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MiR-122-3p regulates the osteogenic differentiation of mouse adipose-derived stem cells via Wnt/β catenin signaling pathway

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Abstract. – OBJECTIVE: To explore the regulatory mechanism of micro-ribonucleic acid (miR)-122-3p in the osteogenic differentiation of mouse adipose-derived stem cells (mADSCs).

MATERIALS AND METHODS: The regulatory mechanism of miR-122-3p in the osteogenic differentiation of mesenchymal stem cells was investigated through its overexpression and knockdown.

RESULTS: The overexpression of miR-122-3p inhibited the osteogenic differentiation of mADSCs. On the contrary, its knockdown promoted the osteogenic differentiation of mADSCs. The further study on the molecular mechanism of miR-122-3p regulating mADSCs' osteogenic differentiation showed that the overexpression of miR-122-3p could activate the Wingless and int-1 (WNT)/β-catenin signaling pathway, but the knockdown of miR-122-3p could repress this signaling pathway.

CONCLUSIONS: MiR-122-3p influences the osteogenic differentiation of mADSCs by modulating the WNT/β-catenin signaling pathway.

Key Words:
MiR-122-3p, Osteogenic differentiation, Adipose-derived stem cells, WNT/β-catenin signaling pathway, Mice.

Introduction

In most cases, bone defects are caused by congenital malformation, trauma, and surgical tumor resection, normally leading to functional defects, and aesthetic and psychological problems. In recent years, numerous studies have focused on the bone regeneration and repair by means of the bone tissue engineering of multipotent stem cells. This technology is recognized as the revolutionary and most promising treatment method to regenerate and repair bone[1]. Adipose-derived stem cells (ADSCs) belong to multipotential progenitor cells with the capacity of self-renewal and multidirectional differentiation, including osteogenesis, lipogenesis, and chondroplasia[2]. ADSCs have become the most promising ones in tissue engineering for the features of easy separation, rich sources, low immunogenicity and low incidence rate among donors[3,4]. To develop the treatment potential of ADSCs and apply them in the tissue engineering as early as possible, it is vital to study the molecular mechanism of ADSCs' differentiation. Micro-ribonucleic acids (miRNAs) are a class of small, non-coding endogenous RNAs, with 19-25 nucleotides in length. Lee et al first discovered that in *Caenorhabditis elegans*[5,6] post-transcriptional miRNAs change the stability of mRNA or its protein translation *via* binding to the 3′ UTR of their target mRNAs. The almost perfect complementary pairing of miRNA with its target gene can directly cause the cleavage of target genes, while part of complementary pairings between them suppress the translation of target genes[5,7-9]. In recent years, large numbers of studies[10-14] have found that miRNAs play an important regulatory role in the osteogenic differentiation of mesenchymal stem cells (MSCs). Based on the literature reports, miR-138, miR-221, miR-433-3p, miR-4739, and miR-548d-5p engage in the regulation of the osteogenic differentiation of MSCs[15]. The study on miRNA gene chip conducted by Wang et al[16] revealed that with the osteogenic differentiation of bone marrow MSCs, miR-122-3p significantly declines in steroid-induced osteonecrosis of the femoral head. In 1980, it was first found that Wingless and INT-1 (Wnt) cascade reaction participates in the embryonic development and maintains homeostasis by regulating cell proliferation, differentiation, and apoptosis[17]. Common Wnt signal transaction pathways include the

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canonical Wnt/β-catenin and non-canonical Wnt/Ca^{2+} pathways. With β-catenin as the core, the Wnt/β-catenin pathway activates the transcription ability of target genes mainly through β-catenin nuclear translocation. The Wnt/Ca^{2+} pathway is activated by Wnt5a and Wnt11 to increase the concentration of Ca^{2+} in cells, further activating protease C, phospholipase C, and NF-AT. In addition, the Wnt/Ca^{2+} pathway can interact with the canonical Wnt/β-catenin signaling pathway. According to large numbers of literature reports, Wnt plays important parts in many physiological and pathological processes. Extensive experiments during the past several years have shown that Wnt signaling pathways feature in orthopedic diseases and participate in osteogenic differentiation[18]. The glycoprotein family encoded by Wnt genes interacts with cell surface receptors and triggers intracellular cascade reactions. The canonical and non-canonical Wnt signaling pathways are key players in bone metabolism and osteogenesis, and closely correlated with the osteogenic pathology. Hence, studying the effect of Wnt signaling pathways on the osteogenic differentiation of ADSCs may help to find out potential regulatory targets. The above study showed that miR-122-3p and β-catenin are likely to take part in modulating the osteogenic differentiation of mADSCs. However, no current experiments have definitely demonstrated the mechanisms of interactions among the Wnt signaling pathway, miR-122-3p, and β-catenin. This work, therefore, explored the regulatory mechanism of miR-122-3p, through its overexpression and knockdown as well as interaction with β-catenin.

Materials and Methods

Culture of mADSCs

A total of 6 male C57BL/6 mice were purchased from Shandong Medical Laboratory Animal Center (Jinan, China) to obtain mADSCs. All procedures were made under the approval of the Animal Experiments Committee in Yantai Yuhuangding Hospital. The inguinal fat pads of mice were obtained in the same method as that reported in the aforementioned literature[19]. The collected inguinal fat pads were added with the phosphate-buffered saline (PBS) containing 1% penicillin, and the tissues were transferred onto the aseptic super-clean bench. After being washed by PBS, the resulting tissues were cut into pieces and digested using 0.2% type I collagenase (bathing in water at the constant temperature of 37°C and shaking for 50 min), followed by centrifugation at 1,000 rpm for 8 min. The precipitate was rinsed by PBS, and then, re-suspended in a 3 mL normal medium [α-MEM (HyClone Laboratories Inc., Logan, UT, USA), 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA)]. The product was inoculated in the culture bottle and cultured in the incubator containing 5% CO_2 at 37°C. The medium was replaced once every 2-3 days.

Induction of Osteogenic Differentiation In vitro

The specific method of inducing the osteogenic differentiation of ADSCs *in vitro* is described the above literature reports. In brief, the culture medium needs to be replaced with that containing 10% FBS, 10 mM β-glycerophosphate, 10^{-9}-10^{-8} M dexamethasone, 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 0.01 μM 1,25-dihydroxyvitamin D3, 100 U/mL penicillin and 100 μg/mL streptomycin so as to induce the osteogenic differentiation of ADSCs *in vitro*. After 7 days of culture, the alkaline phosphatase (Alp) staining was adopted to evaluate the degree of differentiation. After 14 days of culture, the calcium deposition on the cell surface was assessed using the alizarin red staining.

Establishment and Transfection with Lentiviral Vectors

The lentiviral vectors of miR-122-3p, miR-negative control (NC), antimiR-122-3p, and anti-NC were purchased from GENE Biotech Co, Ltd. (Shanghai, China). The primer sequence is shown below: MiR-122-3p: 5'-AACAGCACAAACUACUACCUCA-3'; MiR-NC: 5'-AATTCTTCTCCGAACGTGTCACGTCTTCCTC-3'; AntimiR-122-3p: 5'-UAUUUAGUGUGAUAAUGGCGUU-3'; AntimiR-NC: 5'-AATTCGTGGATATTGTTGCCATCAG-3'; U6: 5'-TGCGGGTGCTCGCTTCGGCAGC-3'. All lentiviruses reached the multiplicity of infection (MOI) of 50.

Analysis Via Real Time-Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

QRT-PCR was performed to detect the miR-122-3p expression and the mRNA expression of osteogenesis-specific genes osteocalcin (Ocn) and Alp. In these experiments, the total RNA was

extracted using a kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR was performed as described in the previous literature[20]. The relative expression levels of miRNA and mRNA were standardized with U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal references. The primer sequences are shown below: Ocn: forward: 5'-GCAATAAGGTAGTGAACAGACTCC-3'; reverse: 3'-GCAATAAGGTAGTGAACAGACTCC-5'; Alp: forward: 5'-ATCTTTGGTCTGGCTCCCATG-3'; reverse: 3'-TTTCCCGTTCACCGTCCAC-5'; GAPDH: forward: 5'-GGTGAAGGTCGGTGTGAAC-3'; reverse: 3'-GACTGTGCCGTTGAATTTG-5'.

Western Blotting Analysis

The expression level of β-catenin protein was measured *via* Western blotting. After protein lysis buffer was added, the products were extracted. The concentration of protein was determined *via* a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Each sample was added with 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) separation gel, followed by transferring onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After that, the protein samples were sealed. Then, they were incubated with the primary antibody, followed by incubation with the secondary antibody. Finally, the proteins were detected *via* the ECL enzyme (PerkinElmer, Waltham, MA, USA), and subjected to squash in a cassette, followed by the observation of the results. The imaging analysis software (Bio-Rad, Hercules, CA, USA) was used for analyzing the images.

Statistical Analysis

All data were expressed as means ± SDs. All experimental operations were repeated for 3 times at least. Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis, by virtue of the Student's two-tailed *t*-test or a one-way ANOVA followed by Post-Hoc-Test (Least Significant Difference). The statistical results were represented as bar charts using GraphPad Prism software (La Jolla, CA, USA). $p<0.05$ suggested that the difference was statistically significant.

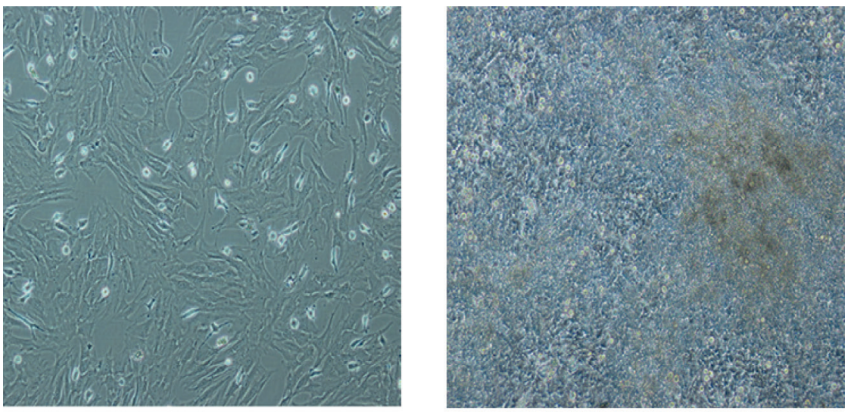
Results

Osteogenic Differentiation of ADSCs

Based on the cytomorphological observation, mADSCs started to osteogenically differentiate, after being put into the medium for inducing differentiation (Figure 1). Alp staining and alizarin red staining verified the osteogenic differentiation in mADSCs (Figure 2).

MiR-122-3p Expression in Four Groups (MiR-NC, MiR-122-3p, AntimiR-NC, and AntimiR-122-3p) Detected Via qPCR

Four lentiviruses (miR-NC, miR-122-3p, antimiR-NC, and antimiR-122-3p) were transfected into mADSCs, so as to promote or inhibit miR-122-3p expression. After transfection, the expres-



A B

Figure 1. Changes in cell morphology during the osteogenic differentiation of mADSCs. ***A,*** Undifferentiated mADSCs under a microscope. ***B,*** Differentiated mADSCs in the medium for inducing osteogenic differentiation under a microscope (magnification 40x).

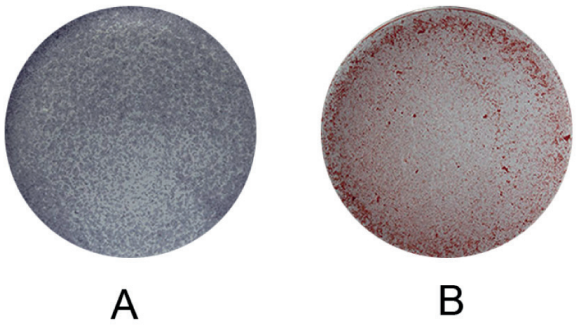


Figure 2. ***A,*** Osteogenic differentiation in mADSCs confirmed *via* Alp staining after 7 days of culture in the induction medium for osteogenic differentiation. ***B,*** Calcium deposition on the surfaces of mADSCs evaluated *via* alizarin red staining after 7 days of culture in the induction medium for osteogenic differentiation.

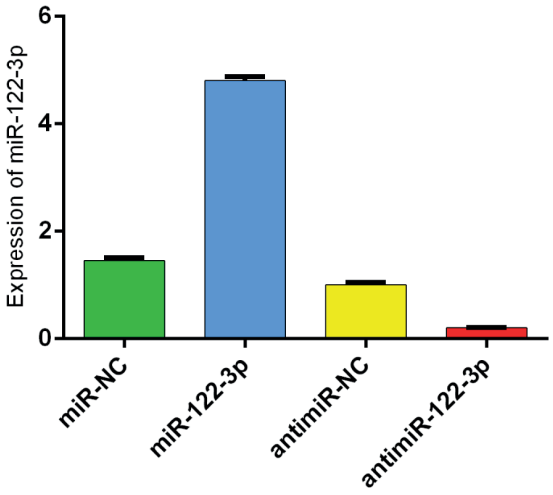


Figure 3. Expression of miR-122-3p in four groups of mADSCs after transfection with four lentiviruses.

sion levels of miR-122-3p in four groups (miR-NC, miR-122-3p, antimiR-NC, and antimiR-122-3p) were determined through qPCR (Figure 3). The miR-122-3p group exhibited significantly raised miR-122-3p expression level, while that in the antimiR-122-3p group was substantially lowered. It suggests that the lentiviral transfection causes the overexpression of miR-122-3p in the miR-122-3p group, and suppresses miR-122-3p expression in the antimiR-122-3p group.

Expression of Osteogenesis-Specific Genes Detected Via qPCR

After transfection with lentiviruses, qRT-PCR was conducted to detect the expression of osteogenesis-specific genes (Alp and Ocn) in four groups (miR-NC, miR-122-3p, antimiR-NC, and antimiR-122-3p). The expression level of osteogenesis-specific gene Ocn was determined *via* qRT-PCR in four groups, and the result showed that the expression of Ocn mRNA in the miR-NC group was markedly decreased, while the antimiR-122-3p group had significantly raised Ocn mRNA expression (Figure 4). The above result holds true for the expression level of osteogenesis-specific gene Alp in four groups (Figure 5). All these results indicate that the overexpression of miR-122-3p may inhibit the osteogenic differentiation of mADSCs, but its knockdown likely promotes the osteogenic differentiation of mADSCs.

Western Blotting Results

Western blotting was adopted to measure the β-catenin expression level in four groups, and the results revealed that the miR-122-3p group had a notably increased β-catenin expression level, but that was markedly decreased in the antimiR-122-3p group, indicating that the overexpression of miR-122-3p may activate the Wnt/β-catenin signaling pathway, but the knockdown of miR-122-3p likely represses this signaling pathway (Figure 6).

Discussion

ADSCs are a class of the most promising MSCs in tissue engineering. Recent studies[21-24]

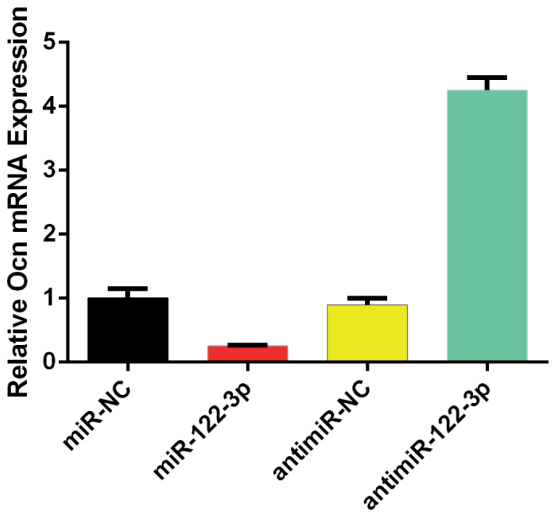


Figure 4. Osteogenesis-specific gene Ocn expression level in four groups detected *via* qRT-PCR.

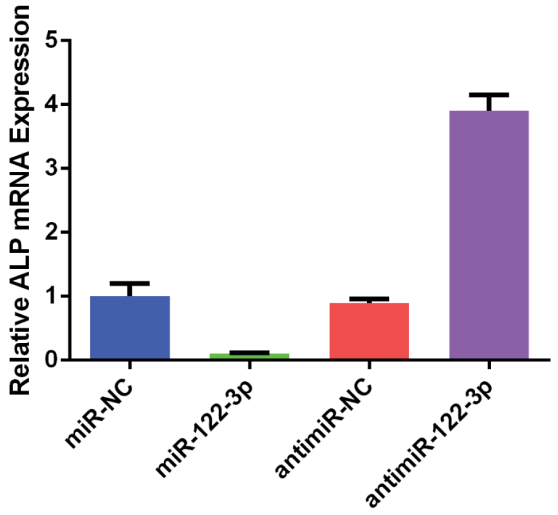


Figure 5. Osteogenesis-specific gene Alp expression level in four groups detected *via* qRT-PCR.

have manifested that ADSCs possess the ability to differentiate osteogenically, and multiple physical, chemical and biological factors can modulate their osteogenic differentiation. In this investigation, the ability of mADSCs to differentiate osteogenically was verified *via* Alp staining and alizarin red staining. Moreover, the regulatory effect of miR-122-3p on the osteogenic differentiation of ADSCs was studied by overexpressing and knocking down miR-122-3p with the lentiviral transfection. The results showed that inhibiting the expression of miR-122-3p can enhance the osteogenic differentiation of mADSCs, but overexpressing miR-122-3p can inhibit their osteogenic differentiation. MiR-122-3p is able to regulate the osteogenic differentiation of mADSCs negatively.

Wnt signaling pathways play important parts in osteogenesis and bone homeostasis[25-27]. Wnt ligands are regarded as the start of limb development and crucial media in the joint formation and limb morphogenesis[28,29]. Some researches[30,31] have revealed that Wnt is a regulatory factor in the osteogenic differentiation of MSCs, because Wnt can promote the further differentiation of osteogenic precursors into osteogenic cells, and also serves as a negatively regulatory factor of lipogenesis. The transcription regulator β-catenin acts as the core of the canonical Wnt/β-catenin signaling pathway, which is mainly composed of Wnt signal proteins, cell membrane receptor FZD family, β-catenin, and endonuclear lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family. Wnt proteins, such as Wnt1, Wnt3a, and Wnt8, bind to FRZ receptor or LRP 5/6 to activate the downstream reactions and form complexes, disabling β-catenin to be degraded by protease without the phosphorylation and resulting in the accumulation of β-catenin in cytoplasm and its translocation to the nucleus. Once binding to the transcription factors TCF/LEF, Wnt proteins can activate the transcription ability of TCF to regulate the expression of target genes[25,32-34]. The canonical Wnt/β-catenin signaling pathway cooperates with osteogenic regulator Runx2 to facilitate the differentiation towards osteogenic progenitors[35,36]. Although the previous studies have demonstrated that β-catenin serves as the core of the canonical Wnt/β-catenin signaling pathway, now, it still remains unclear how miR-122-3p regulates the expression of β-catenin to promote the osteogenic differentiation of mADSCs. Liu et al[37] found that the down-regulation of β-catenin can facilitate the osteogenic differentiation of periodontal stem cells in patients with chronic periodontitis. Besides, according to Li et al[14], inhibiting miR-26a-5p can activate the Wnt/Ca^{+2} signaling pathway and meanwhile repress the expression of β-catenin, thereby promoting the osteogenic differentiation of mADSCs. The above results are similar to those of our work, namely the increased β-catenin expression gives rise to the decrease in the expressions of osteogenesis-specific genes OCN and ALP, thus suppressing the osteogenic differentiation of mADSCs, and the lowered β-catenin expression leads to the opposite results. However, the interaction of the canonical Wnt/β-catenin pathway with the non-canonical Wnt pathway needs further studying, since the previous literature revealed that these two pathways are likely to interact in regulating the osteogenic differentiation of mADSCs.

The previous reports showed that miR-122-3p and β-catenin possibly take part in the regulation of mADSCs. We first demonstrated the roles of these several molecules in the osteogenic differentiation of mADSCs. The findings of this study showed that miR-122-3p overexpression facilitat-

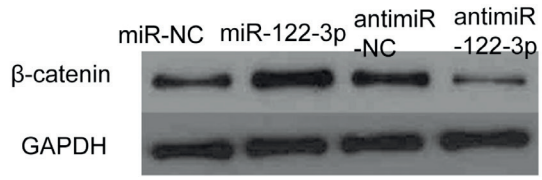


Figure 6. β-catenin expression level in four groups measured *via* Western blotting.

ed the expression of β-catenin, thereby repressing the osteogenic differentiation of mADSCs. Contrarily, its knockdown inhibited the expression of β-catenin, thus promoting the osteogenic differentiation of mADSCs. Because β-catenin acts as the core of the canonical Wnt/β-catenin signaling pathway, it is believed that miR-122-3p affects the osteogenic differentiation of mADSCs by regulating the expression of β-catenin.

Conclusions

We showed that the miR-122-3p influences the osteogenic differentiation of mADSCs through modulating the WNT/β-catenin signaling pathway.

Conflict of interest

The authors declare no conflicts of interest.

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