MicroRNA-495 Inhibits New Bone Regeneration via Targeting High Mobility Group AT-Hook 2 (HMGA2)

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Background: MicroRNAs play critical roles in post-translational gene expression. In this study, we explored the role of miR-495 in new bone regeneration.

Material/Methods: Murine calvarial osteoblasts were isolated and cultured. Microarray was performed to identify differential miRNAs in medicarpin-induced osteoblasts differentiation. Luciferase reporter assay was performed to identify the target gene of miRNA. Murine osteoblast cells were transfected with miC, miR-495, or anti-miR-495. CCK-8 and flow cytometry were performed to detect osteoblasts proliferation and apoptosis. Western blot was used to analyze apoptosis-related proteins. qRT-PCR analysis was performed to detect gene expression. ALP activity and mineralized nodule formation test were used to evaluate bone formation. Dill-hole injury model was constructed and micro CT was utilized to measuring bone healing.

Results: Microarray analysis identified miR-495 as our miRNA of interest and luciferase reporter assay identified HMGA2 as its target gene. Over-expression of miR-495 significantly inhibited ALP activity and mineralized nodule formation as well as the expression of RUNX-2, BMP-2, and Osterix. Also, miR-495 over-expression inhibited osteoblasts proliferation and promoted apoptosis obviously. In this in vivo study, the downregulation of miR-495 promoted murine femur healing.

Conclusions: MiR-495 inhibits new bone regeneration via targeting high mobility group AT-Hook 2 (HMGA2). We propose that targeting miR-495 may be a promising therapeutic approach for bone regeneration.

MeSH Keywords: Bone Regeneration • HMGA2 Protein • Microarray Analysis • MicroRNAs

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/904404
Background

MicroRNAs (miRNAs) are a cluster of small (about 22 nucleotides), noncoding and single-stranded RNAs which were highly conserved in plants, animals, and even some viruses, and they regulates gene expression post-translationally [1–3]. miRNAs function via partial or complete base-pairing with complementary sequences within mRNA molecules [4,5]. The dysregulation of miRNAs is associated with a number of diseases. It was reported that mutations of miRNAs could be found in some inherited diseases such as progressive hereditary hearing loss [6], hereditary keratoconus with anterior polar cataract [7], and skeletal and growth defects [8]. In recent years, an increasing number of miRNAs have been found to be linked with cancers. For example, low levels of miR-324a could serve as an indicator of poor survival [9]. In addition, some specific miRNAs have been associated with certain histological subtypes of colorectal cancer [10]. Also, miRNAs participate in heart development [11], and cholesterol metabolism and regulation [12].

A growing number of studies have revealed that miRNAs also regulate osteoblasts differentiation, bone metabolism, and bone formation. MiR-467g negatively regulates osteogenesis by targeting Ihh (Indian hedgehog)/Runx-2 (runt-related transcription factor 2) signaling pathway [13]. MiR-221 inhibits osteoblast differentiation and new bone regeneration diseases. For example, low levels of miR-324a could serve as an indicator of poor survival [9]. In addition, some specific miRNAs have been associated with certain histological subtypes of colorectal cancer [10]. Also, miRNAs participate in heart development [11], and cholesterol metabolism and regulation [12].

Medicarpin was reported to promote osteoblast differentiation via ER-BMP-2 signaling pathway which deregulates diverse miRNAs [17]. We deduced that miRNAs may play a role in the process of medicarpin-induced osteogenesis. To screen miRNAs changes in osteogenesis, we performed microarray analysis and found that several miRNAs were downregulated. Among those candidates, we selected a markedly downregulated miR-495 as our target. In this study, we revealed that miR-495 inhibited osteoblast differentiation and new bone regeneration. The inhibition of miR-495 stimulated osteoblast proliferation and differentiation in vitro and in vivo. MiR-495 suppressed bone formation directly by targeting HMGA2. Our results identified a novel miRNA in the regulation of osteoblast proliferation and bone regeneration.

Material and Methods

Isolation and culture of mice calvarial osteoblasts

First, one to two day old mice were sacrificed and their calvariae were removed. Calvariae were digested sequentially [18]. Briefly, calvariae were subjected to five sequential digestions at 37°C in 0.1% dispase and 0.1% collagenase P solution and cells were collected, centrifuged and resuspended in α-MEM medium supplemented with fetal bovine serum (10%) as well as 1% antibiotics.

Cell proliferation assay

Transfected osteoblast cells were plated in 96-well plates at a density of 5×10^3 cells per well. Then 48 hours later, 100 µL RPMI-1640 medium mixed with 10 µL CCK-8 solution was added to each well. After incubation at 37°C for 30 minutes, the absorbance of each well was detected at 450 nm. The analysis was repeated at least three times.

Microarray and miRNA target site prediction

Briefly, a total of 100 ng RNA was dephosphorylated and de-denatured. Then T4 ligase was used to ligate the cyanine-3-pCp and the 3’ end of RNAs which were dephosphorylated. The mouse miRNA microarrays (8×15 K) were hybridized with labeled miRNA. After being washed sequentially, slides were promptly scanned with microarray scanner at 5 µm. Finally, the scanned images were quantified with Feature Extraction software and differentially expressed miRNAs were recognized. For the differentially expressed miRNAs, we utilized miRanda (http://www.microrna.org) and Target Scan (http://www.targetscan.org) to find promising target genes.

qRT-PCR analysis

We purchased TaqMan miRNA reverse transcription kit from Applied Biosystems to detect miR-495. Total RNAs were extracted from mice calvarial osteoblasts with TRIzol and reverse transcribed to get cDNAs. TaqMan Universal PCR Master Mix with MicroRNA Assay Mix together with cDNAs were mixed together and synthesized for DNAs with the Step One plus Real Time PCR systems. U6 was used as an internal control. Total RNAs were extracted from cells and reversed transcribed for cDNAs. The relative mRNA levels of RUNX-2, BMP-2, and Osterix were evaluated with GAPDH as an internal reference. These following primers were used: U6 (forward: 5'-AGAGAGAGTAGCATGCCCCCTG-3', reverse: 5'-ATCCAGTGAGGCTTGGCAGG-3'); HMGA2 (forward: 5'-TCCCTCTAAGGACACCTAAA-3', reverse: 5'-ACTGTTGTGGCATTCTCTT-3'); RUNX-2 (forward: 5'-GATGATGACACTGACACCTCCT-3', reverse: 5'-AGAGAGATCTGAGCATGGCCCTT-3');
ciferase was the reporter gene and Renilla luciferase was the
medium (reduced serum and antibiotics-free OptiMEM) sup
or mutant types of 3'UTR of HMGA2 were cloned in special
ZX-MT01 vector was transfected to osteoblast cells where wild
protocols [19]. After osteoblast cells reached about 90%, pE
Luciferase reporter assay was performed according to published
pernatant was measured at 405 nm to analyze mineralization.
Sulfuric acid was used to neutralize the acid. The absorbance of su
fuged; 500 µl supernatant was collected and ammonium hy
was used to overlay the slurry and then the mix was heated, and centri
ted. Mineral oil purchased from Sigma-Aldrich was added

For miRNA PCR analysis, the reaction mixtures were incubated in a
96 well plate at 95°C for 10 minutes followed by 40 cycles of
95°C for 15 seconds and at 60°C for one minute using the Step One plus Real-Time PCR system (Applied Biosystems). For
ostegenic marker PCR analysis, the temperature profile of the
reaction was 95°C for five minutes, 40 cycles of denaturation
at 94°C for 2 minutes and annealing and extension at 62°C for 30
second, extension at 72°C for 30 seconds.

Transfection assay and ALP measurement

As osteoblasts reached a 50% confluence, miR-495 mimics and
miRNA negative control were transfected with Oligofectamine.
ALP activity and its mRNA level were detected after cells were
transfected for 48 hours. Transfected cells were seeded in 96-
well plates and cultured in medium supplied with β-glycerophosphate and ascorbic acid and then incubated for 48 hours. ALP activity was measured with p-nitrophenylphosphate as sub-
strate after induction and detected for colorimetry at 405 nm.

Mineralization nodules formation assay

Cells were plated in differentiation medium supplemented with 10% FBS. Transfected cells were cultured for 21 days and medium changed every 48 hours. Then, after 21 days, cells were
fixed with 4% formaldehyde and rinsed with PBS. Alizarin red-
S was used to stain cells, with the nascent calcium stained.

Tracking gene. In addition, miR-495 mimics or negative control
was transfected to cells. Then, 48 hours later, the strength of luciferase and Renilla were detected in cell lysates by a Dual
Luciferase Reporter Assay kit (Promega).

Drill-hole injury in femur

Six- to seven-week old female Balb/c mice were divided into two
groups (n=8). All mice underwent drill-hole injury. Briefly
speaking, the front skin in the femur diaphysis region was incised longitudinally for 1 cm under anesthetic conditions.
After exposing the femoral surface, a 0.8 mm drill hole was
made 2 cm above the knee joint. The two groups were drill-
hole injury+miC and drill hole injury+anti-miR-495. After treat-
ment for 21 days, all mice were sacrificed to collect femurs to
measure bone micro architectural parameters at injury sites.

Microcomputed tomography analysis

SkyScan 1076micro CT scanner was used to collect bone pa-
rameters. The collected femurs were cleaned of soft tissues and muscles and scanned at 70 kV, 100 mA. Then SkyScan NRecon
software was utilized for reconstruct images. Microarchitectural
parameters including bone volume fraction (BV/TV), thickness
of trabecularized spicules (Tb.Th), and trabecular num-
ber (Tb.N) were assessed.

Western blot assay

After washing with ice-cold PBS, transfected cells were lysed
with PIPA buffer (Beyotime) to get total protein. We determined
protein concentration with a protein assay kit; 10% SDS-PAGE
was used to separate protein, and the samples were applied to
PVDF membranes purchased from Millipore. Then 5% fat-free
milk in TBST buffer was used to block non-specific protein in-
teractions. The membranes were then incubated at 4°C with
case antibody for two hours; then incubated at room tem-
perature with secondary antibody conjugated with horserad-
ish peroxide for two hours. After washing these membranes in
TBST buffer, we developed the membranes using chemilu-
minescence to detect antibody concentrations and used β-ac-
tin as our internal control. The antibodies, anti-Bax, anti-Bcl-
x, and anti-β-actin were purchased from Abcam.

Apoptosis analysis

Cells were grown to about 50% confluence and exposed to
miRC, miR-495, and anti-miR-495 for 24 hours. Annexin V-PI
apoptosis kit was used to detect the apoptotic cells according
to the manufacturer’s instructions.

Luciferase reporter assay

Luciferase reporter assay was performed according to published
protocols [19]. After osteoblast cells reached about 90%, pE-ZX-MT01 vector was transfected to osteoblast cells where wild
or mutant types of 3'UTR of HMGA2 were cloned in standard
medium (reduced serum and antibiotics-free OptiMEM) sup-
plemented with Oligofectamine 2000 for six hours. Firefly luci-
erase was the reporter gene and Renilla luciferase was the
tracking gene. In addition, miR-495 mimics or negative control
was transfected to cells. Then, 48 hours later, the strength of luciferase and Renilla were detected in cell lysates by a Dual
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Apoptosis analysis

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miRC, miR-495, and anti-miR-495 for 24 hours. Annexin V-PI
apoptosis kit was used to detect the apoptotic cells according
to the manufacturer’s instructions.
Statistical analysis

SPSS11.0 was used to analyze our data. Quantitative data was expressed as mean ±SD. Non-paired t-test was used to analyzed data between groups. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA. A p<0.05 was determined as statistically significant.

Results

Selection of interesting miRNAs during medicarpin-induced osteogenesis

Medicarpin has been reported to induce osteoblasts differentiation definitely [17]. Therefore, we used medicarpin as an osteogenesis inducer to explore miRNAs differential expression in medicarpin-induced osteogenesis. Microarray analysis was performed on cells treated with or without medicarpin for 48 hours. Results from the microarray showed that most miRNAs were downregulated while a few were upregulated. We selected miR-495 as our miRNA of interest because it was significantly downregulated (Figure 1A). Furthermore, qRT-PCR analysis verified its downregulation in mice osteoblast cells (Figure 1B).

MiR-495 suppresses osteoblast differentiation

Osteoblasts were transfected with miR-C, mimic miR-495, and anti-miR-495 to evaluate the influence of miR-495 on osteoblast cells differentiation. We measured ALP activity, which is a common osteoblast cells differentiation marker. In miR-495 transfected cells, ALP activity was downregulated obviously while this effect was reversed in the inhibition group compared with cells transfected with miR-C (Figure 2A). In accordance with this effect, miR-495 decreased osteoblasts mineralized nodule formation, whereas this effect was blocked in the anti-miR-495 group (Figure 2B). Expression of osteogenic genes like RUNX-2, BMP-2, and Osterix were inhibited in the miR-495 group (Figure 2C), while this effect was reversed in the anti-miR-495 group. These results indicated that miR-495 suppressed osteoblast cell differentiation.

MiR-495 targets HMGA2 directly

To clarify the mechanisms of miR-495 suppressing osteoblast differentiation, miRanda (http://www.microrna.org) and Target Scan (http://www.targetscan.org) were utilized to identify promising target genes of miR-495. In particular, HMGA2 was a concern among those potential targets, as it has been reported that miR-33-5p promotes osteoblast differentiation by targeting HMGA2 [20]. Also, HMGA2 is associated with highly
Figure 2. MiR-495 negatively regulates osteoblast differentiation. (A) Murine calvarial osteoblasts were transfected with miC, miR-495, and anti-miR-495 for 48 hours in differentiation medium. ALP activity in osteoblasts was measured. (B) Transfected murine osteoblasts were seeded in 12-well plates and stained with Alizarin red-S. Representative images show mineralized nodules in different groups. (C) qRT-PCR and Western blot analysis of osteoblast marker genes such as RUNX-2, BMP-2, and Osterix at 48 hours. * p<0.05; ** p<0.01; *** p<0.001.
MiR-495 over-expression inhibits proliferation and induces apoptosis of osteoblast by targeting HMGA2

CCK-8 assay was performed to test the influence of miR-495 on osteoblasts proliferation. Cells transfected with mimic miR-495 were inhibited proliferation compared with the control group (Figure 4A). However, we did not detect a significant difference between the control group and the anti-miR-495 group in vitro. We used flow cytometry assays to identify whether this was due to induction of apoptosis; Annexin V-PI staining indicated higher apoptosis rates in miR-495 transfected cells (Figure 4B). Furthermore, Western blot was performed to measure the protein levels of Bcl-xl and Bax, which are key regulators of apoptosis. Our results showed that miR-495 over-expression raised the level of Bax and decreased the level of Bcl-xl (Figure 4C). To verify whether the proliferation inhibition effect was dependent on HMGA2, we silenced the HMGA2 expression, and detected no proliferation inhibition effect of miR-495 on osteoblast (Figure 4D).

**Discussion**

New bone regeneration is vital in diverse common bone disorders, including trauma and osteoporosis. Although researchers have explored various treatment methods to overcome these conditions [22], there are almost no extremely effective therapies. In this study, we investigated a novel miRNA involved in osteoblast differentiation. From both in vitro and in vivo studies, we found that miR-495 suppressed bone formation while anti-miR-495 attenuated this effect. The miR-495 upregulation decreased the ALP activity and mineralization nodule formation and also downregulated osteogenic marker expressions such as RUNX-2, BMP-2, and Osterix. To further study the underlying mechanisms, we searched prediction tools to find potential targets of miR-495. We selected to focus our investigation on
3’UTR of HMGA2. We found that miR-495 upregulation inhibited luciferase activity. Using an apoptosis assay, we found that miR-495 induced osteoblast apoptosis by targeting HMGA2. We thus concluded that miR-495 inhibits bone regeneration via targeting HMGA2.

In recent years, miR-495 has been studied as a tumor suppressor gene. It has been reported that miR-495 suppresses the post-transcription of forkhead box C1 (FOXC1) and thus inhibits endometrial cancer progression [23]. Also, miR-495 inhibits the migration and invasion of gastric cancer cells via interacting directly with phosphatase of regenerating liver-3 (PRL-3) [24,25]. Similar results also have been found in non-small cell lung cancer [26], leukemia [27], and prostate cancer [28]. However, little has been studied on the role of miR-495 in osteoblast cells differentiation or bone regeneration. We observed in our study that miR-495 suppressed mice osteoblast differentiation and new bone regeneration. HMGA2,
Figure 4. MiR-495 inhibits osteoblasts proliferation and promotes osteoblasts apoptosis. (A) Cells were transfected with miC or miR-495 and CCK-8 was performed to detect the proliferation. (B) Cells were grown to about 50% confluence and exposed to miC, miR-495 and anti-miR-495 for 24 hours. Flow cytometry was used to measure the apoptotic rate in different groups. (C) Cells were transfected with miC, miR-495, and anti-miR-495. Western blot analysis of Bcl-XL and Bax. Western blot of β-actin was included as a loading control. (D) Cells were silenced for HMGA2 and transfected with miC or miR-495. CCK-8 was used for detecting the proliferation of osteoblasts. **p<0.01.

as a nuclear-binding protein, plays critical roles in cell proliferation and differentiation [29]. In various cancers, it is overexpressed [30]. In osteosarcoma, miR-106a-5p inhibits osteosarcoma cells proliferation, invasion, and migration by targeting HMGA2 [31]. In addition, silencing of HMGA2 by shRNAs inhibits the proliferation of bone marrow-derived mesenchymal stem cells (MSCs) [21]. However, there are controversial ideas about the role of HMGA2 in osteoblast differentiation. It was reported that let-7 promotes osteogenesis of MSCs in vitro and in vivo via repressing HMGA2 expression [32]. In this study, we illustrated, for the first time, that miR-495 restrained HMGA2 expression and thus promoted osteoblast apoptosis.

Conclusions

In conclusion, results from our study indicated that miR-495 negatively regulate osteogenesis by targeting HMGA2 expression which regulates the apoptosis of osteoblast cells. In addition, the inhibition of miR-495 functionally promotes osteoblast proliferation and accelerates bone regeneration in vivo. Hence, we suggest that targeting miR-495 might be a promising therapy for enhancing new bone regeneration.

Conflict of interest

None.
Figure 5. MiR-495 regulates bone regeneration in vivo. (A) Representative micro CT images showing bone healing in each group. (B) Anti-miR-495 restores femoral microarchitecture showing bone volume fraction (BV/TV), Thickness of trabecularized spicules (Tb.Th) and trabecular number (Tb.N). All values represent means ±SD (n=8). ** p<0.01.

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