Inhibition of miR-222-3p activity promoted osteogenic differentiation of hBMSCs by regulating Smad5-RUNX2 signal axis

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ABSTRACT

miRNAs are recently found playing important roles in osteogenesis. In this study, we identified that miR-222-3p decreased during osteogenic differentiation of human mesenchymal stem cells (hBMSCs) using Quantitative Real-Time Reverse Transcription PCR (qRT-PCR). Furthermore, we investigated the effect of miR-222-3p on osteogenic differentiation of hBMSCs. Inhibition of miR-222-3p function in hBMSCs using infection of lentiviruses carrying miR-222-3p specific inhibitor promoted expression of osteoblast-specific genes, alkaline phosphatase (ALP) activity, and matrix mineralization. Whereas, overexpression of miR-222-3p inhibited osteoblast differentiation of hBMSCs in vitro. Moreover, Smad5 and RUNX2, which are the critical transcription factors in osteogenic differentiation, were predicted to be targets of miR-222-3p by bioinformatic analysis. Overexpression of miR-222-3p in hBMSCs significantly suppressed the protein levels of Smad5 and RUNX2, while inhibition of miR-222-3p increased their protein levels. Furthermore, inhibition of miR-222-3p increased phosphorylation of Smad1/5/8, which regulated the expression of osteogenic genes. Our findings suggest that suppression of miR-222-3p activity promoted osteogenic differentiation hBMSCs through regulating Smad5-RUNX2 signaling axis.

Keywords: Mesenchymal stem cells; miR-222-3p; Smad5; RUNX2; Osteogenic differentiation

1. Introduction

Bone marrow-derived human mesenchymal stem cells (hBMSCs) are commonly isolated from adult bone marrow and have the potential to differentiate into adipose tissue, bone, cartilage, tendon and muscle, and thus hold great hope for therapeutic applications [1-2]. However, the molecular mechanisms governing self-renewal and differentiation remain unclear.

MicroRNAs (miRNAs) are non-coding single-stranded RNAs of 21~25 nucleotides that are important negative regulators of cellular gene expression at the post-transcriptional level through binding to targets 3'untranslated region (3'UTR) of a protein-coding gene harboring a "seed" sequence [3]. Certain miRNAs have been shown to play key roles in diverse biological processes, including development [4-5], cell proliferation/differentiation [6-7], and many other physiological and pathological processes [8-12].

The function of miRNA in osteogenesis (bone formation) is now emerging. Some studies have shown that miRNAs played an important role in osteoblast differentiation and bone formation [7, 11-13]. It was recently reported that miR-133 and -135 directly target RUNX2 and Smad5, respectively, and inhibit differentiation of osteoprogenitors of C2C12 mesenchymal cells [14], whereas miR-210 promoted osteoblast differentiation by inhibiting the activity of the TGF- β type I receptor [15]. miR-26a has been reported to repress the translation of the osteogenic marker, SMAD1 [16]. Another research also showed that miR-26a modulates osteoclast formation and function through the regulation of connective tissue growth factor/CCN family 2 (CTGF/CCN2) [17]. In addition, previously studies revealed that miR-29 family of miRNAs were involved in osteogenic differentiation. miR-29 regulated osteoblast differentiation by down-regulating osteonectin expression through the canonical Wnt pathway [18, 19]. miR-31 and miR-145 were reported to suppress *osterix* (*sp7*) expression, leading to osteogenic inhibition [20, 21].

In this study, miR-222-3p was found to be down-regulated during osteoblast differentiation of hBMSC. We identified miR-222-3p as a negative regulator of

osteogenesis. Furthermore, targetscan and miRanda software predicted that the related genes of BMP signaling pathway might be possible targets for the miR-222-3p. Furthermore, we showed that inhibition of miR-222-3p led to enhance the levels of Smad5 and RUNX2 and phospho-Smad1/5/8, suggesting that inhibition of miR-222-3p activity promoted osteogenic differentiation of hBMSCs at early by regulating Smad5-RUNX2 signal axis.

2. Materials and Methods

2.1 Cell culture and induction of osteogenic differentiation

Human fetal MSCs (hMSCs, Cyagen Biosciences Inc., USA) were maintained and expanded in growth medium (Cyagen Biosciences Inc., USA) consisting of hMSC basal medium, pre-selected fetal bovine serum (FBS), penicillin–streptomycin and glutamine). The cells were maintained at 37°C (5% CO₂, 90% humidity) and were used for passages 3. Osteogenesis was induced using differentiation medium (50 µM ascorbate 2-phosphate, 10 mM β-glycerol phosphate, 0.1 µM dexamethasone and 10% FBS in DMEM low glucose medium, all from Cyagen Biosciences Inc., USA). Cells were cultured for up to 14 days in the presence of osteogenic differentiation medium.

2.2 Mineralization assay and activity of ALP

Levels of Alkaline phosphatase (ALPase) activities were determined in cell lysates using p-nitrophenyl phosphate as substrate. Cells were lysed using 1% Triton X-100–containing Tris-HCl buffer (pH 7.0). Aliquots of each sample were incubated with ALP substrate buffer (100 mmol/L diethanolamine, 150 mmol/L NaCl, 2 mmol/L MgCl₂, and 2.5 g/mL *p*-nitrophenylphosphate) for 10 minutes at 37°C, measured at 405 nm, and normalized to the total protein content determined by the bicinchoninic acid method from the same protein extracts. Mineralized matrix formation was assessed by Alizarin Red S staining. Cells were fixed in 4% paraformaldehyde and stained with 40 mmol/L Alizarin Red S (Cyagen Biosciences Inc., USA) for 30 minutes at room temperature.

2.3 Lentiviral vector construction and infection

The full length pre-miR-222 was chemically synthesized by Gene Chem and was introduced into the GV251 lentiviral vector with EGFP (Gene Chem, Shanghai, China) in the unique EcoRI site to construct a lentivirus encoding miR-222 (miR-222), and this construct was confirmed by using nucleotide sequencing. The specific inhibitor of miR-222-3p (miR-222-3p inhibitor) was constructed by cloning the complementary nucleotides of miR-222-3p into the GV159 lentiviral vector with EGFP (Gene Chem)

between the AgeI and Eco-RI sites. The cells were seeded in a 6-well plate at a density of 5×10^4 cells/well and were infected with miR-222 or its inhibitor when the cells reached 30%~60% confluence. Three days after infection, the efficiency of infection was evaluated by observing the EGFP expression by using a fluorescence microscope (Nikon, 80i). The empty lentiviral vector with EGFP was used as a control for infection (miR-222 control or miR-222-3p inhibitor control, respectively).

2.4 RNA extraction and Real-time PCR

Total RNA was extracted from the cultured cells by using the TRIzol reagent (Invitrogen), and microRNA was extracted by using the miRNA pure Mini Kit (CW Biotech, Beijing, China), according to the manufacturers' instructions.

For mRNA, the reverse transcription reactions were performed with the Super cDNA First-Strand Synthesis Kit (CW Biotech). Real-time PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) by using a miScript SYBR Green PCR kit (Qiagen) according to the manufacturer's protocol. For microRNA, reverse transcription was performed with the Taqman microRNA RT Kit (Invitrogen) and the Taqman microRNA Assay with specific stem-loop primers. Real-time PCR was performed with the 2X PCR master mix (Arraystar).

GAPDH was used as an mRNA control and U6 was used as an mRNA control. Each reaction was performed at least in triplicate and repeated in at least three different samples. Relative gene expression was determined by using the comparative $C_T(2^{-\Delta\Delta C_T})$ method. All primers used for qRT-PCR are listed in Table 1.

2.5 Western blot analysis

The harvested cells were suspended in cell lysis buffer. Proteins were separated by 10% SDS-PAGE. Then, the proteins were transferred onto a PVDF membrane (Millipore, Temecula, CA, USA) and blocked with Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% non-fat dry milk for 1 hr. The membrane was incubated with anti-phospho-SMAD1/5/8 (Santa Cruz Biotechnology, Inc.), anti-SMAD5 (Santa Cruz Biotechnology, Inc.), anti-RUNX2 (Santa Cruz Biotechnology, Inc.) and anti-GAPDH (Santa Cruz Biotechnology, Inc.) primary antibodies at 4°C over night and subsequently with horseradish peroxidase-conjugated

secondary antibody at 25°C for 1 h. The proteins were detected by using ECL chemiluminescence.

2.6 miRNA target prediction

A search for predicted target mRNAs was performed using the databases TargetScan (<http://www.targetscan.org>) and microrna (<http://www.microrna.org>).

2.7 Statistical analyses

The results were presented as the mean $\pm$ SEM. Comparisons between groups were analyzed by using a Student's t-test or one-way ANOVA with the SPSS 13.0 software. Statistical significance was defined as P value <0.05.

3. Results

3.1. *miR-222-3p decreased during osteogenic differentiation*

Osteoblast differentiation of hBMSCs was induced by using standard osteoblast-induction medium and evidenced by increased expression of genes associated with osteoblast differentiation, RUNX2, alkaline phosphatase (ALP), and osteocalcin (OC), at days 3, 7, and 14 after induction (Fig. 1A). Increased expression of osteoblast-associated genes were in accordance with previous reports describing hMSCs differentiation into osteoblasts [1-2].

Next, we examined the changes of miR-222-3p expression during osteogenic differentiation using qRT-PCR. The result showed that miR-222-3p was remarkably decreased compared to the undifferentiated human mesenchymal stem cells (0day) during osteogenic differentiation (Fig. 1B). These data suggest that miR-222-3p might play a role during osteogenic differentiation.

3.2. *miR-222-3p suppressed osteogenic differentiation*

We further investigated the role of miR-222-3p on osteogenic differentiation. hBMSCs were infected with miR-222-3p inhibitor or miR-222 or the respective control. After 72h infection, the expression of miR-222-3p was inhibited compared with the control by qRT-PCR (Fig. 2A).

After infection, cells were induced to osteoblast differentiation for 14 days, and expression of ALP, OSX, OC, and RUNX2 was detected using qRT-PCR. All of the osteogenic differentiation markers measured were markedly increased following infection of the miR-222-3p inhibitor (Fig. 2B). In contrast, hBMSCs infected with miR-222 showed decreased expression of osteogenic differentiation markers compared with those infected with the control (Fig. 2C).

Similarly, at 7 d of differentiation, ALP activity showed a significant increased in miR-222-3p inhibitor group compared with the control group (Fig. 2D). Moreover, the Alizarin Red staining (ARS) (Fig. 2E) at 14 d showed the similarly enhance at the matrix mineralization level. In contrast, ALP activity, matrix mineralization was reduced in with miR-222 infected hBMSCs (Fig. 2D and E). These results indicate that miR-222-3p could suppress osteogenic differentiation.

3.3. miR-222-3p targeting BMP signaling molecules

miRNA target analysis tools TargetScan 7.0 and miRanda software were used to explore potential targets of miR-222-3p. Based on the potential for interaction of miR-222-3p with the 3'-UTR of each transcript, several potential targets, BMPRIA, ACVR1B, Smad5 and RUNX2, which are involved in the BMP pathway, emerged from our search. Among these predicted targets, we identified Smad5 and RUNX2, which are the key transcript molecules in this pathway, to validate targeting of Smad5 and RUNX2 by miR-222-3p (Fig.3A). Overexpression of miR-222-3p in hBMSCs significantly suppressed the mRNA and protein level of Smad5 and RUNX2 at 7 days after induction (Fig.3B and C), while inhibition of miR-222-3p increased the mRNA and protein level of Smad5 and RUNX2 (Fig.3B and C). Taken together, these results suggested that the inhibition of miR-222-3p could elevate Smad5 and RUNX2 expression.

3.4. The effects of miR-222-3p inhibitor on the Smad1/5/8 signaling pathway

Because the BMP signal pathway is suggested to activation of Smad1/5/8 to induce osteogenesis, we assessed phosphorylation of Smad1/5/8. Western blot analysis showed markedly increased phosphorylation of Smad1/5/8 in miR-222-3p inhibitor transfected hBMSCs (Fig. 4A). Hence, this result indicates that miR-222-3p inhibitor promote the osteogenesis by activation of Smad1/5/8 signaling pathway.

In addition, Smad5 also is an upstream regulator of RUNX2, further to elevated levels of osteogenic marker genes, including RUNX2. We show that miR-222-3p inhibitor promotes osteoblastic differentiation can be attributed to stimulating Smad1/5/8 signaling way and by directly affecting the functional activity of RUNX2 (Fig.4B).

4. Discussion

Recently, miRNA was found to be able to modulate osteogenic differentiation of MSCs. Recently, an increasing number of research groups are focusing on the involvement of miRNAs in osteogenic differentiation and bone development[10-14].

In the present study, we found that the expression of miR-222-3p decreased during osteogenic differentiation. The infection of miR-222-3p inhibitor led to an elevation in osteoblast markers (RUNX2, OSX, ALP, and OC). Furthermore, ALP staining and ARS assays determined that miR-222-3p negatively regulated osteogenic differentiation of hBMSCs. These data suggest that miR-222-3p might act as a suppressor in osteogenic differentiation.

Bioinformatic analysis was performed to predict the target genes for miR-222-3p to study the molecular mechanism of its regulation on osteogenic differentiation. Targetscan and MiRanda software predicted that Smad5 and RUNX2 might be possible targets with binding sequences for the miR-222-3p within the their 3'UTRs . Previous studies had veriflicated that RUNX2 was a direct target for miR-222. In addition, we showed that miR-222-3p overexpression resulted in suppression of the protein levels of Smad5 and RUNX2, whereas functional inhibition of miR-222-3p led to elevation of Smad5 and RUNX2, strongly suggesting that Smad5 and RUNX2 were regulated by miR-222-3p. It is reported that expression of most genes encoding osteoblastic phenotype-related proteins were induced by BMP signal pathway via either Smad1 or Smad5 in vitro [21]. Smad5 and RUNX2 have been reported to function as key transcription factors during osteoblast differentiation of hMSCs. Moreover, Smad5 also is an upstream regulator of RUNX2 and increased levels of osteogenic marker genes, including RUNX2.

miR-222 was shown to support tumor-associated neoangiogenesis and invasion in many cancers, which is mainly attributed by targeting several key tumor suppressors such as p21Waf1/Cip1, p27Kip1, p57Kip2, PTEN, and PUMA[22-26]. In addition, miR-221/222 also had been found to regulate essential physiological vascular

processes. However, the mechanisms of the miR-222 regulation on osteogenic differentiation remain to be elucidated.

Here, we showed that miR-222-3p may regulate osteogenic differentiation by targeting Smad5-RUNX2 axis. It is of interest to demonstrate a direct interaction between miR-222-3p and 3'UTR of Smad5 in the future.

In conclusion, our data indicate that miR-222-3p was a regulator of osteogenic differentiation, at least in part by regulating Smad5-RUNX2 signaling axis. Our findings provide an experimental basis for further research on miRNA functions during osteogenic differentiation of human MSCs.

Acknowledgments

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Competing interests

The authors declare that they have no competing interests.

Fig. 1. miR-222-3p decreased during osteogenic differentiation. Osteoblast differentiation at indicated time points was confirmed by qRT-PCR analysis of osteoblast marker genes (RUNX2, ALP, and OC) as normalized to day 0 (n=3) (A). The levels of miR-222-3p at indicated time points during osteogenic differentiation by qRT-PCR as normalized to day 0 (n=3) (B). *P < 0.05, ** P < 0.01.

Fig. 2. miR-222-3p suppressed osteogenic differentiation. hBMSCs were infected with miR-222-3p inhibitor or the miR-222 for 72h. Expression of miR-222-3p was determined by qRT-PCR(A). Expression of RUNX2, OSX, ALP and OC was determined by qRT-PCR (B, C) and ALP activity at 7 d (D) and Alizarin Red staining at 14 d (E, original magnification $\times 100$) of osteogenic differentiation after infection with miR-222 or miR-222-3p inhibitor or the respective control. (n = 3). *P < 0.05, **P < 0.01.

Fig.3. miR-222-3p inhibited Smad5 and RUNX2 expression. Schematic of the miR-222-3p target sites within the human RUNX2 and Smad5 3'UTR (A). hBMSCs were infected with miR-222 or miR-222-3p inhibitor or the respective control. mRNA expression of RUNX2 and Smad5 were determined by qRT-PCR (B). Western blot was used to detect the protein levels of Smad5 and RUNX2 (C). (n = 3).* P < 0.05, ** P < 0.01.

Fig. 4. The effects of miR-222-3p inhibitor on the Smad1/5/8 signaling pathway. hBMSCs were infected with miR-222-3p inhibitor or the control. Phosphorylated Smad1/5/8 was assessed by western blot (A). The model diagram of miR-222-3p inhibitor promoted osteogenic differentiation of hBMSCs by regulating Smad5-RUNX2 axis (B).

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Table.1.

Primers used for quantitative RT-PCR analysis

Gene name	Forward (5'– 3')	Reverse (5'– 3')
RUNX2	TGTCATGGCGGGTAACGAT	AAGACGGTTATGGTCAAGGTGAA
ALP	CCACGTCTTCACATTTGGTG	AGACTGCGCCTGGTAGTTGT
OCN	GGCGCTACCTGTATCAATGG	GTGGTCAGCCAACTCGTCA
OSX	CACATCCCTGGCTGCGGCAA	CCGGGTGTGAGTGCGCACAT
Smad5	GAGAGTGGAGAGTCCAGTCT	GGCTGTTTGGAGATAAGGGAAA
GDPDH	CAAGATCATCAGCAATGCC	ATGGACTGTGGTCATGAGT
miR-222-3p	GGGGAGCTACATCTGGCT	TGCGTGTCGTGGAGTC
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

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A **B** **C**

Fig. 2. miR-222-3p suppressed osteogenic differentiation. hBMSCs were infected with miR-222-3p inhibitor or the miR-222 for 72h. Expression of miR-222-3p was determined by qRT-PCR(A). Expression of RUNX2, OSX, ALP and OC was determined by qRT-PCR (B, C) and ALP activity at 7 d (D) and Alizarin Red staining at 14 d (E, original magnification $\times 100$) of osteogenic differentiation after infection with miR-222 or miR-222-3p inhibitor or the respective control. (n = 3). *P < 0.05, **P < 0.01.

A

B

C

Fig.3. miR-222-3p inhibited Smad5 and RUNX2 expression. Schematic of the miR-222-3p target sites within the human RUNX2 and Smad5 3'UTR (A). hBMSCs were infected with miR-222 or miR-222-3p inhibitor or the respective control. mRNA expression of RUNX2 and Smad5 were determined by qRT-PCR (B). Western blot was used to detect the protein levels of Smad5 and RUNX2 (C). (n = 3). * P < 0.05, ** P < 0.01.

A

B

Fig. 4. The effects of miR-222-3p inhibitor on the Smad1/5/8 signaling pathway. hBMSCs were infected with miR-222-3p inhibitor or the control. Phosphorylated Smad1/5/8 was assessed by western blot (A). The model diagram of miR-222-3p inhibitor promoted osteogenic differentiation of hBMSCs by regulating Smad5-RUNX2 axis (B).