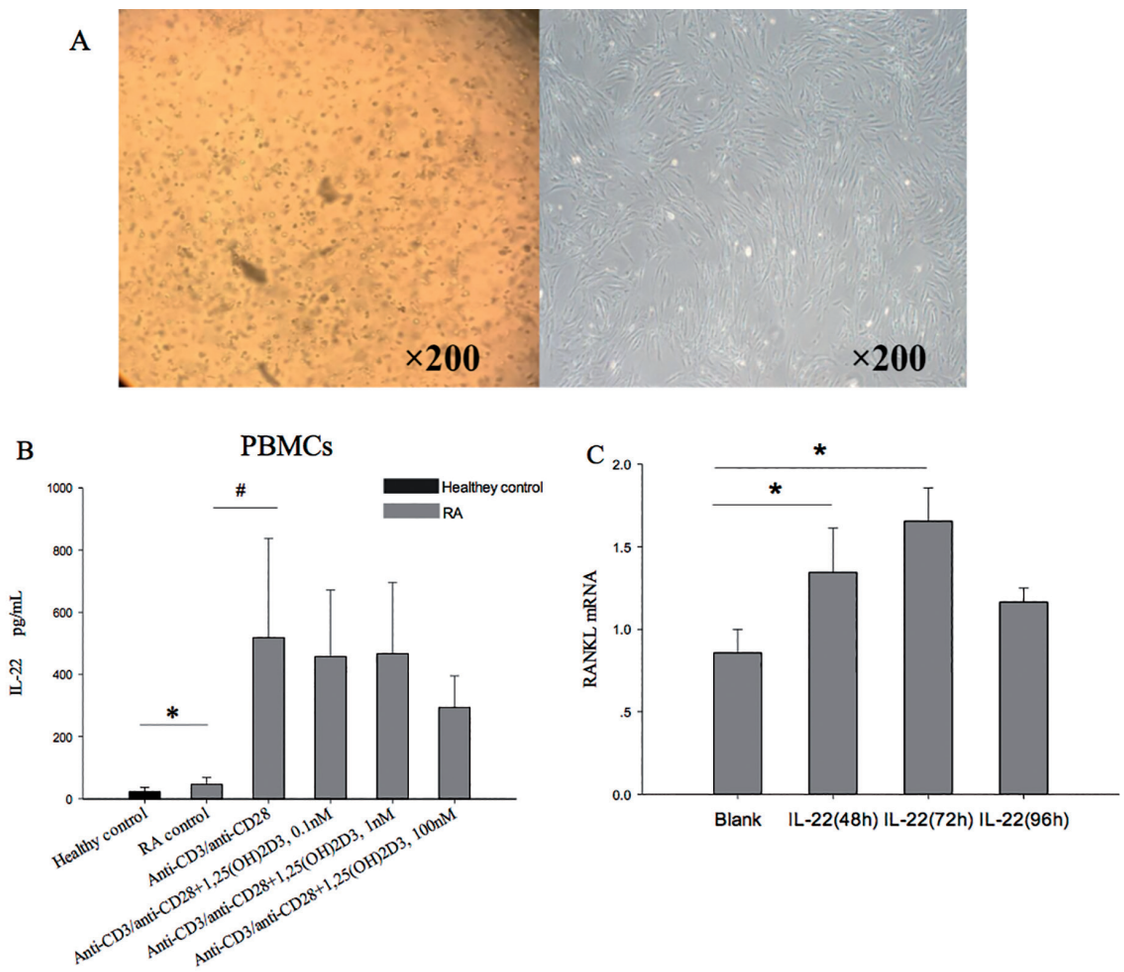

Fig. 1. Effect of 1,25(OH)$_2$D$_3$ on IL-22 secretion in RA-PBMCs stimulated by anti-CD3/anti-CD28. (A) FLS preparation and culture. Left, Synovial tissue pieces were digested by collagenase I for 4-6 hours (Inverted microscope 200 x). Right, FLSs were cultured for 5 days (Inverted microscope 200 x). (B) ELISA assay showed the levels of IL-22 in cultured PBMCs from 18 RA and 18 healthy controls. *=p<0.05 *versus* healthy PBMCs; #=p<0.05 *versus* RA PBMCs. The addition of 0.1 nM, 1 nM, or 100 nM of 1,25(OH)$_2$D$_3$ led to an inhibition of IL-22 secretion in cultured RA-PBMCs compared to that with anti-CD3/anti-CD28-stimulated only, though the results did not reach a statistical degree (p>0.05). Bar show the mean and SD. (C) Optimal time for up-regulation of RANKL in FLSs by IL-22 stimulation. The addition of IL-22 to FLSs at increasing culturing time points led to a time-dependent stimulation and reached the highpoint at 72 hours of the expression of RANKL mRNA, relative to β-actin. *=p<0.05 *versus* blank control.

isolated human monocytes were incubated with RANKL (30ng/mL) and M-CSF (25ng/mL), in the presence or absence of 1,25(OH)$_2$D$_3$ (1nM). The medium was changed every 2 days. On day 14, tartrate-resistant acid phosphatase (TRAP, as a marker of osteoclast differentiation)-positive cells were counted for the number of osteoclasts. Real-time PCR was performed to measure the mRNA expression of osteoclastogenic markers, such as TRAP, matrix metalloprotein-9 (MMP-9), and cathepsin K.

Detection of osteoclast formation

As described above, the isolated human CD14$^+$ monocytes (1×10 ^{6}cells/well) were added to the cultures of stimulated fibroblasts in fresh medium. The cells were incubated for 2 weeks in α-minimum essential medium and 10% heat-inactivated FBS in the presence of 25 ng/ml of M-CSF. The medium was changed at day 3, and every other day thereafter. The cells treated with recombinant human RANKL protein and prepared as described previously were used as the positive control and with M-CSF only as negative control. At day 21, TRAP–positive cells, differentiated osteoclasts, were identified using a leukocyte acid phosphatase kit (Sigma-Aldrich), according to the manufacturer's protocol (24). The mRNA expression of TRAP, MMP-9, and cathepsin K was also measured as above mentioned.

Statistical analysis

Samples were analysed at least in triplicates. All quantitative data were expressed as mean ± standard deviation. Data of normal distribution and homogeneity of variance were compared variance (ANOVA). Student's *t*-test was used to compare multi group variables, or by rank sum test. A *p*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed by SPSS v. 23.0.

Results

Down-regulation of IL-22 secretion after 1,25(OH)$_2$D$_3$ treatment and optimal time of IL-22 stimulation for up-regulation of RANKL in FLSs

IL-22 expression was significantly

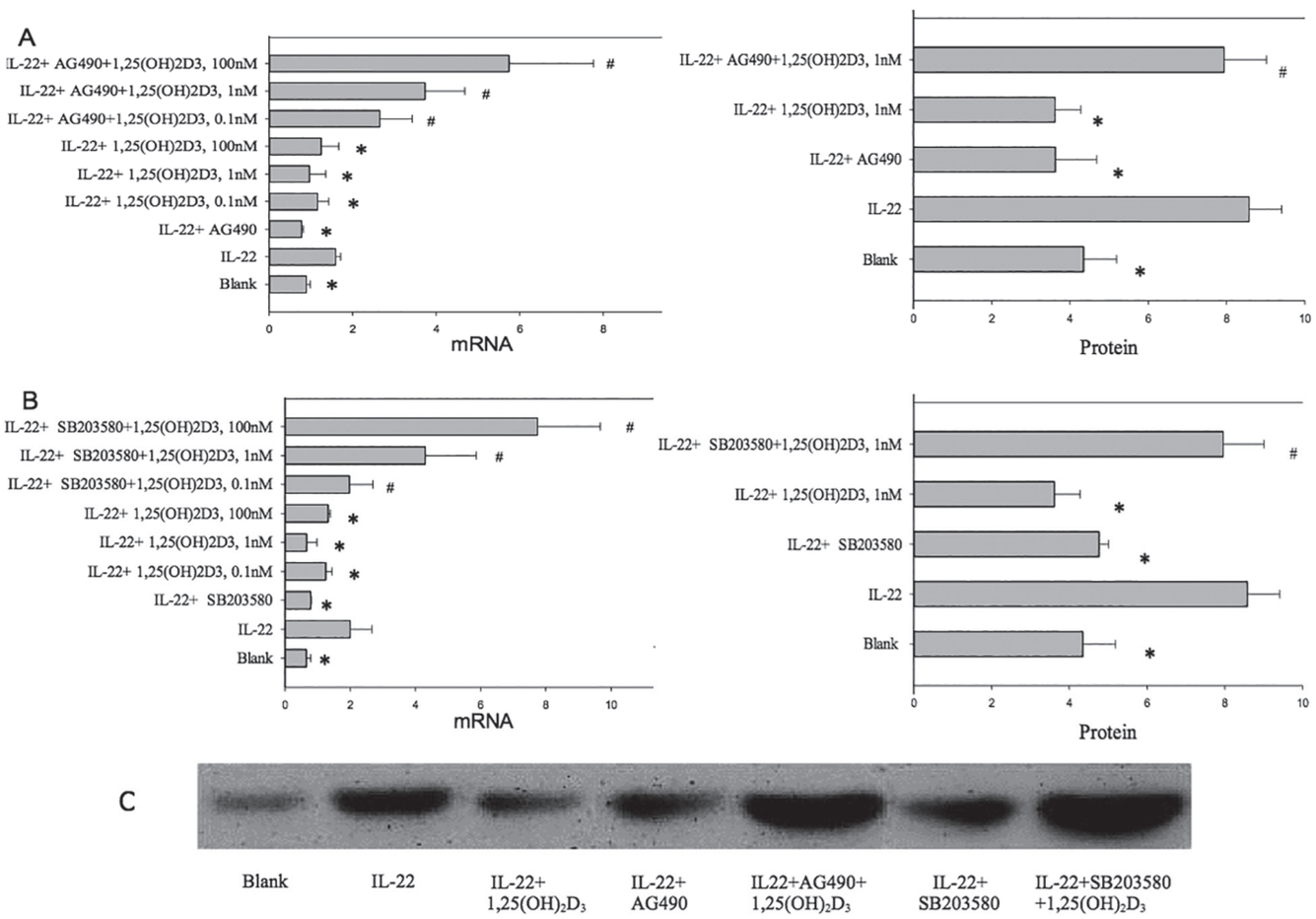

Fig. 2. Inhibitory effect of 1,25(OH)$_2$D$_3$ on the expression of RANKL mRNA and protein mediated by JAK-2/STAT-3 or p38 MAPKκB signaling pathway in IL-22 stimulated RA-FLS. (A and B) RA-FLSs were pretreated for 1 hour with AG490 (JAK-2/STAT pathway inhibitor, 50 μM) (**A**) or SB203580 (p38 MAPK pathway inhibitor, 50 μM) (**B**) and then were cultured with 0.1 nM, 1 nM, or 100 nM of 1,25(OH)$_2$D$_3$ for 72 hours. Bars show the mean ± SD of 3 independent experiments. *=p<0.05 *versus* IL-22 alone. $^{\#}$=p<0.05 *versus* combination of IL-22 and AG490 or SB203580. (**C**) The exposure image of RANKL protein after IL-22 stimulation for 72 hours. A molecular weight of 37kDa band agreed with that of RANKL and β-Actin was used as loading control.

higher in RA-PBMCs than in healthy control (p<0.05) and dramatically increased in RA-PBMCs after anti-CD3/anti-CD28 treatment (p<0.05) (Fig. 1A-B). To assess the effect of 1,25(OH)$_2$D$_3$ on IL-22 secretion, RA-PBMCs were incubated with 1,25(OH)$_2$D$_3$. The addition of different concentrations of 1,25(OH)$_2$D$_3$ led to a decrease of IL-22 secretion in RA-PBMCs compared to that stimulated with anti-CD3/anti-CD28 only, though it did not reach a statistical degree (p>0.05) (Fig. 1B). Furthermore, the experimental doses of 1,25(OH)$_2$D$_3$ at different time point had no cytotoxic effect or proliferative effects on the RA synovial fibroblasts (results not shown). Herein, we examined whether IL-22 induced the expression of RANKL in RA-FLSs and detected the best time for stimulation. The addition of increasing culture time (48 hours, 72 hours or 96 hours) of IL-22 to FLSs (Fig. 1A) led to a time-dependent stimulation and reached the highpoint at 72 hours of the expression of RANKL mRNA (Fig. 1C).

Inhibitory effect of 1,25(OH)$_2$D$_3$ on the expression of RANKL stimulated by IL-22 was mediated by JAK-2/STAT-3 and p38 MAPK/NF-κB signalling pathway in RA-FLSs

Since JAK-2 / STAT-3 and p38 MAPK pathway may mediate the expression of RANKL, we further characterised the relationship between 1,25(OH)$_2$D$_3$ treatment and RANKL expression mediated by JAK-2 / STAT-3 and p38 MAPK. RA-FLSs were stimulated with 10 ng/ml IL-22 for 72 hours and then incubated for 1 hour in the presence or absence of the different signal inhibitors together with or without various concentration of 1,25(OH)$_2$D$_3$. The expression of RANKL mRNA in IL-22 stimulated FLSs decreased significantly after the treatment of 1,25(OH)$_2$D$_3$ (each p<0.05) (Fig. 2A-B). The middle dose (1nM) of 1,25(OH)$_2$D$_3$ showed superior inhibitory activities for RANKL mRNA expression. (Fig. 2A-B). However, the presence of 1,25(OH)$_2$D$_3$ significantly up-regulated RANKL mRNA expression after the activities of JAK-2 and p38 MAPK pathways were inhibited by their inhibitors (each p<0.05). No cytotoxic effects of these chemical inhibitors on RA synovial fibroblasts were observed under the experimental concentrations used (results not shown). We also examined whether 1,25(OH)$_2$D$_3$ regulated RANKL expression in protein level. A similar pattern was observed when the synovial fibroblasts were analysed by Western blotting (Fig. 2A-B-C).

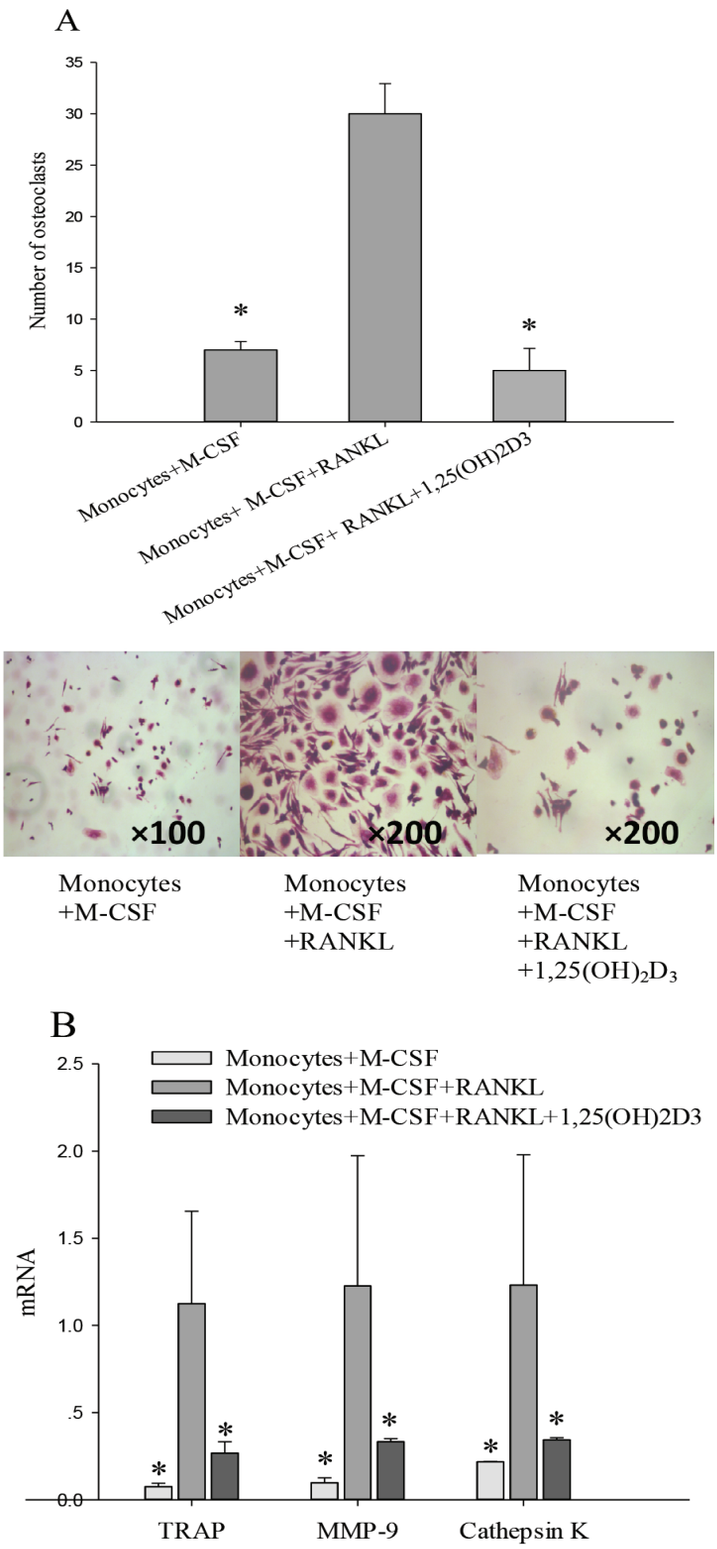

Fig. 3. 1,25(OH)$_2$D$_3$ inhibition of osteoclastogenisis.
A: TRAP was used as a marker of osteoclast differentiation and the TRAP-positive cells were counted (top) and TRAP-positive multinucleated cells were identified using leukocyte acid phosphatase staining (bottom).
B: The presence of 1,25(OH)$_2$D$_3$ could block the process of differentiation of TRAP-positive multinucleated cells (p<0.05).The expression of TRAP, matrix metalloprotein-9 (MMP-9), and cathepsin K mRNA, relative to β-actin, in incubation of monocytes with M-CSF and RANKL, with or without 1,25(OH)$_2$D$_3$. Bars show the mean ± SD of 2 independent experiments. *=p<0.05 *versus* incubation of monocytes with M-CSF and RANKL.

1,25(OH)$_2$D$_3$ inhibition effect on osteoclastogenesis in human CD14$^+$ monocytes

Monocytes can differentiate into TRAP-positive multinucleated osteoclasts in the presence of RANKL and M-CSF. But M-CSF alone had a lesser effect (p<0.05) in the absence of RANKL. When the monocytes were incubated with RANKL and M-CSF, the presence of 1,25(OH)$_2$D$_3$ could block the differentiation of TRAP-positive multinucleated cells (p<0.05) (Fig. 3A). Similar patterns were found using other osteoclastogenic markers, such as TRAP, MMP-9, and cathepsin K (each p<0.05) (Fig. 3B).

1,25(OH)$_2$D$_3$ inhibition of osteoclastogenisis via the JAK-2/STAT-3 and p38 MAPK/NF-κB signalling pathways in the coculture of human CD14$^+$ monocytes and IL-22-stimulated RA-FLS

To determine whether 1,25(OH)$_2$D$_3$ has a role in the intracellular signalling pathways mediating osteoclast differentiation, the isolated monocytes were cocultured with IL-22–stimulated RA-FLSs in medium containing the different signal inhibitors in the presence of 1,25(OH)$_2$D$_3$. A combination of 1,25(OH)$_2$D$_3$ and inhibitor of STAT-3 (AG490) or inhibitor of p38 MAPK (SB203580), significantly increased the differentiation of the cells into osteoclasts (p<0.05). On contrast, the addition of 1,25(OH)$_2$D$_3$ only down-regulated the process of osteoclastogenisis compared with the positive control (coculture of RA-FLS and monocytes with combination of M-CSF and RANKL) (Fig. 4A). The expression of other osteoclastogenic markers, such as TRAP (Fig. 4B), MMP-9 (Fig. 4C), and cathepsin K (Fig. 4D), was also measured by real-time PCR and similar patterns were found as above mentioned (each p<0.05). The relative mRNA expression of each of these markers correlated well with the number of TRAP positive osteoclasts (Fig. 4E).

Discussion

IL-22 plays a dual role in pro-inflammatory process and bone destruction. A high concentration of IL-22A was secreted by activated natural killer cells as

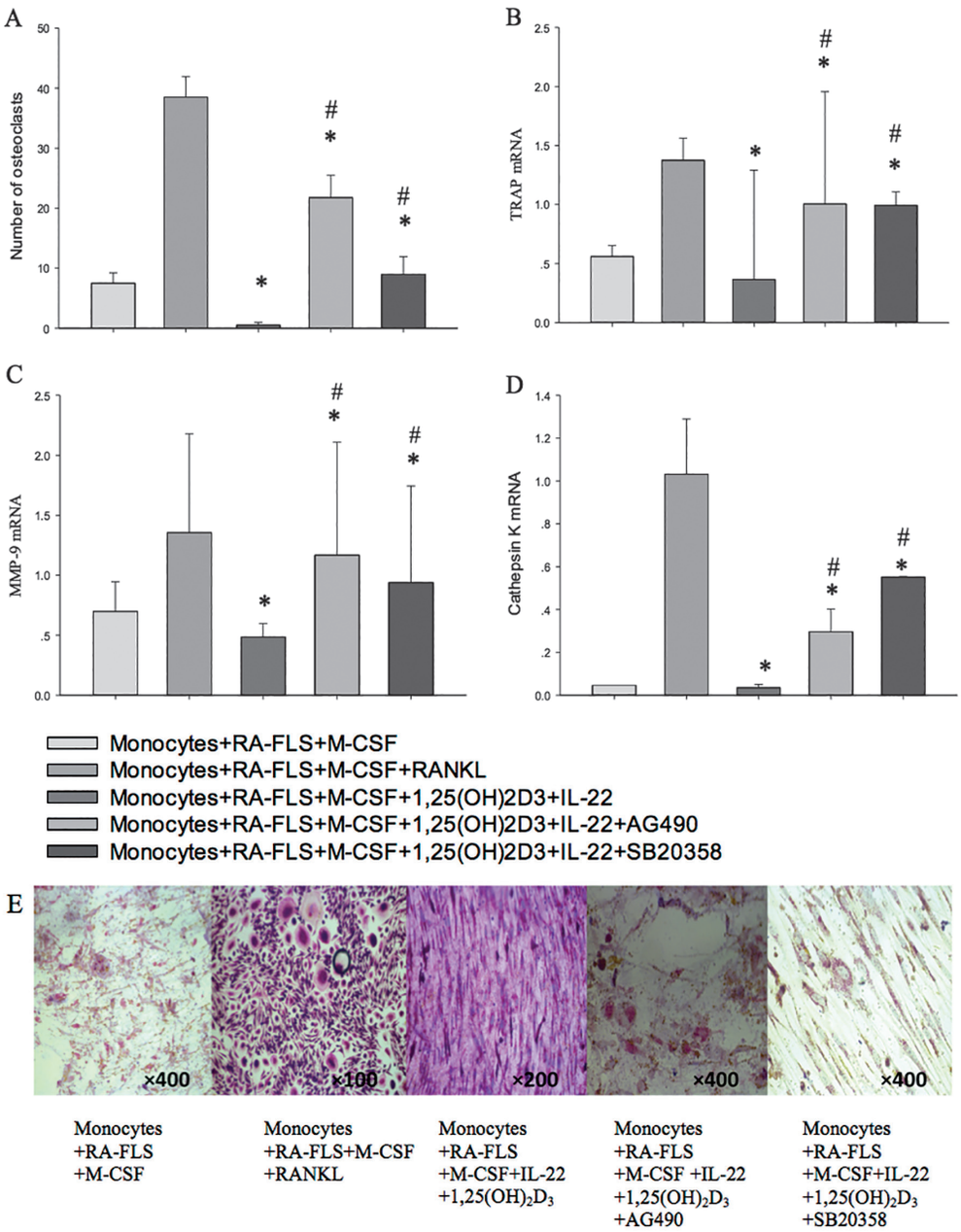

Fig. 4. 1,25(OH)$_2$D$_3$ inhibition of osteoclastogenisis via the JAK-2/STAT-3 and p38 MAPK/NF-κB signalling pathways. The number of osteoclasts (**A**) and the expression of TRAP (**B**), MMP-9 (**C**), and cathepsin K (**D**) mRNA (relative to β-actin), in cocultures of monocytes and RA-FLSs with M-CSF and RANKL, in the presence of 1,25(OH)$_2$D$_3$, with or without various inhibitors. Bars show the mean±SD of 4 independent experiments. *p<0.05 *versus* cocultures with M-CSF and RANKL only. #p<0.05 *versus* cocultures with M-CSF and RANKL, in the presence of 1,25(OH)$_2$D$_3$. (**E**) Osteoclast differentiation was assessed after isolated monocytes and RA-FLSs were cocultured with M-CSF, 1,25(OH)$_2$D$_3$ and specific inhibitors against JAK-2/STAT-3 (AG490) or p38 MAPK (SB203580). TRAP-positive multinucleated cells (differentiated osteoclast) were identified with staining.

well as by other cells, especially by the FLS of RA patients (17). A link should exist between increased expression of IL-22 mRNA and protein in RA (25), and elevated RA disease activity (26). Given the close connection between RA and IL-22, we found that the levels of IL-22 in PBMCs were higher in RA patients than in healthy controls, and dramatically increased in RA-PBMCs after anti-CD3/anti-CD28 treatment. So far, there was not report showing correlation between the levels of IL-22 and 1,25(OH)$_2$D$_3$. We showed firstly that the addition of different concentrations (0.1 nM,1 nM, and 100 nM) of 1,25(OH)$_2$D$_3$ led to a decrease of IL-22 secretion in RA-PBMCs compared to that stimulated with anti-CD3/anti-CD28 only only. Though it did not reach a significantly statistical degree, this down-regulation of IL-22 expression may play a part of the role in the function of 1,25(OH)$_2$D$_3$ inhibiting IL-22.

Danks L *et al*. (27) reported that the expression of RANKL on synovial fibroblasts rather than T cells was predominantly responsible for the formation of osteoclasts and erosions during inflammatory arthritis. To investigate the association of 1,25(OH)$_2$D$_3$ with RANKL expression stimulated by IL-22, based on the research of literatures (16, 28), we treated the cells with IL-22 for 48h, 72h or 96h respectively and revealed that RANKL mRNA reached the maximum value at 72 hours after treatment, which was demonstrated as the most effectively time (16, 28). In our study, FLSs treated with 1nM of 1,25(OH)$_2$D$_3$ were selected for further protein experiment because of the strongest inhibition of RANKL mRNA. And the results indicated that 1,25(OH)$_2$D$_3$ could inhibit the expression of RANKL mRNA and protein induced by IL-22.

Osteoclasts are the primary cells responsible for bone resorption in both

physiologic and pathologic conditions and play a substantial role in bone destruction in RA (29). In incubation of monocytes with M-CSF alone, lesser multinucleated osteoclasts produced and more with combination of M-CSF and RANKL, mirroring the synergistic effects of them on the osteoclast differentiation, consistent with the results of Kim KW's study (16). We found that the presence of 1,25(OH)$_2$D$_3$ could inhibit osteoclast precursors into osteoclasts induced by IL-22 in monocytes. Furthermore, 1,25(OH)$_2$D$_3$ significantly decrease the differentiation of the cells into osteoclasts induced by IL-22 in coculture of RA-FLSs and monocytes, and this is consistent with reduced osteoclast TRAP positivity and osteoclast number in Zarei *et al*. study (30).

Interestingly, the combination 1,25-(OH)$_2$D$_3$ and JAK-2/STAT-3 or p38 MAPK/NF-κB signalling pathway inhibitor resulted in an additive promotion but not inhibition of the expression of RANKL and the differentiation of monocytes cocultured with IL-22 stimulated FLSs into osteoclasts. The observed effect might be partially attributable to the specific action of inhibitors blocking the pathways of 1,25(OH)$_2$D$_3$ induced RANKL expression. Our study indicated that the determination of 1,25(OH)$_2$D$_3$ inhibition or promotion of osteoclastogenesis via activation or inactivation of JAK-2/STAT-3 and p38 MAPK/NF-κB signalling pathways. The mechanism and potential application of this promotion effect on osteoclastogenesis are required to investigate further.

Taken together, the study *in vitro* initially indicated that the presence of 1,25(OH)$_2$D$_3$ could down-regulate RANKL expression in RA-FLS and inhibit osteoclastogenesis mediated by IL-22 through JAK-2/STAT-3 and p38 MAPK/NF-κB signalling, delaying the process of bone destruction of RA. Therefore, 1,25(OH)$_2$D$_3$ may be of value in preventing bone erosions in patients with RA. In addition, we still need more samples and no IL-22 treatment control for further experiments to have a broader relevance and make a guide for clinical application.

Acknowledgements

The authors thank Dr Chong Gao for reviewing the manuscript.

References

1. SCOTT DL, WOLFE F, HUIZINGA TW: Rheumatoid arthritis. *Lancet* 2010; 376: 1094-108.
2. PUCHNER R, HOCHREITER R, PIERINGER H, VAVROVSKY A: Improving patient flow of people with rheumatoid arthritis has the potential to simultaneously improve health outcomes and reduce direct costs. *BMC Musculoskelet Disord* 2017; 18: 7.
3. ANGELOTTI F, PARMA A, CAFARO G, CAPECCHI R, ALUNNO A, PUXEDDU I: One year in review 2017: pathogenesis of rheumatoid arthritis. *Clin Exp Rheumatol* 2017; 35: 368-78.
4. QURAISHI MK, BADSHA H: Rheumatoid arthritis disease activity and vitamin D deficiency in an Asian resident population. *Int J Rheum Dis* 2016; 19: 348-54.
5. ISHIKAWA LL, COLAVITE PM, FRAGA-SILVA TF *et al*.: Vitamin D deficiency and rheumatoid arthritis. *Clin Rev Allergy Immunol* 2017; 52: 373-88.
6. CHEN J, LIU W, LIN Q, CHEN L, YIN J, HUANG H: Vitamin D deficiency and low bone mineral density in native Chinese rheumatoid arthritis patients. *Int J Rheum Dis* 2014; 17: 66-70.
7. ZHENG ZH, GAO CC, WU ZZ *et al*.: High prevalence of hypovitaminosis D of patients with autoimmune rheumatic diseases in China. A*m J Clin Exp Immunol* 2016; 5: 48-54.
8. NEVE A, CORRADO A, CANTATORE FP: Immunomodulatory effects of vitamin D in peripheral blood monocyte-derived macrophages from patients with rheumatoid arthritis. *Clin Exp Med* 2014; 14: 275-83.
9. WEN H, BAKER JF: Vitamin D, immunoregulation, and rheumatoid arthritis. *J Clin Rheumatol* 2011; 17: 102-7.
10. DANKERS W, COLIN EM, VAN HAMBURG JP, LUBBERTS E: Vitamin D in autoimmunity: molecular mechanisms and therapeutic potential. *Front Immunol* 2016; 7: 697.
11. HEWISON M: An update on vitamin D and human immunity. *Clin Endocrinol* (Oxf) 2012; 76: 315-25.
12. LIN J, LIU J, DAVIES ML, CHEN W: Serum Vitamin D Level and Rheumatoid Arthritis Disease Activity: Review and Meta-Analysis. *PLoS One* 2016; 11: e0146351.
13. MARUOTTI N, CANTATORE FP: Vitamin D and the immune system. *J Rheumatol* 2010; 37: 491-5.
14. NIKOOPOUR E, BELLEMORE SM, SINGH B: IL-22, cell regeneration and autoimmunity. *Cytokine* 2015; 74: 35-42.
15. KOMATSU N, TAKAYANAGI H: Inflammation and bone destruction in arthritis: synergistic activity of immune and mesenchymal cells in joints. *Front Immunol* 2012; 3: 77.
16. KIM KW, KIM HR, PARK JY *et al*.: Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts. *Arthritis Rheum* 2012; 64: 1015-23.
17. ZHU J, JIA E, ZHOU Y *et al*.: Interleukin-22 secreted by NKp44$^+$ natural killer cells promotes proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. *Medicine* (Baltimore) 2015; 94: e2137.
18. NAKANO K, OKADA Y, SAITO K, TANAKA Y: Induction of RANKL expression and osteoclast maturation by the binding of fibroblast growth factor 2 to heparan sulfate proteoglycan on rheumatoid synovial fibroblasts. *Arthritis Rheum* 2004; 50: 2450-8.
19. SINGH PK, VAN DEN BERG PR, LONG MD *et al*.: Integration of VDR genome wide binding and GWAS genetic variation data reveals co-occurrence of VDR and NF-kappaB binding that is linked to immune phenotypes. *BMC Genomics* 2017; 18: 132.
20. WEN HY, LUO J, LI XF: Vitamin D levels and associations with disease activity in Chinese Han patients with early rheumatoid arthritis. *J Clin Rheumatol* 2015; 21: 276-7.
21. LUO J, WEN H, GUO H, CAI Q, LI S, LI X: 1,25-dihydroxyvitamin D3 inhibits the RANKL pathway and impacts on the production of pathway-associated cytokines in early rheumatoid arthritis. *Biomed Res Int* 2013; 2013: 101805.
22. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
23. KIM KW, CHO ML, KIM HR *et al*.: Up-regulation of stromal cell-derived factor 1 (CXCL12) production in rheumatoid synovial fibroblasts through interactions with T lymphocytes: role of interleukin-17 and CD40L-CD40 interaction. *Arthritis Rheum* 2007; 56: 1076-86.
24. WU Y, LIU J, FENG X *et al*.: Synovial fibroblasts promote osteoclast formation by RANKL in a novel model of spontaneous erosive arthritis. *Arthritis Rheum* 2005; 52: 3257-68.
25. CONG L, ZHANG C, TU G: Osteoblastic NF-kappaB pathway is involved in 1alpha, 25(OH)2D3-induced osteoclast-like cells formation in vitro. *Int J Clin Exp Pathol* 2015; 8: 5988-96.
26. WOLK K, KUNZ S, ASADULLAH K, SABAT R: Cutting edge: immune cells as sources and targets of the IL-10 family members? *J Immunol* 2002; 168: 5397-402.
27. DANKS L, KOMATSU N, GUERRINI MM *et al*.: RANKL expressed on synovial fibroblasts is primarily responsible for bone erosions during joint inflammation. *Ann Rheum Dis* 2016; 75: 1187-95.
28. COLIN EM, ASMAWIDJAJA PS, VAN HAMBURG JP *et al*.: 1,25-dihydroxyvitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. *Arthritis Rheum* 2010; 62: 132-42.
29. TAKAYANAGI H, IIZUKA H, JUJI T *et al*.: Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 259-69.
30. ZAREI A, MOROVAT A, JAVAID K, BROWN CP: Vitamin D receptor expression in human bone tissue and dose-dependent activation in resorbing osteoclasts. *Bone Res* 2016; 4: 16030.