MicroRNA-148a inhibition protects against ovariectomy-induced osteoporosis through PI3K/AKT signaling by estrogen receptor α

YU XIAO, BING LI and JUN LIU

Department of Joint Surgery, Tianjin Hospital, Tianjin 300211, P.R. China

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Abstract. The present study aimed to investigate the effect of microRNA-148a downregulation on osteoporosis by using an ovariectomized rat model. Reverse transcription-quantitative polymerase chain reaction was used to analyze microRNA-148a expression levels, MTT and flow cytometry assays were used to examine cytotoxicity and apoptosis, respectively. The gap-associated proteins were quantified using western blotting. The expression of microRNA-148a was significantly increased in osteoporosis rat following ovariectomy. Overexpression of microRNA-148a significantly promoted apoptosis and inhibited cell growth, whereas downregulation of microRNA-148a significantly reduced apoptosis and increased cell growth. Overexpression of microRNA-148a significantly reduced estrogen receptor α (ERα) protein expression and suppressed phosphoinositoide-3-kinase regulatory subunit 1 (PI3K) and phosphorylated-protein kinase B (AKT) protein expression in osteoblasts in vitro. The inhibition of ERα increased the microRNA-148a effect on apoptosis in osteoblasts in vitro. Subsequently, LY294002, an PI3K inhibitor, significantly increased the effect of microRNA-148a on apoptosis in osteoblasts in vitro. The findings of the present study revealed that anti-microRNA-148a protected cells against ovariectomy-induced osteoporosis through ERα by PI3K/AKT signaling.

Introduction

Osteoporosis is a metabolic bone disease that is characterized by lower bone mass and bone microstructure and leads to the increase of osteopathsorosis and increased fractures (1). National Institutes of Health defines osteoporosis as a bone disease which the damages bone strength, leads to degeneration of bone tissue microstructure and an increased risk of fractures (2). Osteoporosis may be divided into two types, primary and secondary: Primary osteoporosis occurs in aging patients and is associated with the reduction of hormone secretion and accounts for ~90% of cases (1), whereas secondary osteoporosis accounts for 10% of cases (3). Ovariectomy-induced osteoporosis (OIO) is the most common type of osteoporosis. In postmenopausal osteoporosis, bone formation and bone resorption is imbalanced, and occurs due to reduced estrogen levels following menopause (3,4). Clinical characteristics of postmenopausal osteoporosis include chronic pain in lumbar spinal cord and limbs, humpback, shortened height and fractures in skeleton, vertebra and forearm (5).

The common factors contributing to osteoporosis pathogenesis may be divided as follows: i) Internal secretion; ii) nutritional iii) physical; iv) immune; and v) genetic factors. The reduction of estrogen levels in internal secretion is the primary factor for the occurrence of osteoporosis following menopause (6). Various drugs have various disadvantages, reduced calcium absorption or they may lead to the increase of osteopathsorosis, particularly in estrogen replacement therapy (7). Therefore, there is a great potential to investigate the possible targets of anti-bone absorption drugs. The osteoprotegerin/receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL) system is the primary determinant of bone mass and is a promising target (7,8).

Osteoporosis is a bone metabolism obstructive disease of the whole body, which is characterized by damaging bone tissue microstructure, thickening the bone cortex, reducing the number of trabecula and increasing bone fragility. Osteoblasts and osteoclasts regulate bone formation and bone resorption, respectively (9). The dynamic balance between them maintains normal bone mass (10). As the regulatory factors of bone metabolism, phosphoinositide-3-kinase regulatory subunit 1 (PI3K) and protein kinase B (AKT) may promote osteoblast precursors to differentiate into mature osteoblasts and advocate bone formation (11).

MicroRNAs (miRNAs) are a class of single-strand non-coding RNAs with short sequence. They are extensively distributed in animals and plants, where they have important roles (12). miRNA is a small regulatory molecule at the post-transcription level (13). It may specifically bind to the 3'-untranslated region of target mRNA; therefore, it may regulate the expression of multiple genes. Therefore, it is involved in multiple biological processes, including cell development, differentiation, apoptosis and tumor genesis (13). Intracellular miRNAs expression may be changed to some extent under disease conditions. Changes in miRNA expression may lead to regulatory functional changes at the post-transcription level (14). Therefore, they

Correspondence to: Dr Yu Xiao, Department of Joint Surgery, Tianjin Hospital, 406 Jiefang South Road, Tianjin 300211, P.R. China E-mail: xewu429529@163.com

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may induce a series of biological functional changes in cells. A recent study revealed that miRNAs may regulate biological characteristics of multiple immune cells (13). They may have an important influence on the regulation of the immune function of body. Therefore, to investigate the effect and mechanism of microRNA-148a protection against ovariectomy-induced osteoporosis the present study used in vitro and in vivo models.

Materials and methods

Experimental design and diet. Female Sprague-Dawley rats (6-weeks old; weight, 180-230 g, n=20) were purchased from Animal Experiment Center of Tianjin Medical University and were housed in polycarbonate cages at (22±2˚C) with 55±5% relative humidity, 12-h light/dark cycle with free access to food and water for an adaptation period of 3 days. The experimental protocol was approved by the Animal Care and Use Review Committee of Tianjin Hospital (Tianjin, China). Rats were anesthetized with 2% of isoflurane (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and ovaries were removed bilaterally. Rats were randomly divided into two treatment groups: i) Sham-operated group (control); and ii) ovariectomized rats (model). Rats were anesthetized with 2% of isoflurane (Sinopharm Chemical Reagent Co., Ltd.), and peripheral blood was gathered from caudal vein.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was prepared from blood samples using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). Total RNA (1 µg) was reverse-transcribed at 37˚C for 30 min and 85˚C for 1 min to synthesize complementary cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen ; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the following primers: miR-148a RT-primer, 5'-GCT AGT TGTTCTGAGACACTCCG-3' and U6 RT-primer, 5'-GTCGTTGAGAGTCGAGAGT-3'. qPCR was performed using the SYBR Premix Ex Tag kit (Takara Bio, Inc., Otsu, Japan) by an ABI 7500 Sequencing Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were: 95˚C for 3 min; 95˚C for 30 sec and 60˚C for 30 sec. The following primers were used for qPCR: MicroRNA-148a forward, 5'-GCTGATGTTCTGAGACACTCCG-3' and reverse, 5'-GTCGAGGTCCAGATTTCGACTGGTACGACAACAAAGTT-3' and U6 forward, 5'-GCTCGGCAAGGACTCCGATATCTAAAT-3' and reverse, 5'-GCCTTCAGCAATTTGC-3'. The relative expression was analyzed by the 2-ΔΔCT method (15).

H&E staining. The caput femoris (n=6/group) was collected and fixed with 10% paraformaldehyde for 72 h at room temperature. Formalin-fixed paraffin-embedded sections of caput femoris were sectioned at 10 µM and stained with H&E for 30 min at room temperature. Tissue samples were observed using an Olympus Fluorescent photomicroscope (Olympus Corporation, Tokyo, Japan).

Cell culture and transfection. MC3T3-E1 cells were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and at 37˚C with 5% CO₂. MC3T3-E1 cells treated with RANKL (50 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) for 4 days. MC3T3-E1 cells (1x10⁶ cell/well) were transfected with 100 nM microRNA-148a (5'-GAGGCAAGTCTGAGA-3' and 5'-AGAAGCACTGTC-3'), 100 nM anti-microRNA-148a (5'-CTCCGGTTCAGACTCTGGC-3' and 5'-TCTTGAGAACAGAGGAGA-3'), and negative mimics (5'-CCCCCCCC-3') using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.). MC3T3-E1 cells were treated with RANKL (50 ng/ml; R&D Systems, Inc.) for 3 days, 6 h after transfection. ERα inhibitor, AZD9496 (0.1 nM; MedChemExpress, Shanghai, China) was added to transfected cells for 24 h at 37˚C.

Cytotoxicity assay. Following transfection at 24, 48 and 72 h the cells were seeded onto 96-well plates at a density of 3x10⁴ cells/well. The cells were stained with MTT solution for 4 h and dimethyl sulfoxide was added into the wells for 20 min. The absorbance was detected using a FlexStation 3 Multi-Mode microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 492 nm.

Apoptosis assay. Cells were seeded onto 6-well plates at a density of 1x10⁶ cells/well 48 h after transfection. The cells were then stained with Annexin-V and propidium iodide assay kit (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min. The apoptotic rate was quantified using C6 flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.1 (FlowJo; Tree Star, Inc., Ashland, OR, USA).

Western blotting and caspase-3/9 activity levels. Protein was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Total protein was quantified using Enhanced BCA Protein Assay kit (Beyotime Institute of Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were: 95˚C for 10 min, followed by 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec. The following primers were used for qPCR: MicroRNA-148a forward, 5'-GCTGATGTTCTGAGACACTCCG-3' and reverse, 5'-GTCGAGGTCCAGATTTCGACTGGTACGACAACAAAGTT-3' and U6 forward, 5'-GCTCGGCAAGGACTCCGATATCTAAAT-3' and reverse, 5'-GCCTTCAGCAATTTGC-3'. The relative expression was analyzed by the 2-ΔΔCT method (15).

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Western blotting and caspase-3/9 activity levels. Protein was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Total protein was quantified using Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). Equal quantity (50 µg) of total protein was resolved by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk in tris-buffered saline with 0.1% Tween-20 for 1 h at 37˚C and incubated with various primary antibodies: Bcl2-associated X (Bax; 1:500; sc-6236; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), phosphorlated (p)-AKT (cat. nos. sc-293125 and sc-7985-R, 1:500; Santa Cruz Biotechnology, Inc.), phosphorylated (p)-Akt (cat. nos. sc-293125 and sc-7985-R, 1:500; Santa Cruz Biotechnology, Inc.) and GAPDH (cat. no. sc-25778; 1:2,000; Santa Cruz Biotechnology, Inc.) at 4˚C overnight and subsequently incubated with an anti-mouse immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (cat. no. AAT-16440; 1:5,000; Amoyjet Scientific Inc.) for 1 h at 37˚C. The membranes were visualized using enhanced chemiluminescence kits (GE Healthcare, Chicago, IL, USA) and assessed by densitometry using MacBiophotonics ImageJ version 1.41a (https://imagej.nih.gov/ij/).

Equal quantity (50 µg) of total protein was used to quantify caspase-3/9 activity levels using commercial kits (C1116 and C1158; Beyotime Institute of Biotechnology). The absorbance was detected using a Flexstation 3 Multi-Mode microplate reader at 405 nm.
Luciferase reporter assay. mRNA of ERα 3'UTR was inserted downstream of the luciferase reporter gene in a pMIR REPORT vector (Thermo Fisher Scientific, Inc.). HEK293 cells (1x10^5 cell) were co-transfected with miR-148a mimic and ERα-3'UTR (Promega Corporation, Madison, WI, USA) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Corporation) after 48 h of incubation at 37˚C with 5% CO₂. The ratio of Renilla luciferase of firefly luciferase was calculated for each well.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical significance was determined using Student's t-test or one-way analysis of variance which was followed by Tukey's Honest Significant Difference as a post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of microRNA-148a in osteoporosis rats following ovariectomy. RT-qPCR was used to analyze the changes of
microRNAs in osteoporosis rats following ovariectomy and it was determined that microRNA-148a expression level was significantly upregulated in the osteoporosis rats following ovariectomy when compared the sham group (Fig. 1).

**Effects of microRNA-148a on bone cell growth in vitro.** The function of microRNA-148a on bone cell growth in osteoblasts in vitro was investigated using microRNA-148a and anti-microRNA-148a mimics to upregulate and downregulate microRNA-148a expression in osteoblasts in vitro (Fig. 2). Overexpression of microRNA-148a inhibited bone cell growth and induced apoptosis in osteoblasts in vitro (Fig. 2C and E). However, downregulation of microRNA-148a promoted bone cell growth and reduced apoptosis in osteoblasts in vitro (Fig. 2D and F). Additionally, overexpression of microRNA-148a induced Bax protein expression and caspase-3/9 activity in osteoblasts in vitro (Fig. 3A-D). Downregulation of microRNA-148a reduced Bax protein expression and caspase-3/9 activity in osteoblasts in vitro (Fig. 3E-H).

**Effects of microRNA-148a on bone cell growth in vitro through PI3K/AKT signaling by ERα.** The present study investigated the effect of microRNA-148a on bone cell growth in vitro, and the effect of ovariectomy-induced osteoporosis association with ERα. Bioinformatics and luciferase reporter assays revealed that ERα was a target gene of microRNA-148a (Fig. 4A) and overexpression of microRNA-148a reduced and reduced ERα, PI3K and p-Akt protein expression levels in osteoblasts in vitro (Fig. 4B-E). However, the downregulation of microRNA-148a upregulated ERα, PI3K and p-Akt protein expression levels in osteoblasts in vitro (Fig. 4F-I).

**Inhibition of ERα increases the effect of microRNA-148a on apoptosis in osteoblasts in vitro.** In order to investigate the underlying effect of microRNA-148a on osteoporosis, the present study used ERα inhibitor, 0.1 nM of AZD9496, to reduce reduced ERα expression in osteoblasts in vitro. As presented in Fig. 5A-D, the inhibition of ERα (BHPI, inhibitor used) reduced ERα, PI3K and p-Akt protein expression in osteoblasts in vitro following microRNA-148a transfection when compared with the microRNA-148a only group. The inhibition of ERα increased the effect of the microRNA-148a the inhibition of osteoblast growth and activation of apoptosis in osteoblasts in vitro when compared with the microRNA-148a only group (Fig. 5E and F). Additionally, the inhibition of ERα increased Bax protein expresion and caspase-3/9 activity in osteoblasts in vitro (Fig. 6).
Inhibition of PI3K reduces the effect of microRNA-148a on osteoblast apoptosis in vitro. In order to investigate whether the effect of microRNA-148a on osteoblast apoptosis in vitro by PI3K/AKT signaling the present study used an PI3K inhibitor to reduce PI3K/AKT signaling in osteoblasts in vitro. As presented in Fig. 7A–C, the PI3K inhibitor reduced PI3K/AKT signaling in osteoblasts in vitro following microRNA-148a transfection when compared with microRNA-148a only group. Subsequently, the inhibition of PI3K reduced the effect of microRNA-148a on the inhibition of osteoblast proliferation and increased the apoptotic rate in osteoblasts in vitro when compared with the microRNA-148a only group (Fig. 7D and E). The inhibition of PI3K also increased Bax protein expression levels and caspase-3, caspase-9 activity in osteoblasts in vitro (Fig. 7F-I).

Discussion

Patients with osteoporosis worldwide have exceeded 200 million, including 80% of patients with postmenopausal osteoporosis and fractures in 50% of postmenopausal women are associated with osteoporosis (7). In addition, a previous study revealed that the annual bone loss rate of women after 5-10 years of menopause is 2.4-4% and which is evidently higher...
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than 1% estimated in men (6). OIO is a common disease which frequently occurs in the elderly. The commonest one is postmenopausal osteoporosis (16). As the global population is aging the morbidity of osteoporosis is growing at an alarming rate. Therefore, prevention of osteoporosis may become an important research topic in the future (17). To the best of our knowledge, the present study is the first to demonstrate that microRNA-148a expression was upregulated in osteoporosis rats following ovariectomy.

Thousands of miRNAs have been previously discovered in various organisms with increasingly intensive research on miRNA (12). miRNAs regulate various activities in
organisms, such as growth, development and aging (18). It has been estimated that ~50% human genes are regulated by miRNAs. This is currently verified by using bioinformatics and experiments where one miRNA may specifically bind to multiple genes (18). Additionally, one gene may also be regulated by multiple miRNAs, thus exerting the regulatory function (19). The present study determined that overexpression of microRNA-148a inhibited osteoblast proliferation and induced apoptosis of osteoblasts in vitro.

Although instruments and methods used for detecting the degree of osteoporosis quantitatively domestically and overseas are constantly improving constantly, there is a lack of effective measures in terms of prevention, additionally drug treatments are very limited (20). Currently, ERα remains to be the primary target for the prevention of bone loss in postmenopausal women (21). A previous study revealed that ERα may prevent the occurrence of postmenopausal osteoporosis (22). The present in vitro study revealed that overexpression of microRNA-148a reduced ERα, PI3K and p-AKT protein expression levels in osteoblasts in vitro. Ma et al previously reported that microRNA-148a suppresses estrogen induced viability and migration through ERα expression in breast cancer cells (23). Zhang et al suggested that microRNA-148a promotes cancer cell growth by targeting PI3K/Akt protein expression in osteosarcoma (24).

PI3K in the signaling pathway required for osteoblast differentiation. PI3K stimulates mesenchymal stem cells (MSCs) to differentiate into the osteoblasts and promote bone formation (25). In MSCs, activating PI3K/AKT signal pathway may reduce bone morphogenetic protein-induced alkaline phosphatase (ALP) and osteopontin expression, in order to impact osteoprogenitor cells and reduce osteoblasts (10). PI3K kinase-specific inhibitor prevents the PI3K signaling pathway from excitation. A previous study used ALP staining to observe changes of preosteoblastic cell MC3T3E1 differentiation and revealed that blocking the excitation of the signal pathway may limit ALP activity. Based on the findings of the current study it is possible that the inhibition of ERα or PI3K may significantly increase the effect of microRNA-148a on apoptosis of osteoblasts in vitro.

In conclusion, the present study demonstrated that microRNA-148a significantly increased apoptosis in ovariectomized rats via PI3K/AKT signaling. Therefore, microRNA-148a/PI3K/AKT signaling pathway is a promising candidate for the...
development of future therapeutic agents against osteoporosis in postmenopausal women.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YX designed the experiment, analyzed the data and wrote the manuscript. YX, BL and JL performed the experiment.

Ethics approval and consent to participate

The experimental protocol was approved by the Animal Care Committee of Tianshan Hospital (Tianjin, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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