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MicroRNA-539 promotes osteoblast proliferation and differentiation and osteoclast apoptosis through the AXIN1-dependent Wnt signaling pathway in osteoporotic rats

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Abstract
This study aims to explore the effects of miR-539 on osteoblast proliferation and differentiation and osteoclast apoptosis in a rat model of osteoporosis, and its mechanism involving the regulation of the AXIN1-mediated wingless-Int (Wnt) signaling pathway. A rat model of osteoporosis was successfully established by ovariectomy. With osteoblasts and osteoclasts of rats not receiving ovariectomy in the sham group as control, those of osteoporotic rats were treated with miR-539 inhibitor, miR-539 mimic, and AXIN1 shRNA. The expression of miR-53, AXIN1, the Wnt pathway related-genes, apoptosis related-genes, and osteogenic markers were measured by RT-qPCR and Western blot analysis, respectively. Alkaline phosphatase (ALP) activity in osteoblast and tartrate-resistant acid phosphatase (TRAP) activity in osteoclasts were determined after cell transfection. Osteoblast and osteoclast viability was assayed by CCK-8 assay. Cell cycle and apoptosis of osteoblasts and osteoclasts were detected by flow cytometry. Lastly, alizarin red S staining was used to detect mineralized nodules of osteoblasts. Firstly, we determined that miR-539 was down-regulated in osteoblast and osteoclast of osteoporotic rats and AXIN1 was negatively regulated by miR-539. Additionally, overexpression of miR-539 increased the expressions of β-catenin, LEF1, c-myc, cyclin D1, RUNX2, BGP, BMP-2 in osteoblast as well as β-catenin, RhoA, caspase-3, and Bcl-2 in osteoclasts. Finally, overexpression of miR-539 elevated ALP activity, proliferation, and mineralized nodules in osteoblast and osteoclast apoptosis, with reduced TRAP activity in osteoclasts. Our results demonstrate that miR-539 promotes osteoblast proliferation and differentiation as well as osteoclast apoptosis through the AXIN1-dependent Wnt signaling pathway in osteoporotic rats.

KEYWORDS
axis inhibition protein 1, microRNA-539, osteoblast, osteoclast, osteoporosis, wingless-int signaling pathway
1 | INTRODUCTION

Osteoporosis, a public health problem worldwide as well as a great socioeconomic burden, is considered as a systemic skeletal disease with a low bone mass resulting from an increase in bone resorption.1 This kind of disease mainly results in fragility fractures at typical sites such as the lumbar spine, femoral neck, and the distal radius, which presents a close correlation with a dysmotility and increasing morbidity and mortality.2 According to the statistics, 30-50% of females and 15-30% of males suffer from fragility fracture due to osteoporosis in their lifetime.3 The pain that osteoporosis patients suffers from is associated with bone resorption despite the absence of fractures.4 Bone homeostasis is maintained through the balance between bone resorption by osteoclasts and bone formation by osteoblasts, while imbalance of this process can cause osteoporosis.5,6 The major treatments of osteoporosis are anabolic therapies, which inhibit bone resorption and/or stimulate bone formation through activation of bone modeling, with a prevention of both vertebral and non-vertebral fractures, but given the limitations of current anti-osteoporosis drugs, it still needs efforts for new therapeutics of osteoporosis.7 Therefore, the mechanisms that regulate communication between osteoclasts and osteoblasts are critical to osteoporosis treatments and bone cell biology.

MicroRNAs (miRNAs, miRs), a class of noncoding RNAs 19-25 nucleotides in length, have a regulatory function of gene expression at the post-transcriptional level in skeletal development.8 Moreover, miRNAs play significant roles in physiology and developmental processes such as cell proliferation, differentiation, apoptosis, survival, and pathogenesis in a variety of diseases.9 According to previous studies, a series of miRNAs play crucial roles in the regulation of bone formation and resorption as well as acting as key controllers in the mediation of important transcription factors of osteogenesis.9–11 In addition, miRNAs also function as regulators in bone development and homeostasis, with the involvement of signaling pathways.12 Furthermore, there are previous studies in which miR-539 functions as a suppressor in cell function in multiple kinds of diseases such as osteosarcoma.13,14 However, the mechanism of miRNAs, especially miRNA-539, with tremendous therapeutics potential regulating bone biology has not been explored yet.15 Axis inhibition protein 1 (AXIN1) is considered as one of the degradation complexes of the canonical Wingless-Int (Wnt) signaling pathway (AXIN, APC, GSK3β) and associated with the chondrocyte maturation during endochondral bone formation.16,17 Therefore, the present study is to explore the mechanism of miR-539 on osteoblast proliferation, differentiation, and osteoclast apoptosis through the Wnt signaling pathway by targeting AXIN1 in osteoporosis.

2 | METHODS AND MATERIALS

2.1 | Ethics statement

The study was carried out with the approval of the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (2015-09). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2 | Experimental animals and model establishment

Thirty healthy specific pathogen-free (SPF) female rats (age: 12 weeks; weight: 180-200 g) were purchased from Hunan Normal University (Changsha, China). All rats were housed under specific conditions: at room temperature (21°C), ad libitum access to water and food and in a 12-h light/dark cycle. They were randomly assigned into model (rats receiving ovariectomy) and sham (rats not receiving ovariectomy) groups, with 15 rats in each group. Rats in each group were anesthetized with 2% pentobarbital sodium (45 mg/kg). In the model group, the anesthetized rats were treated with aseptic surgery. An incision at the 1/3 site of bilateral lower abdominal was made, and bilateral ovarian of rats was completely removed and the incisions were closed with a hemostatic suture. In the sham group, rats were removed of the mesentery, with weight equal to ovarian, and then the same procedures were conducted as the model group. Ten to twelve weeks later, rat models of osteoporosis were established successfully, when bone density of rats in the model group was significantly decreased and there was no difference in the bone density in the sham group than that before surgery.

2.3 | Construction of AXIN1-shRNA vector

AXIN1-short hairpin RNA (shRNA) was purchased from Invitrogen Company (Carlsbad, CA). AXIN1-shRNA was induced into the lentivirus-based RNA interference (RNAi) vector pGCSIL-GFP through the endonuclease sites of Age I and EcoR I and then transfected into competent cell of DH5α. The positive clones were screened out for subsequent experimentation.

2.4 | Cell culture and grouping

Twelve weeks after model establishment, the rats in the sham and model groups were sacrificed. Rat skullcap was collected, disinfected by 75% ethanol and washed with Dulbecco's modified Eagle's medium (DMEM). Next, the samples were cut into pieces and treated with trypsin for 3 min, followed by collagenase digestion four times. Subsequently, the samples
were added with phosphate buffer saline (PBS) for the termination of digestion, centrifuged at 1000 r/min for 10 min for cell precipitation purpose, washed twice with PBS, and then incubated with DMEM containing β-glycerophosphate (β-GP) for the induction of osteoblasts. After incubation for 4-7 days, the samples were placed in a culture flask and sub-cultured at 37°C, with saturated humidity and 5% CO₂.

The femur of model rats was collected and the soft tissue on the surface was removed. Cells were scrapped from the inner surface of marrow cavity to femur and filtrated using a screen mesh (300 meshes). Then, the cells were centrifuged at 1000 r/min for 10 min (at 4°C), with the removal of the supernatant, and treated with trypsin containing ethylenediamine tetraacetic acid (EDTA). After washing in PBS, the cells were added with DMEM solution for the termination of digestion, incubated with 1,25-dihydroxyvitamin D3 (10⁻⁸ M) to induce osteoclasts for 3 days at 37°C with 5% CO₂.

Osteoblasts and osteoclasts of model and sham rats were grouped into control (rats in the sham group not receiving ovariectomy), blank (rats in the model group, with no treatment), negative control (NC; rats in the model group transfected with miR-539 inhibitor NC), miR-539 inhibitor (rats in the model group transfected with miR-539 inhibitor), miR-539 mimic (rats in the model group transfected with miR-539 mimic), AXIN1 shRNA (rats in the model group transfected with AXIN1 shRNA vector) and AXIN1 shRNA + miR-539 inhibitor (rats in the model group transfected with AXIN1 shRNA vector and miR-539 inhibitor) groups.

2.5 | Dual-luciferase reporter gene assay

The software including TargetScan and miRanda were applied for the predication of binding site of miR-539 in the 3'-untranslated regions (3' UTR) of AXIN1.

Osteoblasts of model rats were assigned into miR-539 NC + AXIN1 3'UTR WT (co-transfected with wild-type AXIN1-Luc and miR-539 NC plasmid), miR-539 NC + AXIN1 3'UTR Mut (co-transfected with mutant AXIN1-Luc and miR-539 NC plasmid), miR-539 mimic + AXIN1 3'UTR WT (co-transfected with AXIN1-WT-Luc and miR-539 mimic plasmid), and miR-539 mimic + AXIN1 3'UTR Mut (co-transfected with AXIN1-Mut-Luc and miR-539 mimic plasmid) groups. Based on the above potential targets, wild-type AXIN1 3'UTR and mutant AXIN1 3'UTR DNA sequences were designed and synthesized by Beijing Genomics Institute (BGI) in Shenzhen, China. A large segment of pMIR-reporter luciferase vector was collected through electrophoresis of Hind III and Pme I enzyme digestion and wild-type AXIN1 3'UTR and mutant AXIN1 3'UTR DNA sequences were added with the endonuclease sites of Hind III and Pme I. Then, Ligase 4 connects the target gene with the vector, and the lentiviral vector was transfected into DH5α competent cells. The positive clones were screened out and named AXIN1-WT-Luc and AXIN1-Mut-Luc, followed by transfection into cells according to the Lipofectamine 2000 instructions. Forty days later, the luciferase activity at 560 nm was tested by the Firefly Luciferase Reporter Gene Assay Kit (RG005, Beyotime Biotechnology Co., Shanghai, China) and microplate reader (MK3, Thermo Fisher Scientific Inc., Waltham, MA).

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

Total RNA of the osteoblasts and osteoclasts of the rat was extracted using Trizol kit (RP2401, Beijing BioTek Corporation, Beijing, China). The concentration and purity of total RNA were detected using ultraviolet spectrophotometry and agarose gel electrophoresis. The RNA of osteoblasts and osteoclasts in each group (1 μl each) was used as a template for RT reaction into cDNA using the RT-PCR kit (12574026, Thermo Fisher Scientific Inc.), with the reaction condition shown as follows: pre-denaturation at 45-60°C for 15-30 min, at 94°C for 2 min; 40 cycles of 94°C denaturation for 15 s, 55-66°C annealing for 30 s, 68°C extension for 1 min; a final 68°C extension for 5 min. The reaction system was as follows: 25 μl of 2 × Reaction Mix, 1 μg total RNA, 10 μM forward primer, 10 μM reverse primer, 2 μl SuperScript™ III RT/Platinum™ Taq Mix, and autoclaved distilled water, with a total volume of 50 μl. RT-qPCR was performed by PCR kit (11732020, Thermo Fisher Scientific Inc.) with the reaction condition presented as follows: pre-denaturation at 50°C for 15 min and 95°C for 2 min, followed by 40 cycles of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 68°C extension. The reaction system was shown as follows: 100 μl SuperScript™ III RT/Platinum™ Taq Mix; 2 × 1.25 μl of Reaction Mix (a buffer containing 0.4 mM of each dNTP and 6 mM MgSO₄), 1 μl Magnesium Sulfate (MgSO₄, 50 mM), and 100 μl ROX™ Reference Dye (25 μM). All the primers were synthesized by Beijing Genomics Institute (BGI). The 2−ΔΔCt method was used for the quantitative analysis. The primers are listed in Table 1.

2.7 | Western blot analysis

Total protein of the osteoblasts and osteoclasts of the rats in each group was extracted using RIPA buffer (P0013B, Beyotime Biotechnology Co.). The protein samples (30 μg) were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane. Next, the membranes were blocked in 5% skimmed milk for 2 h and then incubated with diluted primary antibodies of goat
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**Table 1**: Primer sequences of related genes for reverse transcription-quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-539</td>
<td>F: 5′-CGCGCGGGGAGAGATATTCCT-3′ R: 5′-GTTCAGGTGGGAGAGGCT-3′</td>
</tr>
<tr>
<td>AXIN1</td>
<td>F: 5′-GAAGACGGCCATCTCAG-3′ R: 5′-GGATGTCTTCAGGCTTCT-3′</td>
</tr>
<tr>
<td>β-catenin</td>
<td>F: 5′-ATCACTGAGGCTGGAATCTG-3′ R: 5′-GGTGCAAAGGCTTCCCATT-3′</td>
</tr>
<tr>
<td>LEF1</td>
<td>F: 5′-CCGGAAGGAAGGGCGATTATGCT-3′ R: 5′-GCTCTGTGAGGTTGCTTGTCT-3′</td>
</tr>
<tr>
<td>c-myc</td>
<td>F: 5′-CTACACAGCACAGAGAAGC-3′ F: 5′-CGTCGGAAGTGCAAGAT-3′</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>F: 5′-GCGTACCTGACAACACTC-3′ R: 5′-GCTCAGGAGCAAAGACG-3′</td>
</tr>
<tr>
<td>Runx2</td>
<td>F: 5′-CACGCGGGGAGAGATATTCCT-3′ R: 5′-TTTCTACAACGGGAGGCATTTC-3′</td>
</tr>
<tr>
<td>BGP</td>
<td>F: 5′-TAACTGAGGCTGGAATCTG-3′ R: 5′-ATTCACCACTTATCTGAGGC-3′</td>
</tr>
<tr>
<td>BMP-2</td>
<td>F: 5′-CCGGAAGGAAGGGCGATTATGCT-3′ R: 5′-ACGGCAAGGCTGCAAGAT-3′</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>F: 5′-CGCTGCGCTGCAAGAAGC-3′ F: 5′-CCGCAGGCTGCAAGAAGC-3′</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>F: 5′-CTTCCAGGCTGCAAGAAGC-3′ R: 5′-CATTCAGGCTGCAAGAAGC-3′</td>
</tr>
<tr>
<td>RhoA</td>
<td>F: 5′-GTTCAGGCTGCAAGAAGC-3′ R: 5′-CTGAGAATGCTGCAAGAAGC-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5′-AGGCTGAGATCCAGATTGCT-3′ F: 5′-TGCCAACAGATGCTTGTCT-3′</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; miR-539, microRNA-539; AXIN1, axis inhibition protein 1; LEF1, lymphocyte enhancer-binding factor 1; RUNX2, runt-related protein 2; BGP, biliary glycoprotein; BMP-2, bone morphogenetic protein-2.

2.8 **Alkaline phosphatase (ALP) activity detection**

Rat osteoblasts at 1, 3, 5, 7, and 9 d after culture were collected, followed by discarding the culture medium and three washes with PBS. After that, the osteoblasts were cultured with Triton X-100 (2 mL/L) in a fridge at 4°C overnight. Next day, the osteoblasts were reacted with 150 μL ALP substrate at 37°C for 30 min. The reaction was terminated by adding KOH solution (0.1 mol/L). The absorbance at 410 nm of the samples was measured using a microplate reader (MK3, Thermo Fisher Scientific Inc.).

2.9 **Tartrate-resistant acid phosphatase (TRAP) activity detection**

Rat osteoclasts in each group were collected and after discarding culture medium, they were washed twice with PBS and lysed with 0.2% Triton-100. Then, osteoclasts were reacted with TRAP (P0332, Beyotime Biotechnology Co.) at 37°C for 30 min. The reaction was terminated by adding 50 mL NaOH solution (0.1 N). The absorbance at 405 nm of the samples was determined using a microplate reader (MK3, Thermo Fisher Scientific Inc.).

2.10 **Cell counting kit-8 (CCK-8) assay**

Rat osteoblasts at 1, 3, 5, 7, and 9 d after culture were collected. The CCK-8 kit (C0038; Beyotime Biotechnology Co.) was used for the determination of osteoblast proliferation. Then, the absorbance at 450 nm of the samples was measured using an automatic quantitative plotting microplate reader (MK3, Thermo Fisher Scientific Inc.).

2.11 **Flow cytometry**

The flow cytometry (AP002, Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China) was applied for the determination of osteoclast apoptosis. Cell culture medium was collected into a centrifuge tube. Osteoclasts were washed with PBS, treated with trypsin for 3 min, followed by trypsin removal, an addition of previously collected cell culture medium and centrifugation at 1000×g for 5 min. Cells were resuspended with 400 μL binding buffer and stained by 5 μL Annexin-V-FITC avoiding light at room temperature for 15 min. After being counterstained with 10 μL propidium iodide (PI), the cells were treated with an ice bath avoiding light for 5 min. Within 30 min, FITC and PI were detected using a flow cytometry (FC 500, Beckman Coulter, Krefeld, Germany) at the excitation wavelength of 488 nm and emission wavelength of 530 nm, and at the wavelength of more than 575 nm, respectively. The percentage of apoptotic cells in total cells was calculated.
PI single staining was performed to detect cell cycle. After absorption of cell culture medium, osteoblasts were treated with trypsin for 3 min and washed twice with PBS. After discarding the supernatant, osteoblasts were fixed with 1 mL 70% pre-cold ethanol at 4°C overnight. After being washed by PBS, cells were centrifuged at 1000 rpm for 5 min, resuspended with 0.5 mL PBS containing PI and RNase (at a final concentration of 50 μg/mL) and then incubated at 37°C for 30 min. Cell cycle was analyzed at the excitation wavelength of more than 575 nm using the flow cytometry (FC 500, Beckman Coulter). The percentage of cell cycle was calculated.

2.12 ALP staining

Osteoblasts at 5 d after culture were collected and the cell culture medium was discarded. After being washed twice with PBS, cells were fixed with 10% neutral formalin for 30 min and washed twice with PBS. Subsequently, ALP staining was performed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) kit. The cells were then added with BCIP/NBT avoiding light for 3-10 min, washed in running water to terminate coloration, washed twice with PBS, and dried overnight. The mineralized nodules were observed under a microscope.

2.13 Alizarin red S staining

Alizarin red S staining was used for staining mineralized nodules of osteoblasts: osteoblasts at 18 d after consecutive culture were collected and the cell culture medium was discarded, followed by two washes with PBS. Next, the osteoblasts were fixed with 95% ethanol, followed by ethanol removal and additional two washes with PBS. The osteoblasts were then stained with 2 mL 0.2% alizarin red S staining for 30 min, slightly rinsed in running water, mounted with a neutral gum and observed under an optical microscope.

2.14 Statistical analysis

SPSS 21.0 software (IBM Corp. Armonk, NY) was applied for data analysis. Measurement data was expressed as mean ± standard deviation. All experiments were conducted at least three times. Comparison between two groups was conducted by t-test. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups. P < 0.05 was considered to be statistically significant. P < 0.01 was considered to be extremely statistical significant.

3 RESULTS

3.1 AXIN1 is a target gene of miR-539

Initially, we performed bioinformatics prediction to explore the binding site of miR-539 and AXIN1. Based on TargetScan and miRanda, the binding site of miR-539 to AXIN1 3′UTR was predicted to be UAAAGAG (Figures 1A and 1B). Therefore, miR-539 might interfere with the expression of AXIN1 through this site. In addition, dual-luciferase reporter gene assay was conducted, which revealed that AXIN1 was a target of miR-539 (Figure 1C): compared with the miR-539 NC + AXIN1 3′UTR WT group, the luciferase activity in the miR-539 mimic + AXIN1 3′UTR WT group was significantly lower (P < 0.05), while there were no significant differences in the luciferase activity in the miR-539 NC + AXIN1 3′UTR WT group and miR-539 NC + AXIN1 3′UTR WT group.

![FIGURE 1 AXIN1 is a target gene of miR-539 by the target prediction program and determination of luciferase activity. A, B, miRanda and TargetScan predict that miR-539 binds to AXIN1 3′UT; (C) the luciferase activity is decreased after treatment by a combination of miR-539 mimics and AXIN1-3′UTR-WT, suggesting that miR-539 regulates AXIN1. *P < 0.05 versus the miR-539 NC + AXIN1 3′UTR WT group. miR-539, microRNA-539; AXIN1, axis inhibition protein 1; WT, wild-type; Mut, mutant; NC, negative control.](image-url)
mimic + AXIN1 3’UTR Mut and miR-539 NC + AXIN1 3’ UTR Mut groups (both $P > 0.05$). Taken together, AXIN1 might be a potential target gene of miR-539.

3.2 | Overexpression of miR-539 activates the Wnt signaling pathway and affects osteogenic markers and apoptosis-related genes in osteoclasts

Subsequently, we investigated relative expression of miR-539, the Wnt signaling pathway-related genes in osteoblasts and osteoclasts, and osteogenic markers and apoptosis-related genes of osteoclasts of rats in each group using RT-qPCR and Western blot analysis. The results of relative expression of miR-539 and the Wnt signaling pathway-related genes in osteoblasts and osteoclasts are shown in Figure 2. Compared with the blank group, there were significant differences in expressions of miR-539 and the Wnt signaling pathway-related genes in osteoblasts and osteoclasts in the NC group (all $P < 0.05$). Compared with the blank group, there was no difference in miR-539 expression in osteoblasts and osteoclasts in the NC and AXIN1 shRNA groups ($P > 0.05$); the miR-539 mimic group showed increased miR-539 expression ($P < 0.05$) and the miR-539 inhibitor and AXIN1 shRNA + miR-539 inhibitor groups showed nearly no miR-539 expression ($P < 0.05$). AXIN1 was considered as one of the degradation complexes of the Wnt signaling pathway (AXIN, APC, GSK3β). In osteoblasts and osteoclasts, there was no difference in AXIN1 expression among the blank, NC, and AXIN1 shRNA + miR-539 inhibitor groups. Compared with the blank group, the mRNA and protein expressions of AXIN, APC, GSK3β were down-regulated in the AXIN1 shRNA group and much more down-regulated in the miR-539 mimic group, but up-regulated in the miR-539 inhibitor group (all $P < 0.05$). Compared with the blank group, the miR-539 mimic and AXIN1 shRNA groups showed increased mRNA and protein expression of β-catenin, LEF1, c-myc, cyclin D1, RUNX2, BGP, and BMP-2 in osteoblasts and increased

![Figure 2](image-url)
mRNA and protein expression of β-catien and RhoA in osteoclasts (all \( P < 0.05 \)); the miR-539 inhibitor group showed the opposite trend; the difference was not significant, however, in the NC and AXIN1 shRNA + miR-539 inhibitor groups (\( P > 0.05 \)). The mRNA expression of osteogenic markers and apoptosis-related genes of osteoclasts are shown in Figure 3. Compared with the control group, the relative mRNA and protein expressions of osteogenic markers and apoptosis-related genes of osteoclasts were of significant difference in other groups (all \( P < 0.05 \)). There was no remarkable difference in the mRNA expression of osteogenic markers RUNX2, BGP and BMP-2 and apoptosis-related genes of osteoclasts caspase-3 and Bcl-2 among the blank, NC, and AXIN1 shRNA + miR-539 inhibitor groups (all \( P > 0.05 \)). The above indexes were obviously increased in the miR-539 mimic and AXIN1 shRNA groups but decreased in the miR-539 inhibitor group in comparison to the blank group (\( P < 0.05 \)). In conclusion, overexpression of miR-539 activates the Wnt signaling pathway and affects osteogenic markers and apoptosis-related genes in osteoclasts.

### 3.3 Overexpression of miR-539 augments ALP activity in osteoblasts

Moreover, we detected ALP activity in osteoblasts (Figure 4). The results showed that ALP activity changed with time. ALP activity was remarkably increased when osteoblasts grew to the stage of extracellular matrix maturation after 1-day proliferation but decreased due to mineralization after 7-day proliferation. Compared with the control group, ALP activity was significantly reduced in other groups (all \( P < 0.05 \)). At day 7, ALP activity in miR-539 mimic group was the highest in comparison to the other groups (\( P < 0.05 \)). There was no significant difference in ALP activity among the blank, NC, and AXIN shRNA + miR-539 inhibitor groups (all \( P > 0.05 \)). Compared with the blank group, ALP activity was elevated in the miR-539 mimic and AXIN1 shRNA groups but declined in the miR-539 inhibitor group (all \( P < 0.05 \)). The aforementioned results demonstrated that overexpression of miR-539 could augment ALP activity in osteoblasts.

**FIGURE 3** Relative mRNA and protein expression of osteogenic markers and apoptosis-related genes in osteoclasts after transfection using RT-qPCR and Western blot analysis. A. Relative mRNA expression of osteogenic markers; (B) relative protein expression of osteogenic markers; (C) relative mRNA expression of apoptosis related-genes in osteoclasts; (D) relative protein expression of apoptosis-related genes in osteoclasts; *\( P < 0.05 \) versus the control group. \( P < 0.05 \) versus the blank group. The data analyzed by one-way ANOVA. The experiment was independently repeated three times. shRNA, short hairpin RNA; NC, negative control; miR-539, microRNA-539; AXIN1, axis inhibition protein 1; LEF1, lymphocyte enhancer-binding factor 1; RUNX2, runt-related protein 2; BGP, biliary glycoprotein; BMP-2, bone morphogenetic protein-2
Overexpression of miR-539 represses TRAP activity in osteoclasts

Next, we detected TRAP activity in osteoclasts after transfection, the results of which are shown in Figure 5. The TRAP activity changed with the time. From the first day to the fifth day of culture, TRAP activity was sharply elevated following osteoclast proliferation, but reduced following osteoclast apoptosis after the ninth day. Compared with the control group, TRAP activity was significantly increased at each day after culture in other groups (all \( P < 0.05 \)). In comparison to the blank group, TRAP activity was increased in the miR-539 inhibitor group but declined in the miR-539 mimic and AXIN1 shRNA groups (all \( P < 0.05 \)). Meanwhile, the suppression of miR-539 almost inhibited the reduction of TRAP activity induced by AXIN1 silencing. The above results demonstrated that overexpression of miR-539 could repress TRAP activity in osteoclasts.

Overexpression of miR-539 promotes osteoblast proliferation

In order to identify the effects of miR-539 on osteoblast proliferation, CCK-8 assay was performed. The results of CCK-8 assay are shown in Figure 6. Compared with the control group, osteoblast proliferation was significantly decreased in other groups (all \( P < 0.05 \)). No significant difference was shown in osteoblast vitality among the blank, NC, and AXIN1 shRNA + miR-539 inhibitor groups (\( P > 0.05 \)). The osteoblast proliferation was inhibited in the miR-539 inhibitor group in comparison to the blank group (\( P < 0.05 \)), while the osteoblast proliferation was promoted in the AXIN1 shRNA and miR-539 mimic groups (both \( P < 0.05 \)). From all the above findings, overexpression of miR-539 could promote osteoblast proliferation.

miR-539 arrests cell cycle of osteoblasts at G2/M stage

For exploration purposes, investigating whether miR-539 could alter cell cycle of osteoblasts after transfection, Annexin-V-FITC/PI double staining with flow cytometry was conducted. The results of PI single staining were shown in Figure 7. Compared with the control group, the other groups had more osteoblasts at G1 stage and less osteoblasts at S and G2-M stages (all \( P < 0.05 \)). Compared with the blank group, more osteoblasts at G1 stage entered into the S stage.
and the G2-M stage in the miR-539 mimic and AXIN1 shRNA groups (both $P < 0.05$). In the miR-539 inhibitor group, the number of osteoblasts at G1 stage was increased, but that at the S stage and G2-M stage was decreased ($P < 0.05$). Besides, AXIN1 knockdown decreased the effect on cell cycle caused by over-expressed miR-539 ($P < 0.05$). All the obtained results revealed that overexpression of miR-539 could arrest cell cycle of osteoblasts at G2-M stage.

### FIGURE 6

Overexpression of miR-539 promotes osteoblast proliferation. Osteoblasts treated with miR-539 mimic and AXIN1 shRNA show significantly increased proliferation on 1, 3, 5, 7, 9 d after treatment. $^aP < 0.05$ versus the control group. $^bP < 0.05$ versus the blank group. The data are analyzed by one-way ANOVA. The experiment was independently repeated three times. shRNA, short hairpin RNA; miR-539, microRNA-539; AXIN1, axis inhibition protein 1; NC, negative control.

#### 3.7 Overexpression of miR-539 accelerates cell apoptosis of osteoclasts

Furthermore, in order to identify the influence of miR-539 on cell apoptosis of osteoclasts, another Annexin-V-FITC/PI double staining with flow cytometry was performed. The results are shown in Figure 8. Compared with the control group, cell apoptosis of osteoclasts was obviously decreased in other groups (all $P < 0.05$). The miR-539 mimic and AXIN1 shRNA groups showed higher apoptosis rate of osteoclasts, while the miR-539 inhibitor group had lower apoptosis rate compared with the blank group (both $P < 0.05$). There was no difference in cell apoptosis among the blank, NC, AXIN1 shRNA + miR-539 inhibitor groups (all $P > 0.05$). All the obtained data indicated that overexpression of miR-539 could accelerate cell apoptosis of osteoclasts.

#### 3.8 Overexpression of miR-539 enhances ALP activity of osteoblast

An investigation was conducted regarding miR-539 and its effects on osteoblast activity using ALP staining. The BCIP was hydrolyzed due to catalysis of ALP enzyme and the obtained product with strong reactivity could react with NBT to produce insoluble and dark blue or royal purple NBT-formazan as shown in Figure 9. Compared with the control group, ALP activity of osteoblast was obviously decreased in other groups (all $P < 0.05$). Compared with the blank group, ALP activity of osteoblast increased in the miR-539 mimic and AXIN1 shRNA groups but decreased in the miR-539 inhibitor group with significant bone formation. There was no difference in ALP activity of osteoblast among the blank, NC, and AXIN1 shRNA + miR-539 inhibitor groups (all $P > 0.05$).

### FIGURE 7

miR-539 arrests cell cycle of osteoblasts at G2-M stage. A, cell cycle mapping of osteoblast determined by flow cytometry; (B) the histogram comparing the cell cycle distribution; $^aP < 0.05$ versus the control group. $^bP < 0.05$ versus the blank group. The data are analyzed by one-way ANOVA. The experiment was independently repeated three times. shRNA, short hairpin RNA; miR-539, microRNA-539; AXIN1, axis inhibition protein 1; NC, negative control.
The above results indicated that overexpression of miR-539 could enhance ALP activity of osteoblast.

3.9 Overexpression of miR-539 induces mineralized nodules of osteoblasts

At last, we used alizarin red S staining to explore whether miR-539 could affect mineralized nodules of osteoblasts after 18 d of consecutive culture. The results showed that all the seven groups had mineralized nodules stained orange red. Compared with the control group, mineralized nodules of osteoblasts were obviously decreased in other groups (all $P < 0.05$). Compared with the blank group, mineralized nodules of osteoblasts increased in the miR-539 mimic and AXIN1 shRNA groups but decreased in the miR-539 inhibitor group (all $P < 0.05$). There was no difference in mineralized nodules of osteoblasts among the blank, NC, and AXIN1 shRNA + miR-539 inhibitor groups (all $P > 0.05$) (Figure 10).

**FIGURE 8** Overexpression of miR-539 accelerates cell apoptosis of osteoclasts. A, osteoclasts in the scatter plots in which the upper left quadrant identifies the necrotic cells (annexin V−/PI+), the upper right quadrant identifies the late apoptotic cells (annexin V+/PI+), the lower left quadrant identifies the live cells (annexin V−/PI−), and the lower right quadrant identifies the early apoptotic cells (annexin V+/PI−). B, histogram of cell apoptosis of osteoclasts. *$P < 0.05$ versus the control group. **$P < 0.05$ versus the blank group. The data are analyzed by one-way ANOVA. The experiment was independently repeated three times. shRNA, short hairpin RNA; miR-539, microRNA-539; AXIN1, axis inhibition protein 1; NC, negative control.

**FIGURE 9** Overexpression of miR-539 enhances ALP activity of osteoblast by ALP staining ($\times 200$, bar = 100 um). A and B, ALP staining and quantitative analysis image reveal that osteoblasts treated with miR-539 mimic and AXIN1 shRNA have enhanced ALP activity of osteoblast, only second to those in the control group. *$P < 0.05$ versus the control group. **$P < 0.05$ versus the blank group. The data are analyzed by one-way ANOVA. The experiment was independently repeated three times. ALP, alkaline phosphatase; shRNA, short hairpin RNA; miR-539, microRNA-539; AXIN1, axis inhibition protein 1; NC, negative control.
The aforementioned results revealed that overexpression of miR-539 could induce mineralized nodules of osteoblasts.

4 | DISCUSSION

Being related to the processes of multiple cancers, miR-539 is demonstrated to be a regulator in cell proliferation and apoptosis, which might be a potential therapeutic target for treatment of various diseases. This study focuses on the effects of miR-539 on osteoblast proliferation and differentiation, and osteoclast apoptosis in osteoporotic rats through the Wnt signaling pathway by binding to AXIN1.

Osteoporosis is characterized by a loss of bone strength, with its main complication in fragility fractures, which results in substantial global morbidity and mortality. The fundamental pathogenetic factors of this disorder are various such as a failure to achieve a skeleton of optimal strength, excessive bone resorption leading to loss of bone mass and the defects in bone formation. Moreover, miRNAs like miR-539 had a significant influence on cell proliferation, metastasis as well as invasion, which might be prospective targets in advancing the treatment for multiple diseases. Similarly, a previous study has provided evidence that miR-539 plays an important role in ameliorating diseases by promoting the expression of target genes. In addition, miRNAs also had potential therapeutic and biomarker functions in maintaining the balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation, thereby treating bone disorders. According to a previous study, the related-gene expression at transcriptional and post-transcriptional levels is closely associated with osteogenesis and osteoblasts differentiation, which play a crucial role in the development of skeletal muscles. In accordance with the findings of our study, miR-539 was overexpressed in rats with osteoporosis and the miR-539 mimic group showed an increased number of rat osteoblasts as well as apoptotic rate of rat osteoclasts compared with the blank group. Thus, miR-539 might play a significant role in the regulation of osteoblast and osteoclast in rats. Also, a recent study has revealed that miRNAs are involved in the regulation of AXIN expression and serve as potential therapeutic targets of diseases. As a multi-domain scaffolding protein, AXIN1 acts not only as a crucial negative regulator of Wnt signaling by the β-catenin destruction complex to enhance cell proliferation, but also a controller of myogenic differentiation. Besides, AXIN1 expression plays a significant role in skeletal and bone development. The results of this study showed that the mRNA and protein expression of AXIN1 in the miR-539 mimic group decreased and AXIN1 was proved a target of miR-539, which suggested AXIN1’s role together with miR-539 in regulating osteoblast proliferation, differentiation, and osteoclast apoptosis in osteoporotic rats.

Another major finding in the present study was that Wnt signaling pathway played a significant role in the mechanism of miR-539 regulating cell growth of osteoblasts and osteoclasts. Wnts consists of secreted, hydrophobic glycoproteins, which play a regulatory role during various development and adult processes such as cellular proliferation, differentiation, and migration. AXIN1 was reported to be a key suppressor of Wnt signaling pathway and an important scaffold for the turnover of β-catenin. With reference to a previous study, miRNAs have regulatory functions on multiple components of Wnt signaling pathway. Also, it has been indicated that the Wnt signaling

![FIGURE 10](image-url)
The Wnt signaling pathway plays an important role in the regulation of both osteoblast and osteoclast differentiation. The crucial step of Wnt signaling pathway is the degradation of cytosolic β-catenin by the APC/AXIN1 destruction complex, and Wnt signaling inhibits β-catenin ubiquitination normally occurring within the complex, resulting in complex saturation through an accumulation of phospho-β-catenin. In accordance with the results of the present study, Wnt signaling pathway was activated by miR-539, and thereby decreasing the expression of its relevant genes like β-catenin, LEF1, c-myc, cyclin D1, RUNX2, BGP, and BMP-2 in osteoblast and β-catenin and RhoA in osteoclast of rats with osteoporosis.

The proteins and targets of Wnt signaling pathway included β-catenin, LEF1, RNUX2, c-myc, cyclin D1, BGP, and BMP-2. The β-catenin is a multifunctional protein, with a regulatory function of cell–cell adhesion at the cell surface. LEF1, one of transcriptional inducers of the Wnt signaling pathway, functions as a suppressor of osteoblast maturation as well as a regulator of RUNX2 protein expression. The experimental results also showed that miR-539 increased ALP activity and decreased TRAP activity, with an enhancement of osteoblasts proliferation and osteoclasts apoptosis. The proliferation related-genes of c-Myc and cyclin D1 demonstrate a significant relationship of cell cycle progression, especially at the G1 to S transition. BMP-2 can activate the Wnt signaling pathway, with a capability of restoring bone defect. Also, BGP and ALP genes play critical roles in osteoblast differentiation. Well-Known as markers of bone formation and disruption, strong ALP and TRAP activity suggests active mineralizing and remodeling as well as mature lamellar bone matrix. According to a previous study, ALP activity was increased with the combination of BMP-2 and dexamethasone synergistically through the activation of signal transducer and activator of transcription 3 (STAT3) signaling pathway in C3H10T1/2 cells. It has been reported that with elevated ALP activity and enhanced mineralization, appropriate plasma irradiation promotes differentiation of osteoblasts, which might be a potential target for bone regeneration. As a proteolytic product, TRAP serves as a suppressor of bone formation by Smad6-mediated RUNX2 inhibition, which suggests that TRAP may function as a mediator of chondrogenic and osteogenic activity, regulating early transcription factors like SOX9 and RUNX2. Taken together the above findings, miRNA-539 might act as an effective regulator in bone resorption and bone formation and enhance osteoblast proliferation and differentiation as well as osteoclast apoptosis.

In conclusion, overexpression of miR-539 in rats with osteoporosis contributed to the promotion of osteoblast proliferation, differentiation and osteoclast apoptosis through the Wnt signaling pathway by binding to AXIN1. Therefore, this study might provide certain value in finding out new molecular targeted therapies for osteoporosis. However, further studies are required to have a full understanding of the specific mechanisms of miR-539 targeting AXIN1 through Wnt signaling pathway by binding to AXIN1 in osteoporosis.

ACKNOWLEDGMENT

We would like show sincere appreciation to the reviewers for critical comments on this article.

CONFLICTS OF INTEREST

None.

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