Brief Report

DOI: 10.5582/irdr.2021.01054

# Loss of miR-23a cluster in skeletal muscle can suppress bone remodeling

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**SUMMARY** Muscle-bone interaction might regulate bone remodeling in an endocrine manner, but the exact mediators have not been identified. Previous in vitro studies suggest that exosomal miRNAs are a candidate for this interaction. Here we present an in vivo study to show that targeted knockout of a muscle-specific miR-23a cluster including miR-23a, miR-27, and miR-24-2 in skeletal muscle tissues can suppress bone remodeling in mice. The effect of miR-23a cluster seem to not be related to aging, but can worsen the pathological extent of osteoporosis in mice. Our findings suggest that muscle-derived miRNAs may contribute to bone metabolism regulation through exosomes in muscle-bone interaction.

***Keywords*** exosome, microRNA, muscle-bone interaction, bone remodeling

## 1. Introduction

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that are known to regulate expression of protein-coding genes (*1*). Among them, several miRNAs located adjacently as miRNA clusters, and dysregulation of these clusters has been identified to be involved in multiple pathogenesis events, such as, cancer, heart disease, and metabolic disorders (*2*). Accumulating evidence indicates that miRNAs also act as mediators of intercellular communications (*3*).

Muscle and bone are anatomically and functionally connected organs. Recent studies suggest that besides biomechanical effects, skeletal muscle and bone could regulate the differentiation of each other by secreting factors (*4*). Our previous study indicated that except for secreting proteins, miRNAs cargoing using exsomes also contribute to bone-muscle interaction (*5*). Our *in vitro* data showed that muscle-abundant miR-27a cargoing by myofibroblasts promotes osteoblast differentiation (*5*), and the level of miR-27a in the serum exosome of Amyotrophic Lateral Sclerosis (ALS) patients was significantly lower than that in non-ALS controls (*6*). Here, we examine if miR-27a knockout in muscle can affect bone remodeling *in vivo*. For miR-27a, miR-23a, and miR-24-2 are adjacently located in a miRNA cluster (miR-23a cluster) and are functionally related. Therefore in this study, the effect of miR-23a cluster knockout on bone modeling was investigated.

## 2. Materials and Methods

### 2.1. Establishment of muscle-specific miR-23a cluster-knockout mice

The core group breeds miR-23a cluster-knockout mice were constructed following the standard protocol as described previously (*7*). A target region was selected at both ends of miR-23 and miR24-2, and the floxp sites were inserted to achieve three miRNA conditional deletions. The miR-23-cluster-floxp mice were mated with skeletal muscle-specific expression of Ckmm-cre mice to achieve conditional knockout of the miR-23a cluster. The animal care and experimental scheme used in this study was approved and conducted under the guidelines approved by the animal ethics committee of our academy. The KO mouse genotype was confirmed using DNA extracted from the tail tissue by mouse direct polymerase chain reaction (PCR) kits (Bimake, Houston, TX, USA). Primers for genotyping PCR amplification were as follows: CKO-X6-LOXP: forward (5′-GTA GAG GAG GGC TAG GGT GTG-3′) and reverse (5′-TGG GAG CGG AGT GTA GCA C-3′); ckmm-cre: forward (5′-GAC AAA AGG TTT TGC CCT CC-3′) and reverse (5′-AGT TTT TAC TGC CAG ACC GC-3′). The weight and length of the mice were measured from 5 weeks.

### 2.2. Expression level of miRNAs in muscle tissues with

and without miR-23a cluster knockout

Total RNA was extracted from muscle tissues of wild-type (WT) and knockout mice (KO) ($n$ = 4 of each group) using an animal tissue total RNA extraction kit (Tiangen, Beijing, China). 2μg RNA was used as starting material. Total miRNA was extracted from total RNA using All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Guangzhou, China). RT-qPCR was performed on Light Cycler®480 Real-time PCR system (Roche Applied Science, Mannheim, Germany). PCR program was performed as follows: pre-denaturation at 95℃ for 10min, amplification for 40 cycles with denaturation at 95℃ for 10 s, annealing at 60℃ for 20 s and extension at 72℃ for 10 s.

2.3. Establishment of osteoporosis model and micro-CT analysis

Twelve weeks old female KO mice and WT mice were used to establish an osteoporosis model by surgically removing both ovaries (OVX). The female KO mice and their age- and sex-matched WT mice were assigned into 4 groups ($n$ = 10 of each group): WT, WT-OVX, KO, and KO-OVX. After 12 weeks of modeling, the bone density and trabecular bone parameters were measured to determine whether the osteoporosis model was successfully constructed. Bone density and trabecular bone were analyzed using high-resolution in-vivo microcomputed tomography (micro-CT) imaging system (PerkinElmer, MA, USA) according to the manufacturer's protocol. The bone density and trabecular bone data analysis of the femurs was analyzed using Analyze 12.0 software. The bone density standard curve is made based on bone density standards. The captured data were loaded into the BMA module, and the bone and trabecula were separated for BMA parameters determination and cross-section analysis.

2.4. Histological analysis of bone tissues

The femurs of mice from the different groups were harvested. The samples were immersed in 4% paraformaldehyde at 4℃ for 24 h, decalcified and then embedded in paraffin. The paraffin-embedded femur sections were deparaffinized using conventional methods. The femurs were sectioned along the longitudinal axis and stained with Van Gieson (VG) and haematoxylin and eosin (H&E) for analysis of histologic differences.

2.5. Statistics

All the data are expressed as means ± standard deviation and compared using $t$-tests between groups. $P < 0.05$ was considered statistically significant. All experiments were repeated in triplicate. All statistics were analyzed with Graphpad Prism 8.0.

## 3. Results and Discussion

The muscle-specifc-miR-23a cluster KO mice were generated by cross-breeding miR-23a cluster-foxed mice with Ckmm-cre mice (Figure 1A-B). To confirm the Ckmm-cre induced miR-23a cluster knockout effect, we found that all the expression of miR-23a, miR-27, miR-24-2 in skeletal muscle tissues was significantly reduced in KO mice compared to their littermate controls (Figure 1C). As seen in Figure 2, muscle-specifc-miR-23a cluster KO mice demonstrated slightly lower body length and weight than WT mice (Figure 2A-B). The micro-CT results show that the KO mice have significantly lower femoral bone mineral density (BMD) than normal mice (Figure 2C), and morphometric parameters except BS/BV notably differs between KO and WT mice, which is consistent with BMD (Figure 2D-H).

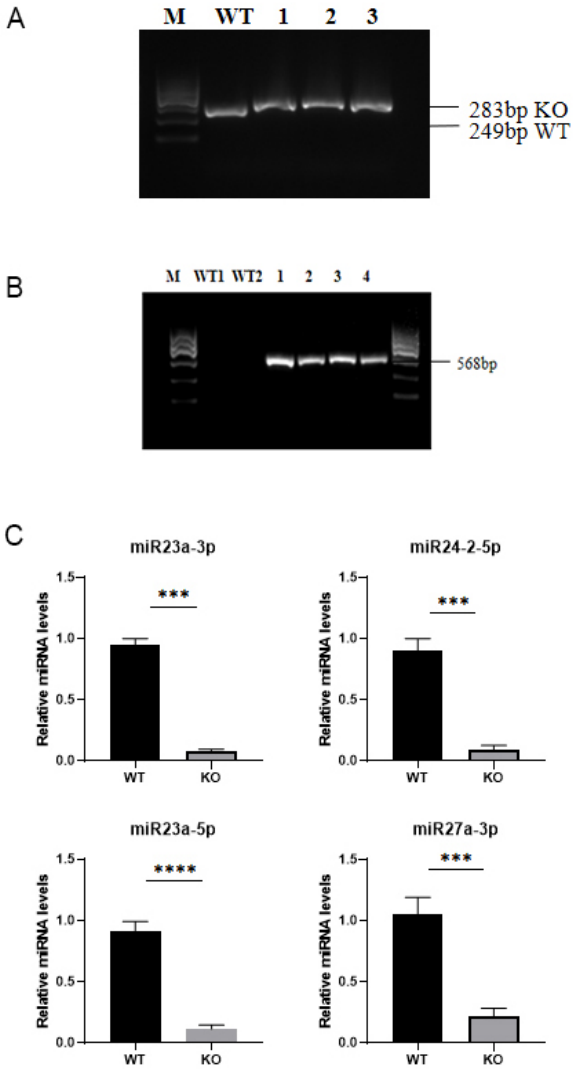


**Figure 1. Confirmation of miR-23a cluster knockout in mice.** Validation of insertion of flop sites **(A)** and Cas9 **(B)** by PCR. **(C)** Validation of loss of miR-23a cluster components in knockout mice tissues by qRT-PCR. Bar represents the mean ± SD. $^{***}P < 0.001$ and $^{****}P < 0.0001$.

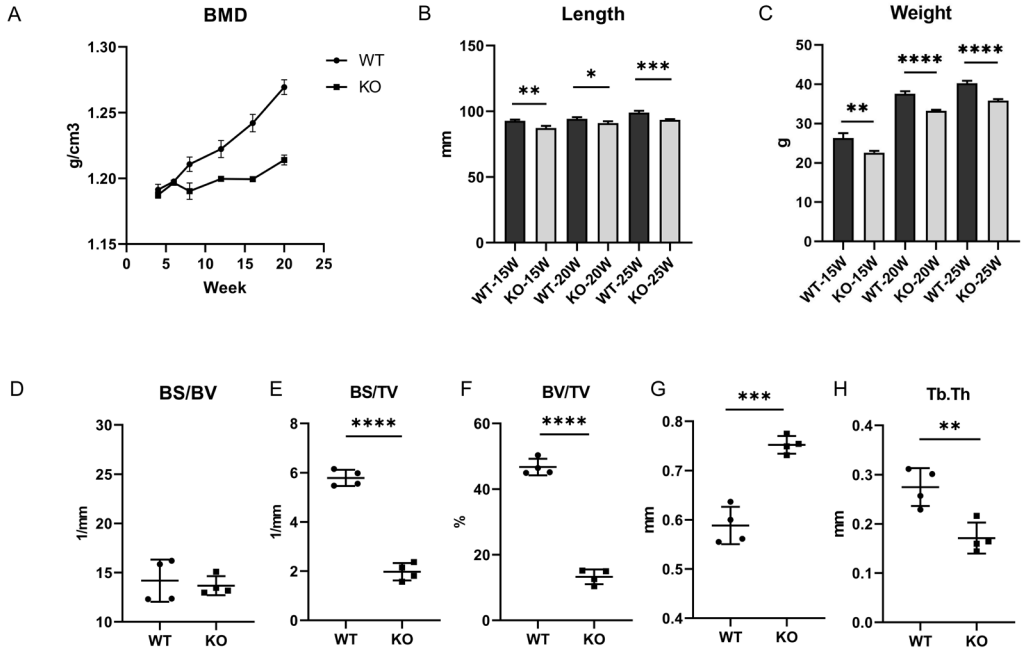


**Figure 2. Bone remodeling phenotype of miR-23a cluster knockout mice. (A)** Variation of bone mineral density (BMD); **(B-C)** Comparison of body length and weight in wild type and knockout mice; **(D-H)** Comparison of trabecular bone parameters measured by micro-CT in wild type and knockout mice. BS/BV, Bone Surface/Bone Volume; BS/TV, Bone Surface/Tissue Volume; BV/TV, Bone Volume/Tissue Volume; Tb.Sp, Trabecular Separation; Tb.Th, Trabecular Thickness. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

We next examined bone modeling in elder KO mice (60 week), and found the same trends observed in younger mice (Figure 3), however, the suppression seems not to be enhanced by aging. Then, we further confirmed if muscle-specifc-miR-23a cluster KO can affect the pathological extent of osteoporosis. As shown in Figure 4, generally, miR-23a cluster KO in muscle enhanced osteoporosis compared with WT controls. These data support that muscle miR-23a cluster KO could suppress bone modeling in both physiological and pathological conditions. Except for having lower BMD and other morphometric parameters, KO mice also demonstrated enhanced micro-architectural deterioration in micro-CT scanning (Figure 4A) and H&E and VG staining (Figure 4H-I).

The interaction between muscle and skeleton is of great significance to understand the remodeling of bones (*8*). In addition to mechanical forces, secretion between muscles and bones may also play an important regulatory role. miR-23a cluster is highly and specifically expressed in muscles. Wada, *et al*. demonstrated that the ectopic expression of miR-23a can reduce muscle atrophy *in vitro* and *in vivo* (*9*). Numerous studies have reported that miR-23a and miR-27a regulate proteins involved in the atrophy process (*10-12*). A previous study by Lee *et al*. (*13*) found that miR-23a cluster knockout has no obvious phenotypic effect on muscles, but no further observations have been made on bone performance of the model. Different from this previous study, we found

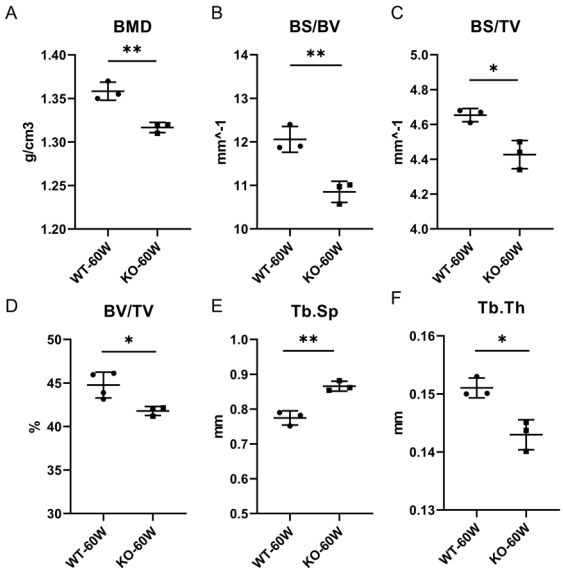


**Figure 3. Bone mineral density and trabecular bone parameters in aged miR-23a cluster knockout mice. (A)** BMD; **(B)** BS/BV; **(C)** BS/TV; **(D)** BV/TV; **(E)** Tb.Sp; **(F)** Tb.Th. $^{*}P < 0.05$, $^{**}P < 0.01$.

that miR-23a cluster knockout exerts a significant effect on bone remodeling, and knockout can also affect the degree of osteoporosis. Combined with our and other previous findings, there is a possibility that miR-23a secreted by muscles may regulate bone remodeling through exosomes or other means (*14*), but the direct

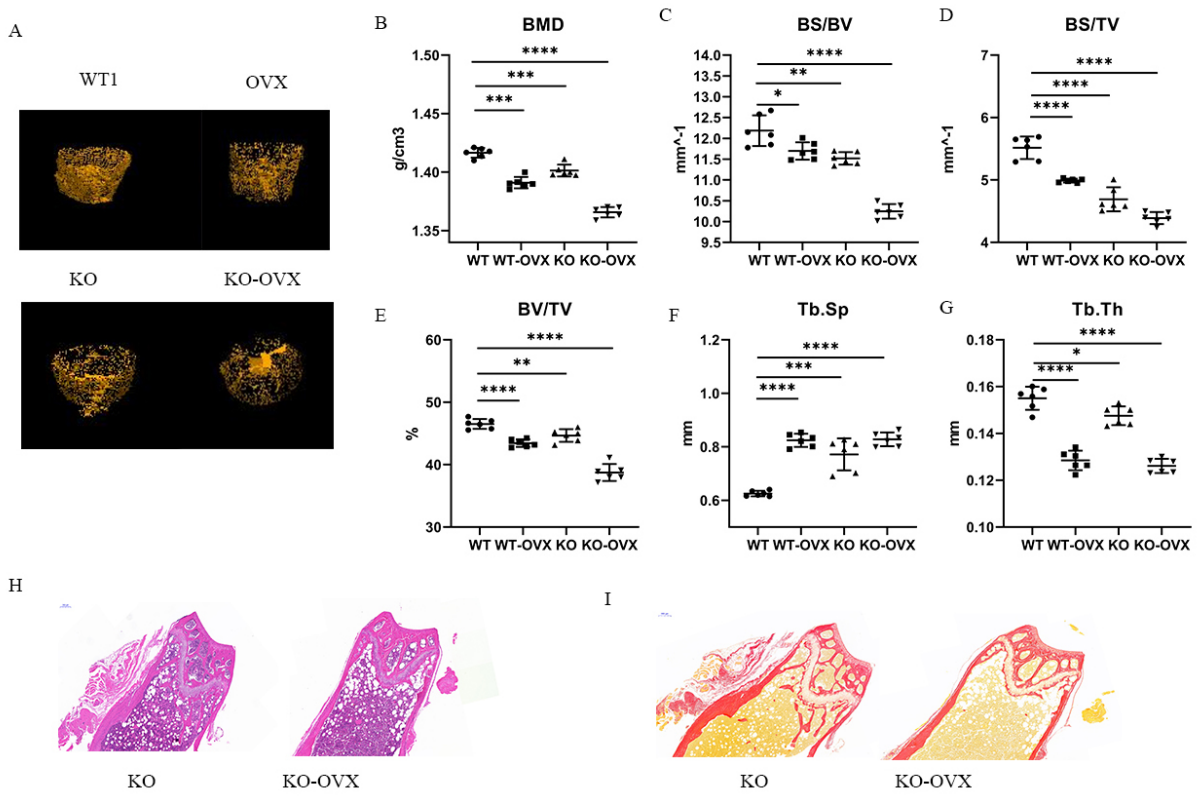


**Figure 4. Bone remodeling phenotype of miR-23a cluster knockout in OVX mice model. (A)** Trabecular bone construction in different groups; **(B-G)** Comparison of BMD and trabecular bone parameters in different groups; **(H-I)** Van Gieson and haematoxylin and eosin (H&E) in WT and KO OVX mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

muscular factors that cause bone abnormalities also need to be eliminated. Moreover, our data also provide indirect evidence that miRNAs may act in a distant manner. The fact that loss of miR-23a cluster can affect bone modeling also suggests combined miR-23a, miR-27, and miR-24-2 can change differentiation of target cells related to osteogenesis. Although our previous study confirmed exosomal miRNAs from myofibroblasts can target osteoblasts and promote its osteogenesis (*5*), osteoblasts might not be the only target cells. Other bone remodeling related cells may also be candidate targets for muscle exosomes such as osteoclasts. Moreover, the key target genes of miR-24a cluster also need to be identified, and in particular, whether some key osteogenesis regulators exist as co-targets for all three miRNAs.

In conclusion, for the first time, we demonstrate that loss of miRNA cluster in muscle tissues can affect bone remodeling, which is independent of aging, but enhanced under osteoporosis progression. These findings suggest an exosomal cargoing miRNAs regulating mechanism might exist in muscle-bone interaction.

*Funding*: This work was supported by National Natural Science Foundation of China (81772300) and the Academic Promotion Programme of Shandong First Medical University (LJ001).

*Conflict of Interest*: The authors have no conflicts of interest to disclose.

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Received March 25, 2021; Revised April 25, 2021; Accepted April 30, 2021.

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Released online in J-STAGE as advance publication May 3, 2021.