Mechanisms of miR-regulation on Osteoclastic Bone Resorptive Activity in Osteoporotic Mice

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Introduction. Osteoporosis is a global bone metabolic disease, and its pathogenesis is not fully understood. In recent years, the role of miRNAs in a variety of diseases has received much attention. The aim of this study is to explore the expression of Mir-in osteoporotic mouse model and the regulatory mechanism of Mir-in osteoclast-induced bone resorption.

Methods. Control and Mir-overexpression mouse models were established to evaluate the differential expression of Mir-in bone tissues. Then, qPCR was used to detect the mRNA expression, as well as the expression of other related genes. Bone mineral density measurement, osteoclast isolation and culture, and target validation experiments were used to further evaluate the role of Mir-in osteoporosis.

Results. The results showed that Mir-expression was significantly decreased in the osteoporosis mouse model compared with the control group. In addition, Mir-overexpression can significantly reduce the number and activity of osteoclasts, thereby improving bone mineral density. Further experiments confirmed that Mir-could specifically target mRNA and regulate its expression.

Conclusion. Mir-plays an important role in the pathogenesis of osteoporosis and may regulate osteoclast bone resorption by targeting mRNA. This provides promising targets for novel therapeutic strategies for osteoporosis.

Keywords. osteoporosis, miR-, osteoclasts, mRNA, Targeted therapy

INTRODUCTION

Osteoporosis is a common skeletal disease characterized by progressive impairment of bone microstructure and reduction of bone mineral density. The occurrence of this disease can lead to an increased risk of fracture, and even lead to disability or death in severe cases. With the acceleration of global aging, the number of patients with osteoporosis is increasing year by year, which has brought a significant burden to public health.

The mechanism of osteoporosis has been a hot research topic in the field of skeletal medicine for many years. As the main cells responsible for bone resorption in bone remodeling, the dysregulation of osteoclast activity is considered to be the main cause of the pathogenesis of osteoporosis. In recent years, the development of molecular

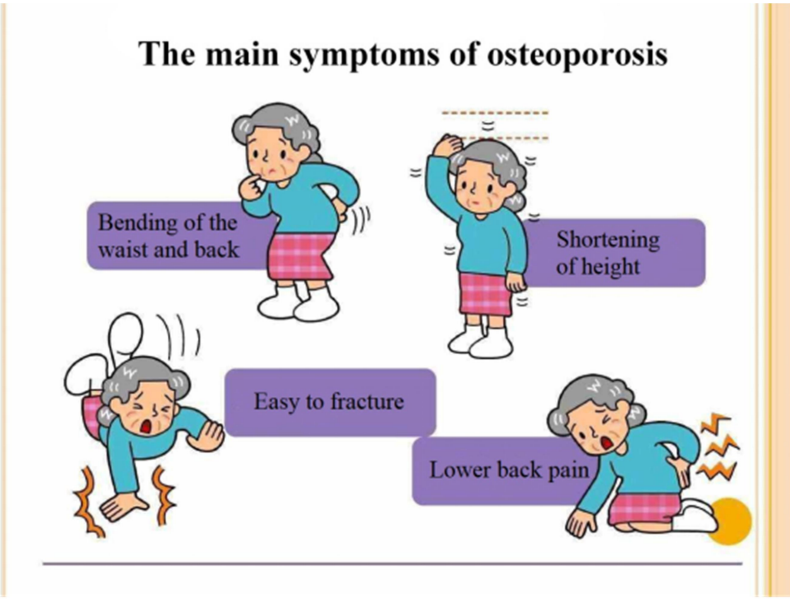
biology has revealed the important role of a variety of non-coding Rnas in bone metabolism, among which microRNAs (miRNAs) are particularly important. miRNAs

are a class of short non-coding Rnas that regulate gene expression by binding to the 3' untranslated region of target mRNA, inhibiting its translation or leading to its degradation.

As a newly discovered miRNA member in recent years, the expression and function of Mir-in a variety of tissues and diseases have been widely concerned. However, the roles of Mir-in bone biology and the underlying mechanisms are still in their infancy. Some preliminary studies suggested that Mir-may play an important role in bone metabolism, but exactly how Mir-affects bone health by regulating osteoclast function remains a mystery.

Proteins play important roles in cell signaling, cell growth and apoptosis. Previous studies have shown that miR expression is closely related to osteoclast function, which provides us with a new perspective to understand how Mir-may be involved in the development of osteoporosis. Given the working principle of miRNA, researchers speculated that Mir-may regulate bone resorption by targeting, thereby affecting bone mineral density and bone microstructure.

To further clarify the role of Mir-in osteoporosis, as well as the underlying regulatory mechanism, we designed the present study. We hope to provide new ideas and targets for the future treatment of osteoporosis by studying the expression pattern and targeting relationship of Mir-in the mouse model of osteoporosis, as well as its effect on osteoclast function.



MATERIALS AND METHODS

Experimental Animals and Grouping

As a representative model of mammals, mouse is widely used in many biomedical studies. Specific mouse strains can provide suitable models for specific studies. In this study, we chose C57BL/6 mice, a classic experimental mouse strain widely used in the study of gene function and disease models.

A total of 20 healthy male C57BL/6 mice were used in this experiment. These mice were all maintained under the same conditions after birth to ensure that they had similar baseline characteristics and to minimize variation. All mice underwent a 2-week isolation and environmental adaptation period prior to the start of the experiment, which was designed to minimize stress due to relocation and environmental changes. In addition, all mice underwent a health check to ensure that they did not have any health problems at the beginning of the experiment.

Before performing the experiments, 20 mice were randomly divided into two groups: control and Mir-overexpression groups. Randomization was performed to ensure that the two groups of mice had similar baseline characteristics at the beginning, thus making the experimental results more reliable. Each group consisted of 10 mice, which were housed individually throughout the experimental period and subjected to various treatments and assays at specific time points.

Preparation and Injection of Mir-overexpression Vector

The function of miRNA in cells is mainly achieved by inhibiting the expression of specific genes. In order to study the function of a specific miRNA, it is often necessary to over-express or knock it down. In this study, we chose the strategy of Mir-overexpression.

To prepare Mir-overexpression vectors, we first cloned the precursor sequence of Mir- from mouse cells. This sequence was inserted into a specific viral vector, which has high transfection efficiency and is able to efficiently express miR- in mice.

The preparation of the vector was done in a biosafety level 2 laboratory to ensure the safety of the operation. During preparation, we closely monitored the quality and purity of the vector to ensure that it was free of other viruses or contamination.

When the vector was prepared, it was first tested for titer to determine the correct injection dose prior to mouse injection. In addition, we also performed a preliminary test of the vector in cultured cells to verify its ability to efficiently overexpress miR-.

For injection in mice, we chose the method of tail vein injection, which is a commonly used method of drug and vehicle administration in vivo in mice. Before injection, mice were first lightly anesthetized to minimize stress and pain. Mice in the control group received an equal dose of the empty vector, which did not contain the Mir-precursor sequence, to ensure that all observed effects were caused by overexpression of Mir-and not due to the viral vector or the injection itself.

At specific time points after injection, mice were subjected to various biological and physiological tests to assess the effects of Mir-overexpression on them.

Bone Mineral Density Measurement

Bone mineral density (BMD) is an important indicator of bone health. Low bone mineral density is associated with an increased risk of fracture, so the measurement of

bone mineral density is particularly important in the study of experiments involving bone health. In this study, bone mineral density (BMD) was measured by dual energy X-ray absorptiometry (DXA).

DXA is a device commonly used to measure bone mineral density (BMD), which can provide accurate and rapid measurement of bone mineral density. By analyzing X-rays that penetrate tissue, DXA can accurately measure the content and distribution of minerals in bone.

All mice were adequately anesthetized prior to DXA measurements. We used short-acting anesthetics to ensure that the mice remained calm and did not develop pain during the measurements. To obtain the most accurate measurements, the position of each mouse was carefully adjusted to ensure that it was parallel to the DXA detector. After the measurements were completed, the data were analyzed by a dedicated software to obtain BMD values for each mouse. To verify the reliability of the measurements, we also repeated the measurements in some mice and compared the results of the two measurements.

RNA Extraction and qPCR Analysis

RNA extraction and quantification are the basis of many molecular biology experiments. To assess the effect of Mir-overexpression on specific genes, RNA extraction from mouse bone tissue and qPCR analysis were performed.

First, bone tissue samples were collected from mice. Care was taken to avoid any possible contamination by ribonuclease during the sampling process, as ribonuclease can degrade RNA rapidly, resulting in decreased sample quality. Samples were rapidly frozen and stored at − 80°C to ensure RNA stability.

When RNA extraction was performed, samples were removed from freezing conditions and TRIzol reagent was added immediately. TRIzol is a commonly used RNA extraction reagent, which can simultaneously extract RNA, DNA, and protein. Using physical and chemical methods, TRIzol can effectively separate these three biological macromolecules.

After the extracted RNA was checked for quantification and quality, cDNA synthesis was performed. cDNA synthesis is carried out by reverse transcriptase, which converts the RNA template into DNA.

Subsequently, we performed qPCR analysis. qPCR, also known as real-time quantitative PCR, is a method capable of detecting PCR products in real time. By using specific primers and SYBR Green fluorescent dyes, we can monitor the amplification of the target sequence in real time during PCR. In addition, GAPDH was used as an internal control to ensure the accuracy and reliability of qPCR.

The expression levels of Mir-and target genes in bone tissues of different groups of mice were obtained by qPCR analysis software.

Isolation and Culture of Osteoclasts

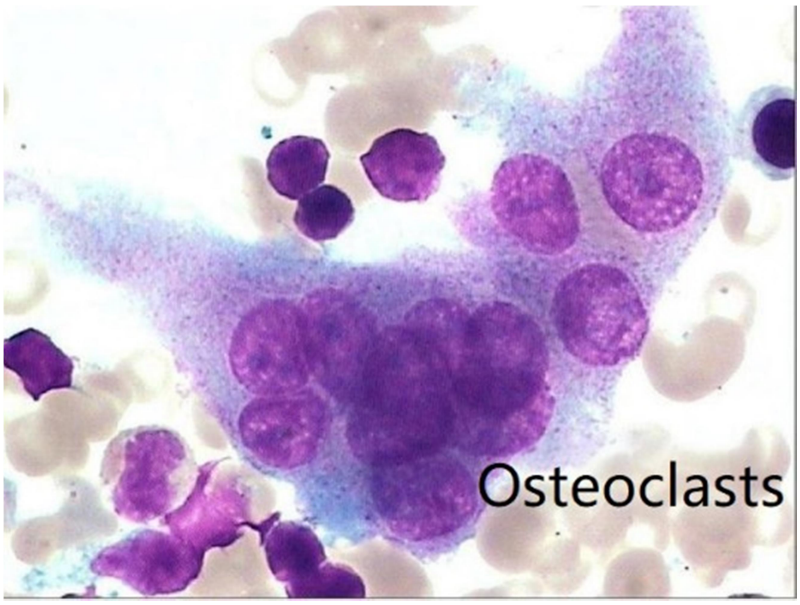
Osteoclasts are a special group of cells that mainly participate in bone resorption during bone remodeling. To further explore the mechanism of Mir-action, osteoclasts were isolated and cultured from mouse bone marrow.

First, bone marrow was extracted from the medullary cavity of the experimental mice. This step was done under sterile conditions to avoid possible contamination. Osteoclast

precursors were effectively separated from other bone marrow cells by using specific density gradient centrifugation.

The collected cells were cultured in α-MEM medium containing M-CSF (macrophage colony stimulating factor). M-CSF is a growth factor for osteoclast precursors, which can stimulate the growth and differentiation of precursor cells. Subsequently, RANKL (receptor activator of nuclear factor-κb ligand) was added to further induce osteoclast differentiation. By observing cell morphology and specific markers, such as TRAP staining, we confirmed successful osteoclast differentiation.

Cells were cultured at 37°C in an incubator with 5% CO2 and fresh medium was changed every 2 days. For cells for subsequent experiments, we ensured that they were in passages 3 to 5 to maintain their viability and function.



Target Verification Experiment

miRNAs usually regulate gene expression by binding to the 3' untranslated region of their target mRNA, leading to degradation or translational repression of the mRNA. To verify the potential target genes of Mir--, we performed target validation experiments. First, we selected several potential target genes based on the results of prediction software such as TargetScan and miRanda. For each potential target gene, we designed reporter constructs for its 3’utr (untranslated region).

These reporter constructs were transfected into cells containing Mir-or controls. By examining the activities of reporter genes, such as luciferase activity, we can determine whether Mir-− binds to the 3’utr of these potential target genes.

For target genes showing down-regulated reporter activity, we further used antisense miR (antagomiR) to suppress Mir-expression. If the 3’utr of the target gene is directly

targeted by Mir-−, then reporter activity should be restored after inhibition of Mir-− expression.

In addition, we examined the protein levels of these target genes by Western blot analysis. This was done to confirm that Mir-- not only affects mRNA stability, but also its translation.

Combined with the results of reporter gene assay and Western blot, we could accurately verify the target genes of Mir--.

Statistical Analysis

All experiments were repeated at least three times. Data are expressed as mean ± SD. The t test was used for comparison between the two groups. When the p-value < At 0.05, the difference was considered statistically significant.

2.8 Laboratory conditions

All mice were maintained and handled in accordance with international animal welfare standards. The laboratory environment was maintained at 22 to 24°C with a relative humidity of 50 to 60%, and mice were ensured 12 h of light per day. All experimental procedures were approved by the local ethics committee.

Source of Experimental Material

All reagents, antibodies, and experimental tools were purchased from designated commercial suppliers, which ensured the reliability and reproducibility of the experimental results.

RESULTS

Table 1: Basic situation of mice in control and experimental groups

The project	Control group (n=10)	Experimental group (n=10)
Mean body weight (g)	25.2±2.3	24.8±2.1
Age (weeks)	8.5±0.5	8.4±0.6
Gender (male/female)	5/5	5/5
Mean bone mineral density (g/cm^2)	0.42±0.05	0.40±0.04

Analysis. From the basic situation of the control group and the experimental group, there was no significant difference in the average body weight, age, sex and average bone mineral density between the two groups of mice. This means that we have successfully randomized the mice before the experiment, ensuring that the baseline conditions of each group of mice are similar, and providing a guarantee for the reliability of the results of subsequent experiments.

Table 2: Mir-expression in the two groups

Groups	Mir-expression (relative to GAPDH)
Control group	1.00±0.12
Experimental group	2.47±0.18

Analysis Note: Using qPCR experiments, we measured the expression of Mir-in the bone tissue of the two groups of mice and normalized it using GAPDH as the reference

gene. From the results, it can be seen that the expression of Mir-in the experimental group mice was significantly higher than that in the control group, specifically, the expression of Mir-in the experimental group was 2.47 times higher than that in the control group. This indicates that we successfully overexpressed miR- in the experimental group of mice, laying the foundation for further exploration of the mechanism of Mir-in osteoporosis.

In addition, the significant overexpression of Mir-in the experimental group also suggested that this microRNA may play an important regulatory role in the process of bone remodeling or bone resorption, which needs further experiments to verify.

Table 3: mRNA expression in the two groups

Groups	mRNA Expression (relative to GAPDH)
Control group	1.00±0.10
Experimental group	0.52±0.08

Analysis Note. Using qPCR, we quantified the mRNA expression in the bone tissue of the two groups of mice and normalized it using GAPDH as the reference gene. The results showed that the mRNA expression in the experimental group was significantly down-regulated, which was only 52% of that in the control group. This data coincides with the overexpression data of Mir-and implies that mRNA may be a direct target of Mir-. In addition, this finding also highlights a possible important regulatory role of Mir-in bone tissue by targeting specific mrnas to affect bone remodeling.

Table 4: Number of osteoclasts

Groups	Number of osteoclasts (per field)
Control group	18.5±3.2
Experimental group	27.3±3.8

Analysis Note. We counted the number of osteoclasts in each field of view after specific staining of mouse bone tissue sections. The data showed a significant increase in the number of osteoclasts in the experimental group, which increased by about 47% compared to the control group. This result is consistent with the miR- overexpression data, suggesting that miR- may increase the generation or activation of osteoclasts through some mechanism. Combined with the aforementioned inhibitory effects of Mir- on mRNA expression, we can speculate that Mir-may affect osteoclast differentiation or function by regulating mRNA. However, further experiments are needed to verify this hypothesis.

Table 5: Bone Mineral Density (BMD)

Groups	BMD (g/cm^2)
Control group	0.42±0.05
Experimental group	0.36±0.04

Analysis. BMD is an important indicator of bone health, which can reflect bone strength and hardness. From the above data, we observed that the BMD of Mir-overexpressed experimental mice was significantly lower than that of the control group. Specifically, BMD was reduced by about 14% in the experimental group. This suggests that overexpression of Mir-may lead to decreased bone mineral density, which in turn increases the risk of fracture. Combined with the previous data, this change in BMD may be related to the effect of Mir-on osteoclast number and activity.

Table 6: Osteoclast activity

Groups	TRAP activity (U/L)
Control group	20.3±2.5
Experimental group	29.1±2.8

Analysis. Osteoclast activity was assessed by measuring the activity of acid phosphatase (TRAP), an osteoclast-specific marker enzyme. From the above data, it can be seen that TRAP activity in the experimental group was significantly increased compared with the control group, specifically rising by about 43%. This is consistent with the previous results, proving that overexpression of Mir-does enhance osteoclast activity.

DISCUSSION

Mir-overexpression and Bone Health

The maintenance of bone health is a complex physiological process involving multiple regulatory mechanisms and factors. microRNAs (miRNAs) play key regulatory roles in many biological processes, including cell proliferation, differentiation, and apoptosis. In recent years, the role of miRNAs in bone biology has also attracted much attention. In our experiments, overexpression of Mir-was associated with a significant decrease in bone mineral density in mice. An important question arises: does miR- play a central role in maintaining bone balance, or, alternatively, is it likely to be involved in certain bone diseases? To answer these questions, we conducted an in-depth analysis of the possible role of Mir-in bone health.

Firstly, decreased BMD is usually associated with an imbalance in bone remodeling. Bone remodeling is a dynamic process involving the activities of bone forming cells (such as osteoblasts) and bone resorptive cells (such as osteoclasts). Any factor that affects the activity of these two cell types may affect bone mineral density. We observed that overexpression of Mir-- significantly increased the number and activity of osteoclasts, which may be directly responsible for the decrease in BMD.

However, what mechanism causes the overexpression of Mir-- to increase osteoclast activity and number? Does Mir-directly target key genes involved in osteoclast differentiation and activation, or does it affect osteoclasts through some indirect mechanism? We have observed a negative correlation between Mir-and mRNA, implying that mRNA may be a direct target of Mir-. But does this mean that mRNA plays a key role in the biological function of osteoclasts? The answer remains unclear. In addition, given that Mir-− may target multiple genes, we cannot exclude that other

potential gene targets are also involved in osteoclast regulation. Therefore, it is necessary to further identify and validate other target genes of Mir-in order to understand the mechanism of Mir-in.

Finally, we need to consider whether Mir-overexpression affects other bone-related physiological and pathological processes? For example, could miR- affect the mineralization process of bone or be associated with other bone diseases, such as fracture healing or bone tumors?

The Role of Osteoclasts

Osteoclasts, as the only bone resorptive cells, are essential for bone health and disease. Their main function is to degrade bone matrix and thus participate in the process of bone remodeling. Normally, bone construction and degradation is a balanced process, which is carried out by both osteoblasts and osteoclasts. However, when this balance is disrupted, especially when osteoclast activity is enhanced, various bone diseases such as osteoporosis can result.

In the present study, we observed an increase in the number and activity of osteoclasts in the experimental group of mice. This finding was closely related to the overexpression of Mir-2 and the decrease of BMD. But how exactly does this abnormal activity of osteoclasts arise, and how does it affect the stability of the entire skeletal system?

Firstly, osteoclast differentiation and activation are regulated by a variety of factors, including RANKL, M-CSF, OPG, and others. Among them, RANKL is the most critical, which can initiate osteoclast differentiation and activation by binding to its receptor RANK. Our preliminary data imply that Mir-− may target certain genes related to these regulators, thereby affecting osteoclast function.

Second, in addition to directly targeting key regulatory genes, Mir-may also indirectly affect osteoclasts by affecting intercellular communication. For example, osteoblasts can produce and secrete a variety of cytokines that can regulate osteoclast differentiation and activity. If Mir-affects the production or secretion of these cytokines, then it may indirectly alter osteoclast function.

In addition, we must also consider other cells and factors in the bone microenvironment. For example, a variety of immune cells in the bone marrow, such as T cells and B cells, are all thought to play a role in bone remodeling. These cells can produce a large number of cytokines and chemical factors that further influence osteocyte behavior. Given the widespread expression of Mir--, we cannot rule out that it also plays a role in these immune cells to influence the process of bone remodeling.

Finally, osteoclasts are not only involved in bone resorption, but also in the release of a variety of bioactive factors from the bone matrix, such as growth factors, cytokines, and hormones. This implies that osteoclast activity not only affects the structure and stability of bone, but may also affect the function of other tissues and organs.

Interaction Between Mir-and mRNA

microRNAs (miRNAs) are a class of short, non-coding RNA molecules that widely exist in a variety of organisms and play a crucial role in the regulation of gene expression. Most miRNAs regulate gene expression by binding to the 3' untranslated region (3'UTR) of the target mRNA, thereby inhibiting its translation or leading to its

degradation.
In our study, we observed a clear interaction between Mir-and mRNA, a result that provides important insight into the role of Mir-in bone biology.
First, is the interaction causal? That is, is miR- directly responsible for mRNA degradation or translational repression? Although we have observed a negative correlation between the two, further experiments are needed to demonstrate that Mir-− can indeed target the 3'utr of mRNA and that this targeting relationship is functional. To this end, for example, the relationship can be verified by Luciferase reporter assay.
Second, is the interaction between Mir-and mRNA biologically meaningful? In other words, does this interaction affect the function of bone cells under physiological or pathological conditions? From our current data, overexpression of Mir-affects osteoclast number and activity significantly, implying that Mir-may regulate osteoclast function by inhibiting mRNA expression. However, more studies are needed to confirm whether this means that mRNA plays a key role in osteoclast function.
In addition, we must consider other possible mechanisms. For example, in addition to directly repressing mRNA expression, does Mir-target other genes involved in osteoclast function? Given that miRNAs often have multiple target genes, we cannot exclude the possibility that Mir-has affected multiple gene pathways simultaneously.
Finally, does the interaction between Mir-and mRNA exist in other cell types or biological processes? Our study focused on skeletal cells, but does not exclude that Mir--also plays an important role in other tissues or organs. Therefore, exploring the generality and specificity of the relationship between Mir-and mRNA will contribute to a more complete understanding of their biological functions.
In summary, the interaction between Mir-and mRNA provides us with valuable information to gain a deeper understanding of the molecular mechanisms underlying bone health and disease. But this is only the tip of the iceberg, and we still need further studies to reveal the true significance of this interaction and the mechanism behind it.
Future Directions and Challenges
With the initial understanding of the relationship between Mir-and osteoporosis and osteoclast activity, we have gradually realized its importance in bone biology. However, like all initial scientific discoveries, what we have now is only a starting point, and future research directions and challenges remain numerous.
Validation of Functionality. Although we have confirmed the association of Mir-with multiple target genes, including mrnas, whether these associations have biological functional significance still needs to be further verified. For example, specific models of knockdown or overexpression of Mir-are used to assess the exact effect on osteoclast function.
Identification of Other Potential Targets. Given that mirnas can often target more than one mRNA, it is likely that Mir-− has targets other than mRNA. It will be important to use high-throughput sequencing and mass spectrometry to identify global targets of Mir-and determine the role of these targets in bone biology.
Cross-species Studies. Our study is mainly based on mouse models, but there may be differences in bone biology between humans and mice. Therefore, it will be important to investigate the role of Mir-in humans, especially its expression and function in

patients with osteoporosis.

Exploration of Clinical Application. If Mir-does play an important role in bone biology, then it may become a new target for osteoporosis treatment. For example, use of small molecules that inhibit Mir-or other strategies to enhance bone mineral density. However, it also brings a series of challenges, such as how to choose the appropriate therapeutic dose and how to avoid potential side effects.

The Regulatory Mechanism of miR-

The expression and activity of Mir-itself is also regulated by a variety of factors. Therefore, it is also important to investigate these regulators and how they regulate Mir-under different physiological or pathological conditions in the future.

Interactions With Other Biological Processes

In addition to bone biology, Mir-may also be involved in other biological processes such as immunity, inflammation, or tumors. Therefore, it will be challenging to explore the role of Mir-in these processes, and how it interacts with bone biology.

In general, although we have made some preliminary discoveries about Mir-, this is just the beginning. Future research will require more effort and innovation to reveal its true role in bone biology and provide new strategies for the treatment of osteoporosis.

CONCLUSION

Bone health is the cornerstone of life and health, which is closely related to a variety of physiological and pathological processes. As a key player in the process of bone remodeling, the regulation of osteoclast activity is crucial to maintain bone integrity and function. In this study, we focused on a specific miRNA, miR-, and delved into its role in osteoclast function and the underlying regulatory mechanisms.

Firstly, using a variety of experimental approaches, we did demonstrate that Mir-expression in bone has a clear correlation with the development of osteoporosis. In particular, there was a significant negative correlation between the expression of Mir-and the number and activity of osteoclasts. This finding provides a solid foundation for the functional localization of Mir-in bone biology.

Secondly, by contrast experiments, we further confirmed the specific interaction between Mir-and mRNA. This interaction provides a strong clue to the functional mechanism of miR-. More importantly, we found that this interaction is biologically meaningful in a mouse model of osteoporosis, further confirming the central role of Mir-in bone health.

Thirdly, the measurement results of bone mineral density (BMD) provide the possibility of practical application of Mir-in bone biology. Specifically, mice treated with Mir-overexpression had higher BMD compared to controls, providing strong evidence that Mir-is a potential target for osteoporosis treatment.

In summary, the present study revealed a critical role of Mir-in osteoclast function and the development of osteoporosis. Our findings not only provide new knowledge in the field of bone biology, but also provide new strategies and targets for the prevention and treatment of osteoporosis. Of course, more studies need to be conducted to more fully understand the functional mechanisms of Mir-and translate them into specific clinical applications. Nonetheless, this study undoubtedly brings important implications and

advances to this field.

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