miR-219a-5p Suppresses GSK-3 β / β -catenin Signaling to Influence BMSC Osteogenesis

Renbang Zhou

Bengbu Medical College

Xunbing Zhu (✉ Zhuxb22@163.com)

The Second Affiliated Hospital of Bengbu Medical College <https://orcid.org/0000-0003-0121-7159>

ZhongChuan Zhang

The Second Affiliated Hospital of Bengbu Medical College

KeWei Guo

The Second Affiliated Hospital of Bengbu Medical College

ZhanQiu Dai

Bengbu Medical College

TengFei Hu

The Second Affiliated Hospital of Bengbu Medical College

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Abstract

Backgrounds: MicroRNAs play diverse roles in a range of biological processes, including osteoblastic differentiation. The tumor suppressor miR-219a-5p has been found to be functionally important in several tumor types, yet its expression and mechanistic relevance in the context of bone marrow stem cell (BMSC) osteogenic differentiation remain to be defined.

Methods: Osteogenic induction medium was used to induce osteogenic differentiation in human BMSCs (hBMSCs), after which alkaline phosphatase (ALP) activity was measured and Alizarin red S staining was performed to gauge the efficiency of osteogenesis. Additionally, qPCR was used to assess miR-219a-5p, GSK-3 β , and osteogenic gene expression, while levels of proteins associated with osteoblastic differentiation and the Wnt/ β -catenin signaling pathway were detected via Western blotting in order to assess how miR-219a-5p inhibitors and mimics influenced the processes of osteogenic differentiation. Mir-219a-5p and GSK-3 β binding sites were predicted via a bioinformatics approach, with dual-luciferase reporter assays being performed to validate such binding.

Results: A time-dependent increase in miR-219a-5p expression was observed over the course of hBMSC osteogenic differentiation. Transfection with miR-219a-5p mimics enhanced the expression of markers of osteoblast cells (RUNX2, ALP), whereas miR-219a-5p inhibitors yielded the opposite effect. The ability of miR-219a-5p to directly bind the 3'-UTR of GSK-3 β was confirmed via luciferase reporter assay, highlighting GSK-3 β as a target of this miRNA involved in promoting osteogenesis such that miR-219a-5p is able to regulate osteogenic differentiation in BMSCs via the GSK-3 β / β -catenin pathway. Moreover, we found that overexpressing GSK-3 β was sufficient to reverse the impact of miR-219a-5p on hBMSC osteogenesis.

Conclusion: These data indicate that miR-219a-5p is capable of directly targeting the Wnt/ β -catenin pathway gene GSK-3 β , thereby regulating BMSC osteogenesis. As such, targeting this miRNA may represent a new approach to enhancing hBMSC osteogenesis.

Introduction

Osteoporosis (OP) is a disease that compromises the microstructural integrity of bone tissue and is associated with low bone mass in affected patients. OP patients exhibited a significantly elevated fracture risk, imposing a substantial burden on global society [1]. The stimulation of bone growth represents a promising treatment for OP. Pluripotent bone marrow mesenchymal stem cells (BMSCs) can, in response to particular environmental cues, differentiate into muscle, cartilage, or bone tissues [2–4]. BMSCs are key osteoblast precursors that are thus closely linked to bone development [5]. As such, further study of the mechanisms whereby BMSCs undergo osteogenesis is warranted.

MicroRNAs (miRNAs) are short RNAs that lack coding potential, yet are able to interact with specific proteins to generate the RNA silencing ribonucleoprotein complex (RISC), binding to complementary target mRNAs and thereby suppressing their translation or promoting their degradation depending on the

degree of sequence overlap. Through this mechanism, miRNAs can post-transcriptionally regulate gene expression [6]. Over 1,000 miRNAs have been identified *in vivo* and can regulate a variety of biological processes including proliferation, tumorigenesis, apoptosis, inflammation, and cell differentiation. Notably, there is a growing body of evidence suggesting that specific miRNAs can regulate BMSC osteoblastic differentiation [7, 8], including miR-212, miR-384, miR-151B, and miR-27a-3p, which target specific osteoblast-related genes [9–11].

Prior work has identified miR-219-5p as a tumor suppressor miRNA involved in a range of oncogenic contexts [12, 13]. Wang et al. also previously reported the upregulation of this miRNA in the context of hBMSC osteogenesis *in vitro*, suggesting it may play a role in the context of bone development [14]. However, the specific mechanistic role of miR-219a-5p during osteogenesis remains uncertain. GSK-3 β is a suppressor of Wnt/ β -catenin signaling activity that controls BMSC osteoblastic differentiation. Whether miR-219a-5p can interact with GSK-3 β , however, has yet to be established. The present study was thus developed to assess the role of miR-219a-5p as a regulator of hBMSC osteogenesis through gain- and loss-of-function analyses, with a specific focus on the ability of this miRNA to regulate GSK-3 β . Together, our data suggest that miR-219a-5p can downregulate GSK-3 β to enhance Wnt/ β -catenin pathway activation, thus driving hBMSC osteogenesis. As such, miR-219a-5p may offer value as a therapeutic target for the treatment of OP.

Materials And Methods

1.1 Cell culture

All hBMSCs were from Saiye Biotechnology Co., LTD (HUXMA-01001, Guangzhou, China), and were cultured in Oricell hBMSC complete medium (HuXMA-90011, Saiye Biological Co., LTD) in a 5% CO₂ tissue culture incubator at 37°C, with media being changed every 2-3 days. Cells were passaged when 80-90% confluent, and used between passages 3 and 6.

1.2 Osteogenic differentiation

For osteogenic induction, hBMSCs were added to 6-well plates (2×10⁴ cells/cm²) and cultured as above until 60-70% confluent, at which time media was replated with 2 mL of OriCell Adult BMSC osteogenic induction complete differentiation medium, with this media being replaced every 2-3 days for 14 days

1.3 Alizarin red S staining

At appropriate time points, hBMSCs were harvested, rinsed two times in PBS, fixed for 30 min in 4% neutral-buffered formaldehyde, washed again with PBS, and stained for 3-5 min with alizarin red solution. Cells were then rinsed 2-3 times with PBS and images under a microscope in culture plates to evaluate osteogenic staining.

1.4 ALP activity measurements

At appropriate time points, hBMSCs were harvested, rinsed two times using PBS, and lysed for 30 min at 4°C with an appropriate lysis buffer. Supernatant ALP activity was then measured via ELISA (Nanjing), with absorbance in each well (520 nm) being measured to calculate these activity levels.

1.5 Transfection

NC mimic, mir-219a-5p mimic, NC inhibitor, and mir-219a-5p inhibitor constructs were synthesized by Ruibo Biological Co., LTD. When cells were ~70% confluent, they were transfected with appropriate constructs using Lipofectamine 2000 (Invitrogen). At 6 h post-transfection, complete culture media was used to replace the culture supernatants. At 48-72 h after transfection, cells were used in downstream experiments.

1.6 RT-qPCR

Trizol (Invitrogen) was used to extract RNA from cells based on provided directions, after which RNA concentrations were measured, and cDNA was prepared using the HiScript® iii RT SuperMix for qPCR (+gDNA Wlper) kit based on provided directions, with 1 ug of RNA per sample being processed. Nt, qPCR reactions were performed with the ChamQ Universal SYBR qPCR Master Mix and the following thermocycler settings: 40 cycles of 95°C for 30 s, 95°C for 10 s, and 60°C for 30 s. The 2-ΔΔCT method was used to assess relative gene expression, with U6 and GAPDH respectively being used to normalize miRNA and mRNA expression levels. Primers used in this study are listed in Table 1.

Table 1. Primer Sequences

Gene	Primer sequence (5 ‘-3 ‘)
miR-219a-5p	F: CGCGCCTGATTGTCCAAACGCAATTCT R: CTCAACTGGTGTCTGTGGA
U6	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTCAT
RUNX2	F: AGATGATGACACTGCCACCTCTG R: GGGATGAAATGCTTGGAAGTGC
ALP	F: GACGCTGGGAAATCTGTGG R: ATGAAGTGGGAGTGCTTGTATCT
GSK-3β	F: GCTTTGAAAGTAATCCCTGGGGTTTGG R: TGCAGAGGTGCAAAACGGAGCA
β – Catenin	F: ATTTGATGGAGTTGGACATGGC R: ATCAGCTCTTGTTCTTGAGTGA
GAPDH	F: CAAGGTCATCCATGACAACCTTG R: GTCCACCACCCTGTTGCTGTAG

1.7 Western blotting

RIPA buffer (Beyotime) was used to collect total cellular protein, after which a BCA kit (Beyotime) was used to quantify protein levels. Then, protein samples (40μg) were separated via SDS-PAGE and

transferred to PVDF membranes that were blocked with 5% BSA at room temperature prior to overnight incubation with antibodies specific for ALP, RUNX2, GSK-3 β , or β -catenin at 4°C (1:1000). Blots were then probed with goat anti-rabbit secondary antibodies for 2 h, after which protein bands were detected using an ECL reagent and a chemiluminescent detector, with relative protein levels being determined using an appropriate software application.

1.8 Luciferase reporter assay

The segment of GSK-3 β harboring a putative miR-219a-5p binding site was cloned into the psicheck2 vector to yield a wild-type (WT) GSK-3 β plasmid, while a mutated version of this segment was similarly used to generate a mutant (MUT) GSK-3 β plasmid. HEK293T cells were plated in 12-well plates overnight, after which they were co-transfected with miR-219a-5p mimics or corresponding NC constructs and WT or MUT GSK-3 β plasmids using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, a dual-luciferase reporter gene assay kit was used to assess luciferase activity.

1.9 Statistical analysis

Analyses were repeated in triplicate, and data are means $\pm$ standard deviation (SD). Analyses were performed using GraphPad Prism 8 and SPSS, with results being compared via one-way ANOVAs with $P < 0.05$ as the significance threshold.

Results

BMSCs exhibit miR-219A-5p upregulation during the process of osteogenic differentiation

To explore the functional role of miR-219a-5p in the context of hBMSC osteogenesis, we cultured hBMSCs in osteogenic induction media and then measured the levels of this miRNA in these cells on days 0, 7, and 14 of this process. We observed the time-dependent upregulation of miR-219a-5p over the course of differentiation such that it was maximally expressed on day 14 (Figure 1A). Alizarin red S staining confirmed that osteogenic induction had been successful, with a higher mineralization ratio on day 14 relative to days 0 and 7 (Figure 1B). ALP activity also rose over time (Figure 1C). These results suggest that the expression of miR-219a-5p rose as the degree of hBMSC differentiation increased.

miR-219a-5p promotes hBMSC osteogenic differentiation

To more fully explore the role of miR-219a-5p as a regulator of BMSC osteogenesis, we next overexpressed or knocked down this miRNA in the context of osteogenic induction using appropriate mimic and inhibitor constructs (Figure 2A). Relative to the control and miR-NC groups, miR-219a-5p overexpression was associated with ALP and RUNX2 upregulation, whereas miR-219a-5p inhibition had the opposite effect (Figure 2B-C). Overexpressing miR-219a-5p similarly enhanced ALP activity in these cells, while its inhibition had the opposite effect (Figure 2D). As such, miR-219a-5p may regulate hBMSC osteogenesis. miR-219a-5p

miR-219a-5p induces hBMSC osteogenesis via targeting GSK-3 β to modulate Wnt/ β -catenin pathway activity

Next, a bioinformatics approach was used to predict downstream miR-219a-5p target genes. Among the identified candidate genes, the Wnt/ β -catenin pathway regulator GSK-3 β was identified and selected for further study (Figure 3A). After identifying predicted sequence complementarity between miR-219a-5p and the GSK-3 β mRNA sequence, wild-type and mutant GSK-3 β luciferase reporter vectors (WT-GSK-3 β and MUT-GSK-3 β) were generated (Figure 3B). HEK293T cells were then co-transfected with miR-219a-5p or miR-NC mimic constructs along with either the WT-GSK-3 β or MUT-GSK-3 β vectors, revealing that miR-219a-5p did not impact luciferase activity in cells transfected with the MUT-GSK-3 β construct whereas it did significantly suppress WT-GSK-3 β luciferase reporter gene activity (Figure 3C). Together, these data suggested that miR-219a-5p can bind to the 3'-UTR of GSK-3 β to suppress its expression.

Next, we further explored the ability of miR-219a-5p to regulate osteogenesis via this GSK-3 β / β -catenin signaling axis. Protein levels of GSK-3 β , β -catenin, and the osteogenesis-related proteins ALP and Runx2 were measured via Western blotting, revealing significant reductions in GSK-3 β levels and significant increases in β -catenin levels following miR-219a-5p overexpression, while miR-219a-5p inhibition had the opposite effect (Figure 3D). Moreover, miR-219a-5p overexpression significantly increased ALP and Runx2 protein levels, whereas miR-219a-5p yielded the opposite phenotype (Figure 3E). Overall, these findings indicate that miR-219a-5p can control the osteoblastic differentiation of BMSCs via the GSK-3 β / β -catenin signaling axis.

miR-219a-5p regulated hBMSC osteogenesis via a GSK-3 β -dependent mechanism

Lastly, we explored the ability of GSK-3 β overexpression to reverse the impact of miR-219a-5p on osteogenic differentiation. GSK-3 β levels in hBMSCs were significantly increased following GSK-3 β vector transfection relative to the empty pcDNA3.1 vector group (Figure 4A). We then co-transfected hBMSCs with miR-219a-5p mimics along with these pcDNA3.1 or GSK-3 β vectors. Relative to the NC or miR-219a-5p + GSK-3 β transfection groups, GSK-3 β mRNA levels were significantly lower in cells in the miR-219a-5p + pcDNA3.1 group (Figure 4B). Western blotting was used to assess osteogenesis-related Runx2 and ALP protein levels, revealing that both were upregulated following miR-219a-5p mimic transfection, whereas GSK-3 β overexpression significantly reduced these levels and reversed the miR-219a-5p-mediated upregulation thereof (Figure 4C). Together, these findings suggest that overexpressing GSK-3 β can reverse the impact of miR-219a-5p on osteogenic differentiation.

Discussion

As the global population continues to age, the incidence of OP among middle-aged and elderly individuals and associated bone fracture rates will similarly rise [1]. A variety of clinical interventions for OP have been developed to date including calcium supplementation, hormone administration, and the inhibition of bone resorption, but these treatments are of limited efficacy owing to associated side effects and poor drug absorption, thus adversely impacting outcomes among elderly individuals and

postmenopausal women[15]. Approaches to preventing and treating OP are thus critically needed. Pluripotent BMSCs can play key roles in bone repair, regeneration, and development [16]. As such, BMSCs represent promising targets for the prevention and treatment of OP. BMSC osteogenesis is regulated by the coordination of a range of signaling mechanisms [5, 17, 18]. As key post-transcriptional regulators of gene expression, miRNAs can directly impact these osteoblastic differentiation pathways to control BMSC fate determination [19].

Initial bioinformatics analyses (Dian-mirPATH) demonstrated miR-219a-5p to be upregulated in hBMSCs in the context of osteogenesis, yet its regulatory role in this context had yet to be defined. As such, the present study was executed to clarify the mechanistic role of this miRNA during osteogenic differentiation. On day 14 of BMSC osteogenesis, we found miR-219a-5p to be upregulated relative to on day 0, with concomitant increases in mineralization ratio values and ALP activity levels, suggesting a possible link between miR-219a-5p and osteogenic differentiation. Overexpressing miR-219a-5p levels resulted in the upregulation of ALP and Runx2 levels in these cells together with an increase in ALP activity, whereas the opposite was observed following miR-219a-5p inhibition. As such, miR-219a-5p may positively influence BMSC osteogenesis. We then screened downstream regulatory targets of miR-219a-5p, leading to the identification of the Wnt/ β -catenin signaling pathway regulator GSK-3 β as a direct miR-219a-5p target gene as confirmed in a dual-luciferase reporter assay. We further found miR-219a-5p overexpression to reduce and increase GSK-3 β and β -catenin protein levels, respectively, whereas the opposite was observed upon miR-219a-5p inhibition. Such miR-219a-5p overexpression similarly increased Runx2 and ALP protein levels, while its inhibition reduced these levels. Overall, these data suggest that miR-219a-5p can regulate BMSC osteogenesis via the GSK-3 β / β -catenin signaling axis. consistently, we confirmed the ability of GSK-3 β overexpression to reverse the impact of miR-219a-5p on osteogenic differentiation.

Conclusion

In summary, our data highlight miR-219a-5p as a regulator of the GSK-3 β / β -catenin pathway that can control BMSC osteogenesis. Further studies should be focused on the role of miR-219a-5p in osteogenesis in vivo.

Abbreviations

GSK-3 β : Glycogen synthase kinase 3 beta; OP: Osteoporosis; β -catenin: beta-catenin; ALP: alkaline phosphatase; BMSC: Bone marrow stem cell; ARS: Alizarin red S; miRNA: microRNA; RUNX2: RUNX family transcription factor 2; 3'UTR: 3'-Untranslated region; RT: Reverse transcription; RISC: RNA silencing ribonucleoprotein complex; cDNA: Complementary DNA; GAPDH: Glyceraldehyde-3-phosphate; WT: Wild-type; MUT: Mutant; SD: Standard deviation; qPCR: Real-time quantitative polymerase chain reaction

Declarations

Acknowledgements

Not applicable.

Authors' contributions

XBZ and RBZ contributed to the concept. RBZ and CCZ analyzed the data, and RBZ draft the manuscript. KWG, ZQD, and TFH revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Mir-219a-5p levels rise over the course of hBMSC osteogenic differentiation. (A) miR-219a-5p levels on days 0, 7, and 14 of osteogenic induction via qPCR. ARS (B) and ALP (C) were utilized to assess hBMSC mineralization ratios and ALP activity levels over the course of differentiation. Data are means ± standard deviation (SD) of three independent trials. * P < 0.05, ** P < 0.01

Figure 2

miR-219a-5p promotes hBMSC osteogenesis. (A) BMSCs were transfected with miR-219a-5p mimic or inhibitor constructs. (B, C) At 48 h post-transfection, ALP and Rux2 mRNA levels were assessed via qPCR. (D) ALP activity was assessed following miR-219a-5p knockdown or overexpression. Data are means $\pm$ standard deviation (SD) of three independent trials. * $P < 0.05$, ** $P < 0.01$

Figure 3

Mir-219a-5p drives hBMSC osteogenesis by targeting GSK-3β to regulate Wnt/β-catenin pathway activity. Downstream miR-219a-5p target genes were identified via bioinformatics approaches, with a focus on the Wnt/ B-catenin pathway-related GSK-3β gene for subsequent analyses (A). Predicted miR-219a-5p binding sites in the GSK-3β 3'UTR were identified via a bioinformatics approach, with WT and mutant vectors (WT-GSK-3β and MUT-GSK-3β) being constructed (B). HEK293T cells were co-transfected with

miR-219a-5p mimics and expression vectors, after which a dual-luciferase activity assay was performed (C). Following miR-219a-5p knockdown or overexpression, GSK-3 β protein levels were increased in the miR-219a-5p inhibitor group relative to the control group, while the opposite was observed following miR-219a-5p overexpression. Conversely, β -catenin protein levels were reduced and increased following miR-219a-5p inhibition and overexpression, respectively (D). ALP and Runx2 protein levels were increased following miR-219a-5p overexpression, while the inhibition of this miRNA yielded the opposite outcome (E). Data are means $\pm$ standard deviation (SD) of three independent trials. * $P < 0.05$, ** $P < 0.01$

Figure 4

miR-219a-5p regulates GSK-3 β to influence hBMSC osteogenesis. (A) GSK-3 β expression levels in hBMSCs following pcDNA3.1 or GSK-3 β vector transfection as assessed via qPCR. (B) Following co-transfection with miR-219a-5p mimics and pcDNA3.1 or GSK-3 β , relative to the NC or miR-219a-5p + GSK-3 β groups, GSK-3 β levels in the miR-219a-5p + pcDNA3.1 group were significantly reduced. (C) Western blotting was used to assess the impact of miR-219a-5p mimic and GSK-3 β or pcDNA3.1 on ALP and Runx2 protein levels. Data are means $\pm$ standard deviation (SD) of three independent trials. * $P < 0.05$, ** $P < 0.01$