Bone 49 (2011) 559–567

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Strontium ranelate inhibits key factors affecting bone remodeling in human osteoarthritic subchondral bone osteoblasts

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ARTICLE INFO

Article history:
Received 2 March 2011
Revised 20 May 2011
Accepted 3 June 2011
Available online 12 June 2011

Edited by: David Burr

Keywords:
Strontium ranelate
Osteoarthritis
Osteoblasts
Subchondral bone

ABSTRACT

Introduction: In osteoarthritis (OA) the progression of cartilage degeneration has been associated with remodeling of the subchondral bone. Human OA subchondral bone osteoblasts were shown to have an abnormal phenotype and altered metabolism leading to an abnormal resorptive process. Bone resorption is suggested to occur, at least in part, through the increased levels of two proteolytic enzymes, MMP-2 and MMP-9, and RANKL, which are mainly produced by osteoblasts. In this study, we investigated in human OA subchondral bone osteoblasts the modulatory effect of strontium ranelate on the above key factors.

Methods: Human subchondral bone osteoblasts were cultured in a medium containing 0.1, 1 and 2 mM of strontium ranelate for 18 h for mRNA and 72 h for protein determination. The effect of strontium ranelate was evaluated on the expression (qPCR) of MMP-2, MMP-9, OPG, RANKL (total), RANKL-1, and RANKL-3, on the production of OPG (ELISA), membranous RANKL (flow cytometry), and MT1-MMP, ADAM17, and ADAM19 (Western blot). After incubation of osteoblasts with pre-osteoclasts (*i.e.*, differentiated human peripheral blood mononuclear cells), the resorbed surface was measured using a sub-micron synthetic calcium phosphate thin film.

Results: Firstly, the expression levels of MMP-2, MMP-9, OPG, and RANKL were determined in normal and OA subchondral bone osteoblasts. As expected, the gene expression of MMP-9 and RANKL were not detectable in normal cells, whereas MMP-2 was very low but detectable and OPG demonstrated high gene expression. Further experiments looking at the effect of strontium ranelate on expression levels, except for OPG, were performed only on the OA subchondral bone osteoblasts. In OA cells, the expression levels of MMP-2 and MMP-9 were significantly decreased by strontium ranelate at 1 mM ($p \leq 0.005$, $p \leq 0.02$, respectively) and 2 mM ($p \leq 0.003$, $p \leq 0.007$), and for MMP-9 only at 0.1 mM ($p \leq 0.05$). In normal cells, the expression of OPG was increased with strontium ranelate at 2 mM, and in OA both the expression ($p \leq 0.02$) and synthesis ($p \leq 0.002$) of OPG were significantly increased with strontium ranelate at 1 and 2 mM. RANKL (total) as well as the isoforms RANKL-1 and RANKL-3 were significantly increased by strontium ranelate at 1 and 2 mM. Of note, it is known that the different RANKL isoforms differentially regulate RANKL membranous localization: RANKL-3, in contrast to RANKL-1, prevents such membranous localization. This is reflected by the significant ($p \leq 0.02$) reduction in the level of membranous RANKL by strontium ranelate at 2 mM. This latter finding was not likely to be related to a proteolytic cleavage of membranous RANKL, as the enzymes known to cleave it, MT1-MMP, ADAM17 and ADAM19, were unaffected by strontium ranelate. In addition, OA osteoblasts treated with strontium ranelate induced a significant ($p \leq 0.002$) decrease in resorbed surface at the three tested concentrations.

Conclusion: This study provides new insights into the mode of action of strontium ranelate on the metabolism of human OA subchondral bone osteoblasts. These data suggest that strontium ranelate may exert a positive effect on OA pathophysiology by inhibiting, in these cells, the synthesis of key factors leading to bone resorption, a feature associated with the OA process.

Introduction

Bone is a specialized connective tissue that is continually remodeled according to physiological events. This bone remodeling results from the activities of various cell lineages including osteoblasts and osteoclasts [1]. Osteoclasts are multinucleated giant cells derived from hematopoietic progenitors of the monocyte-macrophage lineage, whereas osteoblasts are derived from the bone marrow cells and

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doi:10.1016/j.bone.2011.06.005

are responsible for bone matrix apposition. Bone mass is maintained through a delicate balance between formation and resorption. In the bone loss process, regardless of the pathology, the osteoclast is the exclusive resorptive cell but osteoblasts, through the production of factors, can increase or decrease this activity. Some cytokines have been described to govern the maturation of osteoclast precursors and the capacity of these mature resorptive cells to degrade bone matrix. In this context, a molecular triad composed of OPG/RANK/RANKL has been described as a critical system for controlling osteoclast biology. Receptor activator of κB ligand (RANKL), which is synthesized by osteoblasts, is an essential factor for osteoclast differentiation and bone resorption [2]. It stimulates osteoclastogenesis and osteoclast activity by binding to and activating the cell surface RANK located on osteoclast precursors and mature osteoclasts. Such activation leads to the expression of specific genes that are involved during osteoclast differentiation, survival and bone resorption activity [3,4]. The third protagonist, osteoprotegerin (OPG), which is also produced by the osteoblasts, acts as a soluble decoy receptor for RANKL; by interacting with RANKL, it prevents RANK activation and subsequent osteoclastogenesis, resulting in the inhibition of bone resorption [5]. Abnormalities of this system have been implicated in the pathogenesis of various skeletal diseases characterized by increased osteoclastic activity and increased bone resorption.

Osteoarthritis (OA) is characterized by degradation and loss of cartilage, synovial membrane inflammation, and subchondral bone changes [6]. The degeneration of cartilage as a primary pathological mechanism of OA has recently been challenged and the prominent changes in the subchondral bone suggest that this tissue plays a key role in OA. Indeed, both clinical and laboratory evidence indicate that subchondral bone metabolism is altered in OA, a situation that appears to result from abnormal osteoblast behavior [7–12]. Recent evidence suggests that subchondral bone remodeling may be considered a primary attribute of OA and that it may be responsible for cartilage damage [13]. Moreover, it is believed that changes in OA subchondral bone consist of phases of bone resorption and of abnormal bone sclerosis.

Strontium ranelate (SrRan) is a drug used in the treatment of postmenopausal osteoporosis [14,15]. Recently, clinical studies reported SrRan to be of potential interest for OA patients. SrRan was reported to reduce the progression of radiographic features of spinal OA and back pain in women with osteoporosis and concomitant spinal OA [16]. Moreover, in a trial in postmenopausal women with different clinical levels of OA, this drug was found to reduce the urinary level of the type II collagen degradation biomarker (CTX-II) [17]. Interestingly, SrRan is the only therapeutic approach that has been shown to reduce bone resorption at the same time as increasing bone formation, thereby improving bone architecture. This effect results from decreased differentiation and resorption activity of osteoclasts and increased osteoclast apoptosis [18–21]. This mode of action was demonstrated in experimental studies on bone cells and pharmacological studies in animals and in clinical studies. Based on these findings, it could be hypothesized that SrRan may also be effective at reducing the development of structural changes in human OA subchondral bone. We thus investigated the modulation of SrRan on some key factors affecting bone resorption on human OA subchondral bone osteoblasts.

Materials and methods

Specimen selection

Human subchondral bone was obtained from femoral condyles. Normal articular tissues were from individuals within 12 h of death (median age [range] 59 [43;76]; 2F/4M). The tissues were examined macroscopically and microscopically to ensure that only normal tissue was used. OA subchondral bone was obtained from patients undergoing total knee arthroplasty (median age [range] 65 [50;87]; 20F/10M). All patients were evaluated as having OA according to American College of Rheumatology clinical criteria [22]. Generally, at the time of surgery the patients had symptomatic disease requiring medical treatment in the form of acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), or selective cyclooxygenase (COX)-2 inhibitors. Care was taken that none had received intra-articular steroid injections within 3 months prior to surgery, and none had received medication that would interfere with bone metabolism (*i.e.* anti-osteoporotic treatment). The Institutional Ethics Committee Board of the University of Montreal Hospital Centre approved the use of the human articular tissues.

Subchondral bone osteoblast culture

The subchondral bone osteoblast cultures were prepared as previously described [10,12,23]. Briefly, bone samples were cut into small pieces and digested for 4 h with collagenase type I (1.25 mg/mL) and trypsin (0.5 mg/mL) in BGJb medium (both from *Sigma-Aldrich Canada, Oakville, ON, Canada*) without serum at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The bone pieces were then cultured in BGJb medium containing 10% heat-inactivated fetal bovine serum (FBS; *PAA Laboratories Inc., Etobicoke, ON, Canada*) and an antibiotic mixture (100 units/mL penicillin base and 100 μg/mL streptomycin base; *Wisent, St-Bruno, QC, Canada*) at 37 °C in the humidified atmosphere until confluence.

We recently demonstrated [12,23] that human OA subchondral bone osteoblasts could be discriminated into two subgroups based on the metabolic activity of PGE_2 in these cells. One subgroup has low endogenous PGE_2 levels ($PGE_2 \leq 2000$ pg/mg protein; L-OA) and these cells demonstrated a higher level of resorptive activity, whereas those in the other subgroup showed a high production of PGE_2 ($PGE_2 \geq$ 2000 pg/mg protein; H-OA) and higher level of bone formation activity [23]. In this study, we first evaluated the OA osteoblast levels of PGE_2. As the study design included the human OA osteoblasts having resorptive activity only, L-OA osteoblasts were used. The cells used for this study had a PGE_2 level of 658.3 [163.9;1964.9] (median [range]) pg/mg protein.

The experiments were performed by pre-incubating the osteoblasts in the culture medium Dulbecco's modified Eagle's medium (DMEM; *Wisent*)/0.5% FBS for 24 h followed by 18 h (for mRNA determination) and 72 h (for protein determination) incubation with fresh DMEM/0.5% FBS.

Strontium ranelate

The SrRan concentrations were chosen according to previous results obtained in an *in vitro* model with human primary osteoblasts [24]. SrRan is a mixture of strontium chloride ($SrCl_2$, $6H_2O$; *Sigma-Aldrich Canada*) at 0.1, 1 and 2 mM, and sodium ranelate (S12911-5 provided by *Technologie Servier, Orléans, France*) at 1, 10 and 20 μM. Concentrations of SrRan used in this study are expressed in terms of Sr^{2+} (mM).

RNA extraction, reverse transcriptase (RT) and polymerase chain reaction (PCR)

Total cellular RNA from human OA osteoblasts was extracted with the TRIzol™ reagent (*Invitrogen, Burlington, ON, Canada*) according to the manufacturer's specifications. The RNA was quantitated using the RiboGreen RNA quantitation kit. The RT reactions were primed with random hexamers. The primer sequences were: 5′-CACTGTTGGTGGGAACTCAG (antisense), 5′-GTGTAAATGGGTGCCATCAG (sense) (matrix metalloproteinase 2; MMP-2); 5′-CCTTCACTTTCCTGGGTAAG (antisense), 5′-CCATTCACGTCGTCCTTATG (sense) (MMP-9); 5′-GTTTACTTTGGTGCCAGG (antisense), 5′-GCTTGAAACATAGGAGCTG (sense) (OPG); 5′-GGGTATGAGAACTTGGGATT (antisense), 5′-CACTATTAATGCCACCGAC (sense) (RANKL); and 5′-CTGCTCTGATGTGCTGTGATCC (antisense) and

5′-GCCTGCGCCGCACCA (sense) (RANKL1). GAPDH served as housekeeping gene and the primers were 5′-GCTTGACAAAGTGGTCGTGAG (antisense) and 5′-CAGAACATCATCCCTGCCTCT (sense). Real-time quantitation of mRNA was performed in the Rotor-Gene RG-3000A (*Corbett Research, Mortlake, NSW, Australia*) with the 2X Quantitect SYBR Green PCR Master Mix (*Qiagen, Mississauga, ON, Canada*) according to the manufacturer's specifications. The data were given as a threshold cycle (C_T) and calculated as the ratio of the number of molecules of the target gene/number of molecules of GAPDH.

Of note, as described previously [25], it was impossible to target RANKL2 in a specific manner, since the designed primers overlapping the remaining sequence of the 3′ and the 5′ at the deletion region were unable to adequately amplify RANKL2.

Moreover, determination of the expression level of RANKL3 is not possible when using quantitative PCR, as the quantity (50 ng of cDNA) is too low, resulting in non-reliable quantitation as previously described [25]. To this end, RANKL3 determination was performed using semi-quantitative PCR as previously described [25], and the primers were 5′-CTGCTCTGATGTGCTGTGATCC (antisense) and 5′-CGCCTGGCCTATTGAAGG (sense). Briefly, first-strand cDNA was synthesized by incubating 2 μg of total RNA in 20 μL of RT mixture containing 4 μL of $MgCl_2$ 25 mM (*Invitrogen*), 2 μL of PCR buffer 10X (*Invitrogen*), 1 μL random hexamers 50 μM (*Invitrogen*), 1 μL oligo-dT 50 μM (*Roche Perkin-Elmer, Foster City, CA, USA*), 1 μL of 10 mM dNTP (*Invitrogen*), 1 μL of 200 U/μL MMLV-RT (*Invitrogen*) and 0.5 μL of 32,9 U/μL RNAsine (*Invitrogen*) at 42 °C for 15 min, 5 min at 99 °C and then ice-chilled for 5 min. The RT reaction mixture was subjected to PCR using upstream and downstream RANKL3 and GAPDH primers (20 μM each), 5 μL of 10X PCR buffer (*Invitrogen*), 3 μL of 25 mM $MgCl_2$ (*Invitrogen*), 2 μL of 10 mM dNTP (*Invitrogen*), 65.5 μL of sterile water and 0.5 μL of 5 U/μL Taq polymerase (*Invitrogen*). The RT-PCR with denaturation at 94 °C for 1 min and annealing at 60 °C for 1.5 min was performed for 45 and 20 cycles for RANKL3 and GAPDH respectively. PCR amplification products were resolved by electrophoresis on a 2% agarose gel. The relative amounts of PCR products were determined by quantifying the intensity of the bands using the TotalLab TL100 Software (*Nonlinear Ltd, Newcastle, UK*) and data calculated as the ratio of the band intensity of the target gene over the band intensity of GAPDH, and expressed in arbitrary units relative to the control which was given a value of 1.

Membranous RANKL determination

Membranous RANKL determination was performed as previously described [25]. In brief, at the end of the incubation period (72 h), cells were washed once in phosphate buffered saline (PBS), detached with the cell dissociation buffer enzyme-free (*Invitrogen*) at 37 °C for 10 min, and centrifuged at 500 g for 5 min at room temperature. The cells were re-suspended in 1% BSA/PBS and a 500 μL suspension was prepared, having a concentration of 1×10^6 cells/mL. The suspension was incubated for 30 min at room temperature and divided into tubes. One served as negative control to which mouse IgG (15 μg/mL; *Chemicon International, Billerica, MA, USA*) was added, and the other was labeled with the anti-human RANKL antibody (15 μg/mL; *R&D Systems, Minneapolis, MN, USA*) for 30 min at 4 °C. After washing, a goat anti-mouse FITC-conjugated secondary antibody (7.5 μg/mL; *R&D Systems*) was added for another 30 min at 4 °C. Cells were then washed in PBS, re-suspended in PBS, and analyzed using flow cytometry (FACSCalibur; *BD Biosciences, Mississauga, ON, Canada*). The control sample was used to determine background fluorescence and compared to that of the sample incubated with the specific antibody. The level of fluorescence, measured by a FACScan using the CellQuest program (*BD Biosciences*), was calculated as the mean fluorescent intensity of positive cells and data expressed over the control, which was assigned a value of 1.

Western blot

Total proteins were extracted with RIPA buffer (Tris–HCl 50 mM [pH 7.4], NP-40 1%; Na-deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, and Na-orthovanadate 1 mM) supplemented with protease inhibitors as described [26]. The protein level was determined using the bicinchoninic acid protein assay, and 10 μg of the protein electrophoresed on a NuPAGE Novex 4–12% Bis–Tris gel. The proteins were transferred electrophoretically onto a nitrocellulose membrane for 1 h at 4 °C. The efficiency of transfer was controlled by a brief staining of the membrane with Ponceau Red and destained in water and TTBS 1X (Tris 20 mM, NaCl 150 mM [pH 7.5], and 0.1% Tween 20) before immunoblotting.

The membranes were incubated overnight at 4 °C with 5% skimmed milk in SuperBlock Blocking Buffer-Blotting in Tris-buffered saline. The membranes were then washed once with TTBS 1X for 10 min and incubated in TTBS 1X with 0.5% skimmed milk supplemented with the following antibodies, a mouse monoclonal anti-human disintegrin and metalloproteinase (ADAM)17 (1:2000; *Abcam, Cambridge, MA*), a rabbit polyclonal anti-human ADAM19 (1:2000; *Abcam*), a mouse monoclonal anti-human MT1-MMP (1:2000, *Oncogene*), a mouse monoclonal anti-human RANKL (1:1000; *R&D Systems*), and a rabbit anti-human GAPDH (1:50000; *Abcam*) overnight at 4 °C. The membranes were washed with TTBS 1X and incubated for 1 h at room temperature with the second antibody (1:10,000; anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated; *Pierce*) and washed again with TTBS 1X. Detection was performed by chemiluminescence using the Super Signal® West Dura Extended Duration substrate (Pierce) and exposure to Kodak Biomax photographic film. The band intensity was measured by densitometry using TotalLab TL 100 Software and data were expressed as arbitrary unit of the ratio of the target protein over GAPDH relative to the control, which was given a value of 1.

Resorptive activity determination

Resorptive activity was measured using the BD BioCoat Osteologic Bone Cell Culture System (*BD Biosciences*) as described previously [25]. In brief, human peripheral blood mononuclear cells (PBMC; 200,000 cells/well) were inoculated into the wells with culture medium containing DMEM/10% FBS, antibiotics, and 25 ng/mL M-CSF (*R&D Systems*) and incubated for 3 days at 37 °C in order to induce pre-osteoclastic differentiation. Human OA subchondral bone osteoblasts (10,000 cells/well) were then inoculated with the differentiated pre-osteoclasts (PBMC) and incubated for another 3 days. At the end of this period, cells were incubated at 37 °C with fresh DMEM containing M-CSF, 10% FBS and antibiotics for 4 weeks with the SrRan. Media were changed every 3 days.

The effect of SrRan was also tested on the osteoclasts. To this end, PBMC (200,000 cells/well) were inoculated into the wells with culture medium containing DMEM/10% FBS, antibiotics in the presence or absence of SrRan at the above concentrations, M-CSF (25 ng/mL), and sRANKL (100 ng/mL; *Cell Sciences, Canton, MA, USA*) and incubated at 37 °C in a humidified atmosphere for 3 weeks. Media were changed every 3 days.

At the end of the incubation period, cells were bleached (6% NaOCl, 5.2% NaCl) and extensively washed in sterilized water. Von Kossa staining was used as contrast stain for resorption. The quantitation was performed with a light microscope using Bioquant software (*Bioquant Osteo II, v 8.00.20; Nashville, TN, USA*). Results were calculated as the percentage of the resorbed surface and are expressed as the percentage of control, where control is attributed 100%.

Other protein determination

OPG was determined in the culture media by a specific ELISA from Biomedica (*Medicorp, Montreal, QC, Canada*) with a sensitivity of

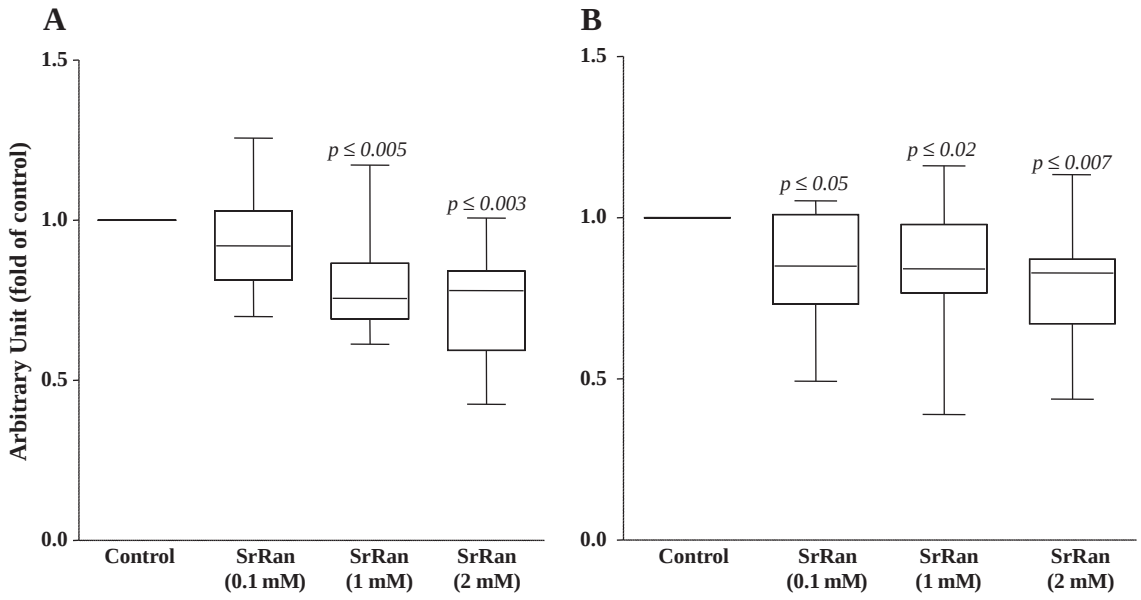


Fig. 1. Effect of SrRan on human OA subchondral bone osteoblast gene expression level of A) MMP-2 (n = 12) and B) MMP-9 (n = 11). Cells were treated for 18 h in the absence (control) or presence of SrRan at 0.1, 1 and 2 mM. At the end of the incubation period, the total RNA was extracted and processed for real-time PCR. Data are expressed as box plots of arbitrary unit over the control which was attributed a value of 1. Box plots represent the first and third quartiles, the line within each box represents the median, and the bars outside the box represent the range of values. Statistical significance was assessed by the Kruskal–Wallis test and the Wilcoxon signed rank test; p values are versus the control group.

2.8 pg/mL, and soluble RANKL (sRANKL) by an EIA (*ALPCO Diagnostics, Salem, NH*) with a sensitivity of 30 pg/mL. Prostaglandin E_2 (PGE_2) level determination was performed by an EIA assay (*Cayman Chemicals, Ann Arbor, MI, USA*) with a sensitivity of 7.8 pg/mL. All determinations were performed in duplicate for each cell culture.

Statistical analysis

Data were expressed as the median and the range of values. Each specimen evaluated was from an independent human subject. Statistical significance was assessed by the Kruskal–Wallis test and by the Wilcoxon signed rank test or the Mann–Whitney *U* test when appropriate versus their autologous control group; p values ≤ 0.05 were considered significant.

Results

Expression levels of factors

Firstly, the expression levels of the factors under study (MMP-2, MMP-9, RANKL, and OPG) were determined in normal and OA subchondral bone osteoblasts. As expected, data showed that in normal, the expression levels of MMP-9 and RANKL were not detectable. However, gene expression of MMP-2 was very low but detectable and OPG demonstrated high gene expression. MMP-2 expression levels were significantly lower ($p \leq 0.0009$) in normal (0.003 [0.002;0.004]) (n = 6) compared to OA (0.25 [0.11;0.67]) (n = 12) subchondral bone osteoblasts, whereas OPG levels were significantly higher ($p \leq 0.0009$) in normal (0.33 [0.19;0.47]) (n = 6) than OA (0.06 [0.006;0.17]) (n = 12) cells.

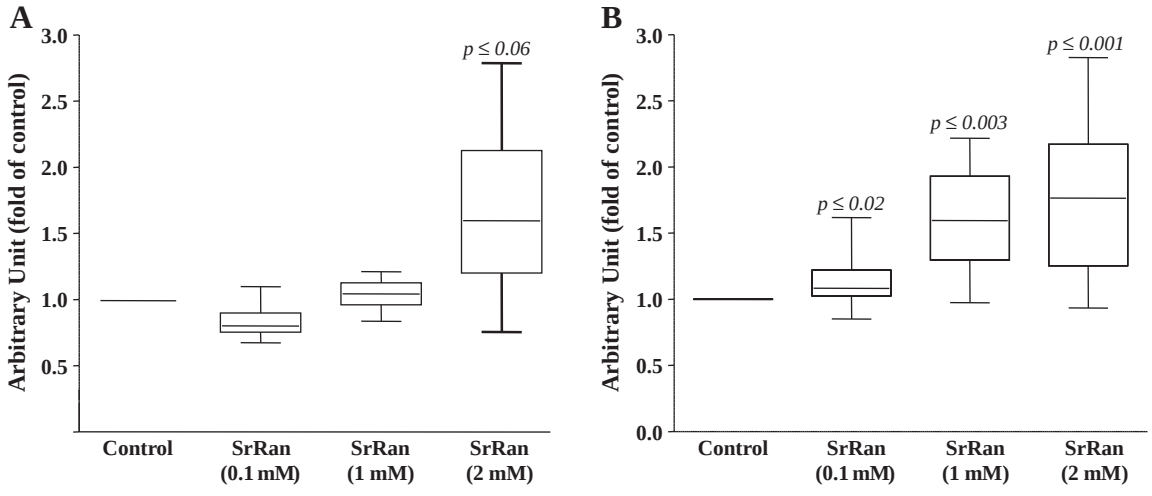


Fig. 2. Effect of SrRan on human A) normal (n = 6) and B) OA (n = 12) subchondral bone osteoblast gene expression level of OPG. Cells were treated for 18 h in the absence (control) or presence of SrRan at 0.1, 1 and 2 mM. At the end of the incubation period, the total RNA was extracted and processed for real-time PCR. Data are expressed as box plots of arbitrary unit over the control which was attributed a value of 1. Box plots represent the first and third quartiles, the line within each box represents the median, and the bars outside the box represent the range of values. Statistical significance was assessed by the Kruskal–Wallis test and the Wilcoxon signed rank test; p values are versus the control group.

Experiments looking at the effect of SrRan on expression levels, except for OPG, were performed only on the OA subchondral bone osteoblasts.

Human OA subchondral bone osteoblast treatment with SrRan showed that the MMP-2 mRNA level was significantly reduced at 1 mM ($p \leq 0.005$) and 2 mM ($p \leq 0.003$) (Fig. 1A), and MMP-9 at all SrRan concentrations tested ($p \leq 0.05$) (Fig. 1B).

In order to appreciate how SrRan affects OPG in normal and OA subchondral bone osteoblasts, data were compared (Figs. 2A, B). In both cell types, SrRan increased OPG, and although significance was reached even at the lower concentration tested for the OA cells ($p \leq 0.02$) (Fig. 2B), in normal cells difference toward significance was reached only at 2 mM SrRan ($p \leq 0.06$) (Fig. 2A).

The modulation of the expression of RANKL revealed that in human OA cells, the total RANKL levels were significantly increased with the 1 and 2 mM concentrations ($p \leq 0.03$; $p \leq 0.02$, respectively) of SrRan (Fig. 3A) as were the RANKL isoform levels, RANKL1 and RANKL3, with SrRan at 1 mM and 2 mM (for both, $p \leq 0.02$) (Figs. 3B, C).

Protein levels of factors

The protein levels of OPG and soluble RANKL released in the culture medium were measured in OA subchondral bone osteoblasts by ELISAs. The level of the OPG protein was significantly increased in OA osteoblasts treated with 1 mM and 2 mM SrRan compared to the control cells ($p \leq 0.002$) (Fig. 4A).

The soluble RANKL levels were at the limit of detection and did not permit reliable measurement in either the absence or presence of SrRan (data not shown). However, the intracellular RANKL level, as determined by Western blot, showed that there was only a slight increase following SrRan treatment but this did not reach statistical significance (Fig. 4B). Interestingly, the membranous levels of RANKL, as determined by flow cytometry, were lower in the SrRan treated groups compared to control cells, and statistical significance was reached with SrRan 2 mM ($p \leq 0.02$) (Fig. 4C).

It is known that some proteases have the ability to cleave RANKL at the membrane. To verify if RANKL reduction observed at the membranous level following treatment with SrRan was due to an increase in its cleavage, we investigated the modulation upon SrRan treatment of the three major proteases involved in this process: MT1-MMP, ADAM17, and ADAM19. These were determined by Western blot, and for each of the proteases, two major bands were seen (Table 1). For MT1-MMP, these corresponded to the latent (66 kDa) or active (54 kDa) forms. ADAM17 encodes proteins with different cytoplasmic domains due to glycosylation and other post-translational modifications, and bands of 92 kDa and 78 kDa were found. For ADAM19, two bands of 105 kDa and 60 kDa were observed, which corresponded to the full length sequence encoding a 956 amino acid protein and to a shorter sequence encoding a 538 amino acid, respectively. Data showed (Table 1) that neither band for any of the proteases was modulated upon SrRan treatment.

Resorptive activity

We then investigated whether SrRan treatment affects the OA subchondral bone osteoblast-induced resorptive activity. Co-culture of pre-osteoclasts and osteoblasts treated with SrRan demonstrated a significantly ($p \leq 0.002$) decreased resorbed surface compared to the control cells (Fig. 5A).

We further explored the direct effect of SrRan on the osteoclasts. To this end, only human PBMC ($n = 2$) were treated with SrRan in the presence of M-CSF and RANKL and SrRan at the three concentrations. Data showed that SrRan inhibits, in a dose-dependent manner, osteoclast resorption activity; inhibitions of 6%, 16%, and 23% were recorded for SrRan at 0.1, 1, and 2 mM (Fig. 5B).

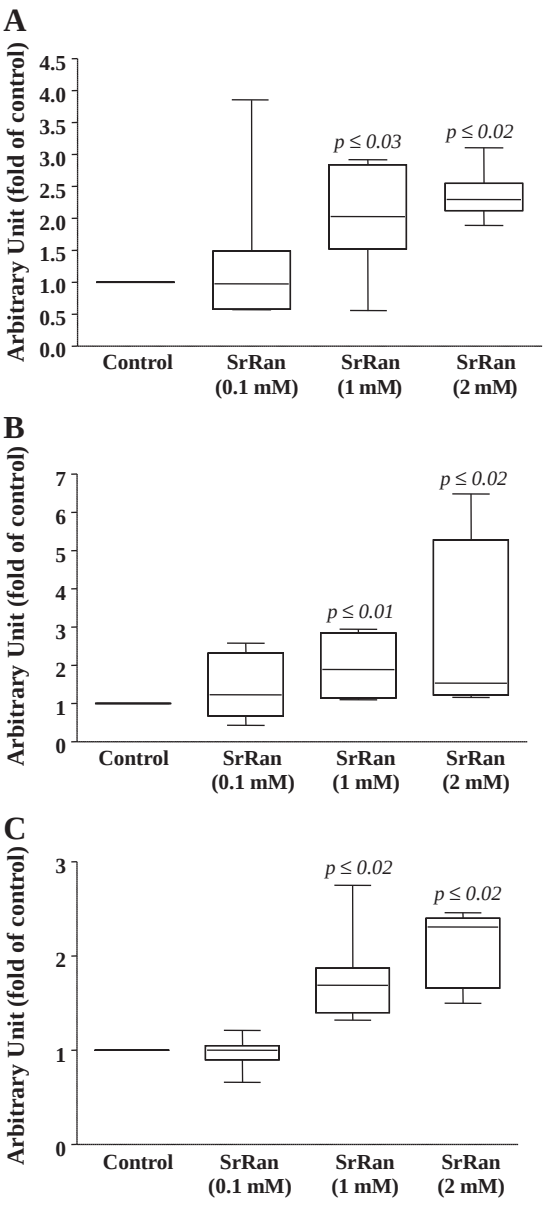


Fig. 3. Effect of SrRan on human OA subchondral bone osteoblasts on the gene expression level of A) total RANKL ($n = 7$), B) RANKL1 ($n = 7$), and C) RANKL3 ($n = 5$). Cells were treated for 18 h in the absence (control) or presence of SrRan at 0.1, 1 and 2 mM. At the end of the incubation period, the total RNA was extracted and processed for either real-time PCR (total RANKL, RANKL1) or semi-quantitative PCR (RANKL3). Data are expressed as box plots of arbitrary unit over the control which was attributed a value of 1. Box plots represent the first and third quartiles, the line within each box represents the median, and the bars outside the box represent the range of values. Statistical significance was assessed by the Kruskal–Wallis test and the Wilcoxon signed rank test (RANKL1) or the Mann–Whitney *U* test for (RANKL3); p values are versus the control group.

Discussion

Although cartilage degradation is the hallmark of OA, other tissues of the joint also show abnormal processes during the disease evolution. In addition to cartilage, there is also the presence of synovial membrane inflammation and subchondral bone remodeling. The latter appears to be a key component in the development and progression of OA [13] and alterations in this tissue were demonstrated to be due, at least in part, to abnormal osteoblast metabolism [9–12,23,27]. Data have also shown that in the early stages of the disease process there is an increase in subchondral bone resorption followed by phases of sclerosis [7,8,28–31].

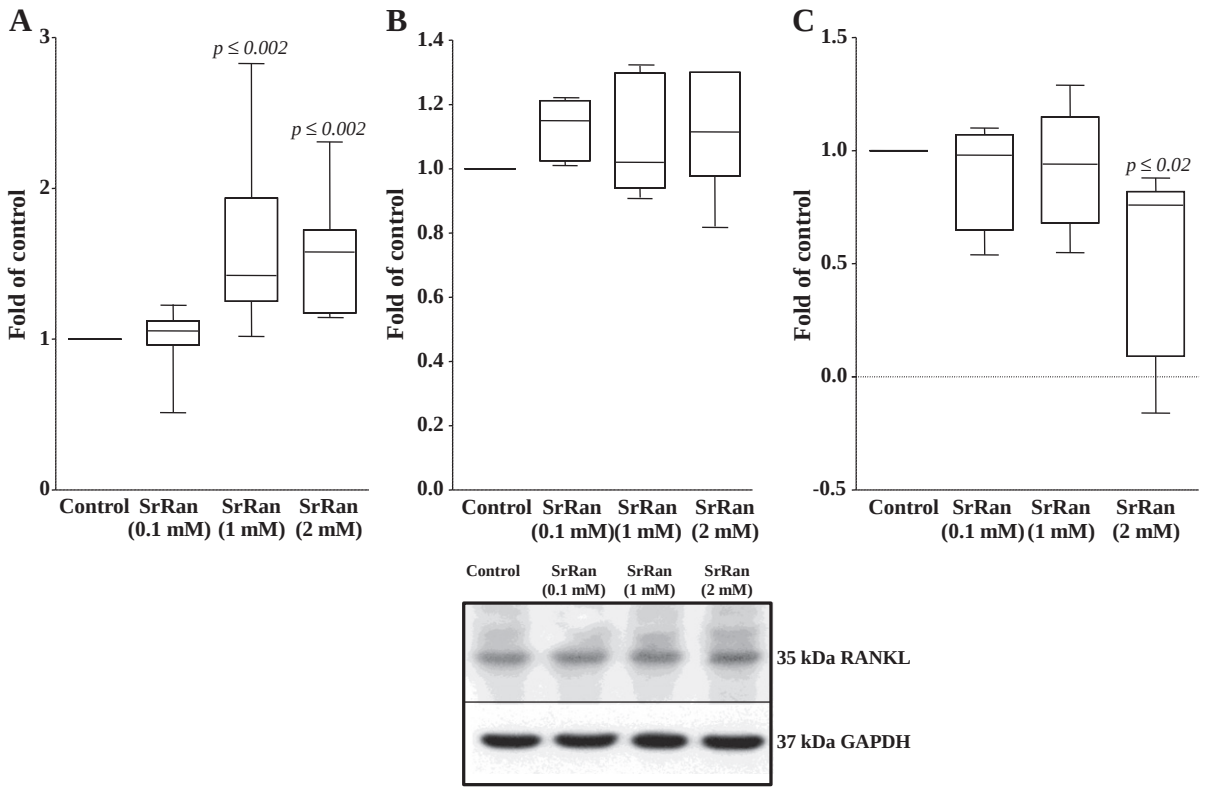


Fig. 4. A) Production of OPG (n = 7), B) graph of intracellular RANKL (n = 6) and a representative Western blot of RANKL and GAPDH, and C) membranous localization of RANKL (n = 7) in human OA subchondral bone osteoblasts incubated in the absence (control) or presence of SrRan at 0.1, 1 and 2 mM. Data are expressed as box plots of arbitrary unit over the control which was attributed a value of 1. Box plots represent the first and third quartiles, the line within each box represents the median, and the bars outside the box represent the range of values. Statistical significance was assessed by the Kruskal–Wallis test and the Wilcoxon signed rank test; p values are versus the control group.

In the context of subchondral bone remodeling and due to the SrRan properties, we investigated whether this agent could be an interesting approach for OA. In this study, the SrRan concentrations used were in the range of a ratio that reflects the relative concentrations of strontium and ranelic acid in the serum of patients treated with SrRan 2 g/day [24]. Although multiple key factors have been reported to be involved in the bone remodeling process, some of them including the proteolytic enzymes MMP-2 and MMP-9 as well as OPG and RANKL have been described as the critical system for controlling bone resorption and are involved in OA pathophysiology. We therefore looked at the effect of SrRan on these factors as well as on the property of osteoblasts to induce bone resorption.

Firstly, we looked at the expression levels of MMP-2, MMP-9, OPG and RANKL in normal and OA subchondral bone osteoblasts. Data showed that MMP-2 and OPG, but not MMP-9 or RANKL, were detectable in these normal human cells. These data agree with those in the literature [23,32]. The effect of SrRan on the expression levels, with the exception of OPG which showed a high expression level in normal, was then investigated in the OA subchondral bone osteoblasts demonstrating resorptive properties (L-OA osteoblasts). Data showed that in human OA subchondral bone osteoblasts, SrRan could play a role in inhibiting subchondral bone resorption by modulating MMP-2 and MMP-9 in addition to its role in the OPG and RANKL system.

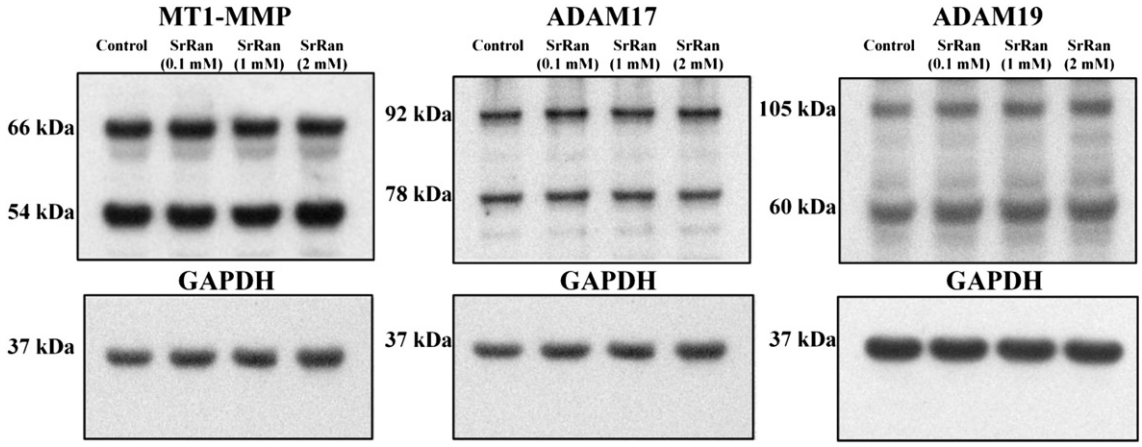
Bone resorption is suggested to occur, at least in part, through the increased levels of the two gelatinases, of which MMP-2 is produced mainly by the osteoblasts and MMP-9 by both osteoblasts and osteoclasts. The significant reduction in both MMP-2 and MMP-9 expression levels by SrRan is thus of importance. Indeed, these MMPs are well-known to induce bone resorption *via* the degradation of extracellular matrix components, including type I collagen as well as other extracellular components such as fibronectin and aggrecans. Moreover, MMP-2 and MMP-9 were observed to be upregulated during OA and *in vivo* experiments performed on MMP-2 and MMP-9 knock-out (KO) mouse models of arthritis demonstrated reduced disease progression, which was much more pronounced in MMP-9 KO mice [33]. The latter data could reflect the fact that MMP-9 was shown to be highly involved in, in addition to osteoblasts, the osteoclastic bone resorption process by facilitating the migration of osteoclasts through proteoglycan-rich matrix [34].

The OPG expression level was increased for both normal and OA cells by the SrRan; data reflected by the increase in the OPG production. In normal cells, the higher concentration of SrRan needed to increase OPG compared to the OA cells could be explained by the fact that in the latter cells the OPG level was much lower (about 5-fold) than the normal. The increased OPG levels with SrRan are in accordance with other *in vitro* studies demonstrating that SrRan increased the OPG mRNA and protein levels in human trabecular osteoblasts [35,36]. Hence, since OPG acts as a decoy receptor of RANKL, preventing it from binding to RANK on osteoclasts, the effect of SrRan on OPG could contribute to limiting the early subchondral bone remodeling process as demonstrated by Kadri et al. [37] in an OA-induced mouse model in which intraperitoneal injections of OPG were found to limit the progression of the disease through an effect on trabecular bone during the early stages of OA.

In contrast to some previous studies in normal osteoblasts [36,38], our data showed that the gene expression level of RANKL was increased following SrRan treatment and that SrRan has no true effect on intracellular RANKL protein. Of note, the normal osteoblasts used by Atkins et al. and Brennan et al. [36,38] were from trabecular bone and those used in the present study, from subchondral bone. Moreover, the difference in SrRan effect could be explained by the possibility that different mechanisms operate in normal and pathological osteoblasts. However, and quite importantly, our data showed that SrRan significantly reduced the membranous localization of RANKL, which remains the most important mechanism for bone resorption.

Table 1
Protein modulation by SrRan of the proteases MT1-MMP, ADAM17, and ADAM19 in human OA subchondral bone osteoblasts.

	SrRan (0.1 mM)	SrRan (1 mM)	SrRan (2 mM)
Pro-MT1-MMP (66 kDa)	1.01 [0.77;1.22]	0.91 [0.67;1.21]	0.92 [0.57;1.37]
Active-MT1-MMP (54 kDa)	1.03 [0.90;1.39]	1.07 [0.91;1.27]	1.04 [0.93;1.30]
ADAM17 (92 kDa)	0.98 [0.93;1.01]	0.83 [0.80;0.84]	0.92 [0.71;0.97]
ADAM17 (78 kDa)	1.13 [0.96;1.56]	0.99 [0.69;1.16]	1.04 [0.70;1.08]
ADAM19 (105 kDa)	1.17 [0.55;1.25]	0.98 [0.86;1.18]	1.04 [0.77;1.27]
ADAM19 (60 kDa)	1.04 [0.87;1.44]	0.96 [0.82;1.28]	1.00 [0.94;1.26]



Protein levels were determined by Western blot and the data are expressed as the median [range] of the ratio of the target protein over GAPDH. Data are expressed in arbitrary units relative to control (without SrRan) which was attributed a value of 1. Representative Western blots of the MT1-MMP, ADAM17, and ADAM19, and the control protein GAPDH.

Such membranous RANKL reduction could have been explained by a higher proteolytic cleavage of its extracellular domain. In fact, the soluble form of extracellular RANKL, which corresponds to the C-terminal domain of the RANKL cell surface, is produced by a proteolytic cleavage of membranous RANKL. Although various proteases have been proposed to be involved in such shedding process, three of them, MT1-MMP, ADAM17, and ADAM19, appear to be the most important [39–42]. We therefore investigated whether these proteases were modulated by SrRan and our data revealed no modulation at any of the three concentrations tested. Moreover, in our study, we were unable to detect the extracellular soluble RANKL even upon SrRan treatment. These data, therefore, strongly support a direct role of SrRan on the decrease in RANKL at the membrane level.

Human RANKL is known to exist as three isoforms: RANKL1, 2, and 3. RANKL1 and 2 encode for transmembrane forms of RANKL. RANKL3 lacks intracellular and transmembrane domains and is released in a soluble form [43–45]. Interestingly, they differentially regulate osteoclastogenesis. Indeed, the three isoforms of RANKL are able to form homotrimeric or heterotrimeric structures between themselves. The trimeric combination of RANKL isoforms is important for orientating the membranous localization and thereby controlling the osteoclast formation and differentiation process. Hence, the trimeric structure of RANKL1 alone or in association with RANKL2 is translated into a membranous localization [43,45]. However, when RANKL1 or 2 is co-transfected with RANKL3, a reduced level of membranous localization is observed, indicating that RANKL3 prevents the membranous localization of RANKL. Thus, RANKL3 acts as an inhibitor of osteoclastogenesis [43,45]. Data on the modulation by SrRan of the different RANKL isoforms revealed that both RANKL1 and RANKL3 were upregulated following treatment. Although it is not possible to directly determine the expression level of RANKL2 for the reasons stated in the Materials and Methods section, the similar level of intracellular RANKL in the presence and absence of SrRan could be explained by a decreased level of RANKL2 by SrRan. However,

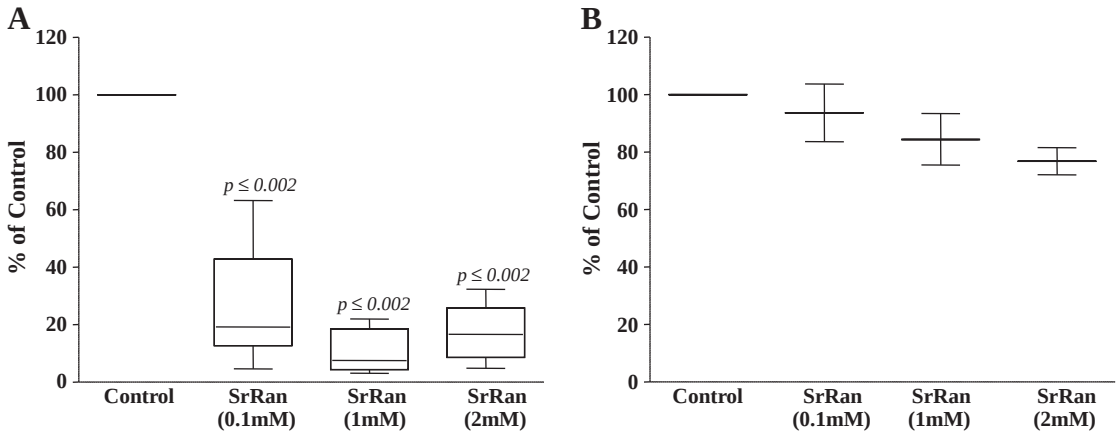


Fig. 5. A) Resorptive activity of human OA subchondral bone osteoblasts co-incubated with human differentiated PBMC (n = 10) in the presence of SrRan at 0.1, 1 and 2 mM. B) Resorptive activity of human differentiated PBMC (n = 2) in the presence of SrRan at 0.1, 1 and 2 mM. Data are expressed as box plots of the total surface resorbed upon treatment with SrRan compared to control which was attributed a value of 100%. Box plots represent the first and third quartiles, the line within each box represents the median, and the bars outside the box represent the range of values. In A) statistical significance was assessed by the Kruskal–Wallis test and the Wilcoxon signed rank test; p values are versus the control group.

importantly, the increased level of RANKL3 could have prevented RANKL membranous localization, thus explaining the decreased RANKL protein level at the membrane. In addition, this decrease could have been due to the elevation of the intracellular level of OPG. Indeed, Aoki et al. [46] recently reported that intracellular OPG regulates RANKL sorting to the plasma membrane. An increased production of intracellular OPG retains RANKL inside the cells and the formed OPG-RANKL complex was predominantly localized in the lysosomes, where the complex is degraded. Thus, upon treatment with SrRan, the upregulation of OPG could prevent the membranous localization of RANKL by forming, intracellularly, a complex with RANKL, which is further directed to the lysosome compartment to be degraded.

Our study showing that SrRan decreases the membranous RANKL level is strongly indicative of decreased resorption activity of the cells. Indeed, Nakashima et al. [47] reported that membranous RANKL is much more effective at inducing bone resorption than soluble RANKL. Furthermore, data showing a decreased membrane-associated RANKL level along with the higher production of OPG protein, suggest a higher OPG/RANKL protein ratio, thus decreased resorptive activity of these cells. Furthermore, the resorptive activity experiments performed on human OA subchondral bone osteoblasts in combination with human PBMC upon treatment with SrRan showed that this drug induced a significant decrease in resorbed surface (more than 70% inhibition), strongly supporting a possible *in vivo* effect of this drug. The effects appear to occur on both osteoblasts and osteoclasts. Indeed, data showed that although more than 70% inhibition was noted when both osteoblasts and osteoclasts were incubated together, only 6–23% was found for osteoclasts, indicating that SrRan also exerts a direct effect on osteoblasts. Such observation reinforces findings from other studies showing that SrRan was able to inhibit osteoclast activity directly by only about 30% [18]. Yet, our results demonstrate that SrRan inhibits both bone cells, osteoclasts and osteoblasts, with a predominant effect on osteoblasts.

In conclusion, this study showed that SrRan significantly decreased the human OA subchondral bone osteoblast-induced resorptive activity, possibly reflecting the combined effect of the downregulation of MMP-2, MMP-9, and the membrane-associated protein level of RANKL, concomitant with the upregulation of OPG. Hence, these data strongly suggest that SrRan could inhibit the subchondral bone resoption during the OA process.

List of abbreviations

ADAM	a disintegrin and metalloproteinase
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
KO	knock-out
MMP	matrix metalloproteinase
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OPG	osteoprotegerin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
RANKL	receptor activator of κB ligand
RT	reverse transcriptase
sRANKL	soluble RANKL
SrRan	strontium ranelate
C_T	threshold cycle
TTBS	Tris-Tween buffered saline

Disclosure

Johanne Martel-Pelletier and Jean-Pierre Pelletier received a grant from Servier, France.

Authors' contributions

All authors have read and approved the contents of this final version of the manuscript.

Study Design: JMP, JPP, SKT
Collection of Data: SKT, FM
Analysis and Interpretation of Data: SKT, JC
Manuscript Preparation: SKT, JMP, JPP

Role of the funding source

Servier, France contributed to the decision to submit this paper for publication.

Acknowledgments

The authors are grateful to Dr Marika Sarfati, Dr Guy Delespesse and Manuel Rubio from the Immunoregulation and Allergy Research Unit of the University of Montreal Research Centre (CRCHUM) for allowing us to use their apparatus and for their expert advice in flow cytometry, François-Cyril Jolicoeur, Changshan Geng and David Hum from the Osteoarthritis Research Unit of the CRCHUM for their expert technical assistance, as well as Virginia Wallis for assistance with the manuscript preparation. This study was funded in part by a grant from Servier, France.

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