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Activation of FGF Receptors is a new Mechanism by which Strontium Ranelate Induces Osteoblastic Cell Growth

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Key Words

Strontium ranelate • MC3T3-E1 • Primary calvaria-derived osteoblast • Growth • Fibroblast growth factor signaling • Cation sensing mechanism • Calcium sensing receptor

Abstract

Background/Aims: Strontium ranelate (SrRan) is an anti-osteoporotic treatment that reduces the risk of vertebral and hip fractures. Recent *in vitro* studies suggest that the effect of strontium ranelate on osteoblastic cell growth likely involves two processes including activation of the calcium sensing receptor (CaSR) and a yet undefined mechanism. In the present study, we investigated the CaSR-independent molecular mechanism by which SrRan stimulates osteoblast growth. Methods: MC3T3-E1 and primary osteoblastic cells, specific inhibitors of receptor tyrosine kinases (RTK) and western blot analysis were used to characterize the CaSR-independent mechanism in osteoblastic cells. Results: a selective inhibitor of FGF receptor but not other RTK inhibitors markedly blunted cell growth induced by SrRan in osteoblastic cells. Associated with this observation, SrRan induced rapid activation of FGFR signaling pathways such as PLCγ, FRS2, Akt, ERK1,2 and p38. FGFR-dependent stimulation of osteogenic cell growth was also observed with other cations but not with neomycin, a selective CaSR agonist. Also, in cultured conditions used in this study, MC3T3-E1 cells and primary osteoblasts did not express the CaSR. Conclusion: data presented in this study suggest that activation of FGFRs is a new potential mechanism by which strontium can stimulate osteoblastic cell growth. Activation of FGFR-dependent cell growth is also observed in response to other cations suggesting that activation of FGF receptors is a new cation sensing mechanism in osteoblasts.

Introduction

Strontium ranelate (SrRan) is a treatment of post-menopausal osteoporosis which reduces the risk of vertebral and hip fractures [1, 2]. The antiosteoporotic action of strontium ranelate is due to a simultaneous increase in bone formation and decrease in bone resorption, thus rebalancing the bone remodelling process in favour of bone formation [3].

SrRan increases bone formation *in vitro* [4] and *in vivo* in rats leading to improvement of bone strength [5,

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6]. *In vivo* and *in vitro* studies also indicate that SrRan inhibits bone resorption [7-9]. Recent *in vitro* studies on primary murine bone cells confirmed the dual action of strontium ranelate on bone remodelling [10] and its effect on osteoblastic cell differentiation [11]. One potential target that has been suggested to mediate SrRan-induced osteoblastic cell growth is the calcium-sensing receptor (CaSR) that is present in bone cells [12]. *In vitro* studies indicate that strontium interacts with the CaSR [13] and a role of the CaSR in mediating SrRan-induced osteoblastic cell growth has recently been documented [14]. More recently, however, evidences for the implication of a calcium sensing-independent mechanism in SrRan-induced osteoblastic cell growth has also been reported [15]. In agreement with this observation, we recently found that activation of signaling pathways by SrRan in osteogenic cells is associated with two signaling responses including a rapid component potentially corresponding to activation of the CaSR and a delayed signal possibly involving the production of an autocrine growth factor [16]. In the present study, we further investigated the molecular mechanism by which SrRan induces the growth of osteogenic cells. We found that in MC3T3-E1 and primary cultured osteoblasts, most of the strontium ranelate effect on cell growth is mediated by activation of Fibroblast Growth factor Receptors (FGFRs) by a cation sensing mechanism independent of the CaSR.

Material and Methods

Reagents and antibodies

Fetal calf serum (FCS), glutamine, antibiotics and trypsin/EDTA were obtained from Gibco (Life Technologies, Basel, Switzerland). Alpha-modified essential medium (α-MEM) was purchased from Amimed (Bioconcept, Allschwill, Switzerland). CaCl2, gadolinium, aluminium chloride and neomycin were purchased from Sigma-Aldrich (Saint Louis, MS, USA). AG1478, PD173074, Vascular Endothelial Growth Factor Inhibitor (VEGFRInhib), Platelet-Derived Growth Factor Inhibitor (PDGFRInhib) were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Recombinant human FGF2, polyclonal anti-p-ERK1,2, anti-p-p38, anti-Fibroblast growth factor receptor substrate 2 (anti-FRS2), anti-p-Akt, were obtained from New England BioLabs (Cell signaling Technology, MA, USA).

For the purposes of this study, strontium ranelate (SrRan) consisted of 1:100 molar ratio of Sr^{2+}, derived from $SrCl_2$ (Sigma Aldrich, St Louis, MS, USA), and ranelate, sourced from SrRan (Technologie Servier, France), to reflect the relative concentration of ranelic acid detectable in the serum of patients receiving the standard dose of 2g/day. Concentrations of SrRan used in this study are expressed in terms of Sr^{2+} (mM).

CaSR	Forward	CAACTGCTCCGAGCACATCC
	Reverse	GACCGAACCCAATGGTACCC
FGFR1	Forward	GGACCFGTCGGATCTGATCTCG
	Reverse	TGTCGGGAAAGCTGGGTGAG
FGFR2	Forward	GAAAGATGCCGCCGTGATCAG
	Reverse	CACCACCATGCAGGCGATTAAG
FGFR3	Forward	ACCGTGCACAAGGTCTCTCG
	Reverse	GGCACGGAGAGGTCCAAGTAC
FGFR4	Forward	TCTACTGCAACGTGGGCATC
	Reverse	CTTCATGGTAGGCGACACTC
GAPDH	Forward	AGGTCATCCCAGAGCTGAACG
	Reverse	GTAGGCCATGAGGTCCACCAC

Table 1. Primers used for mRNA analysis

Cell culture and cell growth

Primary osteoblasts were enzymatically isolated from the calvaria of 2-3 day old C57Bl6 mice by sequential digestion with collagenase, as previously described [17]. Cells obtained from the last three of the five digestion steps were plated in T-75 flasks in α-MEM medium with 10% FCS until reaching subconfluency and then subcultured in 12-well plates. Primary osteoblasts and preosteoblastic MC3T3-E1 cells were cultured in α-MEM containing 10% FCS (vol/vol), 0.5% non-essential amino acids (vol/vol), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at +37 °C in a humidified atmosphere of 5% CO2-95% air. For cell growth analysis, cells were seeded at 20'000 cells/mL in either 12-well plates (one mL/well for cell growth analysis) or 75 cm^2 flasks (15 mL per flask for Westrn blot analysis). At subconfluency, they were switched to α-MEM containing 3% FCS for 24 h prior to analysis of the effects of various factors on either cell growth, signaling analysis or mRNA expression of differents receptors. In experiments aimed at testing the effect of inhibitors and their vehicles, cells were preincubated for 15 min prior to and during experiments with inhibitors. Cell growth was determined after 48 h exposure by cell counting (Beckman Coulter, CA, USA).

Western blotting analysis

Cell layers treated with either vehicle, SrRan, CaCl2 or FGF2 were rapidly frozen in liquid nitrogen and stored at –80°C until used for analysis. For the preparation of lysates, cells were incubated at +4°C in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 2 mM Na3VO4, 1 % of the protease inhibitor cocktail Set V (Calbiochem, Merck Ltd, Norringham, UK), 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS for 10 min. Lysates were then cleared by centrifugation at 6000 g for 30 min. A 75 µL sample of lysate was diluted with an equal volume of 2 x reducing sample buffer containing 125 mM Tris buffer (pH 6.8), 4% SDS, 20% glycerol, 0.05% bromophenol blue and 200 mM dithiothreitol. The mixture was then heated at +70°C for 30 min and subjected to gel

Fig. 1. Effect of selective inhibitors of receptor tyrosine kinases on cell growth induced by strontium ranelate in MC3T3-E1 osteoblastic cells. A, Dose effects of strontium ranelate (SrRan) on cell growth in subconfluent MC3T3-E1 cells. B, Subconfluent cells were preincubated with either vehicle (D = 0.1% DMSO) or selective inhibitors of receptor tyrosine kinases: E = EGFR inhib (AG1478, 1 µM), F = FGFR inhib (PD173074, 1 µM), V = VEGFR inhib (1 µM), P = PDGFR inhib (1 µM). Then, cells were incubated with SrRan (10 mM) for 48 h before measurement of cell growth by cell counting (Coulter Counter). Data are mean ± SEM of triplicates from a representative out of three experiments. a, $p<0.01$ compared with cells not incubated with SrRan. b, $p<0.01$ compared with cells incubated with SrRan alone.

electrophoresis on 6-15 % gels. Following SDS-PAGE electrophoresis, proteins were transferred to Immobilon P membranes and immunoblotted with specific antibodies as previously described [18]. Detection was performed using peroxidase-coupled secondary antibody, enhanced chemiluminescence reaction and visualization by autoradiography (Amersham International plc, Little Chalfont, UK). Reprobed filters were stripped according to the manufacturer's protocol.

mRNA analysis

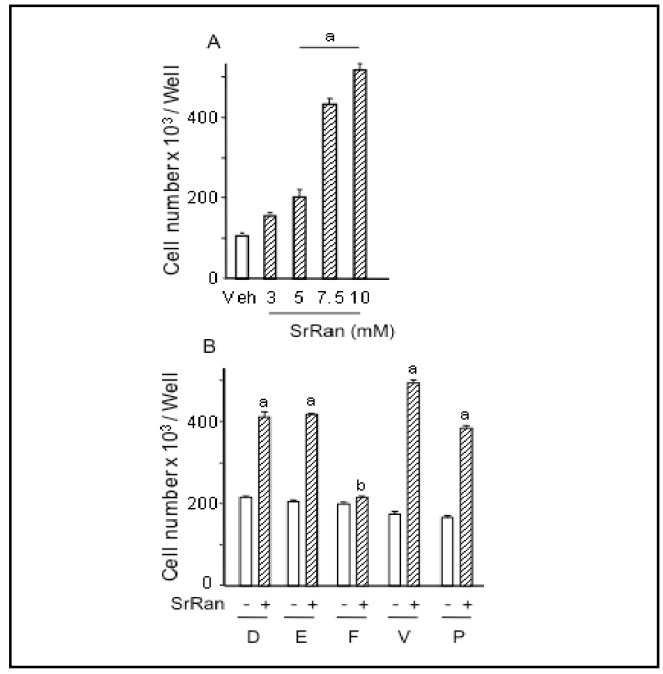
Total cellular RNA from kidney, bone or cells was extracted using peqGOLD TriFast™ (peqlab Biotechnologie GmbH) and purified using an RNeasy Mini Kit (Qiagen). Single-stranded cDNA was synthesized from 2 µg of total RNA using a high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. Two microliters of the cDNA sample was used in a 40-µL-reaction containing a Red Taq® PCR reaction buffer (Sigma), 1.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP, dTTP, 0.4 µM GAPDH, CaSR, FGFR1-4 gene-specific primers (table 1), and 1 unit of Red Taq® DNA Polymerase (Sigma). Primers were amplified using a program starting with 5 minutes of denaturation at +95 ºC, followed by 30 cycles, with 45 s of denaturation at +95 ºC, 45 s of annealing at +58-59 ºC and 30 s of extension at +72 ºC, and 5 minutes of final extension at +72 ºC.

Statistical analysis

All experiments were carried out independently at least two times. Results are expressed as the mean ± SEM of triplicates. Comparative studies of means were performed using one-way analysis of variance followed by a *post-hoc* test (projected least significant difference Fisher) with a significance value of $p < 0.05$.

Results

Recent studies suggested the implication of two molecular mechanisms, a calcium sensing receptor (CaSR)-dependent and a CaSR-independent mechanism for cell growth induced by SrRan [15, 16] with the possible



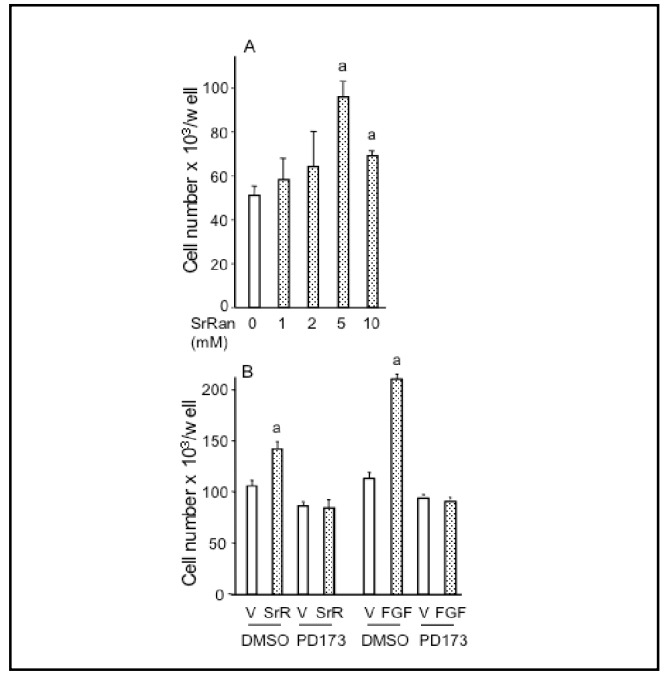
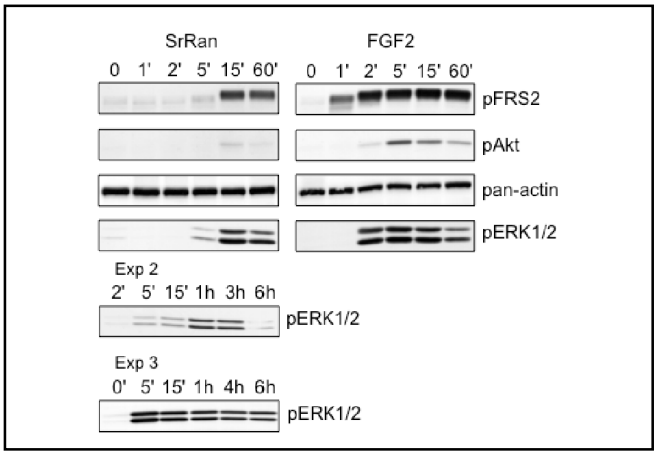


Fig. 2. Effects of strontium ranelate on primary cultured calvaria-derived osteoblast proliferation. A, Subconfluent primary osteoblasts were exposed to either various concentrations of strontium ranelate (SrRan) or B, with strontium ranelate (SrR, 5 mM) or FGF2 (FGF, 10 ng/ml) with or without the selective FGFR inhibitor PD173074 (PD173, 1 µM). After 48 h incubation, cell growth was determined by cell counting (Coulter Counter). Data are mean ± SEM of triplicates of a representative out of two experiments. a, $p<0.01$ compared with vehicle.

implication of an autocrine growth factor in the CaSR-independent mechanism [16]. Among factors suggested

Fig. 3. Effects of strontium ranelate and FGF2 on activation of signaling pathways in MC3T3-E1 cells. Subconfluent cells were exposed to either SrRan (10 mM) or FGF2 (10 ng/mL) for various incubation times. Cell lysates were then harvested and activation of signaling molecules were investigated by Western blot analysis as described in Material and Methods using specific antibodies against phosphorylated molecules. Anti-pan-actin was used to assess the amount of proteins loaded in each lane. Data are representative of three different experiments.



to be involved in this CaSR-independent mechanism, PGE2 and IGF-1 were not found to be involved as autocrine growth factors for SrRan-induced osteoblastic cell growth [16]. Thus, the role of other factors known to induce osteoblastic cell growth was further investigated. For this analysis, we used the 10 mM dose of SrRan that maximally stimulated MC3T3-E1 cell growth (Fig. 1A). To test for the implication of other growth factors in cell growth induced by SrRan, we used several highly specific inhibitors of receptor tyrosine kinases (RTKs). As depicted in Fig. 1B, the selective FGFR inhibitor PD173074 completely blunted the effects of SrRan on MC3T3-E1 cell growth whereas other RTK inhibitors of EGFR (AG1478), VEGFR and PDGFR did not influence this response. To assess whether this FGFR-dependent mechanism might also play a role in bone formation induced by SrRan, its effect was also investigated in primary cultured mouse calvarial osteoblasts. As shown in Fig. 2, SrRan dose-dependently increased cell growth of primary cultured osteoblasts with a maximal effect at 5 mM (Fig. 2A). As found in MC3T3-E1 cells, effects of SrRan and FGF2 were completely blunted by the selective FGFR inhibitor PD173074 (Fig. 2B). The effects of the selective FGR inhibitor strongly suggested an important role of the FGF/FGFR system in mediating changes in cell growth induced by strontium ions. Since FGF2 is the main FGFR ligand produced by and influencing osteoblastic cells [19], we measured the amount of FGF2 produced by MC3T3-E1 cells using a commercial ELISA assay (R&D system, Minneapolis, Minn, USA). Levels of FGF2 were determined after various incubation times ranging from 5 min to 5h in response to 10 mM SrRan in MC3T3-E1 cells. Essentially, FGF2 levels in culture media of cells exposed to SrRan were not changed compared with vehicle-treated cells (data not shown). This observation suggested that SrRan might either induce the production of another FGF or interact with the FGFR system. To gain further insight into the molecular mechanism by which SrRan interacts with the FGF/FGFR system, we compared activation of signaling pathways induced by FGF2 and SrRan in MC3T3-E1 cells. As

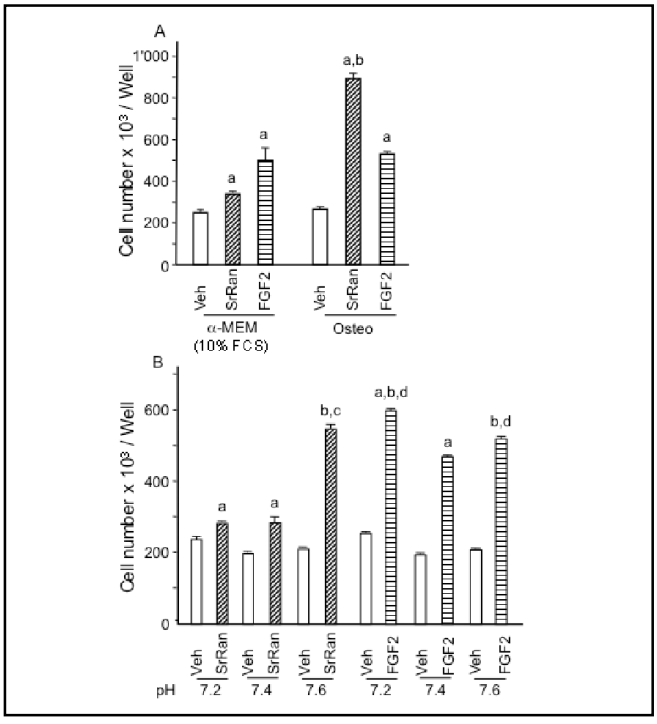


Fig. 4. pH-dependent effects of strontium ranelate on osteoblastic cell. A Subconfluent MC3T3-E1 cells were exposed to either SrRan (10 mM) or FGF2 (10 ng/mL) in either normal (α-MEM-10%FCS) or osteogenic (Osteo) medium supplemented with 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate. a, $p<0.01$ compared with cells not incubated with SrRan or FGF2. b, $p<0.01$ compared with cells incubated with SrRan in αMEM. B Subconfluent MC3T3-E1 cells were exposed to either SrRan (10 mM) or FGF2 (10 ng/mL) in αMEM medium with either normal or slightly alkaline or acidic pH. Cells were incubated with agonists for 48 h before measurement of cell growth by cell counting (Coulter Counter). Data are mean ± SEM of triplicates of a representative out of two experiments. a, $p<0.01$ compared with cells incubated with the respective agonist. b, $p<0.01$ compared with cells incubated with the respective agonist at pH 7.4. c, $p<001$ compared with cells incubated at pH 7.4 or 7.2 with SrRan. d, $p<0.01$ compared with cells incubated at pH 7.4 with FGF2.

shown in Fig. 3, a rapid and sustained activation of FRS2, a typical signaling molecule of FGF receptors, and ERK1/2 were observed in response to 10 ng/mL FGF2 indicating activation of FGF receptors by FGF2 in MC3T3-E1 cells. Interestingly, SrRan also induced activation of FRS2 and ERK1/2 but the response was delayed by approximately 15 min compared with FGF2. This time delay was shorter than the one hour lag time of SrRan-induced signaling activation that we recently reported in these cells [16] suggesting some variability in signaling pathways activation by SrRan. Thus, additional experiments were performed to find out the mechanism of this variability. As shown in the lower part of Fig. 3, signaling by SrRan was indeed quite variable. ERK1/2 activation was observed after one hour in one additional experiment (Fig. 3, exp 2) whereas it was rapid and already maximal after 5 min in a second additional study (Fig. 3, exp 3). This analysis strongly suggested that environmental factors likely influence the effect of SrRan on activation of FGF receptors in MC3T3-E1 cells. To determine which parameter influences the effect of SrRan in MC3T3-E1 cells, we analyzed cell growth induced by SrRan in various experimental conditions including in an osteogenic medium. As shown in Fig. 4A, the effect of SrRan but not that of FGF2 on MC3T3-E1 cell growth was higher in an osteogenic compared with a normal culture medium. The role of ascorbic acid (50 µg/mL) and of β-glycerophosphate (10 mM) that are the two components added to the normal culture medium to make an osteogenic medium were tested separately for their modulatory effects on SrRan-induced cell growth. Data not shown indicated that ascorbic acid had no modulatory effect whereas β-glycerophosphate enhanced the SrRan response. During this analysis, we observed that the pH of the culture medium was slightly more alkaline when β-glycerophosphate was added in the normal culture medium and tested whether the pH could influence the SrRan response. As depicted in Fig. 4B, the extracellular pH had indeed a significant effect on SrRan-induced MC3T3-E1 cell growth with a better response at slightly alkaline pH and a smaller effect at slightly acidic pH. Interestingly, extracellular pH had minimal effect on the FGF2 response. FGFR signaling activated by SrRan and FGF2 was then investigated in a culture medium adjusted at pH 7.6. In this experimental condition, SrRan was almost as rapid as FGF2 to activate FRS2 (Fig. 5A). The time-course analysis of FRS2 phosphorylation by the two agonists indicated that activation of FRS2 by SrRan was transient and maximal at 2 min whereas activation by FGF2 lasted at least 60 min and was more pronounced

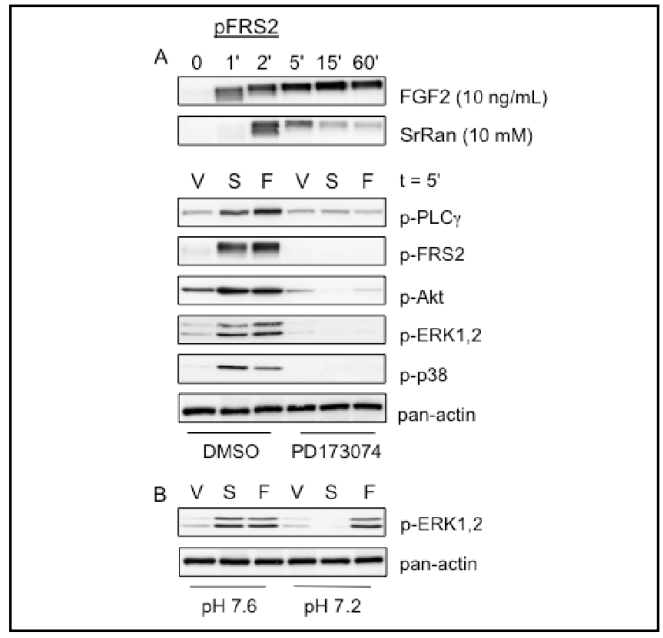


Fig. 5. Kinetics of FGFR signaling by strontium ranelate and FGF2. A, Subconfluent MC3T3-E1 cells were exposed to either FGF2 (10 ng/mL) or SrRan (10 mM) for various incubation times in osteogenic αMEM medium at pH 7.6 (upper panel) or during 5 min with either vehicle (V), SrRan (S) or FGF2 (F) with or without PD173074 (lower panel). B, Cells were incubated with either SrRan (10 mM) or FGF2 (10ng/ml) during 5 min for analysis of ERK1,2 at pH 7.6 and 7.2. Cell lysates were then harvested and activation of signaling molecules were investigated by Western blot analysis as described in Material and Methods using specific antibodies against phosphorylated molecules. Anti-pan-actin was used to assess the amount of proteins loaded in each lane. Data are from one representative out of two experiments. V = vehicle; S = SrRan; F = FGF2.

compared with effects of SrRan. Several other signaling pathways were also activated by SrRan and FGF2 in MC3T3-E1 cells including p-PLCγ, Akt, ERK1/2 and p38. They were all completely blunted when cells were preincubated with the selective FGFR inhibitor (Fig.5A). Also, a slightly acidic pH completely blunted activation of signaling pathways assessed with ERK1/2 induced by SrRan but not that of FGF2 (Fig. 5B). The rapid activation of FGFR signaling by SrRan suggested the possible implication of a cation sensing mechanism. To investigate an implication of the CaSR in this effect through a transactivation mechanism, we studied the effects of other polyvalent cations such as calcium, gadolinium and aluminium as well as of the polycation neomycin that are direct agonists of CaSR [13, 20]. Data presented in Fig. 6 indicate that 10 mM calcium induced a similar effect on MC3T3-E1 cell growth compared with SrRan tested at the same concentration. Gadolinium also enhanced the growth of these cells with a maximal effect at 100 µM

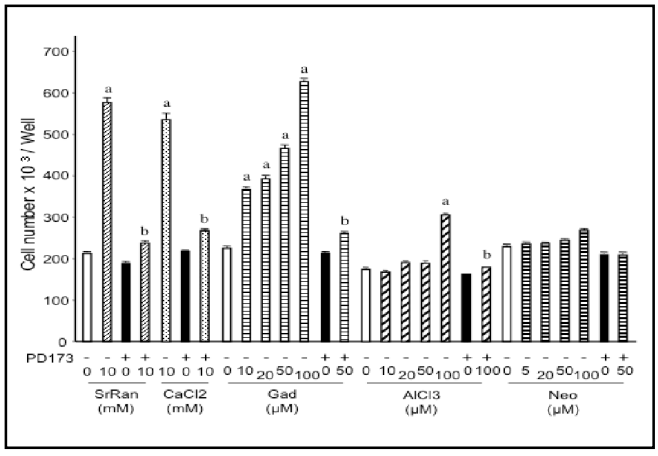


Fig. 6. Effects of various agonists of the calcium sensing receptor on FGFR-dependent MC3T3-E1 cell growth. Subconfluent cells were exposed to either Strontium Ranelate (SrRan), Calcium Chloride (CaCl2), Gadolinium chloride (Gad), Aluminium Chloride (AlCl3) and Neomycin (Neo) at concentrations indicated in the Fig. in αMEM medium (pH 7.6) for 48 h before measurement of cell growth by cell counting (Coulter Counter) with or without the selective FGFR inhibitor PD173074 (PD173). Data are mean ± SEM of triplicates of a representative experiment. a, $p<0.01$ compared with cells not incubated with agonists. b, $p<0.01$ compared with cells incubated with the respective agonist at the same concentration.

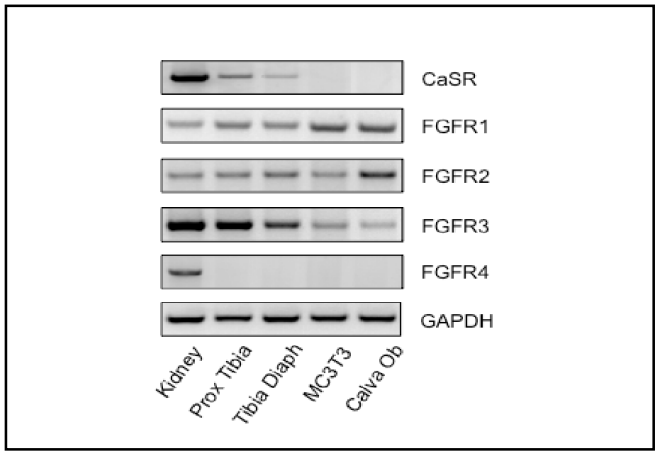


Fig. 7. Analysis of the cation-sensing and FGF receptors mRNA expression in kidney and bone as well as in MC3T3-E1 cells and primary cultured osteoblasts. Expression of the mRNA of the cation-sensing and of various FGF receptors was determined by RT-PCR from RNA isolated from adult mouse kidney, adult mouse proximal tibia (Prox Tibia), adult mouse tibia diaphysis (Tibia Diaph), MC3T3-E1 cells (MC3T3) and primary cultured calvarial osteoblasts (Calva Ob). RNA was isolated as described in Material and Methods and RT-PCR was performed using appropriate primers (Table 1).

whereas aluminium chloride at the same concentration as gadolinium had a much smaller effect. All these cations induced cell growth via a FGFR-dependent mechanism. However, neomycin had no effect on MC3T3-E1 cell growth.

To further assess whether the CaSR contributes to some extent in the growth effect of SrRan in cells used in this study, its mRNA expression was investigated in these cells and compared with tissues known to express this receptor. As shown in Fig. 7, CaSR mRNA was well expressed in kidney, slightly in the proximal and distal tibia but not in MC3T3-E1 and primary cultured calvarial cells assessed in experimental conditions used for analysis of the effects of SrRan. In contrast and as expected from biochemical and signaling pathways analysis presented in this study, several FGFRs were expressed including FGFR1-3 but not FGFR4 that was only expressed in the kidney (Fig. 7).

Discussion

Data presented in this study indicate that activation of the FGF/FGFR system is a new mechanism by which SrRan enhances the number of osteoblasts. This study is a follow up of our recent analysis of the molecular mechanism by which SrRan enhances osteoblastic cell growth. In this previous analysis, we found evidences for the existence of two potential mechanisms for SrRan-induced osteoblastic cell growth including a CaSR-independent mechanism possibly involving an autocrine growth factor [16]. PGE2 and IGF-1 were not found to be involved as autocrine growth factors. To search for the potential growth factor implicated in this CaSR-independent mechanism, we used tyrosine kinase receptor inhibitors of FGFRs, EGFRs, VEGFRs and PDGFRs that are receptors known to mediate osteoblastic cell growth by cognate growth factors [21-24]. Surprisingly, we found a strong inhibitory effect of the selective FGFR inhibitor PD173074 on SrRan-induced MC3T3-E1 cell growth (Fig. 1). A potential role of this FGFR-dependent mechanism in bone formation induced by SrRan is suggested by the observation that the selective FGFR inhibitor completely blunted the growth of primary cultured calvaria-derived osteoblasts induced by SrRan (Fig. 2). Associated with this effect, other RTK inhibitors had no or only a partial inhibitory effect on SrRan-induced cell growth (Fig. 1) suggesting a selective implication of a FGF/FGFR system in osteoblastic cell growth induced by strontium. An implication of a FGF/FGFR mechanism for osteoblastic

cell growth induced by SrRan is quite interesting since this system is well known for its role in skeletal growth and development [25]. For instance, a marked impairment in the mineralization of stromal cells derived from FGF2 knockout mice has been reported [26] with a decreased trabecular bone formation with age in FGF2-knockout compared with wild-type mice. The cellular mechanism by which SrRan and other cations activate the FGF/FGFR system remains to be investigated. The implication of FGF2, one of the main FGF produced by and influencing osteoblastic cells [19] is unlikely since the amount of this factor in the culture medium was not altered in cells exposed to SrRan (data not shown). The involvement of another FGF cannot be ruled out but the rapid increase in FGFR signaling by SrRan (Fig. 5) rather suggests a direct effect of cations on FGFRs. A direct activation of FGFRs by calcium and other cations is a potential mechanism supported by data from a previous report indicating that calcium and other cations can bind to the extracellular domain of fibroblast growth factor receptors [27]. Indeed, high affinity FGF receptors contain a cluster of acidic amino acids in their extracellular domains that is reminiscent of the calcium binding domain of some adhesion molecules that can bind calcium and other cations. Using a calcium blotting technique, Patstone and Maher [27] showed that FGFR-1 binds calcium and that calcium binding is not observed in a mutagenized form of the receptor that lacks the acidic box. Alternative mechanisms, such as trans-activation of a cation membrane target with a FGFR can also be considered. For example trans-activation of the ephrin receptor EphA4 and FGF receptors has been recently described [28]. The EphA4 binds directly and specifically via the N-terminal portion of its protein tyrosine kinase core to the juxtamembrane region of FGFRs leading to synergistic effects of ephrin-A1 stimulation on FGF2-induced cellular responses [28]. Rapid alteration in some intracellular signaling molecules by cations is another potential mechanism that could lead to activation of FGFRs. For instance, the non receptor tyrosine kinase Src is known to be recruited to activated FGFR complexes through the adaptor protein FRS2 [29] and there is evidence that receptor activation can occur indirectly by recruitment and activation of Src kinase [30]. Thus, the molecular mechanism by which cations activate FGFRs requires further investigation.

Results presented in this study also helped to better understand some of our previous data on analysis of the molecular mechanism by which SrRan stimulates the growth of osteoblasts [16]. The finding that signaling induced by SrRan is quite variable in cultured osteoblasts (Fig. 2) led to the observation that SrRan-induced activation of FGFRs is pH-dependent. Thus, the delayed activation of signaling pathway by SrRan is probably best explained by changes in extracellular pH rather than by the production of an autocrine growth factor. As shown in Fig. 5, at slightly alkaline pH, which is the pH that induces a maximal effect of SrRan-induced MC3T3-E1 cell growth, there was practically no more delay in activation of signaling pathways by SrRan. Finally, we also considered that the CaSR may contribute to some extent in osteoblastic cell growth induced by SrRan as previously suggested [14]. Whereas cations such as calcium, strontium, gadolinum, aluminum that are all direct agonists of the CaSR induced MC3T3-E1 cell growth, neomycin, that is a specific CaSR agonist, had no effect strongly suggesting that activation of the FGFR pathway by strontium does not involved CaSR. As presented in Fig. 7, in the subclone of MC3T3-E1 cells selected in this study and in primary calvarial osteoblasts, we could not detect the mRNA expression of this receptor in experimental conditions used for cell growth analysis (subconfluency) whereas as expected it was highly expressed in the kidney and to a lesser extent in bone. Expression of this receptor in MC3T3-E1 cells has not always been found and is probably subclone- and time culture-dependent and its role in mediating SrRan effects in this osteoblastic cell line is controversial [31, 32]. Our observation that FGFRs mediate the growth effect of strontium on MC3T3-E1 cells as well as in primary cultured osteoblasts in absence of CaSR also agrees with the recent report that this cation can stimulate the growth of osteoblasts derived from CaSR null mice [15, 31].

In conclusion, results presented in this study indicate that activation of FGFRs is a new mechanism by which SrRan enhances the growth of osteoblasts. Due to the important role of the FGF/FGFR system in bone biology, it is likely that this new mechanism could play a significant role in the stimulation of bone formation and reduction in fracture risk in osteoporotic patients treated with strontium ranelate.

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