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Original Article

MicroRNA-133b negatively regulates the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells

Wende Xiao, Shifeng Wen, Haoyi Chen, Weipeng Zheng

Department of Spine Surgery, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou, China

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Abstract: Osteogenic differentiation of bone marrow mesenchymal stem cells (BMMSCs) is accurately regulated by essential transcription factors and signaling cascades. However, the precise mechanisms involved in this process remain unknown. MicroRNAs (miRNAs) regulate various biological processes by binding target mRNA to attenuate protein synthesis. The purpose of this study was to investigate the role of miR-133b in regulation of BMMSC proliferation and osteogenic differentiation. Quantitative real time PCR was performed to investigate the expression pattern of miR-133b during osteogenic differentiation of MSCs at different time points. Then the effects of miR-133b downreguation/upregulation on proliferation and osteogenic differentiation were evaluated by MTT, expression levels of osteogenic differentiation markers and Alizarin red S staining. The expression level of miR-133b was downregulated during osteogenic differentiation of BMMSCs ($P<0.01$). miR-133b-specific siRNA promoted proliferation and osteogenic differentiation of BMMSCs, with increased mRNA expression of the osteogenic markers alkaline phosphatase (ALP), runt-related transcription factor (RUNX2), osteocalcin (OCN) and bone morphogenetic protein 2 (BMP-2) as well as stronger intensity of Alizarin red S staining ($P<0.05$, $P<0.01$). Opposite findings were observed when miR-133b was overexpressed. In conclusion, miR-133b plays a important role in regulating the proliferation and osteogenic differentiation of BMMSCs.

Keywords: Bone marrow mesenchymal stem cells, miR-133b, osteogenic differentiation, proliferation

Introduction

Osteoporosis is a systemic disorder resulting in the systemic reduction of bone strength and increasing risk of fragility fractures [1]. Current treatments for osteoporosis are predominantly bone-resorbing drugs that are associated with several side effects. Stem cells especially bone marrow-derived mesenchymal stromal stem cells (BMMSCs) are a population of self-renewing and multipotent cells that have significant clinical potential for treating osteoporosis [2]. However, the molecular signaling pathways that regulate the proliferation and osteogenic differentiation of BMMSCs remain unknown. Therefore revealing the molecular mechanisms accounting for osteogenesis may lead to the development of new therapy for osteoporosis.

MicroRNAs (miRNAs) are a class of endogenous, small non-coding RNAs that have post-transcriptional controls on gene expression *via* either translational repression or mRNA degradation [3, 4]. miRNAs have been shown to be involved in nearly all physiological processes such as proliferation, differentiation, apoptosis, survival and organ development [5]. Deregulation of miRNA expression is closely associated with the pathogenesis of many diseases including osteoporosis [6, 7]. The effects of miRNAs have also been investigated in the bone formation, remodeling, and homeostasis. Huang et al reported that the expression level of miR-144-3p was reduced during osteoblast differentiation of C3H10T1/2 cells. In addition, miR-144-3p was a negative regulator of murine MSC proliferation and osteogenic differentiation by targeting Smad4 [8]. Overexpression of miR-194 significantly enhanced osteoblast differentiation and suppressed adipocytic differentiation, whereas miR-194 inhibition reduced matrix mineralization, and promoted lipid drop-

lets formation, indicating miR-194 was a critical factor in determining the fate of MSCs to differentiate into osteoblasts or adipocytes. In addition, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) was identified as a downstream target of miR-194 [9]. Panizo et al showed that miR-133b might play an important role in regulating the calcification process of vascular smooth muscle cells [10].

Currently whether miR-133b involves in regulating osteogenic differentiation is poorly known. The purpose of this study was to reveal the expression profile of miR-133b during the osteogenic differentiation of BMMSCs and analyze its function by ectopic expression or inhibition of miR-133b.

Materials and methods

Cell culture

Murine BMMSCs (Cyagen Biosciences, Guangzhou, China) isolated from 4-5-week-old female C57/BL6 mice were thawed and expanded based on the supplier's instructions. The BMMSCs used in all experiments were passage 3 cells and the cells were maintained under standard culture condition (37°C, 5% CO_2 and 95% relative humidity).

RNA interference

The miR-133b oligonucleotides (miR-133b mimics, anti-miR-133b and negative control) were designed and purchased by GenePharma (GenePharma Co. Ltd. Shanghai, China). Then miR-133b oligonucleotides were introduced into BMMSCs using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. To ensure the long-term inhibition effects, the miR-133b mimics, the anti-miR-133b and the controls were repeatedly transfected every three days.

MTT

Cell proliferation was determined using an MTT assay. The cells were counted and seeded at a density of 3×10^3 cells per well in 96-well plates. 20 μl MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h at indicated time points. The resulting formazan was dissolved with dimethyl sulfoxide and the solutions were measured at a wavelength of 490 nm with a microplate reader. Each experiment was performed in triplicate.

Quantitative real-time PCR (qRT-PCR)

The cellular RNAs were isolated RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The first strand cDNA was then synthesized from 2 μg of RNA using SuperScript III Reverse Transcriptase (Invitrogen). SYBR Green PCR Master Mix (Applied Biosystem, Carlsbad, CA, USA) was used for the amplification process and PCR was performed using StepOne plus real-time PCR system (Applied Biosystem). The PCR conditions were 94°C for 10 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 10 s. The expression levels of miR-133b and osteogenic mRNA markers were evaluated using the $2^{-\Delta\Delta Ct}$ method and each sample was assayed in triplicate. RNU6B and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for miR-133b and osteogenic mRNA markers respectively.

Alizarin red S staining

The effect of miR-133b upregulation/downregulation on osteogenic differentiation of BMMSCs was evaluated by Alizarin red S staining. The cells were washed three times with PBS and fixed with 70% ice cold ethanol for 20 min, followed by staining with 2% Alizarin red-S (pH 4.2, Sigma-Aldrich) for 15 min at room temperature. The BMMSCs were washed with PBS three times carefully and observed under an optical microscope. For quantification of staining, the deposits were incubated with cetylpyridinium chloride and the absorbance was measured at 540 nm.

Statistical analysis

All experiments were performed at least three times independently, and results were expressed as the means ± standard derivation (SD). Differences among the results were compared using one-way ANOVA. All analyses were performed using the SPSS 21.0 software (SPSS, Chicago, IL, USA) and $P<0.05$ was considered statistically significant

Results

miR-133b expression during the osteogenic differentiation of BMMSCs

The expression level of miR-133b was significantly increased during osteogenic differentia-

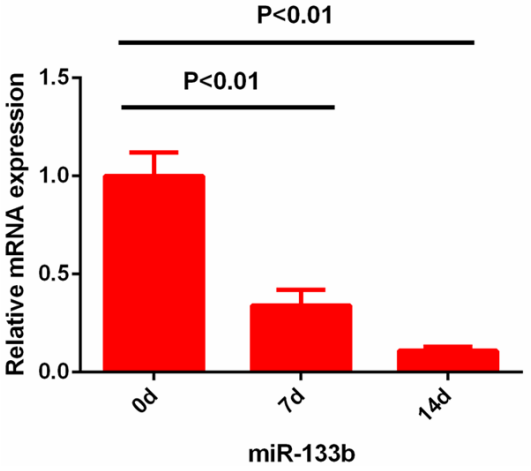

Figure 1. miR-133b expression during the osteogenic differentiation of BMMSCs.

Table 1. The expression level of miR-133b during BMMSC osteogenic differentiation

Time point (day)	Relative miR-133b expression (fold changes)
0	1.00 ± 0.12
7	0.34 ± 0.08
14	0.11 ± 0.02

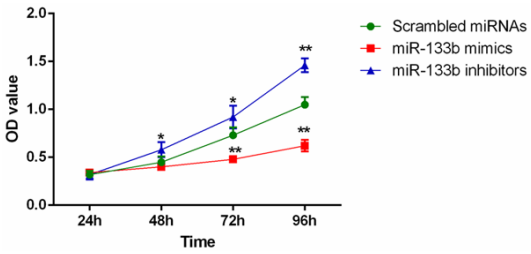

Figure 2. Effects of miR-133b upregulation/downregulation on the proliferation of BMMSCs.

tion of BMMSCs at day 7 and 14 (**P<0.01) (**Figure 1**, **Table 1**).

Effects of miR-133b upregulation/downregulation on the proliferation of BMMSCs

To investigate the role of miR-133b in BMMSC proliferation, we performed gain- and loss-of-function assay. The group with ectopic expression of miR-133b had less cell number at 72 h and 96 h compared with the controls (**P< 0.01). In contrast, downregulation of miR-133b promoted cell proliferation at 48 h, 72 h and 96 h (*P<0.05, **P<0.01) (**Figure 2**, **Table 2**).

Table 2. The OD values of transfected cells in different time points

Time po-int (h)	Mean OD value		
	Scrambled miRNAs	miR-133b mimics	miR-133b inhibitors
24	0.32 ± 0.04	0.34 ± 0.02	0.32 ± 0.05
48	0.45 ± 0.06	0.40 ± 0.03	0.58 ± 0.08
72	0.73 ± 0.08	0.47 ± 0.02	0.92 ± 0.12
96	1.05 ± 0.08	0.61 ± 0.06	1.46 ± 0.07

Table 3. The expression levels of osteogenic differentiation associated markers following-miR-133b upregulation/downregulation

Gene	Relative mRNA expression (fold changes)		
	Scrambled miRNAs	miR-133b mimics	miR-133b inhibitors
ALP	1.14 ± 0.07	0.41 ± 0.08	3.64 ± 0.24
RUNX2	1.25 ± 0.15	0.58 ± 0.06	5.38 ± 0.45
OCN	1.08 ± 0.18	0.13 ± 0.05	3.87 ± 0.58
BMP-2	1.16 ± 0.27	0.78 ± 0.22	10.95 ± 1.35

Effects of miR-133b upregulation/downregulation on osteogenic differentiation of BMMSCs

To investigate the role of miR-133b in regulating the osteogenic differentiation of BMMSCs. We evaluated the expression levels of ALP and other osteogenic differentiation associated markers (RUNX2, OCN, and BMP-2) at day 7 and 14 following mineralization induction respectively. Mineralized nodule formation was determined at day 14. Inhibition of miR-133b significantly increased ALP, RUNX2, OCN, and BMP-2 mRNA expression (**Table 3**), as well as mineralized nodule formation (**Table 4**), mirroring what was seen in overexpression of miR-133b (*P<0.05, **P<0.01) (**Figures 3**, **4**).

Discussion

Various types of miRNAs have been shown to play important roles of MSC osteogenic differentiation [11-13]. In this study, our results showed that the expression level of miR-133b was reduced during the osteogenic differentiation of BMMSCs. In addition, miR-133b was demonstrated to negatively regulated BMMSC proliferation and osteogenic differentiation, indicating that normal expression level of miR-133b was important for maintaining BMMSCs in a undifferentiated state. To the best our knowledge, this is first time to reveal the role of

Table 4. The amount of mineralized nodules in different groups were quantified using cetylpyridinium chloride

	Scrambled miRNAs	miR-133b mimics	miR-133b inhibitors
Relative amount of ARS (fold changes)	1.04 ± 0.30	0.62 ± 0.22	2.71 ± 0.62

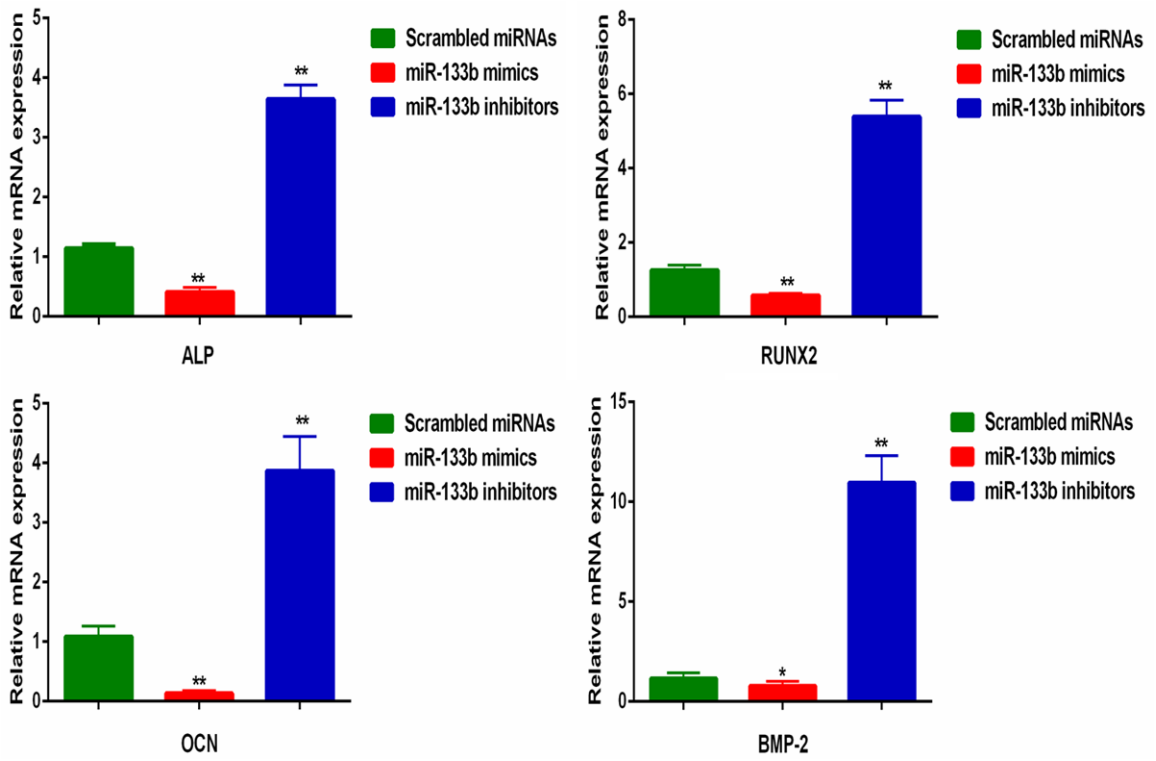

Figure 3. Effects of miR-133b upregulation/downregulation on the expression of osteogenic mRNA markers.

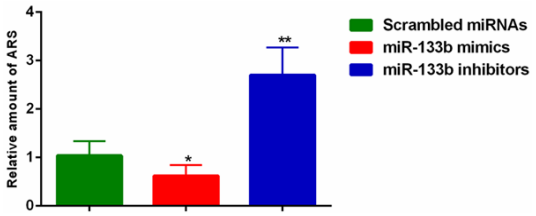

Figure 4. Effects of miR-133b upregulation/downregulation on mineralized nodule formation.

miR-133b in the osteogenic differentiation of MSCs. Consistent with our findings, Panizo et al revealed that the expression level of miR-133b was decreased during the osteogenic differentiation of vascular smooth muscle cells (VSMCs). In addition, downregulation of miR-133b could promote calcium deposition and the gene expression of Runx2, suggesting miR-133b acted as a negative regulator of vascular muscle cell calcification [10].

In addition to osteogenic differentiation, miR-133b has also been involved in various kinds of differentiation processes. Xiao et al reported that miR-133b expression was significantly upregulated when the oocytes were treated with insulin-like growth factor 1 (IGF-1). In addition, miR-133b may play important roles in regulating the growth and maturation of oocytes by targeting TAGLN2 [14]. Feng et al demonstrated that the expression levels of miR-133a and miR-133b were increased during the myogenic differentiation process. The authors also showed that miR-133 could suppress C2C12 cell proliferation while promote its differentiation and through the regulation of the extracellular signal-regulated kinase (ERK) signaling pathway [15]. Koutsoulidou et al showed that the expression level of miR-133b was significantly upregulated during late stages of human foetal muscle development. Moreover, there was a positive association between muscle cell differentiation and miR-133b expression level [16].

As regards to proliferation, the role miR-133b in the proliferation process is very complicated. It seems that the functions of miR-133b are cell type dependent and closely correlated with the microenvironment the cells reside in. Liu et al reported that miR-133b could inhibit the proliferation of non-small-cell lung cancer (NSCLC) cells by downregulating epidermal growth factor receptor (EGFR), suggesting miR-133b might acted as a tumor suppressor in NSCLC [17]. Similarly, ectopic expression of miR-133b could inhibit gastric cancer cell proliferation and colony formation, and fibroblast growth factor receptor 1 (FGFR1) was proved to a dow-nstream target of miR-133b [18]. However, overexpression of miR-133b could promote the proliferation of human Sertoli cells, and opposite findings were observed when the miR133b expression level was reduced. In addition, miR-133b exerted its promotion effect on proliferation by regulating GLI3 directly [19].

One possible limitation of this study is that the concrete molecular mechanisms that responsible for the role of miR-133b in regulating of osteognic differentiation remain poor known. Further studies should reveal the downstream targets of miR-133b in the MSC osteogenic differentiation. In addition, we should also investigate whether miR-133b suppression can promote osteogenic differentiation of BMMSCs *in vivo,* which might provide potential strategy for treating osteoporosis.

In conclusion, our results demonstrated that miR-133b negatively regulated the proliferation and differentiation of BMMSCs *in vitro*, indicating that targeting miR-133b might be a potential therapeutic approach for enhancing bone formation.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shifeng Wen, Department of Spine Surgery, Guangzhou First People's Hospital, Guangzhou Medical University, No. 1. Panfu Road, Guangzhou 510180, China. Tel: +86-20-81048248; E-mail: shifengwengzfph@163.com

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