CELL CYCLE
2022, VOL. 21, NO. 6, 630–640
https://doi.org/10.1080/15384101.2022.2031428

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

miR-30b-5p inhibits osteoblast differentiation through targeting BCL6

Yan Luo[a], Feng Zhou[b], Xiaochun Wu[c], Yi Li[a], and Bin Ye[d]

[a]Department of General Practice, Wuhan Puren Hospital, Wuhan, Hubei, China; [b]Department of Endocrinology, Wuhan Puren Hospital, Wuhan, Hubei, China; [c]Department of Orthopaedics, Wuhan Huangpi People Hospital, Wuhan, Hubei, China; [d]Department of Orthopaedics, Wuhan No. 9 Hospital, Wuhan, Hubei, China

ABSTRACT

Human bone marrow mesenchymal stem cells (hBMSCs) are attractive candidates for new therapies to improve bone regeneration and repair. This study was to identify the function of the miR-30b-5p/BCL6 axis in osteogenic differentiation of hBMSCs. Realtime-quantitative PCR (RT-qPCR) and Western blotting were used to measure the relative expression of ALP, OCN, RUNX2, miR-30b-5p, and BCL6 during osteogenic differentiation of hBMSCs. The relationship between miR-30b-5p and BCL6 in hBMSCs was identified using dual-luciferase reporter system and RNA pull-down assay. Alizarin red S staining (ARS) was used to detect the calcium nodules in hBMSCs. We found that the expression of miR-30b-5p was downregulated, whereas that of BCL6 was upregulated during osteogenic differentiation of hBMSCs. Downregulating miR-30b-5p enhanced the expression of OCN, RUNX2, and ALP, and promoted calcium deposition. Conversely, transfection with si-BCL6 had the opposite effect that it inhibited osteogenic differentiation. However, the inhibitory effect of si-BCL6 was abrogated by miR-30b-5p inhibitor. miR-30b-5p inhibits the osteogenic differentiation of hBMSCs by targeting BCL6.

ARTICLE HISTORY
Received 15 October 2021
Revised 22 December 2021
Accepted 13 January 2022

KEYWORDS
miR-30b-5p; human mesenchymal stem cell; osteogenic differentiation; BCL6; miRNA

Introduction

Human bone marrow mesenchymal stem cells (hBMSCs) are important in bone tissue regeneration or repair due to its strong abilities of self-renewal and multi-directional differentiation[1]. hBMSCs differentiate into many types of cells including adipocytes, chondrocytes, and osteoblasts [2,3]. Improving the differentiation ability of BMSCs is key to bone regeneration, and BMSCs play a crucial role in promoting bone remodeling and repair in patients with osteoporosis[4]. As the differentiation of BMSCs is crucial for the development of osteoblasts, they constitute the ideal seed cells for bone tissue engineering[5].

MicroRNAs (miRNAs) are small single stranded RNA molecules that regulate gene expression by binding to mRNAs, resulting in translational inhibition and/or instability of the target mRNAs [6–8]. miRNAs have been demonstrated to be important regulators of the pathogenesis of clinical diseases[9]. Several miRNAs are involved in the differentiation of MSCs into osteoblasts. miR-19a-3p significantly induced the osteogenic differentiation of BMSCs and limited bone loss in aging mice[10]. Another study reported the potential of miR-223–3p as a therapeutic target for regulating autophagy and thereby promoting osteogenic differentiation and enhancing bone function of BMSCs[11]. miR-30b-5p, as a widely studied miRNA, has been proved to play a certain regulatory function in a variety of cancers (including liver cancer and breast cancer) and cardiovascular related diseases (such as myocardial injury and diabetes) [12–15]. In addition, members of the miR-30 family have been shown to regulate the osteogenesis pathway[16]. Eguchi et al.[17] revealed that the miR-30b-5p expression declined in the later stages of osteo-induction. Studies by Balderma et al.[18] have shown that BMP2 promotes calcification by downregulating miR-30b-5p in vascular smooth muscle cells and upregulating RUNX2. However, the mechanism on miR-30b-5p to regulate osteogenic differentiation remains unclear. Therefore, this study is the first to explore

CONTACT Bin Ye yebin259@163.com Department of Orthopaedics, Wuhan No. 9 Hospital, No. 1 Benxi Street, Qingshan District, Wuhan, Hubei 430080, China
Supplemental data for this article can be accessed here.

the effect of miR-30b-5p on osteogenic differentiation of hBMSCs.

B-cell lymphoma 6 (BCL6), a protein predominantly found in B lymphocytes, plays a crucial role in the development of B lymphocytes [19,20]. BCL6 has been shown to promote osteogenic differentiation, and BCL6-deficient mice show a reduction in bone mass and osteogenesis parameters *in vivo*[21]. Yang et al. reported that BCL6 was upregulated during osteoblast differentiation, and BCL6 knockdown blocked the nuclear translocation of RUNX2[22]. Therefore, it is important to study the specific mechanism of BCL6 in osteogenic differentiation.

The aim of this study was to elucidate the effect of miR-30b-5p on osteoblastic differentiation of hBMSCs, focusing on the mechanism of miR-30b-5p/BCL6 in osteogenic differentiation of hBMSCs.

Methods

Bioinformatics analyses

Two mRNA expression microarrays (GSE35958 and GSE37558) from the gene expression omnibus (GEO) database stored the data on mRNA expression in osteoporosis samples and normal samples, and were used to screen the differentially expressed genes (DEGs) with adjusted $P < 0.05$, and $|\log FC| \geq 1.5$. Then, the STRING database was used to construct a protein-protein interaction network for the DEGs. Finally, Tarbase (http://carolina.imis.athena-innovation.gr/diana_tools/) and TargetScan (http://targetscan.org/vert_72) databases were used to predict the miRNAs that target the key genes in osteoporosis. GSE91033, a miRNA microarray from GEO database, stored the data on miRNA expression in osteoporosis samples and normal samples, and was used to identify the differentially expressed miRNAs with adjusted $P < 0.05$ and $|\log FC| \geq 1.5$. The process of bioinformatics analysis was shown in Supplementary Figure 1.

Cell culture and osteogenic differentiation

HBMSCs were purchased from Cyagen (Guangzhou, China) and cultured in α-minimum essential medium (αMEM; Invitrogen, CA, USA) with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin-streptomycin (Gibco), and 2 mM L-glutamine (Sigma-Aldrich, USA). For osteogenic differentiation, hBMSCs were induced using fresh osteogenic medium containing 0.1 mM dexamethasone, 10 mM β-glycerophosphate, and 0.1 mM ascorbic acid (all from Sigma-Aldrich). The cells were cultured in a cell incubator under the condition of 5% CO_2 and 37°C.

Cell transfection

The miR-30b-5p inhibitor, small-interfering RNA (siRNA) targeting human BCL6 (si-BCL6), and their negative control (si-NC and inhibitor-NC) were purchased from RiboBio (Guangzhou, China). HBMSCs were transfected with NC, si-BCL6, and miR-30b-5p inhibitor using Lipofectamine 3000 (Invitrogen) diluted in Opti-MEM (Thermo Fisher Scientific, MA, USA). 5×10^4 cells/well cells plated in a 6-well plate were transfected once they reached approximately 70% confluence. The cell transfection was performed for 48 h before the subsequent experiments. The sequences are listed in Supplementary Table 1.

RT-qPCR

miRNA was isolated from hBMSCs by the Qiagen miRNeasy FFPE Kit (Qiagen, Hilden, Germany). The miR-X miRNA First Strand Synthesis Kit (Clontech Laboratories, CA, USA) with a specific stem-loop miRNA primer was used for reverse transcription. The miR-30b-5p expression was detected by miR-X miRNA qRT-PCR TB Green Kit from TaKaRa (Japan) and ABI 7500 system from Applied Biosystems (USA). U6 was used as an internal reference of miRNA.

Total RNA was isolated from hBMSCs by RNA high-purity Total RNA Rapid Extraction Kit (Qiagen). The total RNA was then reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (TaKaRa) with gDNA Eraser. The mRNA levels of ALP, OCN, and RUNX2 were detected by SYBR Premix Ex Taq II Kit (TaKaRa) and ABI 7500 system. GAPDH was used as the internal reference of mRNA. The primers are listed in Table 1.

Table 1. Primer sequences for this study.

Gene	Primer sequence
RUNX2	Forward:5′-ATTTAGGGCGCATTCCTCATC-3′ Reverse:5′-GTGGTGGAGTGGATGGATGG-3′
ALP	Forward:5′-CACCATTTTTAGTACTGGCCATCG-3′ Reverse:5′-GCTACATTGGTGTTGAGCTTTGG-3′
OCN	Forward:5′-CAAAGGTGCAGCCTTTGTGTC-3′ Reverse:5′-TCACAGTCCGGATTGAGCTCA-3′
BCL6	Forward:5′-AGACGCACAGTGACAAACCATACA-3′ Reverse:5′-CTCCACAAATGTTACAGCGATAGG-3′
miR-30b-5p	Forward:5`- GCAGTGTAAACATCCTACACTCA – 3` Reverse:5′-ACCTAAGCCAGGGAGGTTA-3′
GAPDH	Forward:5′-AATGG ATTTGGACGCATTGGT-3′ Reverse:5′-TTTGCACTGGTACGTGTTGAT-3′
U6	Forward:5′-CGCTTCACGAATTTGCGT-3′ Reverse:5′-CTCGCTTCG CAGCACA-3′

Cell viability assay

Cells with the density of 1×10^4 cells/well were plated in 48-well plates. After transfection for 24, 48, and 72 h, 10 μl/well of CCK-8 reagent (Dojindo, Japan) was used to incubate cells for 2 h at 37°C. The absorbance was identified by microplate reader (Bio-Rad, USA) at 450 nm.

Alizarin red S (ARS) staining

The mineralization of hBMSCs was assessed using ARS staining. HBMSCs were fixed in 4% paraformaldehyde at room temperature for 20 min and washed with phosphate buffered saline (PBS). The cells were then incubated with 1% ARS staining solution (Sigma-Aldrich) for 20 min followed by rinsing with PBS. An inverted microscope (Nikon, Japan) was used to visualize the cells and obtain representative images.

Western blot analysis

HBMSCs after washing with pre-chilled PBS were lysed with lysis buffer (Beyotime, Jiangsu, China). The BCA Protein Detection Kit (Thermo Fisher Scientific) was used to determine the protein concentrations. The 20 μg protein was separated by 10% SDS-PAGE and transfer to nitrocellulose membranes (Pall, NY, USA). The membrane was incubated by 5% skim milk for 60 min at 25°C, and then was incubated with primary antibodies including anti-BCL6 (1:500; ab203619, Abcam, Cambridge, UK), anti-RUNX2 (1:2000; ab76956, Abcam), anti-ALP (1:2000; ab229126, Abcam), anti-OCN (1:2000; ab93876, Abcam), and anti-GAPDH (1:1000; ab181602, Abcam) for 12 h at 4°C. Following washing the membranes, the corresponding secondary antibodies were continued to incubate membranes for 1 h. A multi-chemiluminescence detection system (Tanon, China) was used to visualize the protein bands. Image J software (NIH, USA) was used to quantify the bands.

Dual-luciferase assay

Sequences with mutations in the binding sequences of BCL6 and miR-30b-5p (BCL6-MUT) and the corresponding wildtype sequences of BCL6 (BCL6-WT) were constructed by TsingKe Int. (Beijing, China). 2.5×10^4 cells hBMSCs were cultured in a 24-well plate and co-transfected 50 nM BCL6-WT or BCL6-MUT together with 50 nM miR-30b-5p mimic or mimic-NC using Lipofectamine 3000. After transfection for 6 h, the culture medium was replaced with antibiotic-free medium for 42 h. The luciferase activities of firefly and Renilla were detected by Dual-Luciferase Reporter Assay System (Promega, WI, USA).

RNA pull-down assay

RNA pulldown assays were performed according to a previously described method[23]. Biotinylated miR-30b-5p (Bio-miR-30b-5p), miR-30 c-5p (Bio-miR-30 c-5p), miR-10b-5p (Bio-miR-10b-5p) and their negative control (Bio-NC) were commercially synthesized by Sangon Biotech and transfected into hBMSCs. The cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer and incubated with T1 Streptavidin magnetic Dynabeads (Invitrogen) after transfection for 48 h. BCL6 RNA levels were analyzed using RT-qPCR.

Statistical analysis

All data from three independent experiments were shown as the mean ± standard deviation, and analyzed by the SPSS V20.0 (IBM SPSS, IL, USA). Differences among groups were analyzed using unpaired Student's *t*-test (for two groups) or one-way ANOVA (for multiple groups).

Pearson correlation analysis was performed to measure the relationship between ALP, RUNX2, OCN, and miR-30b-5p or BCL6 mRNA levels in osteogenic differentiation. Statistically significance was set at $p < 0.05$.

Results

Candidate regulators of osteogenesis

The DEGs in osteogenesis and osteoporosis were screened by analyzing GSE35958 and GSE37558 datasets (selection criteria: adjusted P < 0.05, |logFC| ≥1.5) and eleven DEGs were identified (Figure 1(a)). These DEGs were then uploaded to the STRING database for identify protein-protein interaction networks. BCL6 was associated with CD44 expression (Figure 1(b)). BCL6 has been reported to participate in bone development: BCL6 promotes osteoblastogenesis [21,22] and inhibits osteoclastogenesis [24,25]. By intersecting the candidate regulator miRNAs of BCL6 from Tarbase and TargetScan databases, twelve common miRNAs (hsa-miR-9–5p, hsa-miR-26a-5p, hsa-miR-30b-5p, hsa-miR-30 c-5p, hsa-miR-22–5p, hsa-miR-30a-5p, hsa-miR-30d-5p, hsa-miR -30e-5p, hsa-miR-625–5p, hsa-miR-628–5p, hsa-miR-93–5p, hsa-miR-10b-5p) in Tarbase and TargetScan were identified. Among the twelve miRNAs, three were found to be significantly downregulated in the osteoporosis miRNA expression microarray GSE91033, including miR-10b-5p, miR-30 c-5p, and miR-30b-5p (Figure 1(c)). miR-10b has been found to regulate BCL6 in osteoblast differentiation[22], whereas the miR-30 family has been reported to inhibit osteoblast differentiation [26]. Nonetheless, no study has demonstrated whether the miR-30 family regulates osteoblastogenesis by targeting BCL6. miR-30b-5p was downregulated more than miR-30 c-5p (logFC = −1.73 vs −1.53). In addition, RNA pull-down experiments showed that the levels of BCL6 RNA in bio-miR-10b-5p, bio-miR-30 c-5p, and bio-miR-30b-5p groups were about 6, 3 and 13 times higher

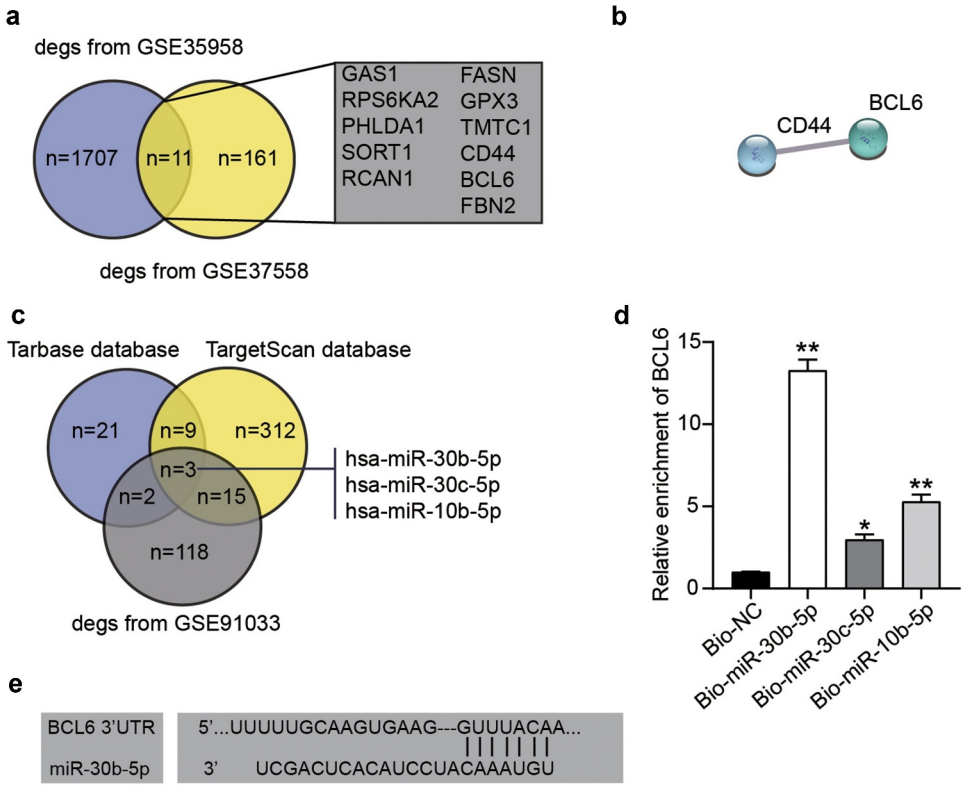

Figure 1. The candidate regulators in osteoblastogenesis. a. The identification of key genes that participate in osteogenesis and osteoporosis. degs: differentially expressed genes. Selection criteria: adjusted P < 0.05 and |logFC|≥1.5). b. The protein-protein interaction network showing the interaction between the genes identified in figure a. c. The identification of candidate regulator miRNAs of BCL6. d. The enrichment of BCL6 on Biotin-labeled miR-10b-5p, miR-30 c-5p and miR-30b-5p determined by RNA pull-down. * $P < 0.05$, ** $P < 0.001$ vs. Bio-NC. E. A schematic diagram of miR-30b-5p binding sites in the 3′ UTR of BCL6 mRNA.

than that in Bio-NC group, respectively (Figure 1(d)). Hence, miR-30b-5p was therefore selected for further studies. Moreover, to elucidate the molecular mechanisms of miR-30b-5p and BCL6, we scanned the TargetScan website and found that they have targeted binding sites (Figure 1(e)).

miR-30b-5p inhibits osteoblast differentiation

The changes in the expression of miR-30b-5p in hBMSCs on days 0, 3, 7, and 16 of osteogenic differentiation were detected by RT-qPCR, showing that miR-30b-5p expression reduced over time during osteogenic differentiation (Figure 2(a)). To confirm the relationship between miR-30b-5p and osteogenic differentiation, we examined the levels of the key osteogenic genes ALP, RUNX2, and OCN. As shown in Figure 2(b), RUNX2, ALP, and OCN expression levels increased during osteogenic differentiation. Pearson's correlation analysis of the relationship between RUNX2, ALP, OCN, and miR-30b-5p revealed that miR-30b-5p was negatively correlated with RUNX2, ALP, and OCN during osteogenic differentiation (Figure 2(c)). These findings revealed that miR-30b-5p is involved in osteogenic differentiation to a certain extent.

After transfecting miR-30b-5p inhibitor to hBMSCs, the expression of miR-30b-5p in hBMSCs decreased by approximately 70% (Figure 2(d)). The viability of hBMSCs, measured using the CCK-8 assay, was increased by approximately 1.5-fold following downregulation of miR-30b-5p downregulated (Figure 2(e)). Furthermore, examination of RUNX2, ALP, and OCN expression levels revealed that transfection with miR-30b-5p inhibitor increased their expression levels by 3.2, 2.6, and approximately 2-fold, respectively (Figure 2(f)). Similarly, Western blot analysis showed that the expression of RUNX2, ALP, and OCN was increased following miR-30b-5p silencing (Figure 2(g)). Finally, mineralized bone matrix formation of hBMSCs was studied by ARS staining. As shown in Figure 2(h), knockdown of miR-30b-5p enhanced the degree of calcium nodules. These results revealed that inhibiting miR-30b-5p promotes osteogenic differentiation of hBMSCs.

BCL6: a target of miR-30b-5p

Based on our previous results, BCL6 was chosen for the follow-up studies. RT-qPCR analysis revealed that BCL6 mRNA levels increased with time during osteogenic differentiation (Figure 3(a)). Pearson's analysis showed that BCL6 expression was negatively correlated with miR-30b-5p and positively correlated with RUNX2, ALP, and OCN (Figure 3(b,c)). BCL6 is involved in osteogenic differentiation, and the expression pattern of BCL6 is contrary to that of miR-30b-5p. Next, we constructed BCL6-WT and BCL6-MUT. Co-transfection of BCL6-WT and miR-30b-5p mimic significantly inhibited luciferase activity, whereas co-transfection of BCL6-MUT and miR-30b-5p mimic had no effect on luciferase activity (Figure 3(d)). To determine whether BCL6 was downregulated by miR-30b-5p, we analyzed the mRNA expression of BCL6. We found an increase in BCL6 mRNA level following transfection with the miR-30b-5p inhibitor and a decrease following si-BCL6 transfection. Furthermore, the miR-30b-5p inhibitor reversed the negative effect of si-BCL6 on BCL6 expression (Figure 3(e)). Additionally, Western blot analysis of BCL6 protein showed similar results to mRNA (Figure 3(f)), suggesting that miR-30b-5p negatively regulates BCL6 mRNA expression in hBMSCs.

miR-30b-5p negatively regulates BCL6 to inhibit osteogenic differentiation

To verify the involvement of BCL6 in miR-30b-5p-mediated osteogenic differentiation of hBMSCs, we transfected BMSCs with either si-BCL6 or miR-30b-5p inhibitor. The CCK-8 assay proved that silencing BCL6 reduced the viability of hBMSCs by approximately 30%, whereas silencing miR-30b-5p partially abrogated the inhibitory effect of si-BCL6 on hBMSC viability (Figure 4(a)). The expression of OCN, RUNX2, and ALP mRNA was decreased by approximately 70%, 60%, and 40%, respectively, following transfection with si-BCL6, whereas the levels were reversed by the miR-30b-5p inhibitor (Figure 4(b-d)). In addition, BCL6 knockdown reduced the protein expression of OCN, RUNX2,

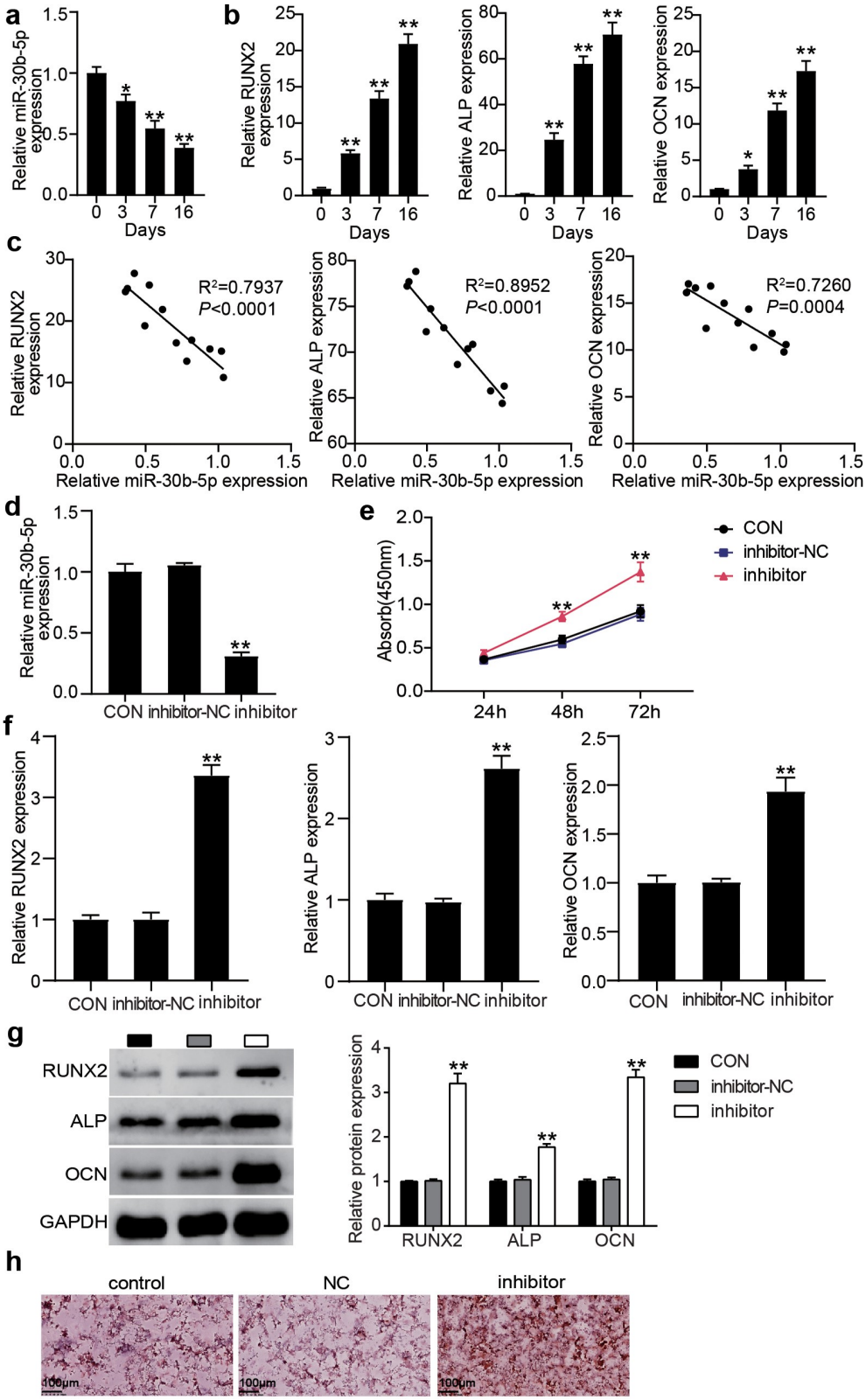

Figure 2. miR-30b-5p inhibits osteoblast differentiation. (a) The expression levels of miR-30b-5p were quantified by RT-qPCR on days 0, 3, 7, and 16 after the osteogenic induction of BMSCs. (b) The expression of RUNX2, ALP, and OCN was measured via RT-qPCR on days 0, 3, 7, and 16 after the osteogenic induction of BMSCs. * $P < 0.05$, ** $P < 0.001$ vs. 0 days. (c) A correlation between miR-30b-5p expression and the expression of RUNX2, ALP, and OCN during the osteogenic induction. (d) The expression of miR-30b-5p in BMSCs transfected with the miR-30b-5p inhibitor, as determined by RT-qPCR. (e) Cell viability was measured by CCK-8 assay in the miR-30b-5p low-expression BMSCs. (f) In BMSCs with down-regulated miR-30b-5p, the expression levels of RUNX2, ALP, and OCN were determined by RT-qPCR. (g) In BMSCs with down-regulated miR-30b-5p, the expression levels of RUNX2, ALP, and OCN were determined by Western blot. (h) ARS staining after low expression of miR-30b-5p transfected BMSCs following 16 days of osteogenic induction. CON, blank control. NC, negative control. Inhibitor, miR-30b-5p inhibitor. * $P < 0.05$, ** $P < 0.001$ vs. CON.

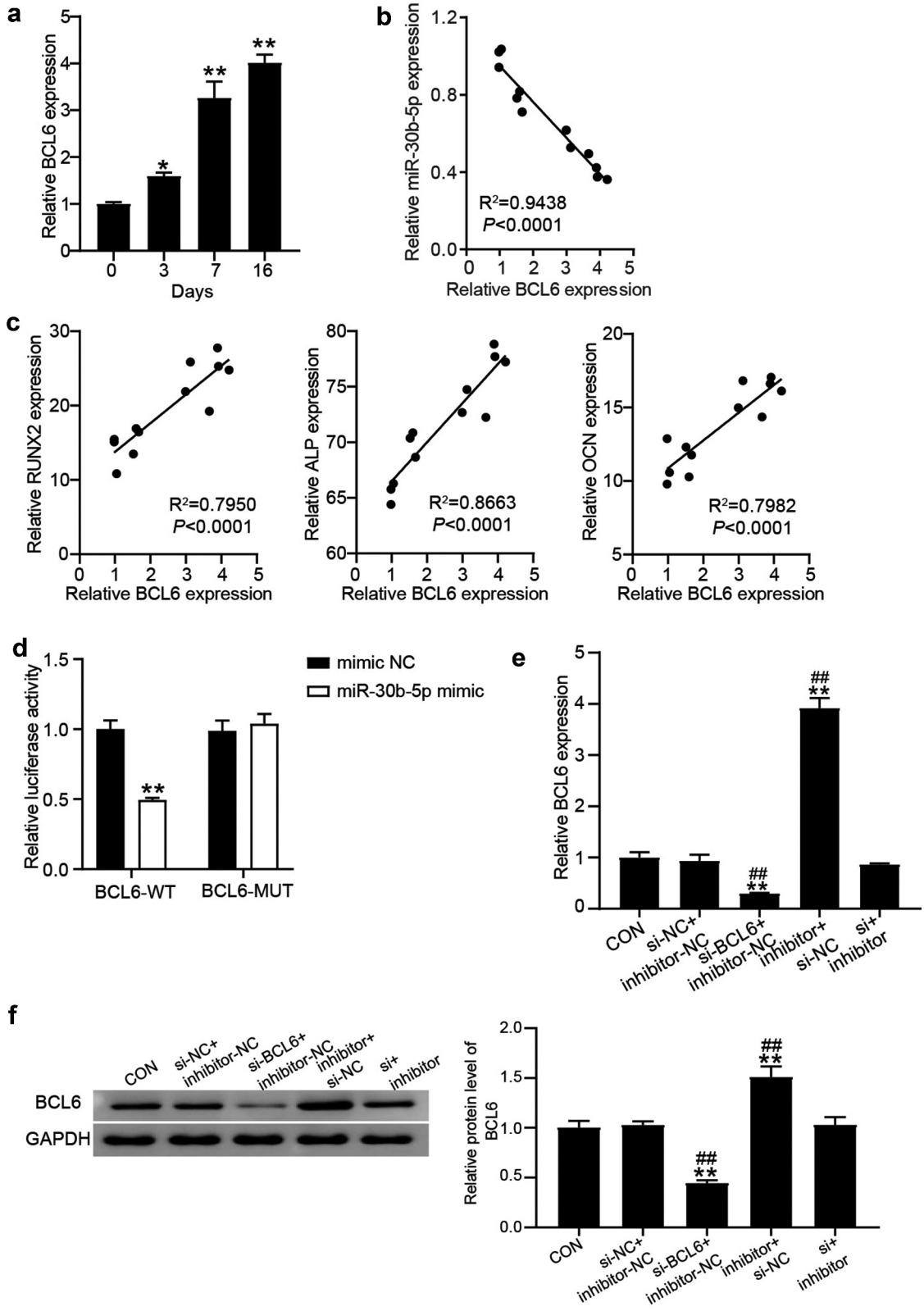

Figure 3. BCL6 is a target of miR-30b-5p. (a) The expression levels of BCL6 were quantified by RT-qPCR on days 0, 3, 7, and 16 after the osteogenic induction of BMSCs. * $P < 0.05$, ** $P < 0.001$ vs. 0 days. (b) The correlation between the expression of BCL6 and miR-30b-5p during the osteogenic induction. (c) A correlation between BCL6 expression and the expression of RUNX2, ALP, and OCN during the osteogenic induction. (d) The luciferase reporter assay was performed to study the relation between miR-30b-5p and BCL6. ** $P < 0.001$ vs. mimic NC. (e) RT-qPCR analyses of BCL6 mRNA expression in BMSCs transfected with miR-30b-5p inhibitor or si-BCL6. (f) BCL6 protein levels were examined by Western blotting in the BMSCs transfected with the miR-30b-5p inhibitor or si-BCL6. CON, blank control. NC, negative control. Inhibitor, miR-30b-5p inhibitor. si, si-BCL6. ** $P < 0.001$ vs. CON. ## $P < 0.001$ vs. si+inhibitor.

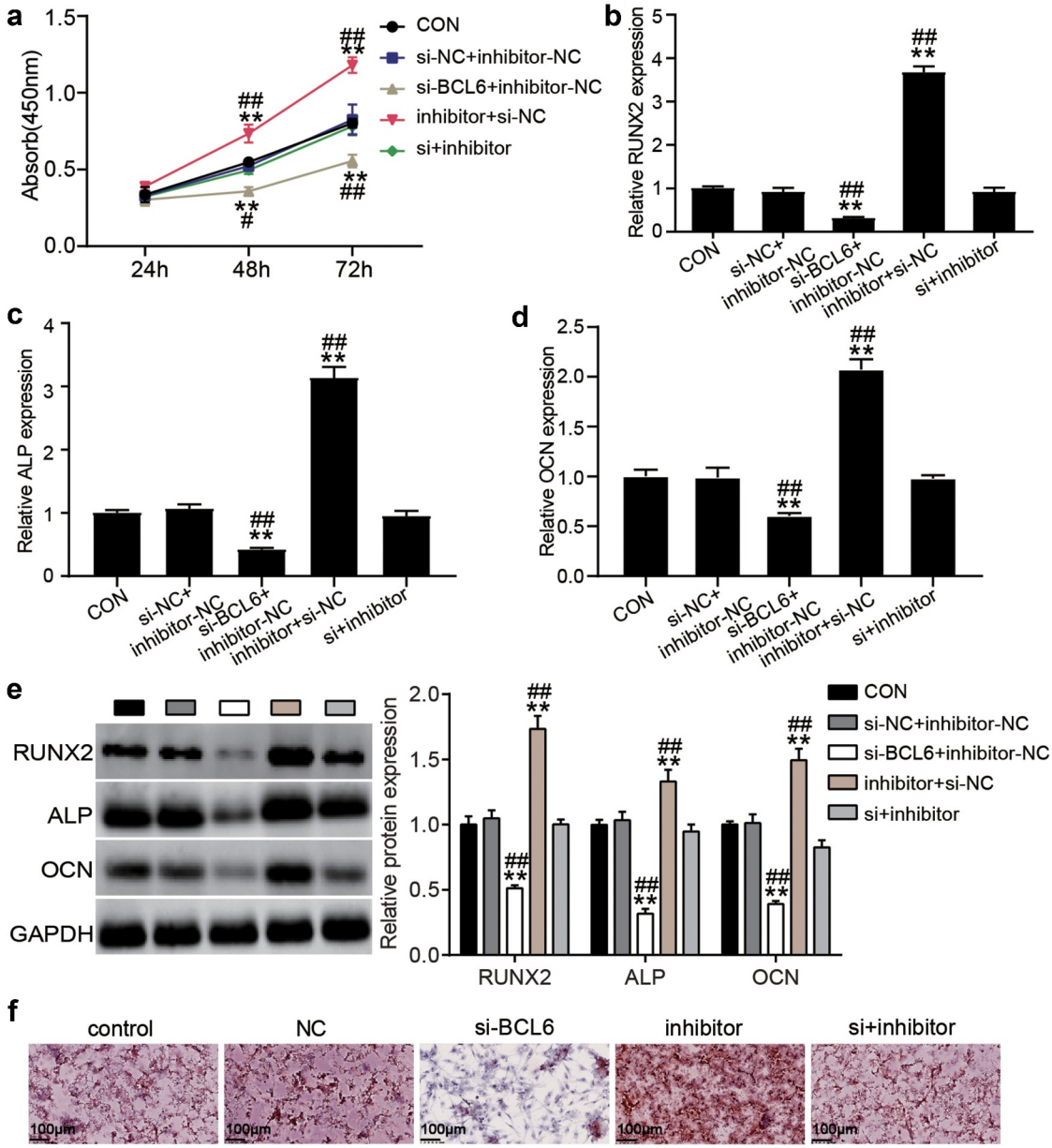

Figure 4. MiR-30b-5p negatively regulates BCL6 to inhibit osteogenic differentiation. (a) Cell viability was measured by CCK-8 assay in the miR-30b-5p or BCL6 low-expression BMSCs. (b-d) In BMSCs with down-regulated miR-30b-5p or BCL6, the expression levels of RUNX2, ALP, and OCN were determined by RT-qPCR. (e) In BMSCs with down-regulated miR-30b-5p or BCL6, the expression levels of RUNX2, ALP, and OCN were determined by Western blot. (f) ARS staining after low expression of miR-30b-5p or BCL6 transfected BMSCs following 16 days of osteogenic induction. CON, blank control. NC, negative control. Inhibitor, miR-30b-5p inhibitor. si, si-BCL6. ** $P < 0.001$ vs. CON. ## $P < 0.001$ vs. si+inhibitor.

and ALP, which was restored by the miR-30b-5p inhibitor (Figure 4(e)). ARS staining showed a similar decrease in the amount of calcium nodules in hBMSCs following downregulation of BCL6, whereas the miR-30b-5p inhibitor reversed the si-BCL6-mediated inhibition of calcium nodules (Figure 4(f)).

Discussion

Because of their strong capacity for bone regeneration, BMSCs hold great potential for application in bone tissue engineering and regeneration. The osteogenic differentiation of BMSCs is mediated via a variety of cellular events that are influenced

by various cellular processes and molecules during bone development [27–29]. Therefore, it is crucial to identify the key factors that are involved in this phenomenon, and to understand the underlying mechanism. Previous studies have shown that miRNAs target different genes involved in signaling pathways that affect bone regeneration[30].

Various miRNAs are differentially expressed during hMSC differentiation[31]. Wu et al.[32] found that miR-30b acts on RUNX2 and negatively regulates osteogenic differentiation. Yang et al.[33] further revealed an increase in miR-30b-5p in exosomes derived from osteoblastic differentiated adipose stem cells (ADSCs) by microarray analysis and RT-qPCR. In this study, we observed a similar phenomenon. First, we analyzed miR-30b-5p expression in hBMSCs during osteogenic differentiation. miR-30b-5p levels decreased with time during osteogenic differentiation. Previous studies have shown that osteoblast-specific genes RUNX2, ALP, and OCN are important markers of osteoblast maturation and differentiation, as well as key genes for osteoblast survival [34,35]. We found that miR-30b-5p was negatively correlated with the expression of ALP, RUNX2, and OCN during osteogenic differentiation. Furthermore, Liu et al.[36] reported that estrogen-mediated suppression of miR-30b inhibited its expression and promoted the proliferation and osteogenesis of BMSCs. To study the effect of miR-30b-5p on hBMSC differentiation, we silenced miR-30b-5p in hBMSC, and found that miR-30b-5p inhibition enhanced hBMSC proliferation, increased the expression of ALP, RUNX2, and OCN, and promoted calcium nodules. As in previous studies, miR-30b-5p was found to inhibit osteogenic differentiation. miRNAs target specific mRNAs by sharing binding sequences, and thereby influence many cellular processes[37]. Previous studies have reported mutations in BCL6-related molecules found in bone pathology such as finger and clavicular deformities[38], and recent studies have shown that BCL6 promotes osteoblast differentiation [21,22]. Bioinformatics analysis revealed that miR-30b-5p and BCL6 contained targeted binding sites. Dual-luciferase reporter and RNA pulldown assays confirmed that miR-30b-5p targets BCL6. In addition, miR-30b-5p inhibitor increased the expression level of BCL6. To our knowledge, this is the first study to show that BCL6 is the target of miR-30b-5p, and promotes the proliferation of hBMSCs and osteogenic differentiation.

The miR-30 family members are proved to be closely associated with osteogenic differentiation [32]. Multiple miRNAs have been shown to co-regulate the same target gene[39]. Therefore, the focus on one miRNA, miR-30b-5p, in this study has its limitations. Therefore, we intend to study the co-regulation of BCL6 by the miR-30 family in hBMSC osteogenesis in the future. Most preclinical and clinical trials that have used BMSCs in treating a variety of diseases, such as osteoarthritis, immune disorders, neurodegenerative diseases, and sports injuries have shown promising results [40]. Thus, it is possible to elucidate the preclinical effect of the miR-30b-5p/BCL6 axis on osteoarthritis and osteoporosis. However, BMSCs tend to senesce and lose their multi-differentiation potential over time in culture[41]. Therefore, it is necessary to overcome the limitations of BMSCs and identify the function of the miR-30b-5p/BCL6 axis in treatment of osteoarthritis, osteoporosis, and other diseases *in vivo* in the future.

In conclusion, miR-30b-5p is downregulated during osteogenic differentiation of hBMSCs, and is negatively correlated with osteogenic gene expression and calcium nodules. The effect of the miR-30b-5p/BCL6 axis on osteogenic differentiation of hBMSCs is explored in this study, which may provide new evidence for the mechanisms on osteogenic differentiation of hBMSCs, thereby facilitating the clinical application of hBMSC in bone tissue regeneration and repair.

Authors' contributions

FZ and BY performed the experiments and data analysis. Yan L conceived and designed the study. XCW made the acquisition of data. Yi L did the analysis and interpretation of data. All authors read and approved the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure statement

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

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Wuhan Puren Hospital (Wuhan, China). The processing of clinical tissue samples is in strict compliance with the ethical standards of the Declaration of Helsinki. All patients signed written informed consent.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

Patient consent for publication

All patients signed written informed consent.

References

[1] Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005;105(4):1815–1822.
[2] Dawson E, Mapili G, Erickson K, et al. Biomaterials for stem cell differentiation. Adv Drug Deliv Rev. 2008;60 (2):215–228.
[3] Sun H, Hu S, Zhang Z, et al. Expression of exosomal microRNAs during chondrogenic differentiation of human bone mesenchymal stem cells. J Cell Biochem. 2019;120(1):171–181.
[4] Li Y, Feng C, Gao M, et al. MicroRNA-92b-5p modulates melatonin-mediated osteogenic differentiation of bone marrow mesenchymal stem cells by targeting ICAM-1. J Cell Mol Med. 2019;23(9):6140–6153.
[5] Zha JP, Wang XQ, Di J. MiR-920 promotes osteogenic differentiation of human bone mesenchymal stem cells by targeting HOXA7. J Orthop Surg Res. 2020;15 (1):254.
[6] Mao L, Liu S, Hu L, et al. miR-30 Family: a promising regulator in development and disease. Biomed Res Int. 2018;2018:9623412.
[7] Gagnon KT, Li L, Chu Y, et al. RNAi factors are present and active in human cell nuclei. Cell Rep. 2014;6(1):211–221.
[8] Bottini S, Hamouda-Tekaya N, Mategot R, et al. Post-transcriptional gene silencing mediated by microRNAs is controlled by nucleoplasmic Sfpq. Nat Commun. 2017;8(1):1189.
[9] Wang F, Wang J, He J, et al. Serum miRNAs miR-23a, 206, and 499 as potential biomarkers for skeletal muscle atrophy. Biomed Res Int. 2017;2017:8361237.
[10] Chen S, Li Y, Zhi S, et al. lncRNA Xist regulates osteoblast differentiation by sponging miR-19a-3p in aging-induced Osteoporosis. Aging Dis. 2020;11 (5):1058–1068.
[11] Long C, Cen S, Zhong Z, et al. FOXO3 is targeted by miR-223–3p and promotes osteogenic differentiation of bone marrow mesenchymal stem cells by enhancing autophagy. Hum Cell. 2021;34(1):14–27.
[12] Qin X, Chen J, Wu L, et al. MiR-30b-5p acts as a tumor suppressor, repressing cell proliferation and cell cycle in human hepatocellular carcinoma. Biomed Pharmacothe. 2017;89:742–750.
[13] Estevão-Pereira H, Lobo J, Salta S, et al. Overexpression of circulating MiR-30b-5p identifies advanced breast cancer. J Transl Med. 2019;17(1):435.
[14] Zhang L, Jia X. Down-regulation of miR-30b-5p protects cardiomyocytes against hypoxia-induced injury by targeting Aven. Cell Mol Biol Lett. 2019;24(1):61.
[15] Zang J, Maxwell AP, Simpson DA, et al. Differential expression of urinary exosomal microRNAs miR-21–5p and miR-30b-5p in Individuals with diabetic kidney disease. Sci Rep. 2019;9(1):10900.
[16] Croset M, Pantano F, Kan CWS, et al. miRNA-30 family members inhibit breast cancer invasion, osteomimicry, and bone destruction by directly targeting multiple bone metastasis-associated genes. Cancer Res. 2018;78(18):5259–5273.
[17] Eguchi T, Watanabe K, Hara ES, et al. OstemiR: a novel panel of microRNA biomarkers in osteoblastic and osteocytic differentiation from mesencymal stem cells. PloS One. 2013;8(3):e58796.
[18] Balderman JA, Lee HY, Mahoney CE, et al. Bone morphogenetic protein-2 decreases microRNA-30b and microRNA-30c to promote vascular smooth muscle cell calcification. J Am Heart Assoc. 2012;1(6): e003905.
[19] Chang CC, Ye BH, Chaganti RS, et al. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. Proceedings of the National Academy of Sciences of the United States of America 1996; 93:6947–6952.
[20] Jardin F, Ruminy P, Bastard C, et al. The BCL6 proto-oncogene: a leading role during germinal center development and lymphomagenesis. Pathologie-biologie. 2007;55(1):73–83.
[21] Fujie A, Funayama A, Miyauchi Y, et al. Bcl6 promotes osteoblastogenesis through Stat1 inhibition. Biochem Biophys Res Commun. 2015;457(3):451–456.
[22] Yang J, Wang S, Wang F, et al. Downregulation of miR-10b promotes osteoblast differentiation through targeting Bcl6. Int J Mol Med. 2017;39(6):1605–1612.
[23] Liu L, Cui S, Wan T, et al. Long non-coding RNA HOTAIR acts as a competing endogenous RNA to promote glioma progression by sponging miR-126–5p. J Cell Physiol. 2018;233(9):6822–6831.
[24] Miyauchi Y, Ninomiya K, Miyamoto H, et al. The Blimp1-Bcl6 axis is critical to regulate osteoclast

differentiation and bone homeostasis. J Exp Med. 2010;207(4):751–762.
[25] Ihn HJ, Lee T, Lee D, et al. Inhibitory Effect of KP-A038 on Osteoclastogenesis and inflammatory bone loss is associated with downregulation of Blimp1. Front Pharmacol. 2019;10:367.
[26] Zhang L, Li G, Wang K, et al. MiR-30 family members inhibit osteoblast differentiation by suppressing Runx2 under unloading conditions in MC3T3-E1 cells. Biochem Biophys Res Commun. 2020;522(1):164–170.
[27] Guan M, Yao W, Liu R, et al. Directing mesenchymal stem cells to bone to augment bone formation and increase bone mass. Nat Med. 2012;18(3):456–462.
[28] Li J, Huang Y, Song J, et al. Cartilage regeneration using arthroscopic flushing fluid-derived mesenchymal stem cells encapsulated in a one-step rapid cross-linked hydrogel. Acta Biomater. 2018;79:202–215.
[29] Liu C, Zhang H, Tang X, et al. Mesenchymal stem cells promote the Osteogenesis in Collagen-Induced arthritic mice through the inhibition of TNF-α. Stem Cells Int. 2018;2018:1–10.
[30] Zhang S, Chu WC, Lai RC, et al. Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. Osteoarthritis Cartilage. 2016;24(12):2135–2140.
[31] Schoolmeesters A, Eklund T, Leake D, et al. Functional profiling reveals critical role for miRNA in differentiation of human mesenchymal stem cells. PloS One. 2009;4(5):e5605.
[32] Wu T, Zhou H, Hong Y, et al. miR-30 family members negatively regulate osteoblast differentiation. J Biol Chem. 2012;287(10):7503–7511.
[33] Yang S, Guo S, Tong S, et al. Promoting osteogenic differentiation of human adipose-derived stem cells by altering the expression of exosomal miRNA. Stem Cells Int. 2019;2019:1351860.
[34] Jang WG, Kim EJ, Kim DK, et al. BMP2 protein regulates osteocalcin expression via Runx2-mediated Atf6 gene transcription. J Biol Chem. 2012;287(2):905–915.
[35] Salingcarnboriboon R, Tsuji K, Komori T, et al. Runx2 is a target of mechanical unloading to alter osteoblastic activity and bone formation in vivo. Endocrinology. 2006;147(5):2296–2305.
[36] Liu G, Lu Y, Mai Z, et al. Suppressing MicroRNA-30b by estrogen promotes osteogenesis in bone marrow mesenchymal stem cells. Stem Cells Int. 2019;2019:1–13.
[37] Cloonan N. Re-thinking miRNA-mRNA interactions: intertwining issues confound target discovery. BioEssays. 2015;37(4):379–388.
[38] Ng D, Thakker N, Corcoran CM, et al. Oculofaciocardiodental and Lenz microphthalmia syndromes result from distinct classes of mutations in BCOR. Nat Genet. 2004;36(4):411–416.
[39] Kumar P, Luo Y, Tudela C, et al. The c-Myc-regulated microRNA-17~92 (miR-17~92) and miR-106a~363 clusters target hCYP19A1 and hGCM1 to inhibit human trophoblast differentiation. Mol Cell Biol. 2013;33(9):1782–1796.
[40] Chu DT, Phuong TNT, Tien NLB, et al. An Update on the progress of isolation, culture, storage, and clinical application of human bone marrow mesenchymal stem/stromal cells. Int J Mol Sci. 2020;21(3):708.
[41] Derubeis AR, Cancedda R. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. Ann Biomed Eng. 2004;32(1):160–165.