miR-199b-5p modulates BMSC osteogenesis via suppressing GSK-3β/β-catenin signaling pathway

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1. Introduction

Efficacious bone regeneration could revolutionize the management of bone and musculoskeletal disorders. Bone is one of the few organs that retains its potential for continued regeneration into adult life and is the only tissue that undergoes continual remodeling throughout life [1,2], and bone remodeling is precisely coordinated by the interplay of osteoblasts and osteoclasts. The loss of equilibrium between osteoblastic and osteoclastic functions may result in bone diseases, such as osteoporosis or osteopetrosis [3].

Osteoblasts in the bone derive from bone marrow stromal cells (BMSCs). BMSCs are multipotent stem cells that can differentiate into bone, cartilage and adipose cells as well as into many other cell types. In vivo and in vitro evaluations have demonstrated differentiation of BMSCs along these lineages under the influence of local stimuli, in many pre-clinical models, and the osteogenic capacity of BMSCs has also been studied [4,5]. The differentiation of uncommitted BMSCs to osteoblasts is a fundamental process, which is positively or negatively regulated by numerous activators and repressors [6,7]. A better understanding of the mechanisms by which BMSCs osteogenesis is regulated is essential for its further application.

Several molecules possess a role in regulating the differentiation of BMSCs [8–10]. The GSK-3β/β-catenin signaling pathway appears to be of particular importance, since its activation was required in the BMSC differentiation into osteoblast cells [11]. In particular, β-catenin stabilization and induction of its translocation into the nucleus play an important role in promoting BMSC osteogenic differentiation.

Recently, several studies have investigated that miRNAs might also be involved in the BMSCs differentiation into osteoblast cells [12,13], of which 5 miRNAs (miR-199b-5p, miR-218, miR-148a, miR-135b, and miR-221) are more than 2-fold change in exosomes isolated from BMSCs culture when compared with the maximum changes at 0.5–7 days with the values at 0 day. In more detail, the expression level of miR-199b-5p in BSMC exosomes is 3.75 ± 0.81 folds increased at day 4 of osteogenic differentiation compared to that of day 0, which is the most significantly altered. Moreover, GSK-3β has been regarded as a direct target of miR-199b-5p. Given all these, we hypothesized that miR-199b-5p might exert its regulatory effect by modulating GSK-3β/β-catenin signaling in BMSCs.

To prove our hypothesis, we investigated the effect of miR-199b-5p on BMSC differentiation and bone formation. The expression level of miR-199b-5p during BMSCs osteogenesis was examined. Next, the effects of knockdown and overexpression of miR-199b-5p on osteoblast differentiation were determined, with regard to alkaline phosphatase (ALP) expression and activity, and expression of osteogenic marker gene, Runx2. Furthermore, the expression levels of GSK-3β, β-catenin, ALP and Runx2 were determined by using Western blot assay. Our results showed that miR-199b-5p promoted BMSC osteogenic potential through the regulation of...
the GSK-3β/β-catenin signaling pathway. These results advocate miR-199b-5p and its analogs as rather promising drugs against bone and musculoskeletal disorders.

2. Material and methods

2.1. Isolation and culture of human BMSCs

Human BMSCs were isolated and expanded using a modification of methods previously reported [14]. The study has been approved by the Xiangya Hospital Central South University; written informed consent was obtained from all subjects or their parents in the case of children. This work received approval from the institution ethics committee and conformed to the tenets of the Declaration of Helsinki. Eleven subjects (F/M = 6/5; Age = 25 ± 7) are with no metabolic disease, inherited diseases and other diseases which may affect the current study. Bone marrow aspirates were obtained from all subjects or their parents in the case of children. This work received approval from the institution ethics committee and conformed to the tenets of the Declaration of Helsinki. Eleven subjects (F/M = 6/5; Age = 25 ± 7) are with no metabolic disease, inherited diseases and other diseases which may affect the current study. Bone marrow aspirates were obtained during routine orthopedic surgical procedures. Marrow aspirates (20 ml volumes) were harvested using a bone marrow biopsy needle inserted through the cortical bone; aspirates were immediately resuspended in α-MEM (Life technologies; Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/l streptomycin (Life technologies), and cultured in a humidified 37 °C/5% CO2 incubator. hBMSC were selected on the basis of adhesion and proliferation on tissue culture plastic substrate. The obtained BMSCs were cultured and expanded for further experiments. The BMSCs prior to passage four were used in the following experiments. To induce osteoblastic differentiation, BMSCs were cultured in an osteogenic medium (α-MEM supplemented with 10% FBS, 50 mg/ml l-ascorbic acid, 10 mM glycerophosphate and 100 nM dexamethasone) and antibiotics (Sigma; St. Louis, MO) for 7 days.

2.2. Cells culture and transfection

BMSCs were incubated in DMEM (Gibico, Invitrogen) with 10% fetal bovine serum (Gibico, Invitrogen) and 100 U/mL penicillin/streptomycin (Invitrogen). BMSCs were maintained at 37 °C and 5% CO2. miR-199b-5p mimics, miR-199b-5p inhibitor, and relevant controls were purchased from Invitrogen. BMSCs were inoculated into 6-well plates at a density of 4.0 × 105 per well and were transiently transfected using Lipofectamine RNAiMAX (Invitrogen) when they reached 60% confluence. Cells were collected at 0 and 7 d after transfection, and 3 independent repeats were performed for all experiments.

2.3. Quantitative real time PCR (qRT-PCR)

After treating with experimental test agents, total cellular RNA was extracted using TRizol reagent (Invitrogen). cDNA was synthesized from 1 μg total RNA using a reverse transcription kit (Takara). qRT-PCR with SYBR Green was performed using a Bio-Rad real time PCR system following the manufacturer’s protocol. Melt-curve analysis was conducted to verify that only one product was produced. The mRNA levels of specific genes were calculated relative to the GAPDH levels using the 2−ΔΔCt method.

2.4. Western blotting

Total cellular protein was collected using RIPA Lysis Buffer according to the manufacturer’s instruction (Beyotime, China). Aliquots of protein from each sample were used to assay the protein levels of ALP, Runx2, GSK-3β and β-catenin with GAPDH as the internal control. Antibodies were purchased from Abcam or Santa Cruz Biotechnology. Proteins were subjected to SDS-PAGE on a 12% polyacrylamide gel and transferred onto a PVDF membrane (Millipore, MA). After blocking with 5% nonfat milk in TBS containing 0.05% Tween-20, the membrane was incubated overnight at 4 °C with the primary antibody. Subsequently, the appropriate secondary antibody (horseradish peroxidase-conjugated) was added, and bound antibody was visualized via chemiluminescence using an ECL kit (Amersham, Germany).

Fig. 1. The expression of miR-199b-5p was altered during osteogenesis (A) The expression of miR-199b-5p was determined during BMSCs osteogenesis. The expression levels of miR-199b-5p were up-regulated in a differentiation time-dependent manner, and reached the highest level on day 7 compared to day 0. (B) The formed mineral nodules were identified by ARS staining in treated cells on day 0 and day 7. Results showed that after 7 days induction, the proportion of mineralization was increased compared with day 0. The data were presented as mean ± SD of three independent experiments.
2.5. ALP activity and ARS staining assay

On day 7, treated cells were washed twice with PBS and lysed by two cycles of freezing and thaw. ALP activity in the cells was measured colorimetrically by monitoring the release of p-nitrophenol from p-nitrophenyl phosphate at 37 °C using an ALP assay kit (Nanjing Jiancheng Biotech., China). Production of 1 mg p-nitrophenol at 37 °C in 15 min was described as 1 unit (U). ALP activity was normalized to total protein, which was determined using a BCA assay kit (Beyotime, China), and expressed as U/g protein.

Following a 7-d treatment with the specified agents, ARS staining was performed to detect the mineralized nodules in BMSCs, as previously described [15]. Additionally, the bound dye was dissolved with 10% cetylpyridinium chloride, and ARS in samples was quantified by measuring absorbance at 550 nm. Parallel wells in culture were used for DNA isolation using a standard kit (Beyotime, China). The stained ARS was normalized to total DNA content, and expressed as µmol ARS/µg total DNA.

2.6. Statistical analysis

Three independent repeats were performed for all experiments. Data were expressed as MEAN ± SD. Origin software was used to analyze differences between samples, either by the two-sample student t-test or by one-way ANOVA for differences between selected pairs of samples. P < 0.05 was considered significant.

3. Results

3.1. The expression of miR-199b-5p was altered during osteogenesis

Firstly, we tested whether the expression of miR-199b-5p changes during BMSCs osteogenesis. Osteoblast identification was achieved by using ARS staining assay, and the results were shown in Fig. S1. BMSCs were cultured to induce osteoblastic differentiation for 7 d, and the expression levels of miR-199b-5p and the degrees of differentiation were determined on day 0, day 3 and day 7. Results showed that the expression levels of miR-199b-5p were up-regulated in a differentiation time-dependent manner, and reached the highest level on day 7 compared to day 0 (Fig. 1A). The formed mineral nodules were identified by ARS staining in treated cells on day 0 and day 7. Results showed that after 7 days induction, the proportion of mineralization was increased compared with day 0 (Fig. 1B). These data indicated that miR-199b-5p expression increased with the degree of BMSCs differentiation.

Fig. 2. miR-199b-5p modulates osteogenic differentiation in BMSC
(A) miR-199b-5p overexpression was achieved by miR-199b-5p mimics transfection, and miR-199b-5p inhibition by miR-199b-5p inhibitor transfection. (B and C) The expression levels of ALP and Runx2 were up-regulated in response to miR-199b-5p overexpression while down-regulated after miR-199b-5p inhibition, compared to control and miR-NC group. (D) ALP activity in the cells was significantly promoted by miR-199b-5p overexpression while reduced by miR-199b-5p inhibition in the mean time, compared to control and miR-NC group. The data were presented as mean ± SD of three independent experiments.
3.2. miR-199b-5p modulates osteogenic differentiation in BMSC

To further investigate the effect of miR-199b-5p on BMSCs differentiation, we determined the expression and activity of ALP, and the expression of Runx2 during BMSCs osteogenesis. miR-199b-5p overexpression was achieved by miR-199b-5p mimics transfection, and miR-199b-5p inhibition by miR-199b-5p inhibitor transfection (Fig. 2A). Results showed that the expression levels of ALP and Runx2 were up-regulated in response to miR-199b-5p overexpression while down-regulated after miR-199b-5p inhibition, compared to control and miR-NC group (Fig. 2B and C), in addition, ALP activity in the cells was significantly promoted by miR-199b-5p overexpression while reduced by miR-199b-5p inhibition in the mean time, compared to control and miR-NC group (Fig. 2D). These data suggested that miR-199b-5p plays an important role in stimulating BMSCs osteoblast differentiation.

3.3. GSK-3β/β-catenin signaling pathway was involved in osteogenesis

Since GSK-3β has been regarded as a direct target of miR-199b-5p, we further detected whether the GSK-3β/β-catenin signaling pathway was associated with osteogenesis. We determined the mRNA and protein expression of GSK-3β and β-catenin on day 0 and day 7 of the BMSCs differentiation. Results showed that GSK-3β mRNA expression was down-regulated on day 7 compared to day 0 (Fig. 3A), while β-catenin mRNA expression was up-regulated on day 7 compared to day 0 (Fig. 3B). Consistent results were observed with the protein expression of GSK-3β and β-catenin (Fig. 3C). GSK-3β protein expression was down-regulated on day 7 compared to day 0, while β-catenin protein expression was up-regulated on day 7 compared to day 0. These data suggested that GSK-3β/β-catenin signaling pathway was involved in osteogenesis.

3.4. miR-199b-5p regulates the BMSCs osteoblast differentiation through GSK-3β/β-catenin signaling pathway by directly targeting GSK-3β during osteogenesis

To further elucidate whether miR-199b-5p regulates the BMSCs osteoblast differentiation through GSK-3β/β-catenin signaling pathway during osteogenesis, we determined the protein expression of GSK-3β, β-catenin, ALP and Runx2 in response to miR-199b-5p overexpression and inhibition by using Western blot assay. Results showed that GSK-3β protein expression was negatively regulated by miR-199b-5p, in other words, up-regulated by miR-199b-5p inhibition while down-regulated by miR-199b-5p overexpression; β-catenin protein expression was up-regulated by miR-199b-5p overexpression while down-regulated by miR-199b-5p inhibition; compared to control and miR-NC group (Fig. 4A). In addition, the protein expressions of ALP and Runx2 were both up-regulated by miR-199b-5p overexpression while down-regulated by miR-199b-5p inhibition, compared to control and miR-NC group (Fig. 4B). In order to investigate the association of miR-199b-5p with GSK-3β, we created a wild type GSK-3β 3’ UTR luciferase reporter vector (wt-GSK-3β), as well as two mutant GSK-3β 3’ UTR luciferase reporter vectors (mut-GSK-3β) by sequentially mutating two predicted miR-199b-5p binding sites in the GSK-3β 3’ UTR (Fig. 4C). The wt-GSK-3β/mut-GSK-3β vectors and miR-NC/miR-199b-5p mimics were co-transfected into cells. The luciferase activity of the wt-GSK-3β vector was significantly reduced by miR-199b-5p mimics transfection, compared to control groups (Fig. 4C). Moreover, miR-199b-5p-mediated repression of GSK-3β 3’UTR luciferase reporter activity was abolished by mutation of the putative miR-199b-5p binding sites in the GSK-3β 3’UTR (Fig. 4C). These data suggested that miR-199b-5p regulates the BMSCs osteoblast differentiation through GSK-3β/β-catenin signaling pathway by directly targeting to GSK-3β 3’UTR during osteogenesis.

**Fig. 3.** GSK-3β/β-catenin signaling pathway was involved in osteogenesis (A) GSK-3β mRNA expression was down-regulated on day 7 compared to day 0. (B) β-catenin mRNA expression was up-regulated on day 7 compared to day 0. (C) GSK-3β protein expression was down-regulated on day 7 compared to day 0, while β-catenin protein expression was up-regulated on day 7 compared to day 0. The data were presented as mean ± SD of three independent experiments.
4. Discussion

The physiological role of microRNAs (miRNAs) in osteoblast differentiation remains elusive. Recently, emerged evidences have revealed that miRNAs are correlated with osteogenesis. Baglio et al. demonstrated that primary MSCs release small RNAs via exosomes, which are increasingly implicated in intercellular communications [16]. Bioinformatic analysis by DIANA-mirPath demonstrated that several miRNAs expression altered during osteogenic differentiation, suggesting exosomal miRNA is a regulator of osteoblast differentiation. The role of miR-199b-5p in regulating the osteoblast differentiation was further explored in this study. The results showed that miR-199b-5p regulates the BMSCs osteoblast differentiation through GSK-3β/β-catenin signaling pathway during osteogenesis.
establish GSK-3 as a critical transcription factor in osteogenesis. Previous study has osteoblast differentiation of BMSCs still remained unclear. Runx2 is changes of Runx2 and ALP, and the activation of ALP activity. 

miR-199b-5p functions as a positive regulator of osteogenesis, since knockdown of miR-199b-5p inhibited, whereas over-expression of it enhanced, the expression levels of ALP and Runx2.

In the course of BMSC osteogenesis, miR-199b-5p was significantly up-regulated on day 7, compared to that of day 0. The meantime, we also found a higher proportion of mineralization on day 7 than that of day 0. The expression alternation of miR-199b-5p indicated that it might be involved in osteoblast differentiation. This was consistent with the previous study, which indicated that miR-199b expression was up-regulated in BMSC [12]. This was consistent with the previous study, which indicated that miR-199b expression was up-regulated in BMSC [12].

miR-199b-5p expression was up-regulated on mRNA and protein levels on day 7 compared to day 0, while β-catenin mRNA expression was up-regulated on mRNA and protein levels on day 7 compared to day 0, indicating that GSK-3β/β-catenin signaling pathway was involved in osteogenesis. To further investigate the association between GSK-3β and miR-199b-5p, we further detected whether miR-199b-5p regulates the BMSCs osteoblast differentiation through GSK-3β/β-catenin signaling pathway during osteogenesis. GS-3β protein expression was up-regulated by miR-199b-5p inhibition while down-regulated by miR-199b-5p overexpression; β-catenin protein expression was up-regulated by miR-199b-5p overexpression while down-regulated by miR-199b-5p inhibition. In addition, the protein expression of ALP and Runx2 were both up-regulated by miR-199b-5p overexpression while down-regulated by miR-199b-5p inhibition. These data suggested that miR-199b-5p regulates the BMSCs osteoblast differentiation through GSK-3β/β-catenin signaling pathway during osteogenesis.

In summary, miR-199b-5p is induced during osteoblast differentiation of BMSCs. miR-199b-5p acts as an activator in osteoblast differentiation, because knockdown of miR-199b-5p reduces, while overexpression of it enhances, the differentiation process. miR-199b-5p associates with ALP and Runx2 expression, as well as ALP activity. Moreover, miR-199b-5p regulates the BMSCs osteoblast differentiation through GSK-3β/β-catenin signaling pathway during osteogenesis. Collectively, miR-199b-5p functions as a positive regulator in osteoblast differentiation of BMSCs.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.06.130.

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