Regulatory effects of miR-28 on osteogenic differentiation of human bone marrow mesenchymal stem cells

Min Wang, Tianming Dai, Qingqi Meng, Wen Wang, and Siming Li

ABSTRACT
We aimed to assess the regulatory effects of miR-28 on the osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMMSCs). HBMMSCs isolated, cultured and induced (at P3) to undergo osteogenic induction. The expressions of miRNAs were detected by gene micro-array, and differentially expressed miRNAs in HBMMSCs compared with induced cells were obtained by significance analysis of microarrays. The microarray findings were confirmed by RT-PCR. TargetScan showed that signal transducer and activator of transcription 1 (STAT1) was the downstream target gene of miR-28. The relationship between miR-28 and STAT1 was validated using dual-luciferase reporter gene assay. HBMMSCs were transfected with miR-28 mimics and STAT1 siRNA, respectively. Samples were collected on day 10 after osteogenic differentiation, and the alkaline phosphatase (AKP) activity, Runt-related transcription factor 2 (RUNX2, a key regulator of osteogenic differentiation) and STAT1 expressions were determined using kits, PCR and Western blotting, respectively. During the osteogenic differentiation of HBMMSCs, the expression level of miR-28 increased. MiR-28 specifically bound the 3'-untranslated region (3'UTR) of STAT1 mRNA. It inhibited STAT1 expression in a targeted manner during osteogenic differentiation. Interference with STAT1 partially mimicked the regulatory effects of miR-28 overexpression on the osteogenic differentiation of HBMMSCs. Interference with STAT1 or overexpression of miR-28 did not affect proliferation or migration. MiR-28 has gradually increased expression during the osteogenic differentiation of HBMMSCs, which can directly bind STAT1 3'UTR and inhibit its expression, thereby up-regulating AKP and RUNX2, and promoting osteogenic differentiation.

ARTICLE HISTORY
Received 23 September 2021
Revised 24 November 2021
Accepted 25 November 2021

KEYWORDS
MiR-28; human bone marrow mesenchymal stem cell; osteogenic differentiation

Introduction
Bone defects affect the quality of patients’ life and cause huge social and economic losses.1 Severe bone injuries caused by fractures cannot be repaired by the self-healing system alone.2,3 Currently, there is still an insufficient demand of bone transplantation for bone repair and reconstruction due to donor-autologous bone graft, potential epidemic transmission, immune rejection of allogeneic and xenograft bone, and low biological function of synthetic bone.4,5

Human bone marrow mesenchymal stem cells (HBMMSCs) are multipotent progenitor cells with self-renewal and multidirectional differentiation potential, such as osteogenesis, chondrogenesis and lipogenesis.6 They have well-documented therapeutic effects on a variety of degenerative diseases, such as osteoarthritis, rheumatoid arthritis, and bone defects.7 Moreover, hBMMSCs are effective for treating non-skeletal diseases.8 However, the molecular mechanism regulating the osteogenic differentiation of hBMMSCs remains elusive.

MiRNAs are a class of small RNAs involved in post-transcriptional regulation, which can lead to degradation or translational repression by base pairing with target gene mRNA, thereby participating in the regulation of growth and development, signal transduction, cell growth, differentiation and apoptosis, and occurrence of diseases.9 They dominate in the regulation of stem cell self-renewal and multidirectional differentiation.10,11 Besides, miRNAs can also participate in regulating the osteogenic differentiation of HBMMSCs.

In this study, therefore, a model of HBMMSCs and those cultured by osteogenic induction was used, the
differential expression of miRNAs was observed through gene microarray, miRNAs possibly involved in the osteogenic differentiation of hBMMSCs were screened, and their possible regulatory effects on osteogenic differentiation were investigated, aiming to provide a potential target for the prevention and treatment of diseases related to osteogenic regeneration and development.

Materials and methods

Materials

Percoll solution (1.073 g/mL) was provided by Sigma (USA). L-DMEM and H-DMEM were obtained from Gibco (USA). Fetal bovine serum (FBS) was bought from Hangzhou Sijiqing Biotechnology Co., Ltd. (China). Ampicillin, streptomycin, pancreatic and phosphate buffered saline (PBS) were purchased from Xi’an Maojian Biotechnology Co., Ltd. (China). Mouse anti-human signal transducer and activator of transcription 1 (STAT1) monoclonal antibody (Jingmei Bioengineering Co., Ltd., China), dexamethasone, vitamin C, transforming growth factor-β1 (TGF-β1), insulin, transferrin and bovine serum albumin (BSA) (Sigma, USA) were used. Toluidine blue ethanol solution (0.2 g of toluidine blue (Shanghai YBio Biotechnology Co., Ltd., China) dissolved in 100 mL of 30% ethanol) and 4% paraformaldehyde were prepared in-house. TRIZol solution was from Invitrogen (USA), miRNA Isolation Kits were from Roche (Switzerland), and miRNA microarray V2.0 was purchased from Beijing CapitalBio Corporation (China; 576 miRNAs labeled in human, 238 in rats, and 358 in mice). LightCycler FastStart DNA Master SYBR Green I (Roche, Switzerland) and PCR primers (Shanghai Sangon Biotech Co., Ltd., China) were also used.

Isolation and culture of hBMMSCs

This study has been approved by the ethics committee of our hospital, and written informed consent has been obtained from all subjects. Bone marrow (10 mL) was extracted from volunteers (3 cases aged 30–45 years old, (36.45 ± 4.58) years on average) during iliac bone graft surgery, and mixed with an equal volume of Percoll solution. The mononuclear cell layer was separated by density gradient centrifugation, washed twice with L-DMEM, and resuspended in L-DMEM containing 100 U/mL ampicillin and 100 U/mL streptomycin with 10% FBS. After the concentration was adjusted to 2 × 10^6/mL, the cell suspension was seeded into a 50 cm^2 culture flask, and the medium was replaced on day 5 after culture, followed by replacement every other day. The growth status of adherent cells was observed. HBMMSCs isolation/cultures were conducted independently in triplicate. At confluence, the cells were trypsinized and subcultured (recorded as P1, P2, P3 . . . ).

Identification of hBMMSCs

The third-passage hBMMSCs at 50%–70% confluence were digested with 0.25% trypsin-EDTA and prepared into single-cell suspension. After the cell concentration was adjusted to 2 × 10^6/mL, they were seeded into a 6-well plate, and incubated in a 5% CO_2/37°C incubator overnight. On the next day, immunofluorescence staining was performed as follows: (1) The cells were rinsed with PBS 3 times, fixed with 4% paraformaldehyde for 30 min at room temperature, and rinsed again with PBS 3 times. (2) They were blocked with PBS containing 1% BSA for 1 h, and rinsed with PBS 3 times. (3) Primary antibodies (mouse anti-human CD34 hematopoietic stem cell surface molecule or mouse anti-human CD44 MSC surface molecule) were added for incubation at 37°C for 2 h. (4) Fluorescent secondary antibodies were added for incubation at 37°C for 60 min in the dark, the cells were washed with PBS 3 times, and residual PBS was sucked dry using a pipette tip. (5) Hoechst (1 mg/mL; Yeasen Biotechnology (Shanghai) Co., Ltd., China) was added for nuclear staining for 15 min in the dark, and the final Hoechst concentration was 1 µg/mL. The expression of cell surface markers was observed under a fluorescence microscope.

Osteogenic induction of hBMMSCs

The suspension (1 mL) of P3-passage hBMMSCs at a concentration of 4 × 10^6/mL was harvested, centrifuged at 500 × g for 15 min in a 15 mL plastic centrifuge tube to obtain cell micro-mass. Then the micro-mass was cultured with osteogenic induction medium, that is, serum-free H-DMEM containing
1 ng/mL TGF-β1, 10−7 mmol/L dexamethasone, 50 μg/mL vitamin C, 6.25 ng/L insulin, 6.25 μg/mL transferrin and 1.25 μg/mL BSA. The medium was replaced for the first time after 4 days, followed by replacement every other day until day 21.14

**MiRNA microarray**

P3-passage hBMSCs and those cultured by osteogenic induction for 21 days were collected, from which total RNA was extracted by TRIzol one-step method. Then, 1–2 μg of total RNA was taken, and small RNA fragments less than 100 nt were separated using miRNA Isolation Kits, followed by poly(A) tailing and reverse transcription. The amplified RNA samples were fluorescently labeled, hybridized with gene microarrays (mammalian miRNA microarray V2.0) at 42°C overnight, washed, and scanned using a Lux Scan 10 K/A dual-pathway laser scanner.15

**Data processing and analysis**

Lux Scan3.0 software was used to convert the image signals into digital signals. After data pre-processing, differentially expressed miRNAs in hBMSCs compared with those cultured by osteogenic induction for 21 days were obtained by significance analysis of microarrays (SAM, version 2.1). The screening conditions were as follows: False discovery rate (expressed as q-value after SAM) was controlled within 5%, and the fold change was not less than 2.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

The total RNA of P3-passage hBMSCs and those cultured by osteogenic induction for 21 days were reversely transcribed into cDNA, followed by PCR using real-time quantitative miRNA-specific primers and cDNA as templates with LightCycler FastStart DNA Master SYBR Green I. The reaction conditions were as follows: primer concentration: 0.25 μmol/L, denaturation at 95°C for 15 s, and annealing at 60°C for 30 s, 40 cycles in total. The melting curve was plotted at 70–95°C, and PCR amplification was performed by electrophoresis.16 The primer sequences are shown in Table 1.

| Table 1. Primer sequences. |
|---------------------------|
| Gene | Primer sequence |
|------|-----------------|
| miR-149 | F: 5′-TCGGGCTCCGTTCTCCTAAA-3′ |
|      | R: 5′-AGTGGTGCTCTGTCCTGA-3′ |
| miR-21 | F: 5′-TTGACCAAAACTCTCCGC-3′ |
|      | R: 5′-CAAGGCTTCCGGTAACAT-3′ |
| miR-572 | F: 5′-GGGGAGGTCGCTCCCGCA-3′ |
|      | R: 5′-CTCCTGGGTTCTGACCATTA-3′ |
| miR-130b | F: 5′-CAGAGTTGTCAAGAGAAGAAAA-3′ |
|      | R: 5′-CCGGCGAGTTGATGTGATTA-3′ |
| miR-193b | F: 5′-TGAGGCGAACGAAAGAGGATGAA-3′ |
|      | R: 5′-GACACATGCCGATGAATGAGA-3′ |
| miR-152 | F: 5′-GGCCATCTTCTGCTCCTGAA-3′ |
|      | R: 5′-AAAGCTTGGAAAGGGCGAGG-3′ |
| miR-560 | F: 5′-CTCTGGTGCGACCATA-3′ |
|      | R: 5′-AAGCTTTCAAGAATTCTCGT-3′ |
| miR-28 | F: 5′-GATGGTGAAGCTCGGTGTA-3′ |
|      | R: 5′-TGAACCTGCGTGTTGAGA-3′ |
| miR-424 | F: 5′-TTATGGGCTCAATAAGAAGAG-3′ |
|      | R: 5′-TTTACCTTGACGTGGTC-3′ |
| miR-122a | F: 5′-ACTTTTCTCTACGCCCTTT-3′ |
|      | R: 5′-TACCCTTCTCTTCTTATGATG-3′ |
| RUNX2 | F: 5′-GGTAGGTTAAGGCTCGTGATA-3′ |
|      | R: 5′-CGTGTGTTGGCAGCTTGTCG-3′ |

**Luciferase activity assay**

Construction of mutant-type STAT1 3′-untranslated region (3′UTR) luciferase reporter vector (STAT1-Mut): With wild-type STAT1-Mt luciferase reporter vector as the template, miR-28 binding site on Smad4 3′UTR was subjected to site-directed mutagenesis by PCR using QuickChange Site-Directed Mutagenesis Kit, and the template plasmid was removed specifically using Dpn I enzyme, followed by identification through sequencing.

HBMMSCs were seeded into a 24-well plate, and co-transfected with the luciferase reporter vector and miR-28 mimics, control mimics or miR-28 inhibitors upon reaching 60–80% confluence, with pRL-TK transfection as the standard internal quality control. At 36 h after transfection, the cells were harvested, and the luciferase activity was detected according to the instructions of dual-luciferase assay kit (Promega) as follows. After the medium was discarded, the cells were washed twice with PBS, lysed with diluted 1× passive lysis buffer for 15 min, and scraped into a 1.5 mL EP tube, followed by centrifugation at 12,000 r/min for 2 min. Then, 20 μL of supernatant was taken and added with 100 μL of human luciferase assay reagent II. The luciferase activity was detected using a single-photon detector (Centro XS3 LB 960, Berthold Technologies, UK), and the firefly luciferase activity was recorded. After 10 μL of Stop&Glo reagent was
added, the Renilla luciferase activity was detected using the single-photon detector. Finally, the relative luciferase activity was calculated as firefly luciferase activity/Renilla luciferase activity.17

**Cell transfection**

The cells in good growth status were seeded into a 6-well plate, and transfected upon reaching 50–70% confluence. Afterward, 5 μL of liposomes were added into 250 μL of serum-free medium, mixed evenly and left at room temperature for 5 min. Then, 100 pmol of miRNA mimics (miR-28 mimics) or siRNA (STAT1 siRNA) was added into 250 μL of serum-free medium in another tube. The contents in the two above tubes were mixed and left at room temperature for 45 min. The cells were washed twice with Hank’s solution, and centrifuged at 12,000 r/min for 2 min. Then the cells were resuspended by adding 1.5 mL of serum/antibiotic-free medium. The gene solutions were mixed with P3000 reagent and incubated with diluted lipofectamine 3000 reagent for 15 min at room temperature. Subsequently, hBMMSCs were incubated with the gene solution and lipid complex together for 48 h.18

**Western blotting**

Total protein was extracted from hBMMSCs by RIPA buffer (Thermo Fisher, USA) and quantified using BCA protein assay kit (Thermo Fisher, USA). Then, 40 μL protein and 5 μL marker (Beijing Solarbio Science & Technology Co., Ltd., China) was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Sigma-Aldrich, USA). The membrane was thereafter blocked by 5% skimmed milk in Tris-buffered saline with 1% Tween 20 (TBST, Thermo Fisher, USA) and incubated at 4°C overnight with primary antibodies against STAT1 (1:1000) or GAPDH antibody (1:4000) at 4°C overnight. After the membrane was washed with TBST, it was incubated again with secondary antibody (1:8000) at room temperature for 1 h, and washed again with TBST. Finally, chemiluminescence was developed with ECL and exposed to X-ray film, followed by image development and fixation.19

**Alkaline phosphatase (AKP) activity assay**

The cells were collected, and total protein was extracted. The AKP activity was detected according to the instructions of AKP assay kit (Beijing Biolab Technology Co., Ltd., China), and the protein concentration was determined using Bradford method. The ratio of AKP activity to the corresponding protein concentration indicated the specific activity of AKP.20

**Cell counting kit-8 (CCK-8) assay**

HBMMSCs were seeded into a 96-well plate (100 μL/well), added with 10 μL of CCK-8 reagent (Dojindo, Japan) in each well, and incubated in a 5% CO2/37°C incubator for 1 h. The absorbance at 450 nm was measured as point 0 using a microplate reader. At the same time point every day, the sample was loaded and the absorbance was detected for 96 h. The growth curve was plotted.21

**Transwell assay**

Transwell chambers (24 mm) with 8.0 μm pore polycarbonate membrane insert (Corning, USA) were used to assess the invasive capacity of hBMMSCs. HBMMSCs were digested and counted, and the cell concentration was adjusted to 2 × 10^4/mL. Then, 100 μL of cell suspension was added into the upper chamber, while the medium was added into the lower chamber. After culture in the 5% CO2/37°C incubator for 24 h, the medium in the upper chamber was aspirated. The basement membrane of the chamber was rinsed twice with PBS, the cells on the inner membrane were wiped off with cotton swabs, and the membrane was fixed with 4% paraformaldehyde for 30 min. Then, the outer membrane was rinsed with PBS once or twice, and stained with 0.1% crystal violet for 15 min. After the crystal violet was aspirated, the membrane was washed with PBS, and observed and photographed under a microscope.22

**Statistical analysis**

SPSS23.0 software was used for statistical analysis. Measurement data were expressed as mean ± standard deviation, and compared by t test between
two groups and by one-way analysis of variance among groups. P < 0.05 was considered statistically significant.

Results

Culture and identification of hBMMSCs

It was observed by immunocytochemical staining under an inverted fluorescence microscope that hBMMSCs were positive for CD44 expression (Figure 1c) and negative for CD34 expression (Figure 1d), proving that hBMMSCs originated from mesenchyme, rather than hematopoietic stem cell tissues. No positively stained cells were discerned in blank and negative control groups incubated with ddH₂O and PBS instead of primary antibody (Figure 1a and b), indicating the binding specificity of secondary antibody.

Microarray results

MiRNAs are important regulatory molecules widely participating in the regulation of various life processes. Many kinds of miRNAs have been involved in bone metabolism, and their participation in the epigenetic process is critical for the differentiation of related cells toward osteogenesis.23 HBMMSCs and those cultured by osteogenic induction for 21 days were detected using miRNA microarrays, and differentially expressed miRNAs were screened by SAM. Compared with hBMMSCs, those cultured by osteogenic induction had eight highly expressed miRNAs (miR-149, miR-21, miR-572, miR-130b, miR-193b, miR-152, miR-560, and miR-28), among which miR-28 was upregulated more obviously. Compared with hBMMSCs, two miRNAs (miR-424 and miR-122a) were down-regulated in those cultured by osteogenic induction (Figure 2).

RT-PCR results

The results of RT-PCR revealed that miR-149, miR-21, miR-572, miR-130b, miR-193b, miR-152, miR-560, and miR-28 were up-regulated, while miR-424 and miR-122a were down-regulated after induction compared with those before induction, in which miR-28 was up-regulated most obviously. Therefore, miR-28 was selected for subsequent studies (Figure 3).

Figure 1. Immunocytochemical staining. A: Negatively stained hBMMSCs for blank control; B: negatively stained hBMMSCs for negative control; C: negatively stained hBMMSCs for CD34; D: positively stained hBMMSCs for CD44.
Expression of miR-28 significantly increased during osteogenic differentiation of hBMMSCs

AKP is an exoenzyme of osteoblasts, and its expression marks osteoblast differentiation. The main physiological function of AKP is to hydrolyze phosphate esters in the process of bone formation, providing phosphoric acid required for the deposition of hydroxyapatite. Meanwhile, AKP hydrolyzes pyrophosphate to prevent it from inhibiting bone salt formation, which is conducive to osteogenesis.24 Runx2 is a transcription factor required to determine the osteoblast lineage, and Sp7 and Wnt signaling

Figure 2. miRNA microarray results of hBMMSCs (A1/A2/A3) and those cultured by osteogenic induction for 21 days (B1/B2/B3).
transduction block the differentiation of mesenchymal cells into chondrocytes by forming osteoblasts instead.\textsuperscript{25} HBMMSCs were cultured in osteogenic differentiation medium, and whether miR-28 was involved in the regulation of osteogenic differentiation was validated. During the osteogenic differentiation of hBMMSCs, the expressions of osteoblast-specific markers (e.g. RUNX2 and AKP) significantly increased (Figure 4a and b). Besides, the expression of miR-28 at different time points was dynamically monitored during osteogenic differentiation. The results showed that miR-28 expression gradually increased during the osteogenic differentiation of hBMMSCs, confirming that miR-28 may be involved in regulating this process (Figure 4c).

![Figure 3. RT-PCR results (n = 3).](image)

![Figure 4. Expression of miR-28 significantly increased during the osteogenic differentiation of hBMMSCs. A: RUNX2 mRNA expression on days 1, 10 and 15 detected by qRT-PCR; B: AKP activity detected by AKP assay kits; C: miR-28 expression on days 1, 5, 10 and 15 detected by qRT-PCR (n = 3).](image)
MiR-28 inhibited luciferase activity of STAT1 mRNA 3'UTR

The results of luciferase activity assay showed that compared with control mimics, miR-28 mimics significantly suppressed the luciferase activity of STAT1-Mt reporter vector (P < 0.05), while miR-28 inhibitor did not down-regulate the activity. Besides, miR-28 mimics did not obviously inhibit the luciferase activity of STAT1-Mut reporter vector (Figure 5). Hence, miR-28 can specifically bind STAT1 mRNA 3'UTR.

MiR-28 down-regulated STAT1 mRNA and protein expressions during osteogenic differentiation of hBMMSCs

HBMMSCs were transfected with miR-28 mimics or siRNA, and cultured by osteogenic differentiation medium for 10 days. Then, STAT1 mRNA and protein expressions were determined using qRT-PCR and Western blotting, respectively. The results of qRT-PCR showed that compared with Control group, miR-28 mimics group had significantly decreased expression of STAT1 mRNA (P < 0.05) (Figure 6a). The results of Western blotting were basically consistent with those of PCR, and the degree to which miR-28 mimics down-regulated the expression of STAT1 was comparable to that of STAT1 siRNA, indicating that miR-28 can regulate the expression of STAT1 during the osteogenic differentiation of hBMMSCs in a targeted manner (Figure 6b).

Interference with STAT1 or overexpression of miR-28 promoted osteogenic differentiation of hBMMSCs

HBMMSCs were transfected with synthetic STAT1 siRNA and added with inducers to induce osteogenic differentiation. The samples were collected on day 10 to detect the AKP activity. Transfection of STAT1 siRNA significantly enhanced the AKP activity (P < 0.05) (Figure 7). The results of PCR showed that STAT1 siRNA up-regulated the expression of RUNX2 (P < 0.05) (Figure 8). Our findings suggest that interference with STAT1 can partially mimic the regulatory effects of miR-28 on the osteogenic differentiation of hBMMSCs.

Interference with STAT1 or overexpression of miR-28 did not affect proliferation or migration of hBMMSCs

HBMMSCs were transfected with synthetic STAT1 siRNA and added with inducers to induce cell differentiation. There were no significant differences in cell proliferation and migration among the three groups (P > 0.05), suggesting that interference with STAT1 or overexpression of miR-28 did not affect the proliferation or migration of hBMMSCs (Figure 9).

![Figure 5. MiR-28 inhibited luciferase activity of STAT1 mRNA 3'UTR (n = 3).](image-url)
MiR-28 has gradually increased expression during the osteogenic differentiation of hBMMSCs, which can directly bind STAT1 3’UTR and inhibit its expression, thus up-regulating AKP and RUNX2, and facilitating osteogenic differentiation. HBMMSCs are a class of

**Discussion**

MiR-28 has gradually increased expression during the osteogenic differentiation of hBMMSCs, which can directly bind STAT1 3’UTR and inhibit its expression, thus up-regulating AKP and RUNX2, and facilitating osteogenic differentiation. HBMMSCs are a class of
adult stem cells with multidirectional differentiation ability, which can differentiate into osteoblasts, chondroblasts, and adipocytes under different induction conditions. HBMMSCs, characterized by strong proliferation capacity, great differentiation potential, low immunogenicity and easy culture in vitro, have become ideal seed cells for bone tissue engineering. They are clinically applicable in the promotion of bone repair and reconstruction. Improving the osteogenic differentiation ability of HBMMSCs is the key to bone regeneration.26

MiRNAs play important regulatory roles in stem cell self-renewal and multidirectional differentiation.27,28 Goff et al. screened several differentially expressed miRNAs from MSCs isolated and cultured from the bone marrow of 3 volunteers using microarray, and found that hsa-miR-638 and hsa-miR-663 were highly expressed in chondrocytes under differentiation induction.29 Laine et al. reported differentially expressed miRNAs in BMMSCs during osteogenic and adipogenic differentiation, and showed that hsa-miR-199a was highly expressed in chondrocytes and osteoblasts under differentiation induction.30 Hsa-miR-574-3p31 and hsa-miR-23b32 have also been related to the osteogenic differentiation of MSCs. Due to the different sources and differentiation induction methods of MSCs, however, miRNAs involved in osteogenic differentiation are greatly different. In this study, several differentially expressed miRNAs were screened from hBMMSCs and those cultured by osteogenic induction, including eight highly expressed ones (miR-149, miR-21, miR-572, miR-130b, miR-193b, miR-152, miR-560, and miR-28), in which miR-28 was up-regulated most obviously. Besides, two miRNAs (miR-424 and miR-122a) were lowly expressed in hBMMSCs cultured by osteogenic induction, so miR-28 was selected for subsequent studies. Given that miR-28 expression gradually increased during the osteogenic differentiation of hBMMSCs, it may be involved in regulating this process.

To study the regulatory mechanism of miR-28 for the osteogenic differentiation of BMMSCs, all the target genes of miR-28 were searched by bioinformatics prediction. The target genes closely related to the osteogenic signaling pathway were found, and the possible targeted regulatory effect of miR-28 on STAT1 was confirmed. STAT1, a member of the STAT protein family, maintains the intrinsic specificity of signals in cells and mediates a wide range of cellular responses caused by various cytokines and growth factors.33,34 In this study, the luciferase
activity assay revealed that miR-28 specifically bound 3’UTR of STAT1 mRNA, and transfection of STAT1 siRNA significantly promoted both AKP activity and RUNX2 expression, indicating that interference with STAT1 can partially mimic the regulatory effects of miR-28 on the osteogenic differentiation of hBMMSCs.

Conclusions
In conclusion, miR-28 has gradually increased expression and exerts a positive regulatory effect on the osteogenic differentiation of hBMMSCs. Probably, miR-28 directly binds STAT1 3’UTR and inhibits its expression, thereby facilitating the osteogenic differentiation of hBMMSCs. In
addition, STAT1 expression further declines due to the overexpression of miR-28, promoting the osteogenic differentiation of hBMMSCs. Therefore, miR-28, as a potential target for regulating the osteogenic differentiation of hBMMSCs, is of great significance for the prevention and treatment of diseases related to osteogenic regeneration and development.

**Highlights**

1. MiR-28 facilitates the osteogenic differentiation of hBMMSCs, as a potential target.
2. MiR-28 directly binds the STAT1 3’UTR and inhibits its expression.
3. MiR-28 is valuable for diseases related to osteogenic regeneration and development.

**Acknowledgements**

This study was financially supported by Guangzhou Traditional Chinese Medicine and Integrated Traditional Chinese and Western Medicine Project (No. 20202A011011) and General Guidance Project of Western Medicine in Science and Technology Project of Guangzhou Municipal Health Commission (No. 20211A011021).

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This study was financially supported by Guangzhou Traditional Chinese Medicine and Integrated Traditional Chinese and Western Medicine Project (No. 20202A011011), and Guangzhou Municipal Health Commission Science and Technology Project Western Medicine General Guidance (No. 20211A011021).

**Authors’ contributions**

Min Wang and Siming Li designed this study and prepared this manuscript; Tianming Dai, Qingqi Meng and Wen Wang collected and analyzed experimental data. All authors approve the submission and publication of this manuscript.

**References**

[1] Cao C, Wang F, Wang EB, et al. (Application of β-TCP for bone defect restore after the mandibular third molars extraction: a splitmouth clinical trial). Beijing Da Xue Xue Bao Yi Xue Ban. 2020;52:97–102.
[2] Wang J, Yin Q, Gu S, et al. Induced membrane technique in the treatment of infectious bone defect: a clinical analysis. Orthop Traumatol Surg Res. 2019;105:535–539.
[3] Han L, Fang WL, Jin B, et al. Enhancement of tendon-bone healing after rotator cuff injuries using combined therapy with mesenchymal stem cells and platelet rich plasma. Eur Rev Med Pharmacol Sci. 2019;23:9075–9084.
[4] Piacentini F, Ceglia MJ, Bettini L, et al. Induced membrane technique using enriched bone grafts for treatment of posttraumatic segmental long bone defects. J Orthop Traumatol. 2019;20:13.
[5] Marechek A, AliShare A, Pack S, et al. Nonvascularized bone grafts for reconstruction of segmental mandibular defects: is length of graft a factor of success? J Oral Maxillofac Surg. 2019;77:2557–2566.
[6] Ono M, Kosaka N, Tominaga N, et al. Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. Sci Signal. 2014;7:ra63.
[7] Zahedi M, Parham A, Dehghani H, et al. Stemness Signature of Equine Marrow-derived Mesenchymal Stem Cells. Int J Stem Cells. 2017;10:93–102.
[8] Robey PG, Kuznetsov SA, Ren J, et al. Generation of clinical grade human bone marrow stromal cells for use in bone regeneration. Bone. 2015;70:87–92.
[9] Gasparello J, Papi C, Allegretti M, et al. A distinctive microRNA (miRNA) signature in the blood of Colorectal Cancer (CRC) patients at surgery. Cancers (Basel). 2020;12:2410.
[10] Bi X, Guo XH, Mo BY, et al. LncRNA PICSAR promotes cell proliferation, migration and invasion of fibroblast-like synoviocytes by sponging miRNA-4701-5p in rheumatoid arthritis. EBioMedicine. 2019;50:408–420.
[11] Gruzdev SK, Yakovlev AA, Druzhkova TA, et al. The missing link: how exosomes and mirnas can help in bridging psychiatry and molecular biology in the context of depression, bipolar disorder and schizophrenia. Cell Mol Neurobiol. 2019;39:729–750.
[12] Munshi A, Mehic J, Creskey M, et al. A comprehensive proteomics profiling identifies NRIP1 as a novel identity marker of human bone marrow mesenchymal stromal cell-derived small extracellular vesicles. Stem Cell Res Ther. 2019;10:401.
[13] Chen X, Wang L, Hou J, et al. Study on the dynamic biological characteristics of human bone marrow mesenchymal stem cell senescence. Stem Cells Int. 2019;2019:9271595.
[14] Bloise N, Petecchia L, Ceccarelli G, et al. The effect of pulsed electromagnetic field exposure on osteoinduction of human mesenchymal stem cells cultured on nano-TiO2 surfaces. Plos One. 2018;13:e0199046.
[15] Pradervand S, Weber J, Thomas J, et al. Impact of normalization on miRNA microarray expression profiling. RNA. 2009;15:493–501.
[16] Wall SJ, Edwards DR. Quantitative reverse transcription-polymerase chain reaction (RT-PCR): a comparison of primer-dropping, competitive, and real-time RT-PCRs. Anal Biochem. 2002;300:269–273.
[17] Choi S, Matta H, Gopalakrishnan R, et al. A novel thermostable beetle luciferase based cytotoxicity assay. Sci Rep. 2021;11:10002.
[18] Duckert B, Vinkx S, Braeken D, et al. Single-cell transfection technologies for cell therapies and gene editing. J Control Release. 2021;330:963–975.
[19] Laidoudi Y, Sereme Y, Medkour H, et al. SARS-CoV-2 antibodies seroprevalence in dogs from France using ELISA and an automated Western blotting assay. One Health. 2021;13:100293.
[20] Yu L, Feng L, Xiong L, et al. Rational design of dual-emission lanthanide metal–organic framework for visual alkaline phosphatase activity assay. ACS Appl Mater Interf. 2021;13:11646–11656.
[21] Zhang J, Wang J, Jiang JY, et al. Tanshinone IIA induces cytochrome c-mediated caspase cascade apoptosis in A549 human lung cancer cells via the JNK pathway. Int J Oncol. 2014;45:683–690.
[22] Omar Zaki SS, Kanesan L, Leong MY, et al. The influence of serum-supplemented culture media in a transwell migration assay. Cell Biol Int. 2019;43:1201–1204.
[23] Cheng F, Yang MM, Yang RH. MiRNA-365a-3p promotes the progression of osteoporosis by inhibiting osteogenic differentiation via targeting RUNX2. Eur Rev Med Pharmacol Sci. 2019;23:7766–7774.
[24] Yorgan TA, Peters S, Amling M, et al. Osteoblast-specific expression of Panx3 is dispensable for postnatal bone remodeling. Bone. 2019;127:155–163.
[25] Chen PC, Liu JF, Fong YC, et al. CCN3 Facilitates Runx2 and Osterix Expression by Inhibiting miR-608 through PI3K/Akt Signaling in Osteoblasts. Int J Mol Sci. 2019;20:3300.
[26] Safari F, Fani N, Eglin D, et al. Human umbilical cord-derived scaffolds for cartilage tissue engineering. J Biomed Mater Res A. 2019;107:1793–1802.
[27] Kundrotas G, Gasperskaja E, Slapyshe G, et al. Identity, proliferation capacity, genomic stability and novel senescence markers of mesenchymal stem cells isolated from low volume of human bone marrow. Oncotarget. 2016;7:10788–10802.
[28] Gothelf Y, Kaspi H, Abramov N, et al. miRNA profiling of NurOwn#: mesenchymal stem cells secreting neurotrophic factors. Stem Cell Res Ther. 2017;8(1):249.
[29] Goff LA, Boucher S, Ricupero CL, et al. Differentiating human multipotent mesenchymal stromal cells regulate microRNAs: prediction of microRNA regulation by PDGF during osteogenesis. Exp Hematol. 2008;36(10):1354–1369.
[30] Laine SK, Alm JJ, Virtanen SP, et al. MicroRNAs miR-96, miR-124, and miR-199a regulate gene expression in human bone marrow-derived mesenchymal stem cells. J Cell Biochem. 2012;113:2687–2695.
[31] Guérit D, Philipot D, Chuchana P, et al. Sox9-Regulated miRNA-574-3p Inhibits chondrogenic differentiation of mesenchymal stem cells. PLoS ONE. 2013;8:e62582.
[32] Ham O, Song BW, Lee SY, et al. The role of microRNA-23b in the differentiation of MSC into chondrocyte by targeting protein kinase A signaling. Biomaterials. 2012;33:4500–4507.
[33] Choi YS, Eto D, Yang JA, et al. Cutting Edge: STAT1 Is required for IL-6–Mediated Bcl6 induction for early follicular helper cell differentiation. J Immunol. 2013;190:3049–3053.
[34] Choi YS, Eto D, Yang JA, et al. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. J Immunol. 2013;190:3049–3053.