# 学 位 論 文 の 要 旨

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学 位 論 文 名　The Calcium-sensing Receptor (CaR) is Involved in Strontium Ranelate-induced Osteoblast Differentiation and Mineralization

発 表 雑 誌 名　Hormone and Metabolic Research
（巻，初頁～終頁，年）　42: 627-631, 2010

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## 論 文 内 容 の 要 旨

### Introduction

Strontium ranelate is widely used in European countries for osteoporosis treatment in postmenopausal women. It is known to have anabolic effects on bone by increasing bone formation as well as decreasing bone resorption, which reduces the risk of vertebral and hip fractures. However, precise mechanisms of these anabolic effects are not fully elucidated.

The calcium-sensing receptor (CaR) plays an important role in calcium homeostasis, predominantly through its effects on the regulation of parathyroid hormone secretion by parathyroid glands and on urinary calcium excretion by the kidney. The CaR senses not only $Ca^{2+}$ but also other divalent cations including $Sr^{2+}$. It is ubiquitously expressed in various tissues including bone cells, and is also physiologically relevant in bone turnover. Several studies using cultured osteoblasts have shown that activation of the CaR stimulates proliferation and differentiation of osteoblasts, while a recent study using osteoblast-specific CaR-deficient mouse has shown that the mouse demonstrated significant reductions in bone volume and bone mineral density due to impaired osteoblast differentiation. These findings indicate that the CaR is important for promoting osteoblast differentiation and bone formation. Thus, strontium ranelate might possibly exert its anabolic effect on bone by activating the CaR on osteoblasts.

In this study, to clarify this issue, we examined whether or not strontium would stimulate signal pathways in HEK293 cells (Human Embryonic Kidney cell line) transiently transfected with the CaR or mouse osteoblastic MC3T3-E1 cells. We also examined the effect of strontium ranelate on differentiation and proliferation of MC3T3-E1 cells.

## Materials and Methods

### Cell culture

MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS and 1% penicillin streptomycin in 5% $CO_2$ at 37℃.

### Western blot analysis

Rabbit polyclonal antibodies specific for the phosphorylated form of ERK and for its total forms were purchased from New England Biolabs, Inc. (Beverly, MA, USA). Subconfluent HEK cells transfected with the CaR or MC3T3-E1 cells in 6-well plates were incubated overnight with α-MEM containing 0.5% bovine serum albumin (BSA), and were then treated with strontium ranelate, strontium chloride, or calcium chloride in fresh medium with 0.5% BSA. Preparation of cellular extracts and Western blot analyses were carried out.

### Real-time PCR quantification of gene expression

Total RNA was isolated using Trisol reagent, and first-strand cDNA was synthesized using oligo-dT primer and SuperScript Ⅲ cDNA synthesis kit. The cDNA was amplified using an ABI PRISM 7000 sequence detection system.

### Cell mineralization assay

Mineralization of the cells was determined using Alizarin red staining after cells were grown in α-MEM supplemented with 10mM β-glycerophosphate for 2 weeks after reaching confluency.

### Cell proliferation assay

MC3T3-E1 cells ($1 \times 10^5$ cells/well, 6-well plate) were cultured for 24 hr before adding strontium ranelate 1 or 2 mM. The cells were further cultured up to 5 days, and the cell number was counted with the microscope and hemocytometer after dissociating the cells with trypsinization.

### Statistical Analysis

Results are expressed as a mean ± SEM. Statistical evaluations for differences between groups were carried out using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). For all statistical tests, a value of $p < 0.05$ was considered to be a statistically significant difference.

## Results

Strontium ranelate, strontium chloride, as well as calcium chloride (1 and 2 mM) stimulated ERK phosphorylation in the CaR-transfected cells with 15 min treatment more potently than in the cells transfected with the empty vector. In osteoblastic MC3T3-E1 cells expressing the CaR endogenously, both strontium ranelate and calcium chloride (1-2 mM) dose-dependently stimulated ERK phosphorylation with 15 min treatment. Strontium ranelate (2 mM) also time-dependently stimulated ERK phosphorylation at times up to 15 min in MC3T3-E1 cells .

Next, since strontium ranelate is used for osteoporosis treatment in humans, we examined the effects of this compound on differentiation of MC3T3-E1 cells. We analyzed mRNA

expression of OCN and BMP-2, a potent stimulant to osteoblast differentiation, by real-time PCR. Both OCN and BMP-2 mRNA levels were significantly increased by strontium ranelate (1 mM) on days 14 and 21, respectively, compared to the controls (vehicle alone) ($p<0.05$) .

Treatment of MC3T3-E1 cells with strontium ranelate (2 mM) for 21 days also enhanced mineralization of the cells by alizarin red staining . Twenty-four hours pretreatments of the cells with NPS2390 (10 $\mu$ M), a CaR inhibitor, significantly and almost totally antagonized this enhancement by alizarin red staining and its quantification ($p<0.001$).

Next, we examined the effect of strontium ranelate on proliferation of MC3T3-E1 cells by cell counting. Strontium ranelate (1 and 2 mM) significantly stimulated proliferation of MC3T3-E1 cells to the similar extent up to day 5 compared to the controls (at least $p<0.01$). Twenty-four hours pretreatments of the cells with NPS2390 (10 $\mu$ M) significantly and almost totally antagonized this enhancement by strontium ranelate (1mM) on day 5 ($p<0.05$).

## Discussion

In this study, we found that $Sr^{2+}$ itself as well as $Ca^{2+}$ stimulated ERK phosphorylation in both CaR-transfected HEK293 cells and osteoblastic MC3T3-E1 cells. Strontium ranelate also stimulated differentiation and proliferation of MC3T3-E1 cells, and NPS2390, a CaR inhibitor, antagonized such strontium-induced reactions. These findings suggest that activation of the CaR may be involved in strontium ranelate-induced differentiation and proliferation of osteoblasts, and may contribute to its therapeutic efficacy for osteoporosis.

ERK is one of the family of mitogen-activated protein kinases (MAPKs), and plays an important role in osteoblast differentiation and skeletal development by phosphorylating and activating RUNX2. We also found that strontium ranelate stimulated ERK phosphorylation in the cells, suggesting that its beneficial effect on bone could be at least partially mediated by this cascade.

Although strontium ranelate is widely used for osteoporotic patients in clinical practice, its exact mechanism of ameliorating osteoporosis has not been fully elucidated. Only recently, a couple of studies have indicated that the CaR may be involved in the pharmacologic action of strontium ranelate. However, these experiments have not clarified whether or not the CaR would be involved in strontium ranelate-induced osteoblast differentiation and mineralization. The present study for the first time showed that strontium ranelate could also promote this process with increasing OCN and BMP-2 mRNA expression as well as cellular mineralization by activating the CaR.

## Conclusion

Both physiological $Ca^{2+}$ and pharmacological $Sr^{2+}$ may activate osteoblastic bone formation by interacting with the CaR on the cells, and confirm that the CaR may be a target of strontium ranelate. Thus, the therapeutic efficacy of strontium ranelate for osteoporosis may be at least partly mediated by the CaR.