Strontium Promotes Osteogenic Differentiation of Mesenchymal Stem Cells Through the Ras/MAPK Signaling Pathway

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Key Words

Strontium • Osteogenesis • Runx2 • Mitogen-activated protein kinase (MAPK) pathway • Ras

Abstract

Strontium ranelate is a new anti-osteoporosis agent. The cellular and molecular mechanism underlying the anabolic effect of strontium on bone remains to be elucidated. Osteoblasts, the main bone forming cells are known to be derived from bone marrow mesenchymal stem cells (MSCs). The present study therefore aimed to investigate the possible effects of strontium on MSCs and signaling pathways possibly involved. It was firstly demonstrated that strontium treatment significantly increased osteoblast-related gene expression and alkaline phosphatase (ALP) of osteogenic-differentiating MSCs. Accompanying the enhanced osteogenic differentiation, the increased phosphorylation of mitogen-activated protein kinase (MAPK) ERK1/2 and p38 was detected in strontium-treated MSCs. PD98059 and SB203580, selective inhibitors of ERK1/2 kinase and p38, attenuated the effect of strontium on osteogenesis. Furthermore, it was demonstrated that Rat Sarcoma viral oncogene homolog (RAS), an upstream regulator of ERK1/2 and

p38, was activated by strontium treatment and siRNA-mediated Ras knockdown inhibited strontium-stimulated expression of osteogenic markers. Finally, the transcriptional activity and phosphorylation level of Runx2 was significantly increased in response to strontium treatment in MSCs. PD98059 and Ras siRNA inhibited the effect of strontium on Runx2 activation. Taken together, these results indicated that strontium can promote osteogenic differentiation of MSCs through activating the Ras/MAPK signaling pathway and the downstream transcription factor Runx2.

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Introduction

Strontium ranelate is a new anti-osteoporotic agent and has the potential to decrease vertebral and non-vertebral fracture risk in postmenopausal women [1-3]. The beneficial effect of strontium on osteoporotic bone in clinical trials is consistent with results of several preclinical studies, which demonstrated that strontium ranelate could prevent bone loss in ovariectomized or immobilized osteopenia rats [4-6]. The anabolic effect of strontium on bone metabolism is also observed in the intact

animals, such as mice [7], rats [8] and monkeys [9]. Our recent *in vivo* study demonstrated that following oral administration of strontium compound for half a year in an ovariectomy-induced osteoporosis goat model, bone mineral density (BMD) and the expression of osteoblast-related genes, such as alkaline phosphatase (ALP) and collagen type I (COL1A), were significantly up-regulated in long bones (manuscript submitted).

To understand the mechanism for the anabolic effect of strontium on bone, several recent studies have been conducted to characterize the effect of strontium at the cellular level [10-12]. Strontium has been shown to enhance the replication and the functional activities, including bone matrix synthesis and ALP activity of mouse and rat preosteoblasts and osteoblasts [10-13]. Several osteogenic genes can be up-regulated upon strontium treatment, such as Runt-related transcription factor 2 (Runx2), bone sialoprotein (BSP), ALP and osteocalcin (OCN). Among of these osteogenic genes, Runx2 is a key transcription factor to initiate osteogenesis and specifically expressed in osteoblasts [14]. It remains unclear whether and how strontium can enhance Runx2 activation and its transcriptional activity. Osteoblasts originate from bone marrow mesenchymal stem cells (MSCs), therefore, the commitment and differentiation of MSCs towards osteoblast lineage play an essential role in bone formation. Whether the promotive effect of strontium on bone formation is fulfilled, at least partly, through an effect on MSCs is of great interest as this information will give new insights into identifying cell types and the determining factors in bone tissues that respond to strontium treatment. Such insights may in turn promote our understanding on the strontium actions at the molecular level within these cells.

So far, the signaling pathway possibly involved in strontium effects remains poorly understood. It is known that mitogen-activated protein kinase (MAPK) can directly enhance osteogenesis through the phosphorylation and subsequent activation of Runx2 [15]. Previous studies have proposed that strontium probably, like calcium, enhance ERK1/2 MAPK signaling, as evidenced in human embryonic kidney (HEK293) cells and murine calvaria osteoblasts [16, 17]. However, whether strontium can activate the same pathway in MSCs is unknown.

In this study, we hypothesized that strontium can promote osteoblast lineage differentiation of MSCs through the MAPK signaling pathway. Our results indeed showed that strontium treatment enhanced the phosphorylation of ERK1/2 and p38, but not of C-Jun N-terminal Kinase (JNK) in osteogenic-differentiating

MSCs. The upstream regulator RAS was also identified to be activated during osteogenesis by strontium treatment. Furthermore, the activity of the key osteogenic factor Runx2 was shown to be enhanced in response to strontium treatment and this effect is dependent on the Ras/ MAPK signaling pathway.

Materials and Methods

Cell cultures

Murine mesenchymal stem cell-like C3H10T1/2 cells were obtained from ATCC (Rockville, MD, USA). Primary bone marrow MSCs from mice were collected as previously described [18]. Briefly, mice tibia and femurs were removed and dissected free of soft tissues, the marrow cavity was flushed with α -MEM (calcium concentration 1.8 mM, Invitrogen, San Diego, USA) and centrifuged at 1000 rpm for 10 mins. The medium was suspended in growth medium, containing 10% fetal bovine serum (FBS), 100U/ml penicillin, 100mg/ml streptomycin, 2.5 μ g/ml fungizone and 2 mM L-glutamine (Invitrogen) and seeded into T75 flasks (Becton, Dickson and Company, USA) at the density of $1-3 \times 10^5$ cells/cm². C3H10T1/2 and primary bone marrow MSCs were incubated at 37°C in a humidified atmosphere with 5% CO₂ and medium change was carried out every three days. After the confluence, MSCs were grown in osteogenic induction medium, containing 10^{-8} M dexamethasone, 0.2 mM ascorbic acid-phosphate and 10 mM β -glycerophosphate (Sigma-Aldrich, Co., St Louis, USA). Strontium chloride (Sigma-Aldrich) was added into osteogenic induction medium during differentiation process of C3H10T1/2 and MSCs. As a soluble compound, strontium chloride concentration was expressed as strontium concentration (Sr 1.0 mM and 3.0 mM). For control, MSCs were treated with solvent vehicle (DMSO, 0.1%). For the purpose of inhibiting signaling molecular activities, PD98059 at 25 μ M, SB203580 at 10 μ M, SP600125 at 10 μ M (all from Sigma-Aldrich) were added into the differentiation medium, respectively.

Gene expression by real-time PCR analysis

Total RNA was extracted from cultured C3H10T1/2 cells and bone marrow MSCs at different time points (Day 7, 14 and 21) after the treatment with DMSO or strontium chloride and the extracted RNA was dissolved in RNase-free distilled water. Two microgram of total RNA was first reverse transcribed into cDNA using the High Capacity cDNA Kit (Applied Biosystems, Foster City, USA) following the manufacture's instructions. Quantitative PCR for gene expression was performed in 96-well plates and the reaction volume is 20 μ l per well, including 2 x SYBR green master mix (Applied Biosystem), diluted gene primers and cDNA. The primer sequences were as follows: Runx2 forward 5'-TCA CTA CCA GCC ACC GAG AC; reverse, 5'-ACG CCA TAG TCC CTC CTT TT. BSP forward 5'- CAG AGG AGG CAAGCG TCA CT; reverse, 5'- CTG TCT GGG TGC CAA CAC TG. OCN forward 5'- AAG CAG GAG GGC AAT AAG GT; reverse, CAA GCA GGG TTA AGC TCA CA and GAPDH forward 5'-CGT CCC GTA GAC AAAATG GT; reverse,

5'-AAT GGC AGC CCT GGT GAC. The q-PCR was run in ABI Prism 7700 sequence Detection System instrument (Applied Biosystem) using universal thermal cycling parameters. Quantitative analysis was performed according to the ABI protocol. The threshold cycle (Ct) value was calculated from amplification plots. Data were analyzed using the delta delta Ct relative quantification approach. The control calibrator was a pool of reverse transcribed samples with each sample being normalized to internal control GAPDH. Each sample was run in three replicates and was expressed relative to control calibrator at each time point.

Alkaline phosphatase (ALP) staining and activity assay

ALP staining was carried out using an Alkaline Phosphatase Kit (Sigma-Aldrich). Primary murine bone marrow MSCs cultures after treatment with strontium chloride for 14 days were incubated in a mixture of naphthol AS-MX phosphate alkaline solution with fast red salt. The resulting red granular dye deposit indicates sites of alkaline phosphatase activity. For the quantitative determination of alkaline phosphatase activity, the cultured cells were incubated with p-nitrophenol phosphate as a substrate and then washed with PBS buffer and lysed with 0.1% Triton X 100 in 10 mM Tris HCL, PH 9.0. Absorbance was determined at 405 nm and compared with p-nitrophenol standard titration curve. ALP activity was normalized to total protein measured with the Bio-Rad protein assay (BioRad, Hercules, USA).

Alizarin Red staining and quantification

To evaluate the effect of strontium on the matrix mineralization of MSCs, Alizarin Red staining of MSCs cultures was carried out at day 21 after the treatment of strontium chloride. Cells were fixed with formalin (10%, v/v) at room temperature for 15 mins, rinsed with PBS and stained with 40 mM Alizarin Red S (Sigma-Aldrich) with PH 4.2 for 15 mins at room temperature. For the quantification assay, the stained plates were washed with distilled water to remove non-specific staining. The stained cells were then incubated for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using a spectrophotometer. The concentration of Alizarin Red staining in the samples was determined by comparing the absorbance values with those obtained from Alizarin Red standards.

Luciferase assay

C3H10T1/2 cells were cultured for 48 hours and serum starved for 12 hours. The osteocalcin gene promoter fused to firefly luciferase was cotransfected with Runx2, Cbfb expression vector into C3H10T1/2 cells with strontium treatment. 48 hours after incubation, cells were lysed and assay was performed using a dual luciferase assay kit (Promega) according to the manufacturers instructions. Luciferase activity was determined using specific substrates in a luminometer (Promega). As an internal control, pRL-CMV vector (Promega) fused with renilla luciferase was used to determine the transfection efficiency, values of luciferase activity were normalized by values obtained from renilla luciferase activity.

Immunoprecipitation and immunoblots

C3H10T1/2 cells were treated with strontium chloride for the indicated times. For immunoblots, protein extraction was carried out by harvesting serum-deprived cells with ice-cold lysis buffer [150 mM NaCl, 50 mM Tris, 1% Triton-X, 0.3 mM sodium vanadate, 1% deoxycholic acid, 0.2% sodium dodecyl sulfate (SDS), pH 7.4]. The lysate was centrifuged at 12,000 rpm for 10 minutes before the supernatant was subjected to the protein concentration determination. An equal amount of protein was denatured at 100°C for 5 minutes, resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad). The membranes were incubated with blocking solution (5% nonfat dried milk in phosphate-buffered saline (PBS) and 0.2% Tween-20 (PBST)) for 1 hour, then incubated overnight at 4°C with the following primary antibodies: anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK (all from Cell Signaling, Beverly, USA). After three washes with PBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary IgG for 1 hour. Immunoreactive bands were detected using the SuperSignal Chemiluminescent reagent (Pierce, Rockford, USA).

For analysis of Runx2 activity, immune complexes were precipitated with protein A-agarose beads (Sigma Chemical Co., St. Louis, USA). The beads were washed three times with PBST. Proteins were eluted from the beads by boiling for 5 minutes and then subjected to SDS-PAGE and immunoblots. Runx2 activity were recognized by specific mouse anti-phosphotyrosine antibody (Oncogene, Boston, USA) and then followed by the detection of secondary antibodies. The activated protein levels were visualized with chemiluminescence agents.

Ras activation assay

Serum-starved C3H10T1/2 cells in 10cm dish were treated with or without strontium chloride (3 mM) for 1 h. Cells were lysed in 1.5 ml ice-cold lysis buffer supplemented with 20 µg/ml GST-Raf-1-RBD (Raf's Ras binding domain fused to GST) (Upstate Biotechnology, NY, USA). The activated Ras bound to Raf-1 agarose beads and the bead-associated proteins were analyzed by SDS gel electrophoresis, followed by immunoblot using anti-Ras antibody (Upstate Biotechnology).

Small interference RNA (siRNA) assay

Ras siRNA assays were performed using a commercially available Ras siRNA/siAb assay kit (Dharmacon, Chicago, USA). C3H10T1/2 cells were grown to 50% confluence and transfected with ON-TARGET smart pool Ras siRNA duplexes or non-target control siRNA duplexes using Lipofectamine RNAi Max (Invitrogen), according to the manufacturer's instructions. 48 hours after transfection, cells were cultured with serum free osteogenic induction medium for 24 hours and then subjected to strontium chloride (3.0 mM) for 1 hour, ERK1/2, p38 and Runx2 phosphorylation level was detected using western blotting assay. ALP activity and OCN gene expression was analyzed at day 14 and 21 after strontium treatment in C3H10T1/2 cells transfected with siRNA.

Fig. 1. Effect of strontium on the expression of early, middle and late osteogenic genes in C3H10T1/2 and primary murine bone marrow MSCs. (A) Runx2 mRNA expression at day 7 (B) Bone sialoprotein (BSP) mRNA expression at day 14 and (C) Osteocalcin (OCN) mRNA expression at day 21 after treatment with osteogenic induction medium supplemented with DMSO, Sr 1.0 mM and Sr 3.0 mM, respectively. GAPDH was used as the internal control. Relative fold change of target gene in a sample to a control calibrator sample was analyzed. * $P < 0.05$. Data shown are representative of three independent experiments.

Statistic analysis

All data values were expressed as mean $\pm$ standard deviation (SD). One way analysis of variance (ANOVA) with post hoc test was used to evaluate the differences of variables among groups. A value of $P < 0.05$ was considered a significant difference. All statistical evaluations were performed with SPSS version 13.0 (SPSS, Chicago, USA).

Results

Strontium promoted osteogenic differentiation of mesenchymal stem cells

The osteogenic differentiation of MSCs is driven by a series of specific genes. In this study, the expression level of Runx2, BSP and OCN, representative of early, middle and late stages of osteogenic differentiation of MSCs, respectively, were detected to monitor the differentiation progress. It was found that at day 7, strontium at the dosage of 3.0 mM significantly increased Runx2 mRNA expression in both C3H10T1/2 cells and primary bone marrow MSCs (Fig. 1A). The middle-phase gene marker, BSP was significantly increased in MSCs instead of C3H10T1/2 cells at day 14 in response to strontium treatment (Fig. 1B). OCN gene expression was similarly up-regulated in these two cells at day 21 (Fig. 1C).

Increased ALP activity is a reliable indication for the differentiation of MSCs into the osteoblastic lineage. As shown in Fig. 2A, treatment of MSCs with strontium chloride obviously increased the intracellular red granular dye. In the quantitative assay, strontium at both dosages showed little effect on ALP activity at day 7. Continuous treatment with strontium at 3.0 mM for 14 days, however,

significantly increased the ALP activity. The effect could be observed when cells were exposed to strontium for even longer time, when strontium at both dosages exhibited promoting effects (Fig. 2C).

Alizarin Red S staining in this study indicated mineralization of extracellular matrix occurred when MSCs underwent osteogenesis and the supplement of strontium chloride further enhanced the calcium deposition (Fig. 2B). The quantification assay demonstrated that calcium nodules were significantly increased by strontium chloride at both doses at day 21 (Fig. 2D).

Strontium enhanced ERK1/2 and p38 MAPK signaling pathway during osteoblast lineage differentiation of MSCs

Total protein was extracted from C3H10T1/2 cells cultured in serum-free osteogenic induction medium for 24 hours and exposed to strontium chloride for 2 hours. The treated cells were then subjected to the detection of MAPK signaling molecules, ERK1/2, p38, JNK and their phosphorylated forms, namely p-ERK1/2, p-p38 and p-JNK. Enhancement of ERK1/2 phosphorylation in C3H10T1/2 cells was detectable after the treatment with strontium for 15 minutes and the phosphorylation level decreased at half an hour after strontium treatment. Another high level of phosphorylation was detected at 1 hour and then decreased gradually. Similarly, strontium exerted a rapid and transient increase of p38 phosphorylation after treatment for 15 minutes. Another delayed stimulation of p38 was detected at 2 hours after treatment. However, the phosphorylation of JNK, another MAPK molecule, was not further enhanced by strontium treatment (Fig. 3A).

Fig. 2. Effect of strontium on ALP activity and matrix mineralization of primary murine bone marrow MSCs. The cells were cultured with osteogenic induction medium supplemented with DMSO, Sr 1.0 mM and Sr 3.0 mM. (A) ALP staining was performed at day 14 (scale bar: 50 μ m). (B) Alizarin Red S staining was performed at day 21 (scale bar: 50 μ m). (C) ALP activity at day 7, 14 and 21 was normalized to the total protein as described in materials and methods. (D) Quantitative determination of Alizarin Red S staining at day 21 by extraction with cetyl-pyridinium chloride. * $P < 0.05$, ** $P < 0.01$. Data shown are representative of three independent experiments.

In subsequent experiments, C3H10T1/2 cells were pretreated with ERK1/2 kinase (MEK1/2), p38 and JNK specific inhibitors, PD98059 (PD), SB203580 (SB) and SP600125 (SP), respectively, before the supplementation of strontium chloride in the induction medium. Osteocalcin gene expression, ALP activity and matrix mineralization in differentiating C3H10T1/2 cells were then evaluated. PD and SB, the potent inhibitor of MEK1/2 and p38, blocked strontium-enhanced ERK1/2 and p38 activation.

Fig. 3. Cell lysates were immunoblotted with anti-ERK1/2, anti-p38 and anti-JNK antibodies or their phosphorylated form (p-ERK1/2, p-p38 and p-JNK). (B) Serum deprived C3H10T1/2 cells were pretreated with ERK1/2, p38 and JNK specific inhibitors, PD98059 (PD), SB203580 (SB) and SP600125 (SP) respectively for 1 hour, followed by the treatment of DMSO or Sr (3.0 mM) for 1 hour, cell lysates were immunoblotted with the same antibodies mentioned above. (C) OCN relative gene expression was analyzed at day 21 with real-time PCR assay. (D) ALP activity at day 14 was normalized to the protein level. (E) Quantitative determination of matrix mineralization by Alizarin Red at day 21. * $P < 0.05$, ** $P < 0.01$.

The phosphorylation level of JNK has not changed with the pretreatment of SP (Fig. 3B). Consistent with this, pretreatment with PD and SB strongly suppressed strontium-induced up-regulation of osteocalcin gene expression, ALP activity and mineralization in C3H10T1/2 cells, whereas SP did not result in any significant changes (Fig. 3C, D and E). Therefore, these data further indicated that ERK1/2 and p38 might play critical roles in strontium-promoted osteogenic differentiation of MSCs.

Fig. 4. Requirement of Ras activation for strontium-enhanced ERK1/2 and p38 signaling. (A) Ras activation was measured with a pull-down assay using the Ras-binding domain of Raf-1 in cell lysates from serum-deprived C3H10T1/2 cells exposed to strontium treatment (3.0 mM) for 1 hour. Cell lysates were immunoblotted with anti-ERK1/2, anti-p38 and their phosphorylated form. The results from immunoblot analysis of the same samples with anti- α -Tubulin antibody are shown. (B) C3H10T1/2 cells transfected with H-Ras siRNA was cultured with DMSO or Sr (3.0 mM) until day 21. The effect of H-Ras knockdown on OCN gene expression was analyzed by real-time PCR. (C) ALP activity at day 14 was normalized to the total protein as described in materials and methods. (D) Quantitative determination of matrix mineralization by means of Alizarin Red S staining at day 21 by extraction with cetylpyridinium chloride. * $P < 0.05$, ** $P < 0.01$. Data shown are representative of three independent experiments.

Involvement of Ras signaling in strontium-enhanced osteoblast lineage differentiation of C3H10T1/2 cells

To explore whether Ras, an upstream mediator of ERK1/2 and p38 MAPK, is involved in effects of strontium on osteogenic differentiation, we tested whether Ras was activated after the treatment with strontium in C3H10T1/2 cells. It is known that only GTP-bound Ras interacts with Raf-1 kinase and stimulates the latter to initiate MAPK signaling cascade. Therefore, the level of GTP-bound Ras represents the activation of Ras. We examined the GTP loading of Ras in C3H10T1/2 cells and found that strontium increased the binding of Ras to the Raf-1 Ras-binding domain (RBD) (Fig. 4A), suggesting that strontium has the potential to stimulate Ras activation.

To explore the role of Ras in strontium-enhanced ERK1/2 and p38 MAPK activation, we used siRNA strategy to examine the effect of H-Ras down-regulation on strontium-mediated osteogenesis. C3H10T1/2 cells were transfected with specific H-Ras siRNA and then exposed to strontium treatment. H-Ras knockdown decreased the basal level of Ras protein expression. Knockdown of H-Ras also inhibited strontium-induced enhancement of ERK1/2 and p38 phosphorylation level in C3H10T1/2 cells (Fig. 4A). Consistent with the diminished ERK1/2 and p38 phosphorylation, H-Ras knockdown significantly reduced strontium-induced up-

regulation of osteocalcin gene expression, ALP activity and mineralization (Fig. 4B-D). To avoid the off-target effect of siRNA, we also assessed the role of Ras by using ON-TARGET smart pool Ras siRNA (siRNA2), which exerted similar effects as that of Ras siRNA on strontium-mediated osteogenic differentiation of MSCs (data not shown). All together, these results demonstrated that Ras played an essential role in ERK1/2 and p38 MAPK signaling activation and subsequent osteogenic differentiation in response to strontium treatment in C3H10T1/2 cultures.

Strontium increased Runx2 activation through Ras/MAPK signaling pathway in C3H10T1/2 cells

As Runx2 is the essential transcription factor for osteoblasts and osteocalcin is the target gene of Runx2, we decided to assess the effect of strontium on transcriptional activity and activation of Runx2. As demonstrated in Fig. 5A, strontium significantly increased Runx2 luciferase activity in C3H10T1/2 cells. PD, instead of SB can significantly inhibit the promotive effect of strontium on Runx2 luciferase activity (Fig. 5B and C). Then Runx2 activation was examined by means of immunoprecipitation. Consistent with the increased transcriptional activity, we found that strontium enhanced Runx2 phosphorylation. However, only PD but not SB abrogated strontium-mediated enhancement of Runx2 phosphorylation (Fig. 5D and E). Thus, ERK1/2 MAPK

Fig. 5. Involvement of Ras/MAPK signaling pathway in strontium-mediated Runx2 activation in C3H10T1/2 cells. (A) A reporter construct containing the osteocalcin gene promoter was cotransfected with Runx2 in C3H10T1/2 cells. Runx2 transcriptional activity was determined with or without strontium. Relative luciferase activities were determined 24 hours after transfection. (B) and (C) ERK1/2 kinase inhibitor PD98059 (PD) or p38 inhibitor SB203580 (SB) was added into the cell culture 1 hour before strontium treatment and then luciferase assay was performed. (D) and (E) Before DMSO or strontium treatment, the cell culture was treated with or without PD or SB, the total amount of Runx2 and the phosphorylated Runx2 were detected by anti-Runx2 and anti-phosphoserine antibody by western blotting. (F) C3H10T1/2 cells were transfected with H-Ras siRNA and then treated with Sr (3.0 mM), the total amount of Runx2 and the phosphorylated Runx2 were detected by anti-Runx2 and anti-phosphoserine antibody by western blotting. * $P < 0.05$, ** $P < 0.01$. Data shown are representative of three independent experiments.

signaling is involved in Runx2 activation by strontium. Finally, the blockade of Ras by siRNA decreased the enhanced phosphorylation of Runx2 in response to strontium treatment in C3H10T1/2 cells (Fig. 5F). Taken together, these results suggested that strontium would increase the transcriptional activity and phosphorylation of Runx2 in C3H10T1/2 cells and this effect is dependent on the Ras/ MAPK signaling pathway.

Discussion

Strontium ranelate has the potential to reduce osteoporotic bone fracture by increasing bone formation markers and decreasing bone resorption markers in postmenopausal women [1-3] as well as a number of animal models [4, 7, 8]. The beneficial effect of strontium ranelate on bone resulted from the bioactive component of strontium and ranelate was just a carrier [19]. Other strontium-containing compounds, such as strontium carbonate or strontium chloride, can also exhibit the similar effects as strontium ranelate in animal studies [20, 21]. Therefore, in our study, strontium chloride was adopted to investigate the mechanism of strontium on bone as this strontium salt is easy to dissolve in water. The cell proliferation assay using MTT method demonstrated that strontium at 1 and 3 mM exerted a dose dependent effect on the proliferation of C3H10T1/2 cells. There was no significant difference on the OD values in C3H10T1/2

cells after treatment with 3 mM and 5 mM (data not shown). Therefore, in our study, the dosage of strontium at 1 and 3 mM was used in the following study.

Bone formation is a spatial cascade process, which initiates from the differentiation of bone marrow MSCs into osteo-progenitor cells and then preosteoblasts and osteoblasts, followed by matrix maturation and matrix mineralization [22]. The commitment and differentiation of bone marrow MSCs towards osteoblast lineage, therefore, is the critical step in osteogenesis and initiated by the activation of Runx2 gene. The importance of the gene has been proved by the study that Runx2 gene knockout results in skeletal development with defects in bone formation because of the maturational arrest of osteoblasts [23]. During the differentiation towards mature osteoblasts, a number of extracellular matrix genes are expressed by MSCs, such as BSP, which is regarded as the middle stage gene marker of osteogenesis. At the late stage of bone formation, OCN is up-regulated, which represents the maturation of osteoblasts and matrix mineralization processing.

As shown in murine primary bone marrow MSCs, strontium treatment could increase BSP and OCN gene expression. In addition, we also detected that strontium could increase calcium nodule in differentiating C3H10T1/2 cells by alizarin red staining, suggesting that the matrix mineralization was also promoted by strontium treatment in progenitor cells. The up-regulated expression of osteogenic genes as well as increased ALP activity and

matrix mineralization suggested that strontium has the positive effect on osteogenic differentiation of MSCs. This finding is consistent with the recent report that showed the up-regulation of ALP, osteocalcin gene expression and mineralization in murine bone marrow stromal cells in response to strontium ranelate treatment [13]. However, it remained elusive whether the early differentiation related genes were affected by strontium in MSCs. In this study, Runx2 was significantly increased after 7 days of treatment with strontium both in C3H10T1/2 and primary bone marrow MSCs, suggesting that strontium could promote osteogenesis at the initial stage, i.e. the commitment of undifferentiated MSCs into osteoblast lineage.

Multiple signaling pathways including the one mediated by MAPK have been suggested to be activated in osteogenesis [24-27], although the role of MAPK signaling in regulating osteoblast differentiation still seems to be rather controversial. Some studies suggested that MAPKs may act as an antagonist in the regulation of osteogenesis [25, 26]. Other reports, on the contrary, demonstrated that MAPK signaling pathway was both essential and advantageous for osteogenic differentiation [27-30]. Particularly, Xiao et al reported that activation of ERK1/2 MAPK can directly result in the phosphorylation of Runx2 in MC3T3E1 preosteoblasts and this pathway played an important role in the control of osteoblast-related gene expression [15]. Wang et al reported that p38 was involved in the regulation of osterix expression during osteoblastic differentiation induced by bone morphogenetic proteins [30]. The basal ERK1/2 and p38 phosphorylation was observed under the osteogenic induction conditions applied in this study and strontium exhibited an enhancing effect on ERK1/2 and p38 activation to promote osteogenic differentiation. The use of the ERK1/2 and p38 specific inhibitor, PD98059 and SB203580, significantly inhibited osteogenic differentiation, further support this conclusion. Interestingly, it was noted that the phosphorylation of p38 was also inhibited by SB203580 (Fig. 3B) although autophosphorylation of p38 has never been described. It could be speculated that this inhibition might be due to the changed feedback loops of other involved signaling pathways. However, the phosphorylation of ERK1/2 and JNK was not significantly affected by SB203580 in C3H10T1/2 cells (data not shown). Thus, the inhibition of p38 phosphorylation by SB203580 might be the result of the activity change of signaling molecules other than ERK1/2 and JNK, which remain to be investigated. In line with our finding, two previous reports also suggested that strontium was able

to activate ERK1/2 signaling pathway in rat or murine osteoblasts [16, 17]. Caverzasio suggested that strontium increase osteoblast replication through at least two mechanisms: one is the rapid activation of ERK, p38 and PKC and the other is the delayed activation of ERK, p38, PKC and PKD in C3H10T1/2 cells [31]. The delayed response of MAPK signaling in C3H10T1/2 cells after strontium treatment is suggested to be due to the release of growth factors by strontium [31]. In our study, we also detected the rapid response of ERK1/2 and p38 in as soon as 15 mins after strontium treatment and the delayed response of ERK1/2 and p38 occurred at 1 hour and 2 hours after strontium treatment, respectively.

Ras is a small GTPase that regulates a number of signaling pathways, including those controlling growth and differentiation by converting its inactive GDP bound form to its active GTP bound form [32]. Many targets have been identified as downstream of Ras, including the other small GTPases Rac and Ral as well as ERK1/2, p38, JNK, phosphatidylinositol 3 phosphate kinase (PI3-K) and NF- κ B pathways [33]. It has long been recognized that Ras/MAPK is implicated in osteoblastic differentiation [34, 35]. In agreement with these notions, this study showed that Ras is critical for the effect of strontium on MSCs, as Ras activation was detected following the treatment with strontium in C3H10T1/2 cells. Ras activity in C3H10T1/2 is diminished with siRNA targeting strategy and this molecular manipulation abrogated the enhancement of ERK1/2 and p38 phosphorylation and the up-regulation of osteoblast-related gene expression and ALP activity in response to strontium treatment. These results indicate that the Ras/MAPK signaling cascade plays an essential role in strontium-mediated osteogenic differentiation of MSCs.

Runx2 is a key transcription factor that regulates osteoblast differentiation. During osteoblast differentiation, Runx2 directly regulates osteogenic genes such as OCN, OPN and COL2A by means of its DNA binding element, OSE2 [23]. Runx2 transcriptional activity can be regulated by MAPK signaling pathway, which can phosphorylate Runx2 and result in increased osteoblastic differentiation [15, 36]. In our study, strontium greatly increased Runx2 transcriptional activity and phosphorylation. In addition, ERK1/2 kinase inhibitor PD98059 significantly decreased strontium-mediated up-regulation of Runx2 transcriptional activity and phosphorylation, suggesting that strontium mediated Runx2 activation in C3H10T1/2 cells is dependent on MAPK signaling. Interestingly, strontium-mediated enhancement of Runx2 transcriptional activity

phosphorylation could not be blocked by pre-treatment with SB203580 (Fig. 5C and D), suggesting that strontium-mediated Runx2 activation may be independent on p38 signaling. This raises a question: how strontium-activated p38 signaling promotes osteogenesis of C3H10T1/2 cells, as observed earlier? As a transcription factor, Runx2 can be activated by a numerous of up-stream regulators, including growth factors/MAPK signaling, BMP/Smad signaling, PTH/PKA signaling, and even mechanical stimuli. A number of previous reports have demonstrated that the Runx2 activation may be dependent on either ERK1/2, p38, or the both [36-38], depending on the type of stimuli and the cell type applied in a particular study. In our experiments using murine mesenchymal stem cell line C3H10T1/2, other signaling molecules that are in parallel or synergize with the p38 pathway definitely exist. Recent report demonstrated that strontium can also enhance ERK1/2 and p38 signaling pathway in osteoblastic cells such as MC3T3E1 [31], whether p38 signaling is involved in strontium-mediated Runx2 activation of the cells may deserve further investigation.

In addition to ERK1/2, suppression of Ras signaling by means of siRNA also abrogated the enhancement of Runx2 phosphorylation in response to strontium treatment in C3H10T1/2 cells. The data therefore suggest that activation of Ras/MAPK acts as an upstream signaling pathway of Runx2 transcription factor in strontium-mediated osteoblastic differentiation of MSCs.

The limitation of this study is that we only investigated the cellular and molecular mechanism of the anabolic effect of strontium on bone. As the new anti-osteoporosis

agent, strontium ranelate is reported to exert dual effect on bone remodeling, that is, to promote osteoblasts-mediated bone formation and concomitantly to inhibit osteoclasts-mediated bone resorption [1-4, 7, 8]. Strontium ranelate has been reported to inhibit osteoclasts activation and differentiation directly [12, 37, 38], but the mechanism remains unclear. It has been recognized that MAPK signaling molecules (ERK, p38 and JNK) played important roles in osteoclast activation, differentiation and survival [39-42], whether MAPK signaling is also involved in the inhibitory effect of strontium on osteoclastogenesis deserves further investigation.

In summary, this study demonstrated that 1) strontium can promote the osteogenic lineage differentiation of MSCs by enhancing expression of multiple genes regulating different osteogenic stages and matrix maturation, 2) this effect is, at least partly, mediated through the activation of Ras/MAPK signaling pathway in MSCs, 3) strontium-induced up-regulation of Runx2 transcriptional activity and phosphorylation is mediated by Ras/MAPK signaling. These results may promote our understanding on the molecular mechanisms underlying the anabolic effect of strontium on bone and will lay down a primary basis for the further optimized use of strontium as an anti-osteoporosis reagent.

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