Strontium inhibits osteoclastogenesis by enhancing LRP6 and β -catenin-mediated OPG targeted by miR-181d-5p

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Abstract

Strontium is a drug with the bone formation and anti-resorption effects on bone. The underlying mechanisms for the dual effect of strontium on bone metabolism, especially for the anti-resorption effects remain unknown. Thus, we aim to investigate the mechanisms of effects of strontium on osteoclastogenesis. Firstly, we found that strontium decreased the levels of important biomarkers of receptor activator of nuclear factor kappa-B ligand (RANKL) which induced osteoclast differentiation, indicating that strontium might directly inhibit osteoclast differentiation. Next, we revealed that strontium enhanced Low Density Lipoprotein Receptor-Related Protein 6 (LRP6)/ β -catenin/osteoprotegerin (OPG) signaling pathway in MC3T3-E1 cells. The signaling pathway may negatively regulate osteoclastogenesis. Thus, strontium indirectly inhibited RANKL induced osteoclast differentiation. Finally, we revealed that OPG was targeted by miR-181d-5p as determined by luciferase reporter assay and downregulated by miR-181d-5p at both mRNA and protein levels as determined by western blot.

Keywords Strontium · Osteoclast · MiR-181 · Osteoprotegerin · β -catenin

Abbreviations

RANKL nuclear factor kappa-B ligand
LRP6 Low Density Lipoprotein Receptor-Related Protein 6
OPG osteoprotegerin

RBPI Recombining binding protein suppressor of hairless
HES hairy and enhancer of split
HEY hairy/enhancer-of-split related with YRPW motif
miRNAs MicroRNAs
3'UTR 3'-untranslated region
CALCR calcitonin receptor
MMP9 matrix metalloproteinase 9
c-Fos FBJ osteosarcoma oncogene
TRAP tartrate-resistant acid phosphatase
CTSK cathepsin K

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Introduction

Strontium could be used for treating osteoporosis and the drug strontium ranelate has been approved by European Union, China and other countries. Our previous studies showed that strontium regulated the biological processes of osteoclasts (Peng et al. 2011a). However, the studies of direct and indirect effects of strontium on osteoclasts are few (Bonnelye et al. 2008; Buehler et al. 2001; Caudrillier et al. 2010; Hurtel-Lemaire et al. 2009; Mi et al. 2017; Peng et al. 2011b; Takahashi et al. 2003; Wornham et al.

2014; Yamaguchi and Weitzmann 2012). Wnt signaling pathways including the co-receptor LRP6 and β -catenin are essential for osteogenesis (Burgers and Williams 2013; J. Q. Chen and Long 2013; Zhong et al. 2015). LRP6/ β -catenin signal regulated the expression of OPG (Matsuzaki et al. 2013). The roles of LRP6 in strontium-mediated osteogenesis are not very clear. Thus, we hypothesize that strontium might exert its effects by modulating Wnt signaling pathways such as LRP6 and β -catenin. Osteoblasts were thought to be the critical player in regulating osteoclast differentiation through expressing OPG and RANKL (Theoleyre et al. 2004; B. Wang et al. 2015). We have shown that OPG is involved in the dual effect of strontium on bone (Peng et al. 2011b). OPG acts as the cross-talk mediator between osteoblasts and osteoclasts after strontium treatment. However, the exact mechanisms of this dual effect are not well known.

Previous studies showed that Notch signaling pathway affected bone homeostasis including bone cell proliferation and apoptosis (Deregowski et al. 2006; Muguruma et al. 2016; Tezuka et al. 2002). The Notch intracellular domain modulates gene expression by binding to RBPJ (Capaccione and Pine 2013). Notch target genes include human hairy and enhancer of split (HES) families and hairy/enhancer-of-split related with YRPW motif (HEY) families. As the important signaling pathway in bone formation, Notch signaling pathway has the potential to regulate osteoclastogenesis by means of up-regulation of OPG in osteoblasts, which is also an important mechanism for bone homeostasis. Therefore, we hypothesize that Notch signaling pathways might be involved in strontium-mediated inhibition of osteoclastogenesis and bone resorption.

MicroRNAs (miRNAs) decrease the expression of genes by binding to the 3'-untranslated region (3'UTR) of target mRNAs. For instance, our previous studies showed that miR-375-3p negatively regulated osteogenesis by targeting β -catenin (Sun et al. 2017b). miRNAs play critical roles in cell proliferation and apoptosis (Jovanovic and Hengartner 2006; Lee et al. 2005; Sun et al. 2017a). For example, our previous studies found miR-9 could suppress osteoblast precursor MC3T3-E1 cell proliferation (Sun et al. 2016). Thus, miRNAs can be used as the biomarkers and potential therapeutical drugs.

In the present study, we investigated the effects of strontium on osteoclastogenesis. Then we further studied the binding relationship between OPG and its regulatory miRNAs. The data from this paper will shed light on a novel mechanism for the anti-resorptive effect of strontium on bone.

Materials and methods

Cell culture, reagents and animals

Cell culture of MC3T3-E1 was described in our previous studies (Sun et al. 2016). The RAW 264.7 cells and HEK 293 cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics. The miR-181d-5p mimics and inhibitors were purchased from Exiqon. The strand for miR-181d-5p mimics and inhibitors were 5'-AACATTCA TTGTTGTCGGTGGGT-3' and 5'-CCACCGACAACAAT GAATGT-3' respectively. The strontium chloride (Wako, Osaka, Japan) was used in this study. LRP6 knockout mice were bred and maintained under specific pathogen-free conditions in the Animals Unit. The transgenic mice were euthanasia by cervical dislocation following our previous methods (Riddle et al. 2013). All animal procedures were approved by the Ethics Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (CULATR No. 3500–14).

Osteoblast and osteoclast differentiation

The differentiation and osteogenesis of MC3T3-E1 cells were induced by the growth medium supplemented with 10 mM β -Glycerophosphate disodium salt (Sigma-Aldrich, St. Louis, USA) and 50 μ g/ml L-Ascorbic acid (Sigma-Aldrich, St. Louis, USA). To induce the differentiation of RAW 264.7 cells, 40 ng/ml of RANKL (Sigma-Aldrich, St. Louis, USA) was added. RNA was extracted from RAW 264.7 cells after four days differentiation. The HiPerfect (Qiagen, Duesseldorf, Germany) was used for miRNA mimics and miRNA inhibitors transfection.

RNA extraction, reverse transcription and qPCR

TRIzol was used for RNA extraction. For the detection of gene expression, the RNA was mixed with random reverse transcription primer. The methods of qPCR were described before (Sun et al. 2017a). The sequences of primers were listed in the Table 1.

Bone slices resorption assay

To study the role of strontium in inhibition of bone resorption, RAW 264.7 cells (2.0×10^3 /well) were cultured in bone slices (BioVendor, Asheville, USA) in a 96-well plate in the presence of RANKL. After 14 days of culture, cells were removed from the bone slices with sonication for 5 min. The slices were observed under a light microscope. Then the slices were stained with 1% toluidine blue for 2 min and washed with distilled water and observed under microscope.

Table 1 Sequences of all primers

Primer Name	Sequences (5'-3')
<i>Gapdh</i> (<i>Mus musculus</i>) Forward	ATTGTCAGCAATGCATCCTG
<i>Gapdh</i> (<i>Mus musculus</i>) Reverse	ATGGACTGTGGTCATGAGCC
<i>Calcr</i> (<i>Mus musculus</i>) Forward	TGCAGACAACCTTTGGTTGG
<i>Calcr</i> (<i>Mus musculus</i>) Reverse	TCGGTTTCTTCTCCTCTGGA
<i>Mmp9</i> (<i>Mus musculus</i>) Forward	CTGGACAGCCAGACACTAAAG
<i>Mmp9</i> (<i>Mus musculus</i>) Reverse	CTCGCGGCAAGTCTTCAGAG
<i>c-Fos</i> (<i>Mus musculus</i>) Forward	GGTTTCAACGCCGACTACGAG
<i>c-Fos</i> (<i>Mus musculus</i>) Reverse	CTGACACGGTCTTCACCATTC
<i>Trap</i> (<i>Mus musculus</i>) Forward	GCTGGAAACCATGATCACCT
<i>Trap</i> (<i>Mus musculus</i>) Reverse	GAGTTGCCACACAGCATCAC
<i>Ctsk</i> (<i>Mus musculus</i>) Forward	GACGCAGCGATGCTAACTAA
<i>Ctsk</i> (<i>Mus musculus</i>) Reverse	CCAGCACAGAGTCCACAACCT
<i>Opg</i> (<i>Mus musculus</i>) Forward	CGGAAACAGAGAAGCCACGCAA
<i>Opg</i> (<i>Mus musculus</i>) Reverse	CTGTCCACCAAAACACTCAGCC
<i>Rbpj</i> (<i>Mus musculus</i>) Forward	CGGCCTCCACCCAAACGACT
<i>Rbpj</i> (<i>Mus musculus</i>) Reverse	TCCAACCACTGCCATAAGATACA
<i>U6</i> (<i>RNU6B</i>) Forward	ATGACACGCAAATTCGTGAAGC
<i>miR-181d-5p</i> Forward	AACATTCAATTGTTGTCGGTGGGT
<i>miRNAs</i> Reverse	GCGAGCACAGAATTAATACGAC

To study the role of miR-181d-5p mimics and inhibitors in inhibition of bone resorption, RAW 264.7 cells (2.0×10^3 /well) were cultured in bone slices (BioVendor, Asheville, USA) in a 96-well plate in the presence of RANKL. For transfection of miR-181d-5p mimics, we used HiPerfect (Qiagen, Duesseldorf, Germany) as the transfection reagent. In brief, we added HiPerFect to the medium without serum and gave a final HiPerFect concentration of 0.5% (v/v) after adding cells. We diluted miRNA mimics in medium, mixed, incubated for 10 min at room temperature, and added the cells. The concentration of miR-181d-5p mimics and inhibitors were maintained for 14 days. Then the cells were processed with the above procedures.

Hydroxyapatite resorption assay

RAW 264.7 cells were plated in hydroxyapatite-coated Corning Osteo Assay Surface 24-well Plates (Sigma-Aldrich, St. Louis, USA). After 14 days of culture hydroxyapatite coated plates were washed with 15% sodium hypochlorite solution for 5 min to remove cells, rinsed with distilled water, and air-dried. For staining, plates were treated in dark at room temperature with 5% (w/v) silver nitrate solution for 30 min. Wells were then aspirated and washed for 5 min in distilled water. Wells were aspirated again, and 5% (w/v) sodium thiosulfate was added. After a 5-min incubation at room temperature, plates were aspirated and air-dried. The resorption pits were imaged using a light microscope.

TRAP staining

We seeded RAW264.7 cells at a concentration of 1×10^4 cells/well in total 150 μ l medium into 12 wells chamber (ibidi, Martinsried, Germany) and treated the cells with strontium. TRAP staining was conducted according to the manual of the Acid Phosphatase kit (Sigma-Aldrich, St. Louis, USA).

Plasmid construction and luciferase reporter assays

The methods for plasmid construction and luciferase assay were described in our previous study (Sun et al. 2017b). In brief, we annealed the designed oligonucleotides and the DNA fragments were digested with restriction enzymes at the sites of SacI and XhoI, and then ligated into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation, Madison, USA). The exact sequences and length of oligonucleotides were listed in the Table 2.

Colony PCR, agarose gel electrophoresis and sequencing the successful ligation. Thus, the wild type and mutant fragments of the 3'UTR of OPG (shown in Table 2) containing the binding sites of miR-181d-5p were successfully inserted into the report vector. The plasmids and miR-181d-5p mimics were co-transfected into MC3T3-E1 cells. The Dual-Glo® Luciferase Assay System (Promega Corporation, Madison, USA) was used to measure the firefly luciferase activities when after 48 h transfection.

Table 2 Sequences of all oligonucleotides

Name	Sequences (5'-3')
OPG1287 Forward	CAT GCT CTG AGA AAT TGA ATG TAC
OPG1287 Reverse	TCG AGT ACA TTC AAT TTC TCA GAG CAT GAG CT
OPG1287 Mutant Forward	CAT GGT GGC TGT TTT ACT TAC AAC
OPG1287 Mutant Reverse	TCG AGT TGT AAG TAA AAC AGC CAC CAT GAG CT
OPG1149 Forward	CTC AGT TTT CCA GTG TGG AAT GTT C
OPG1149 Reverse	TCG AGA ACA TTC CAC ACT GGA AAA CTG AGA GCT
OPG1149 Mutant Forward	CTC AGT TTT CCA GTG TGC TTA CAT C
OPG1149 Mutant Reverse	TCG AGA TGT AAG CAC ACT GGA AAA CTG AGA GCT

Fig. 1 Strontium directly inhibited osteoclast differentiation. Relative expression levels of CALCR (a), MMP9 (b), c-Fos (c), TRAP (d), CTSK (e) in RANKL induced RAW264.7 cells treated with different concentrations of strontium. The results are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, independent Student's t-test. $n = 3/\text{group}$

Fig. 2 Strontium directly inhibited osteoclast differentiation. **a**, **b**, and **c** Representative images of osteoclasts by TRAP staining to evaluate the RANKL-induced osteoclastogenesis in RAW 267.4 cells treated with strontium

Western blot

In brief, the MC3T3-E1 cells were seeded in 6 well plate (5×10^5 cells/well). The cells were treated with a serial dilution of miR-181d-5p mimics or inhibitors. The lysis buffer was Radio Immunoprecipitation Assay (RIPA) buffer with SIGMAFAST Protease Inhibitor Cocktail Tablets (Sigma-Aldrich, St. Louis, USA). The Laemmli buffer was used to denature the samples. The PVDF membrane was blocked by 5% BSA solution for one hour. Secondary antibodies included Goat Anti-Rabbit

IgG H&L (HRP) (ab6721, Abcam, Cambridge, UK). For beta actin staining, the membrane was incubated with the first antibody (1:10000 dilution) for one hour at room temperature, rinsed with TBST, and then incubated with the second antibody (1:50000 dilution) for one hour at room temperature. For OPG staining, the membrane was incubated with the first antibody (1 μ g/ml) overnight at 4 $^{\circ}$ C, rinsed with TBST, and then incubated with the second antibody (1:2000 dilution) for one hour at room temperature. ECL Prime Western Blotting Reagents (GE Healthcare, Little Chalfont, UK) were used to

Fig. 3 Strontium inhibited functions of osteoclast. **a** Representative images of bone slices to evaluate the RANKL-induced osteoclastogenesis in RAW 267.4 cells treated with strontium. **b** Representative images of bone slices to evaluate the pit formation in RAW 267.4 cells treated with strontium. The green arrows indicated the pits generated by osteoclasts. **c** Representative images of osteoclasts. The green arrows indicated the osteoclasts with multinucleuses. **d** Representative images of osteoclasts by von Kossa staining to evaluate the functions of osteoclasts in RAW 267.4 cells treated with strontium

develop. The developer was myECL Imager (Thermo Fisher Scientific, Waltham, USA).

Immunocytochemistry and immunofluorescence

The expression levels of β -catenin were detected by ICC and IF. We seeded cells at a concentration of 1×10^4 cells/well into chamber slides (ibidi, Martinsried, Germany) and treated the cells with strontium. The cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, blocked with 10% Normal Goat Serum, incubated with primary antibody β -catenin Antibody (Abcam) overnight, and then secondary antibody Goat Anti-Rabbit Ig G (Alexa Fluor 488) (Abcam).

Statistical analysis

Statistical analysis was performed by a t-test or paired t-test, and $p < 0.05$ was considered statistically significant. The software GraphPad Prism was used to draw the charts.

Results

Strontium directly inhibited osteoclast differentiation

Firstly, to explore the effects of strontium on osteoclast differentiation, we treated the osteoclast precursors RAW264.7 cells with strontium and then detected total five representative biomarkers relating to osteoclast differentiation, namely calcitonin receptor (CALCR), matrix metalloproteinase 9 (MMP9), FBJ osteosarcoma oncogene (c-Fos), tartrate-resistant acid phosphatase (TRAP), and cathepsin K (CTSK), according to previous studies (C. Chen et al. 2015; Cheng et al. 2013; Li et al. 2014; Zeng et al. 2016). Results showed that strontium decreased the expression levels of the biomarkers when its concentration was at 8 mM and more than 8 mM (Fig. 1a, b, c), or at 16 mM (Fig. 1d), indicating that strontium inhibited the osteoclast differentiation at the relatively high concentration. In addition, this effect of inhibition was higher with the increase of concentration (Fig. 1a, b, c). On the other hand, we also found that strontium increased the expression levels of

Fig. 4 Strontium indirectly inhibited osteoclastogenesis by enhancing LRP6/ β -catenin/OPG signal in MC3T3-E1 cells. **a** The MC3T3-E1 cells were treated with strontium (6.0 mM) for 5, 15, 30 and 60 min respectively. The expression levels of phosphorylation of LRP6 (p-LRP6), LRP6, and β -catenin were compared by western blot analysis. β -actin was used as a control. Strontium induced phosphorylation of LRP6 and stabilization of β -catenin. **b** Representative staining images of MC3T3-E1 cells. The intensity and the degree of green represented the expression levels of β -catenin. After treatment of strontium, the nucleus of cells were greener. In other words, the degree of green was higher in strontium-treated group than the control group. Strontium

increased the nucleus translocation of β -catenin. **c** The MC3T3-E1 cells were treated with strontium for 5, 15, 30 and 60 min respectively. The expression levels of p-LRP6, LRP6, and β -catenin were compared by western blot analysis. Strontium induced LRP6 phosphorylation and β -catenin stabilization while strontium failed to increase LRP6 phosphorylation and β -catenin levels after LRP6 deletion. **d** The MC3T3-E1 cells were treated with strontium for 15 and 30 min respectively. No effects of strontium on β -catenin was observed with HEK 293 cells while strontium induced β -catenin stabilization in LRP6 expressed HEK 293 cells

the biomarkers when its concentration was at 4 mM and lower than 4 mM (Fig. 1a, b, c), or at 8 mM and lower than 8 mM (Fig. 1d), indicating that strontium promoted the osteoclast differentiation at the relatively low concentration. Additionally, strontium increased the levels of CTSK (Fig. 1e). To further confirm the effects of strontium, we conducted TRAP staining in RAW264.7 cells. There were less

TRAP positive cells and less multinuclear cells in strontium-treated cells (Fig. 2a, b, c), indicating that strontium inhibited osteoclastogenesis. Taken together, these data supported previous studies (Bonnelye et al. 2008; Roy and Bose 2012; Roy et al. 2013; Takahashi et al. 2003; Wornham et al. 2014).

Fig. 5 Strontium indirectly inhibited osteoclastogenesis by enhancing LRP6/ β -catenin/OPG signal in MC3T3-E1 cells. Relative expression levels of OPG (**a**), RBPJ (**b**) in MC3T3-E1 cells treated with different concentrations of strontium. **c** Relative expression levels of TRAP in RAW264.7 cells treated with strontium. **d** Relative expression levels of OPG in the procedure of MC3T3-E1 cell differentiation induced by β -Glycerophosphate disodium salt and L-Ascorbic acid. RNA extraction was conducted after the cells were induced for four or five days. The results are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, independent Student's t-test. $n = 3/\text{group}$

Strontium inhibited functions of osteoclast

To further study the effects of strontium on functions of osteoclast, we conducted bone slices resorption assay and hydroxyapatite resorption assay. For the bone slices resorption assay, if the activity of osteoclast was higher, the bone slices were absorbed more adversely and they are thinner, so the transmittance of light of microscopy was higher and the whole view was brighter. Figure 3a showed that the view of microscopy of RAW 264.7 treated with strontium was darker than control, indicating that strontium (3 mM) inhibited functions of osteoclast. Then we stained the pits generated from osteoclasts and Fig. 3b showed that there were more pits in the control group than in the strontium treated group, suggesting that strontium inhibited pit formation of osteoclasts. We then further conducted hydroxyapatite resorption assay by using Corning Osteo Assay Surface 24-well Plates. Firstly, we identified the formation of osteoclasts because they had multinucleuses (Fig. 3c). Then we stained the plates and results showed that there were larger areas of brown and black staining (Fig. 3d), indicating that strontium (3 mM) inhibited the resorption function of osteoclasts.

Strontium indirectly inhibited osteoclastogenesis by enhancing LRP6/ β -catenin/OPG signal in MC3T3-E1 cells

Currently our results showed that strontium directly inhibited RANKL induced osteoclast differentiation. Additionally, our previous studies indicated that active molecules produced by osteoblast modulated osteoclastogenic response to strontium (Peng et al. 2011a). Therefore, we further studied the mechanisms of this indirect regulation. We detected the levels of LRP6/ β -catenin/OPG signals because this pathway played vital roles in osteogenesis (Burgers and Williams 2013; Cai et al. 2016; J. Q. Chen and Long 2013; J. Q. Chen et al. 2014; Fromigue et al. 2010; Peng et al. 2011b; Sun et al. 2016; Sun et al. 2017b; Yang et al. 2011). Western blot analysis showed that strontium increased phosphorylation of LRP6 instead of the expression levels of LRP6, stabilized β -catenin (Fig. 4a), and increased the nucleus translocation of β -catenin in MC3T3-E1 cells (Fig. 4b), suggesting that strontium activated Wnt signaling pathways. It was further confirmed by the results of animal experiments showing that strontium activated β -catenin signaling by inducing phosphorylation of LRP6 rather than the expression levels of LRP6 in calvarial cells from mice (Fig. 4c). Strontium failed to increase LRP6

Fig. 6 OPG was targeted by miR-181d-5p. **a** The binding sites between miR-181d-5p and the 3'UTR of OPG. **b** The relative luciferase activity of MC3T3-E1 cells transfected with luciferase-wild type (or mutant) OPG plasmids and miR-181d-5p mimics. **c** Relative expression levels of OPG in MC3T3-E1 cells upon transfection of miR-181d-5p mimics. **d** The expression levels of OPG were compared in MC3T3-E1 cells by western blot analysis. β -actin was used as a control. The results are

expressed as mean $\pm$ SD. * $p < 0.05$. ** $p < 0.01$, NS, not significant, independent Student's t-test. $n = 3$ /group. **e** Relative expression levels of miR-181d-5p in MC3T3-E1 cells treated with strontium. **f** Representative images of osteoclasts by TRAP staining to evaluate the RANKL-induced osteoclastogenesis in RAW 267.4 cells transfected with miR-181d-5p mimics or inhibitors

phosphorylation and β -catenin levels after LRP6 deletion (Fig. 4c). To further confirm the positive roles that LRP6 played in the activation of β -catenin, we overexpressed LRP6 in the HEK 293 cells. Results showed that strontium induced β -catenin stabilization in LRP6 expressed HEK 293

cells, while no effects of strontium on β -catenin was observed without transfection of LRP6-VSVG plasmid (Fig. 4d). Taken together, the above data suggested that strontium enhanced the LRP6/ β -catenin signal.

We then detected the levels of OPG and results showed that strontium increased the levels of OPG especially at the 3 mM (Fig. 5a). In addition, we also found the levels of RBPJ, an important molecule in Notch signaling pathway in osteoclasts (Ma et al. 2013; Zhao et al. 2012), increased after the cells were treated with strontium (Fig. 5b), indicating that strontium activated Notch signal in MC3T3-E1 cells.

OPG was a cross-talk mediator between the osteoblast and osteoclast in response to strontium treatment. Thus, we further investigated the role of OPG in the inhibitory effect of strontium on osteoclastogenesis. We added different concentrations of strontium into the medium of MC3T3-E1 cells and collected the conditional medium after culturing the cells for two days. We then used the conditional medium to culture RAW264.7 and found that the mRNA levels of osteoclast differentiation biomarker TRAP decreased significantly (Fig. 5c), indicating that strontium could indirectly inhibit osteoclast differentiation by upregulating OPG and RBPJ in MC3T3-E1 cells.

Taken together, the above results suggested that strontium enhanced LRP6/ β -catenin/OPG signaling pathway and RBPJ signal in MC3T3-E1 cells. These signals negatively regulated osteoclastogenesis.

Given that OPG played vital roles in the crosstalk between osteoblast and osteoclast, we further studied its expression levels during MC3T3-E1 differentiation. We induced MC3T3-E1 differentiation and found that the levels of OPG significantly increased during this process (Fig. 5d). Specifically, after the cells were induced for four days and five days, the mRNA levels of OPG rose significantly (Fig. 5d). Because OPG was the osteoblastic marker (Lumetti et al. 2016; Takai et al. 1998), its upregulation might promote MC3T3-E1 differentiation.

OPG was targeted by miR-181d-5p

Because OPG served as the critical bridge between osteoblast and osteoclast, we further studied its upstream regulatory miRNAs. We used the prediction tool miRanda (<http://www.microrna.org/microrna/home.do>) to search for these regulatory miRNAs. And miR-181d-5p was predicted to be the miRNA (Fig. 6a). Notably, there were two binding sites (1287 site and 1149 site) located in the 3'UTR of OPG (Fig. 6a), indicating that miR-181d-5p might strongly target and inhibit OPG. We then cloned these two sites and their mutant controls into the Luciferase miRNA Target Expression Vector. Transfection of miR-181d-5p mimics resulted in a significant reduction in luciferase activity in MC3T3-E1 cells transfected with wild type plasmids (Fig. 6b), while the inhibition of luciferase activity by miR-181d-5p mimics was abrogated in cells transfected with mutant plasmids (Fig. 6b). In other words, miR-181d-5p inhibited its wild type targets more significantly than mutant ones, so resistance of mutant luciferase

vectors to miRNAs confirmed the specific targeting. In addition, the relative luciferase activity was lower at the 1287 site than the 1149 site (Fig. 6b). The reason should be that the number of paired and binding nucleotides at the 1287 site is more than that of 1149 site (Fig. 6a). Taken together, these results suggested that miR-181d-5p directly targeted OPG in MC3T3-E1 cells.

Given that miR-181d-5p targeted OPG, it is expected that this miRNA might decrease the expression of OPG. Thus, we

Fig. 7 miR-181d-5p promoted bone resorption. **a** Representative images of bone slices to evaluate the RANKL-induced osteoclastogenesis in RAW 267.4 cells treated with miR-181d-5p mimics. **b** Representative images of bone slices to evaluate the RANKL-induced osteoclastogenesis in RAW 267.4 cells treated with miR-181d-5p inhibitors. **c** Representative images of bone slices to evaluate the pit formation in RAW 267.4 cells treated with miR-181d-5p mimics or inhibitors. The green arrows indicated the pits generated by osteoclasts

firstly detect the mRNA levels of OPG and results showed that the expression levels of OPG were significantly decreased by miR-181d-5p mimics (Fig. 6c). Then we conducted western blot to detect the protein levels and results showed that miR-181d-5p mimics (2.5 nM) downregulated the levels of OPG while miR-181d-5p inhibitors upregulated OPG in a dose dependent manner (Fig. 6d), suggesting that miR-181d-5p inhibited protein levels of OPG. Taken together, all these data suggested that OPG was downregulated by miR-181d-5p.

We then studied the relationship between effects of strontium and miR-181d-5p. Results showed that strontium decreased the expression levels of miR-181d-5p (Fig. 6e), indicating that strontium might upregulate OPG by downregulating miR-181d-5p. Finally, we studied the physiological functions of miR-181d-5p by using TRAP staining. The pictures showed that miR-181d-5p mimics increased the osteoclastogenesis while miR-181d-5p inhibitors inhibited osteoclastogenesis (Fig. 6f).

miR-181d-5p promoted bone resorption

To further study the effects of miR-181d-5p on bone resorption, we performed bone slices resorption assay. Figure 7a showed that it is brighter in the miR-181-5p mimics treated group, suggesting that the bone slices were thinner and there were more bone resorption in this group. In contrast, it is darker in the RAW 264.7 cells treated with miR-181-5p inhibitors (Fig. 7b). Then we stained the slices and results showed that there were more pits in the miR-181d-5p mimics group while less pits in the miR-181d-5p inhibitors group (Fig. 7c). Taken together, these data suggested that miR-181d-5p promoted bone resorption.

Discussion

In the present study, we revealed the important role of LRP6 in the previously documented effects of strontium in activating the beta-catenin/OPG signaling pathway in osteoblast precursor cells. The OPG subsequently suppressed osteoblast differentiation. Then we further investigated the upstream regulatory miRNA of OPG. miR-181d-5p targeted and suppressed the expression of OPG at both mRNA and protein level. These main factors studied in this study and their regulatory relationships were summarized in the Fig. 8.

The binding and regulatory relationship between the 3'UTR of OPG and miR-181d-5p was supported by three main aspects. Firstly, the prediction tool predicted that there were two binding sites located in the 3'UTR of OPG. Secondly, the decline of luciferase activity confirmed the specific binding. Thirdly, the mRNA and protein expression of OPG were downregulated by miR-181d-5p.

There were some studies of miR-181 and it was reported that miR-181 influenced carcinogenesis (B. Wang et al. 2010). Upregulation of hepatic transforming growth factor beta (TGF- β) in the liver correlated with elevated miR-181d in mice (B. Wang et al. 2010). Another study also reported that miR-181 was upregulated by TGF- β (Y. Wang et al. 2011). However, the studies of its roles in bone were very few (Dige et al. 2013; Su et al. 2015). Thus, we explored the relationships between miR-181d and OPG, an important molecule in bone remodeling.

Wnt signaling pathways and Notch signaling pathway were two very important pathways regulating bone remodeling and related diseases (J. Q. Chen and Long 2013; Jin et al. 2015; Zhong et al. 2015). Our previous studies showed that low-density lipoprotein receptor-related protein 5, a co-

Fig. 8 The flowchart of the main study factors and their relationships

receptor of Wnt pathways, was essential for osteogenesis (Sun et al. 2016; Sun et al. 2017b). OPG overexpression in osteoblasts in β -catenin knockout mice could normalize the decreased bone mass (Yao et al. 2017), indicating that OPG might be regulated by Wnt signaling pathways. Further studies of these aspects are needed.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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