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MiR-182 antagonist alleviates glucocorticoid-induced secondary bone degeneration and osteoclast differentiation

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ABSTRACT

In this study, the expression of miR-182 in secondary bone degeneration was investigated, and the effect of its antagonist on glucocorticoid-induced osteoclast differentiation and its mechanism was studied. For this purpose, PBMC cell lines were selected for cultivation, and the changes were observed by hematoxylin-eosin (HE) staining. Real-time fluorescence quantitative (qRT-PCR) was used to detect mRNA expression. The protein expressions of RANKL, OPG and CXCL10 were detected by Western blot. CCK-8 and flow cytometry was used to detect cell proliferation and apoptosis. The results showed that protein expression levels of RANKL, OPG and CXCL10 in the miR-182 group were significantly higher than those in other groups (P>0.01). The miR-182 can promote RANK signal transduction in osteoclasts by regulating RANKL/NFκB signaling pathway, accelerating osteoclast proliferation and differentiation, and slowing down the process by miR-182 inhibitor. In general, miR-182 alleviates OP by inhibiting the activity of osteoclast via RANKL/NFκB signaling.

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Introduction

Osteoporosis (OP) is a common chronic bone disease, and osteoblast apoptosis induced by glucocorticoids (GC) is an important pathological mechanism of massive or long-term use of glucocorticoids to lead to osteoporosis (1). At present, after postmenopausal and senile Osteoporosis, the incidence of glucocorticoid-induced Osteoporosis (GIOP) ranks third and ranks first in secondary Osteoporosis (2). As the most widely used and effective anti-inflammatory and immunosuppressant in clinical practice, how to take safe and effective measures to prevent glucocorticoid-induced osteoporosis has become a hot research issue.

Bones are the main connective tissue in the body and are constantly being renewed and remodeled. Osteoporosis is a systemic metabolic bone disease. In the process of bone reconstruction, bone resorption by osteoclasts and bone formation by osteoblasts are very important for maintaining mineral homeostasis and the integrity of the bone structure (3). When this coordination is out of balance and bone resorption exceeds bone formation, osteoporosis is caused (4). It is characterized by decreased bone mass and bone microstructure damage, resulting in decreased bone strength and increased risk of fracture, which seriously affects the health of postmenopausal women and the elderly. In aging and some diseases (such as osteoporosis, periodontitis, periapical periodontitis), the balanced state of bone metabolism is destroyed, and the amount of bone absorption is greater than the amount of bone formation, resulting in bone destruction (5). Studies have shown that the occurrence of osteoporosis is closely related to the regulation of a variety of signaling pathways (6). At present, the drugs used to prevent and treat osteoporosis mainly involve the following two mechanisms: inhibiting bone turnover and promoting protein anabolism. The signaling pathways involved in this process include RANKL, Wnt signaling pathway and cathepsin K. It plays an important role in cell proliferation, apoptosis and differentiation, and is

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closely related to the occurrence and development of bone-related diseases. Although there are many drugs available to treat osteoporosis. However, most osteoporosis drugs still have many disadvantages such as poor safety, large side effects and ineffective reduction of fracture risk. Osteoclasts are derived from blood monocytes and are multinucleated giant cells fused with monocytes. They are small in number and have great effects, and cannot proliferate themselves (7-9).

Micro RNA regulates the expression of protein molecules. It is widely found in the eukaryotic cells of plants and animals and participates in the regulation of biological growth. miRNA can maintain the metabolic balance of bone by regulating biomolecules. Phenotypic differentiation of mesenchymal stem cells ultimately affects metabolic homeostasis and bone formation. miRNA-182 can inhibit osteoblast apoptosis and improve glucocorticoid-induced osteoporosis, but its effect on glucocorticoid-induced osteoblast apoptosis and its specific mechanism is not clear. At present, there are no reports on the function of miRNA-182 in GIOP.

Therefore, in this study, glucocorticoid-induced osteoporosis model was established in rats, and the effect of Mir-182 regulating the RANKL/NFκB signaling pathway on long-term glucocorticoid-induced secondary bone degeneration and osteoclast differentiation was analyzed to study the antagonistic effect of Mir-182 antagonist on glucocorticoid-induced osteoporosis and its possible mechanism and to provide a new reference for the basic research of miRNA-182 and GIOP.

Materials and methods

Reagents and instruments

The used reagents and instruments were ALP staining kit, Caspase-3 activity assay kit, apoptosis detection kit, Total RNA extraction kit and general reverse transcription kit (article Number: BC3830, R1200, RP1105, Beijing Solebao), PCR primers such as miRNA-182 (Shanghai Jima Biological Company),ELISA kit, Trizol total RNA extraction reagent (B511311, Shanghai Shenggong Bioengineering Co., LTD.), Cell Counting Kit-8, RANKL/NFκB signaling pathway activator and inhibitor (P605, SC0330, Shanghai Biyuntian Biotechnology Co., LTD.), Glyceraldehyde-3-phosphatedehydrogenase (GLYCERaldehyde-3-phosphatedehydrogenase), GAPDH, anti-nuclear factor κB receptor activating factor ligand (RANKL), OPG, CXCL10 antibody (item number: AB181602, AB239607, AB73400, AB214668 Abcam), Gel imager, fluorescence quantitative PCR and flow cytometry (Model: VersaDoc 3000, CFX96Touch, ZE5, BIO-RAD, USA) Carbon dioxide cell incubator (Model: Herocell 180, US Thermo), GEHealthcare Locus SP MicroCT, Roche Cobas C311 Automatic Biochemical Analyzer, Biotek Synergy HT Plate Reader (Bio Tek, Winooski, VT,USA).

Cell culture, grouping and treatment

Thawed and revived PBMC cells were inoculated into culture flask containing DMEM medium (supplemented with 10% fetal bovine serum) and routinely cultured in a humidity saturated, 37°C and 5% carbon dioxide incubator. After cell adherence, the cells were digested with 0.25% trypsin.

The experiment was divided into: Control group (NC): normal culture; GIOP group: cultured in medium containing 400 μmol/L GC for 48 h; Mir-182 interference group: culture medium containing 400 μmol/L GC and 5μmol/L Mir-182 for 48 h. Mir-182 antagonistic group cultured in medium containing 400 μmol/L GC and 5μmol/L Mir-182 inhibitor for 48 h; Three replicates were set for each group.

Induction and detection of osteoclasts

PBMC was induced to differentiate into osteoclasts by RANKL reagent. The isolated PBMC was cultured and inoculated into a six-well plate with a cell density of 3×10^4 / well. 30 ng/mL macrophage colony-stimulating factor (M-CSF) was added, and the supernatant was discarded after incubation overnight. 10 ng/mL and 50 ng/mlRANKL reagent were added, respectively, and transferred to 37°C. Then they were cultured in a 5% CO2 incubator. The osteoclasts were identified by HE staining and observed by an inverted light microscope.

After 72 h treatment with lentivirus, cells of each group were collected and then inoculated into 6-well plates at a cell density of 4×10^4 / well. Staining reagent was added according to the requirements of the ALP staining kit, and incubated for 24 h under light protection, and observed under an optical microscope.

Detection of mRNA expression level in cells by real-time fluorescence quantitative PCR

Real-time immunofluorescence quantitative polymerase chain reaction (RT-PCR) was used to detect miRNA expression levels in the two groups. Total RNA was extracted from PBMC cells using the mirVana miRNA extraction kit, and the reverse transcription reaction was carried out after uv spectrophotometer measurement. Reverse transcription and RT-PCR were performed using the Taqman miRNA reverse transcription kit and the RT-PCR detection kit according to the relevant instructions. The reverse transcription reaction conditions were 95°C for 30 s, 95°C for 5 s and 60°C for 34 s, with a total of 40 cycles. PCR reaction was preheated at 95°C for 10 min. Then 40 cycles of denaturation were performed at 95°C, 10 s each, annealing at 60°C for 20 s, and extension at 72°C for 10 s. The amplification and dissolution curves were checked using 7500 System SDS Software and Ct values were automatically analyzed. Three multiple Wells were performed for each sample and each sample was repeated 3 times. Using GAPDH as an internal reference, the mRNA expression levels of RANKL and OPG in PBMC cells were detected by the $2^{-\Delta\Delta Ct}$ method. This process is repeated three times. $2^{-\Delta\Delta Ct}$ < 0.5 was defined as low expression, 0.5~2.0 was defined as normal expression, > 2.0 was defined as high expression.

MiR-182 forward primer for 5'- CTTGCTATACA AGGGCAAGCACGAA-3', reverse primer for 5 '- CTTGAGTACGACCAAATCCCGTC -3.
The GAPDH forward primer was 5'-CTCGCTTCGGCAG-CACA-3 '; The reverse primer was 5'-AACGCTTCACGAATTTG-CGT-3'.
The forward primer of RANKL was 5'-CATCGG GTTCCCATAAAG. The reverse primer is 5'-GAAGCAAATGTTGGCGTA '.
OPG forward primer for 5'- AGCACCCTGTAGG AAACACACCAACT; Reverse primer for 5'- ACTGTCCACCAGAACACTCAGCCAAT'.

Detection of protein expression in cells by western blotting

PBMC cells were collected after treatment, washed with phosphoric acid buffer for two times, and then added cell lysate to extract total protein. The protein samples were mixed with the same volume loading buffer and then placed in a boiling water bath and boiled for 5 min. The denatured protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transformed into membranes. Polyvinylidene fluoride (PVDF) was sealed with 5% skimmed milk powder for 2h, and RANKL, OPG and CXCL10 primary antibodies diluted at the ratio of 1 - 1000 were added and incubated at room temperature for 2h. After incubation at room temperature for 2h with a secondary antibody solution diluted at the ratio of 1 ∶2000, the exposure was developed with a chemical luminescent agent. Using GAPDH as an internal reference, the protein expression levels of RANKL, OPG and CXCL10 in PBMC cells were analyzed by a gel imaging analysis system. This process is repeated three times.

CCK-8 was used to determine cell viability

Follow instructions for the CCK-8 kit. Cells of different transfected groups (5×103) at the logarithmic growth stage were digested by trypsin and cultured in 96-well plates for 24h. At the beginning of the measurement, 10μL CCK-8 solution was added to each well and incubated at 37°C for 2h. The absorbance value of each well was measured at 450nm with an absorbance meter. Cell viability was the absorbance fraction compared with control. Cells in each group were tested repeatedly with 6 reproes.

Cell apoptosis rate detected by flow cytometry

PBMC cells were collected after treatment, and cell precipitation was washed with pre-cooled phosphoric acid buffer. . 600μL 1×Binding Buffer was used to adjust cell concentration. Annexin V-FITC and PI working solution 5μL each were added to 105 cell suspensions. After mixing, the cells were incubated at room temperature under dark conditions for 15 min. The apoptosis rate of PBMC cells was detected by flow cytometry within 60 min.

Statistical Analysis

SPSS 20.0 was used as statistical analysis software, and the measurement data consistent with normal distribution were presented in the form of mean ± standard deviation. The Shapiro-wilk method was used to test the normal distribution of quantitative

data. If the variance of the data is homogeneous, the paired sample T-test is used; those that do not conform to the normal distribution are expressed in the form of the mean (value range), and the Mann-Whitney U test is used for comparison. Analysis of variance and $\chi 2$ test were used to measure the cell viability. Test level α=0. 05. $P < 0.05$ was considered as a statistically significant difference.

Results and discussion

Identification of osteoclasts

HE staining was used to identify osteoclasts and the results showed that monocytes could transform into osteoclasts under RANKL induction. HE staining showed that the number of nuclei was ≥3. As shown in Figure 1.

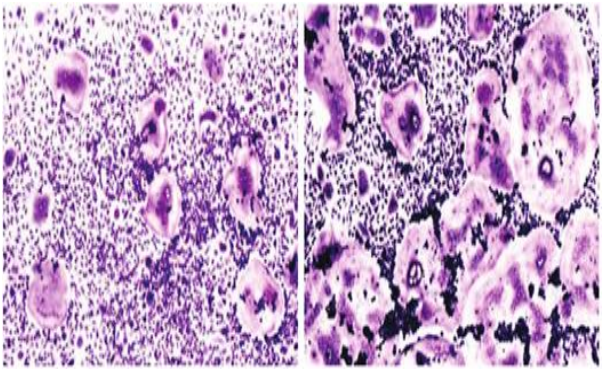

Figure 1. Identification of osteoclasts by HE staining (×200)

Lentivirus transfection

PBMC was transfected with lentivirus-mediated blank and RNA plasmids. The results showed that lentivirus-mediated blank plasmids (left) and RNA plasmids (right) had high transfection efficiency, both > 90%. As shown in Figure 2.

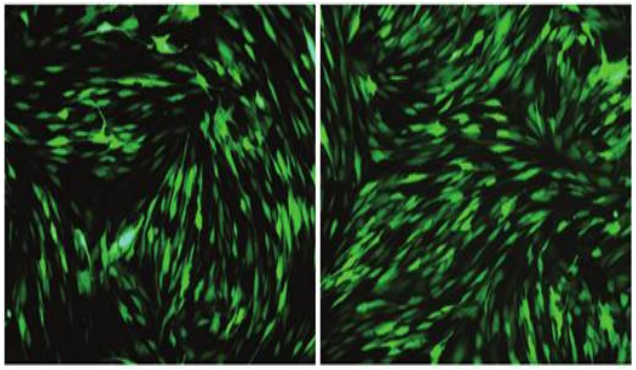

Figure 2. The PBMC transfected with lentivirus（×200）

ALP staining results in PBMCs co-culture system

The results showed that RNA plasmids can promote the osteogenic differentiation of PBMCs. The relative expression of ALP in Mir-182 interference group was 0.51 ± 0.06, which was significantly higher than that in control group (0.21 ± 0.09) and inhibition group (0.28 ± 0.08) (Q = 2.983, 2.895, P = 0.009, 0.012 < 0.05). There was no significant difference between the control group and the inhibition group (P > 0.05), as shown in Figure 3.

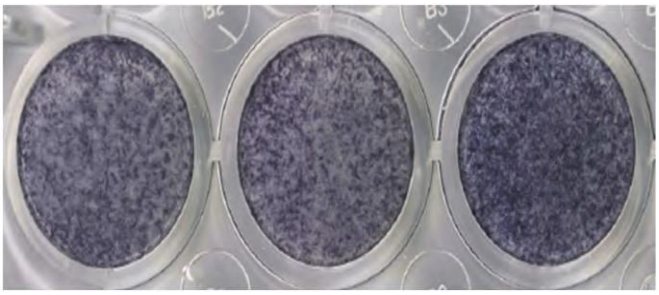

Figure 3. The results of ALP staining. Note: A is NC; B is miR-182 inhibitor C miR-182

Comparison of Mir-182 expression levels in each group

The expression level of the Mir-182 group (0. 527 + 0. 116) was significantly higher than that of the control group (0. 125 + 0. 012) and inhibition group (0. 130 + 0. P<0. 01), there was no statistical difference between control group and inhibition group (P > 0. 05)(Figure 4).

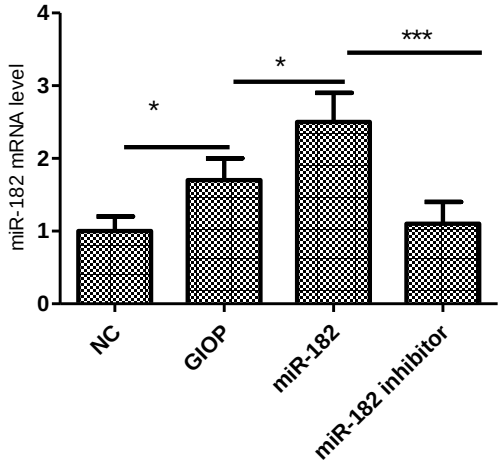

Figure 4. The expression of miR-182 in each group. Note: * P < 0. 05, *** P < 0.05

CCK-8 kit was used to detect cell proliferation

Cck-8 proliferation test results showed that the proliferation rate of PBMC cells increased gradually with the extension of miRNA-182 treatment time, and there was statistical significance in the proliferation rate of PBMCs cells in the three groups at each time point (F = 9.881, P = 0.009 < 0.05). At 48 h, the proliferation rate of PBMCs in the dry disturbance group was significantly higher than that in the control group and inhibition group, and the difference was statistically significant (Q = 2.457, 2.904; P = 0.032, 0.016 < 0.05). As shown in Figure 5.

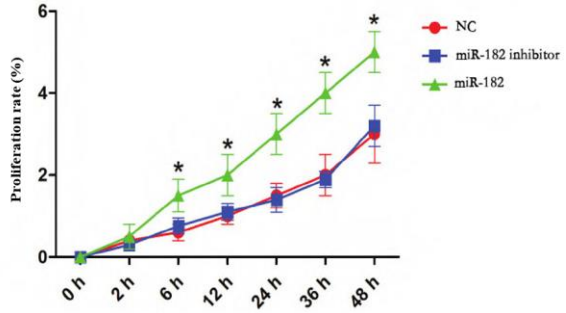

Figure 5. Results of the cell proliferation (*P<0.05)

Western blot detection of protein expression in cells

The protein expression levels of RANKL, OPG and CXCL10 in Mir-182 group were significantly higher than those in other groups (P> 0.01), the levels of RANKL, OPG and CXCL10 in the Mir-182 inhibition group were lower than those in the GIOP group, but higher than those in the control group (P >0.05)(Figure 6).

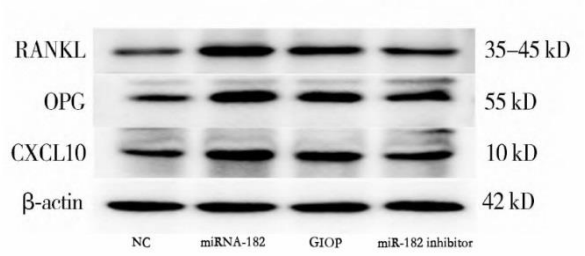

Figure 6. Expression of RANKL、OPG related factors in each group

Comparison of cell survival rate between each group and control group

The survival rate of Mir-182 cells was the lowest among all groups. After mir-182 inhibition, the cell survival rate was significantly increased, and the difference was statistically significant compared with GIOP (P > 0. 05) (Tabe1).

Table 1. Comparison of cell survival rate in each group (n = 10, $\bar{x}\pm s$)

Group	Cell survival rate /%
NC	98. 68±7. 26
miR-182	48. 27±2. 25a
GIOP	71. 12±4. 23b
miR-182 inhibitor	85. 56±5. 30c
F value	148. 364
P value	＜0. 001

Note: compared with control group, A P < 0. B P < 0.05; Compared with GIOP group, C P<0.05.

Comparison of apoptosis rate among all groups

The apoptosis rate of the Mir-182 group was significantly higher than that of the control group (P<0.05), while the apoptosis rate of the Mir-182 inhibition group was significantly lower than that of the Mir-182 group (P<0.05). Compared with the GIOP group, the apoptosis rate of mir-182 was significantly increased after treatment and significantly decreased after mir-182 inhibition (P < 0. 05), as shown in Figure 7.

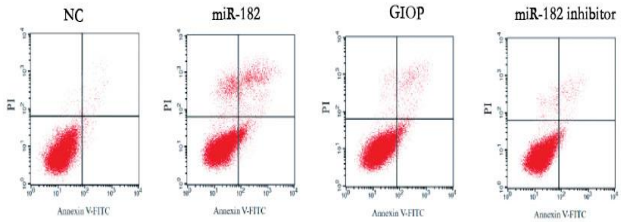

Figure 7. Flow cytometry to detect cell apoptosis in each group

Glucocorticoids are mainly secreted by the adrenal cortex and are widely used in many diseases such as nephrotic syndrome, rheumatoid arthritis (10), rheumatoid polymyalgia, colitis and chronic obstructive pulmonary disease due to their cheapness, anti-inflammatory and immunosuppressive effects. However, improper use can lead to hormonal osteonecrosis, and GIOP is one of the common side effects of GC. Long-term and extensive use of GC can inhibit the proliferation and differentiation of Osteoblast (OB), and GC down-regulates the expression of OPG by inhibiting the β-catenin protein of the Wnt signaling pathway. Thus, the expression of

RANKL and M-CSF can be induced to increase the RATIO of RANKL/OPG, promote bone resorption and OB apoptosis, reduce OB function, and also inhibit the apoptosis of Osteoclast (OC), thus breaking the active homeostasis of Osteoclast and osteoblast. The activity of osteoclasts is significantly increased, which delays and reduces bone formation, resulting in bone loss and ultimately osteoporosis (11, 12).

Mir-182 is a member of the Mir-17-82 family. Mir-182 regulates specific anti-proliferation and/or pro-apoptotic genes [including B-cell chronic lymphocytic leukemia/ lymphoma-2-like 11, BCL2L11) and transforming growth factor-β (TGF-β) expression are beneficial to cancer cells in some environments (13). Adverse prognostic markers were in oral squamous cell carcinoma (14). The presence of exosomes provides a pathway for signal exchange between osteoblasts and osteoclasts. Related studies have shown that osteoblast exosomes contain bone regulation-related proteins, which can stimulate the structure of osteoclasts, thus realizing signal exchange between bone tissue cells. The combination of the Wnt classical signal transduction pathway and rank-RANKL plays an important role in the signal exchange network of osteoblast exosomes regulating the repair of steroid-induced femoral head necrosis (15).

RANKL, a member of the tumor necrosis factor family, regulates the activation of osteoclasts. In this process, the receptor activator of the NF-κB ligand (RANKL) plays an important role (16). When RANKL binds to RANK, the activated RANK sends the stimulation signal to the nucleus via nuclear transcription factor κB, and c-FOS expression is enhanced (17). After binding with recombinant nuclear factor of Activated T-cell cytoplasmic 1 (NFATC), the signal is further transmitted to promote the transcription of osteoclasts. Thus, osteoclasts or their precursors can be induced to differentiate, further promote their differentiation, proliferation and inhibit their apoptosis, and ultimately promote bone resorption (18-20), leading to impaired bone homeostasis caused by reduced osteoblast differentiation (21). Therefore, it is speculated that Mir-182 of osteoclast-derived exosomes can promote bone resorption through the interaction of multiple pathways, and ultimately affect the repair of osteonecrotic tissue. In addition to Mir-182, RANKL was also found to induce the expression of Mir-23a-5p in osteoclast derived exosomes by inhibiting RUNX2 or by yES-associated protein 1. YAP1 inhibits osteoblast activation by activating MT1DP, a long non-coding RNA member of the Metal-Lothioneins (MTs) gene family.

The current cell co-culture system is a more reasonable cell culture mode, which can simulate the microenvironment of cell symbiotic growth to the maximum extent, and thus better evaluate the biological behaviors of cells, such as cell proliferation, apoptosis and differentiation (22,23). In this study, PBMC was purified and induced by RANKL to successfully construct an osteoclast model in vitro. Meanwhile, PBMCs derived from bone marrow were cultured and co-cultured with PBMCs to observe the proliferation, differentiation and apoptosis of PBMCs under co-culture mode. At the same time, the interference plasmid of Mir-182 was constructed and the PBMCs were transfected with lentivirus. The purpose of this study was to explore the effects of Mir-182 on the proliferation, differentiation and apoptosis of PBMCs in the co-culture system. The results indicate that inhibition of Mir-182 can inhibit PBMCs differentiation into bone cells, thus slowing down the formation of bone. These results suggest that Mir-182 can promote bone formation and improve the activity of osteoclasts, thus leading to the occurrence of osteoporosis in patients.

The protein expression levels of RANKL, OPG and CXCL10 in the Mir-182 group were significantly higher than those in other groups ($P>0.01$), compared with the GIOP group, the levels of RANKL, OPG and CXCL10 in the Mir-182 inhibition group were decreased. But compared with the control group, the level was higher and the difference was statistically significant ($P>0.05$). These results indicate that Mir-182 can regulate the RANKL/NFκB signaling pathway, promote the expression of corresponding RANKL, OPG and CXCL10 proteins, and further activate the Wnt signaling pathway to promote the RANK signaling transduction in osteoclasts and accelerate the proliferation and differentiation of osteoclasts. Cck-8 kit detection results showed that Mir-182 can promote the proliferation of PBMCs. The results of the AV-PI kit showed that Mir-182 could inhibit the apoptosis of PBMCs. The results indicate

that Co-culture of PBMCs transfected with Mir-182 can promote the osteogenic differentiation of PBMCs.

In general, after research comparison, it is concluded that: Clinically, the corresponding genetic material Mir-182 content in exosomes can be regulated to activate the Wnt signaling pathway to promote the expression of downstream osteogenic markers and inhibit ranK-RANKL interaction to inhibit the RANK signaling in osteoclasts, slow down the proliferation and differentiation of osteoclasts, and provide effective guarantee for the treatment of patients.

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None.

Interest conflict

The authors declare no conflict of interest.

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