Original Article
miR-193-3p ameliorates bone resorption in ovariectomized mice by blocking NFATc1 signaling

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Abstract: Background: Nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) as a key transcription factor contributes to osteoclast differentiation and bone resorption. However, the post-transcriptional mechanisms of microRNAs (miRNAs) targeted to NFATc1 have not been completely clarified in postmenopausal osteoporosis (PMO). In our study, we aimed to investigate the role of miR-193-3p in ovariectomy (OVX)-induced bone loss by regulating the NFATc1 pathway. Methods: Female C57BL/6J mice underwent sham or OVX operation. Injection of Agomir-Control or Agomir-miR-193-3p was performed in OVX mice. Serum, urine and tibia were collected for experimental measurements, including biochemical markers, RT-qPCR and western blotting assays. Results: We identified NFATc1 as a direct target of miR-193-3p. Up-regulation of NFATc1 and down-regulation of miR-193-3p were found in the tibia of OVX mice. Gain-of-function of miR-193-3p resulted in the reduction of NFATc1 mRNA and protein expression in vivo and in vitro. Furthermore, injection of Agomir-miR-193-3p markedly ameliorated OVX-induced Ca\textsuperscript{2+} dyshomeostasis and bone loss by inhibiting the expression of NFATc1 and its downstream targets of osteoclast-specific genes, Ctsk, TRAP and Car2. Conclusion: Overexpression of miR-193-3p had an osteoprotective effect in OVX mice by suppressing NFATc1 pathways.

Keywords: Ovariectomy, osteoporosis, miR-193-3p, NFATc1, post-transcriptional

Introduction

Postmenopausal osteoporosis (PMO), as an estrogen deficiency-induced metabolic bone disorder, is one of the most prevalent forms of primary osteoporosis and occurs in approximately 50% of postmenopausal women worldwide [1]. At present, estrogen loss-induced osteoclastogenesis followed by increased bone resorption and decreased bone mass is closely associated with overactivation of immune system activity, inflammatory response, and cytokines, including interleukin-6 (IL-6), IL-7, tumor necrosis factor-\alpha (TNF-\alpha) and macrophage colony-stimulating factor (M-CSF) [2-4]. Numerous studies have reported that estrogen deficiency-induced osteoporosis in both ovariectomized rodent models and women can be prevented by hormone therapy replacement (HTR) [5, 6]. However, HTR reportedly increases the risk of endometrial cancer, cardiovascular diseases, and atypical fractures [7, 8]. Therefore, understanding of the underlying molecular mechanisms in PMO will contribute to the development of safe therapeutic schemes for the treatment of PMO.

Nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) is a key transcription factor for osteoclast differentiation and is initially induced by tumor necrosis factor receptor-associated factor 6 (TRAF6)-activated nuclear factor kappa B (NF-\kappa B) and NFATc2 [9]. NFATc1 stimulates the expression of various osteoclast-specific genes, including tartrate-resistant acid phosphatase (TRAP), cathepsin K (Ctsk), and carbonic anhydrase II (Car2) [9, 10]. Recently, inhibition of NFATc1 was proposed as a novel therapeutic target for preventing bone loss in OVX-induced osteoporosis [11, 12].

MicroRNAs (miRNAs) are single-stranded non-coding, ~22-nucleotide RNAs that induce translational repression or cleavage of the target mRNAs by binding to their 3’-untranslated regions (3’-UTRs) [13]. miRNAs perform a variety of biologic processes, including osteoblast or
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osteoclast differentiation [14, 15]. For example, miR-34a, miR-141, and miR-219a-5p block bone resorption [15-17], while miR-133a, miR-214 and miR-376c accelerate osteoclastogenesis and inhibit bone formation [14, 18, 19]. In the present study, utilizing on-line prediction algorithms, we found that NFATc1 was a direct target of miR-193-3p. The aim of the present study was to investigate whether miR-193-3p as a post-transcriptional mediator alleviated ovariectomy (OVX)-induced bone loss by inhibiting the NFATc1 pathway.

Materials and methods

Animal treatment

Female C57BL/6N mice (8-weeks-old and body weight 18-22 g) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and allowed to acclimate for 1 week in a temperature-controlled environment (21 ± 2°C; humidity, 55 ± 5%) under an artificial 12-h light/dark cycle with free access to food and tap water. Mice were randomly divided into four groups (n = 6/group) as follows: (1) Sham group, sham-operated mice were treated with normal saline; (2) OVX group, OVX-operated mice were treated with normal saline; (3) Agomir-Control group, OVX-operated mice were injected with Agomir-Control; (4) Agomir-miR-193-3p group, OVX-operated mice were treated with Agomir-miR-193-3p (100 mg/kg; twice) by tail vein injection for 4-weeks. At week 4, mice were euthanized, and serum, urine and tibia samples were immediately collected and maintained at -80°C for further analysis. Agomir-control (miR-Con) and miR-193-3p mimics were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China) and were inserted into multiple cloning sites of the luciferase expressing pMIR-REPORT vector (Ambion; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol.

Luciferase reporter assay

For the luciferase activity assay, C3H10T1/2 cells were co-transfected with luciferase reporter vectors containing WT or Mut 3'-UTR (0.5 μg) of NFATc1, and miR-Con or miR-193-3p mimics (100 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 48 h at 37°C at final concentrations of 100 nM, according to the manufacturer’s protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. TaqMan® RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan® MicroRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to perform RT-qPCR of miR-193-3p, according to the manufacturer’s protocol. U6 small nuclear RNA was used as an endogenous control.

Cell culture

The murine mesenchymal cell line C3H10T1/2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO₂ and 95% air (Thermo Fisher Scientific, Inc.).
perform RT-qPCR of OPG, RANKL, NFATc1, TRAP, Ctsk, and Car2 using the Applied Biosystems 7300 Real-Time PCR system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. The relative miR-193-3p and mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method [20]. The primers were synthesized by Sangon Biotech (Shanghai, China), shown in Table 1.

**Western blotting**

Proteins were extracted with radio immunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China). Western blotting was done as previously described [21]. The membranes were incubated with the primary antibody for NFATc1 (cat. no. sc-7294; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 2 h. Then, the membrane was incubated at room temperature for 1 h with the appropriate horseradish peroxidase-conjugated anti-mouse secondary antibody (cat. no. sc-516102; 1:10,000; Santa Cruz Biotechnology, Inc.) and visualized using chemiluminescence (Thermo Fisher Scientific, Inc.). β-actin (1:2,000; cat. no. sc-130065; Santa Cruz Biotechnology, Inc.) was used as the control antibody. Signals were analyzed with Quantity One® software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA).

**Immunohistochemical (IHC) and hematoxylin and eosin (H&E) staining**

Tibias were collected immediately following sacrifice and fixed with 4% formalin at room temperature for 24 h, and then decalcified in 0.5 M EDTA (pH = 8.0) and embedded in paraffin, which was cut into 3 μm sections and mounted on glass slides for staining by immunoperoxidase, and the procedures of immunohistochemical staining for NFATc1 (cat. no. sc-7294; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were performed as previously described [22]. The pictures were visualized under a microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany). Image Pro-Plus 6 software (Media Cybernetics, Inc., Rockville, MD, USA) was used for the analysis of the integral optical density (IOD) of NFATc1 IHC positive staining.

Sections at 3 μm were cut and stained with an H&E staining kit (Beyotime Institute of Biotechnology, Haimen, China) and visualized under a microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany).

**Ca levels in serum and urine**

The levels of calcium (Ca) and creatinine (Cre) in serum or urine were measured by kits (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China) according to the manufacturer’s protocol.

**Ca content in tibia**

Ca content in the tibia was incinerated using a muffle furnace (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 800°C for 12 h, and then 10 mg of bone ash was dissolved in 1 ml of 37% HCl diluted with Milli-Q® water. The calcium content was determined using a kit (Nanjing Jiancheng Biology Engineering Institute) according to the manufacturer’s protocol.

**ELISA assays for calciotropic hormones**

Serum intact parathyroid hormone (PTH) was measured using chemiluminescence immunoassays (Roche Diagnostics, Mannheim, Germany). Serum calcitonin (CT) was measured using

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**Table 1. Primers for PCR assays**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-193-3p</td>
<td>ACACCTCAGCTGGGAAGCTAAGGCCTACAAAGT</td>
<td>TGCGTGCTGGAGAGTGC</td>
</tr>
<tr>
<td>U6</td>
<td>CGCTTCGGCAAGCATATATAA</td>
<td>TATGGAGCCTTCAGAATTTC</td>
</tr>
<tr>
<td>OPG</td>
<td>CACACACACTGGGAGCTCTG</td>
<td>CAGCTCTGAGAGGAGGAAAGG</td>
</tr>
<tr>
<td>RANKL</td>
<td>CAGGCCCTCTTCTTGAGC</td>
<td>GACTGTCACCCCTTCATA</td>
</tr>
<tr>
<td>Ctsk</td>
<td>AGGGAGGGTGCCCCG</td>
<td>CAGTCGACCCCTTCAGGT</td>
</tr>
<tr>
<td>TRAP</td>
<td>GCTACTTGGGGTICATGGA</td>
<td>TGCGTATTCTGGGGGCTATTCT</td>
</tr>
<tr>
<td>Car2</td>
<td>CATTACGTGACAGGCGAGCA</td>
<td>GACGCCAGTTGTCCACCACAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CACCATGGGAGAGGCCCTAGG</td>
<td>GACGGACAGCATGGGGGTAG</td>
</tr>
</tbody>
</table>
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ELISA kit (LifeSpan BioSciences, Inc., Seattle, WA, USA). Serum 1,25(OH)_{2}VD_{3} was measured using ELISA kit (Elabscience Biotechnology, Wuhan, China) with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA), according to the manufacturers’ protocols.

Statistical analysis

Data are presented as the means ± standard error of the mean. Statistical analysis was performed using SPSS Statistics version 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student’s t-test was used to analyze differences between two groups. Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. \( P < 0.05 \) was considered significant.

Results

NFATc1 is a direct target of miR-193-3p

To investigate post-transcriptional regulatory mechanism by miRNAs in OVX-induced osteoporosis by targeting NFATc1, we used Targetscan (http://www.targetscan.org) to predict the binding sites of miRNAs in 3′-UTR regions of NFATc1. We found that a conserved complementary pairing region was identified between NFATc1 and miR-193-3p, as shown in Figure 1A. To show whether miR-193-3p directly targeted NFATc1, we transfected miR-193-3p mimics or negative control miR-Con sequences into C3H10T1/2 cells, and a luciferase reporter assay was performed. The results demonstrated that the luciferase activity was reduced by ~60% in C3H10T1/2 cells co-transfected with plasmids containing WT 3′-UTR of NFATc1 and miR-193-3p mimics; however, miR-193-3p mi-
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miR-193-3p regulates Ca homeostasis in OVX-induced osteoporotic mice

To investigate the role of miR-193-3p on Ca²⁺ metabolism, we measured the levels of Ca²⁺ in serum, urine, and tibia. Results demonstrated a decrease in serum Ca²⁺ and an increase in urinary Ca²⁺ of OVX mice, and the Ca²⁺ content inhibited by over-expression of miR-193-3p by binding to its 3'-UTR.

miR-193-3p regulates NFATc1 expression in OVX-induced osteoporotic mice

To investigate the function of miR-193-3p in OVX-induced bone resorption, mice underwent sham operation, or OVX-operated mice received Agomir-Con or Agomir-miR-193-3p treatment by tail vein injection. Our results revealed that miR-193-3p was significantly decreased in the tibia of OVX-operated mice compared with that of the sham group (Figure 2A). However, the expression of miR-193-3p was markedly elevated in the tibia of OVX-operated mice injected with Agomir-miR-193-3p compared with OVX-operated mice injected with Agomir-Control (Figure 2A). Moreover, IHC staining, RT-qPCR, and western blotting showed that after mice were operated on by OVX, the mRNA and protein of NFATc1 were increased in the tibia, whereas Agomir-miR-193-3p injection had the ability to reverse OVX-induced up-regulation of NFATc1 (Figure 2B-D). All these studies confirmed that injection of Agomir-miR-193-3p could up-regulate the expression of miR-193-3p and suppress NFATc1 expression in vivo.

Figure 2. miR-193-3p inhibits NFATc1 expression in OVX-induced osteoporotic mice. After injection of Agomir-miR-193-3p into OVX mice, the expression levels of miR-193-3p in the tibia were detected using RT-qPCR (A); IHC staining (B), RT-qPCR (C), and western blotting (D) were performed to measure the expression levels of NFATc1 in the tibia. n = 6 in each group, *P < 0.05.
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in treated tibia was dramatically reduced compared with that of the sham group. However, Agomir-miR-193-3p injection markedly up-regulated serum Ca\(^{2+}\) and inhibited OVX-stimulated Ca\(^{2+}\) loss (Figure 3A).

To investigate whether miR-193-3p improved Ca dyshomeostasis by regulating calcitropic hormones levels, three main calcitropic hormones, including PTH, CT and 1,25(OH)\(_2\)VD\(_3\) in serum were measured using ELISA. Serum PTH was elevated approximately 3-fold in OVX mice, whereas CT and 1,25(OH)\(_2\)VD\(_3\) were down-regulated in serum from OVX mice. The OVX mice being injected with Agomir-miR-193-3p resulted in a decrease of serum PTH and an increase of serum CT and 1,25(OH)\(_2\)VD\(_3\) compared with that of OVX mice treated with Agomir-Control (Figure 3B).

miR-193-3p inhibited bone resorption in OVX-induced osteoporotic mice

To investigate the role of miR-193-3p in OVX-induced bone resorption, H&E staining was performed to assess the change of the trabec-
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Figure 4. miR-193-3p inhibits bone resorption in OVX-induced osteoporotic mice. After injection of Agomir-miR-193-3p into OVX mice, H&E staining was performed to assess the change of the trabecular bone microstructure in the proximal epiphysis of the tibia (A); osteoclast differentiation-related factors, OPG and RANKL, and osteoclast-specific genes, Ctsk, TRAP and Car2, were measured using RT-qPCR (B). n = 6 in each group, *P < 0.05.

Discussion

Recently, there has been a growing number of publications focusing on the function of miRNAs in the regulation of bone homeostasis [14, 15, 23]. There is clear evidence that miRNAs contribute to osteoblast or osteoclast differentiation in accelerating or suppressing bone formation [14, 15, 23]. For example, miR-34b/c and miR-214 decrease bone mass by inhibiting osteoblastogenesis and bone formation [14, 24, 25]. In contrast, miR-34a and miR-503 block osteoporosis by suppressing osteoclastogenesis [15, 23]. These findings indicate that multiple miRNAs may be involved in bone resorption and remodeling by targeting different genes and signaling pathways.

In our study, we identified that miR-193-3p was markedly reduced in the tibia of OVX mice compared with that of the sham group. Gain-of-function of miR-193-3p treatment markedly reversed OVX-induced down-regulation of OPG/RANKL ratio and abolished OVX-induced upregulation of osteoclast-specific genes in the tibia (Figure 4B).

Certain in vivo and in vitro evidence strongly suggests that NFATc1 pathway as a downst-
靶标miRNAs与miRNAs介导的破骨细胞分化有密切关系[26-28]。过表达miR-7b或miR-30a部分抑制RANKL介导的破骨细胞分化在骨髓单核细胞（BMMs）中由DC-STAMP介导的抑制[NFATc1]通路[26, 27]。此外，miR-124调节破骨细胞分化由NFATc1介导的抑制机制[28]。这些结果表明NFATc1直接或间接参与miRNAs介导的破骨细胞分化。在本研究中，生物信息学和实验测量结果证实NFATc1是miR-193-3p的直接靶标。过表达miR-193-3p抑制NFATc1的mRNA和蛋白表达在活体中和体内。在OVX小鼠中，与miR-193-3p表达降低有关，NFATc1的mRNA和蛋白表达在胫骨显著升高。然而，miR-193-3p过表达缓解OVX诱导的钙排泄和骨丢失并抑制NFATc1和破骨细胞特异性基因Ctsk, TRAP和Car2的表达。

Ca²⁺是骨架的必要成分，血清Ca²⁺水平和Ca²⁺排泄与雌激素的稳态相关，涉及肾脏、肠和骨[29]。高钙尿症在雌激素缺乏的绝经后女性中已被报道，并由HTR[30]。在OVX小鼠或大鼠中，低钙血症和高钙尿症是雌激素缺乏的响应[31, 32]。一致的是，我们发现血清Ca²⁺降低和尿液Ca²⁺升高在OVX小鼠中。更重要的是，miR-193-3p过表达有抑制OVX诱导的钙代谢失衡，以及潜在的机制被调节，至少部分地，通过调节钙调素激素水平。

破骨细胞特异性基因Ctsk, TRAP和Car2，是破骨细胞分化级联反应的组成部分，在OVX小鼠中，破坏了骨小梁骨微结构。这可能与NFATc1, Ctsk, TRAP,和Car2的降低有关。在结论中，我们发现miR-193-3p过表达缓解了OVX诱导的骨丢失。miR-193-3p在骨质疏松症中起着关键作用，可能是一个缓解骨质疏松症的治疗策略。

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Disclosure of conflict of interest

None.

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