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Original Article

The relationship between miR-106a and bone morphogenesis proteins and its role in bone marrow mesenchymal stem cell osteogenesis differentiation

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Abstract: Osteoblast is the major functional cell for osteogenesisr responsible for bone matrix synthesis, secretion and mineralization. Osteoblast mainly derives from undifferentiated mesenchymal stem cells (MSCs). Osteogenesis differentiation process of MSCs is critical for human bone formation, differentiation and regeneration. This study aimed to investigate the relationship between miR-106a and bone morphogenesis, as well as its role in bone marrow MSCs osteogenesis differentiation. Percoll approach was used to separate MSCs from human bone marrow for osteogenesis differentiation induction. qRT-PCR was used to test miR-106a expression. Bioinformatics analysis was performed to predict target genes of miR-106a, followed by luciferase reporter gene assay. The effects of miR-106a on MSCs osteogenesis were analyzed. Western blot was used to analyze expression of osteogenesis differentiation related proteins including RUNX2, OCN and ALP. Cells separated by Percoll method expressed CD44 and CD25 as demonstrated by flow cytometry. MSCs showed features of osteoblast after osteogenesis differentiation induction, as miR-106a expression level was significantly suppressed ($p<0.05$). Bioinformatics prediction and luciferase reporter gene assay confirmed that miR-106a could suppress BMP2 gene expression. Cell transfection for inhibiting miR-106a expression facilitated MSCs osteogenesis differentiation, and significantly elevated RUNX2, OCN and ALP expression level ($p<0.05$ compared to control group). In conclusion, During osteogenesis differentiation of MSCs, miR-106a is down-regulated to enhance expression level of its target gene BMP2, eventually facilitating MSCs osteogenesis differentiation.

Keywords: Mesenchymal stem cells, MiR-106a, BMP2, osteogenesis differentiation

Introduction

Osteoblast and osteoclast play important roles in maintaining normal human bone density and quality. Osteoblast is the major functional cell for osteogenesis, and is responsible for bone matrix synthesis, secretion and mineralization [1]. Osteoblast is differentiated from mesenchymal stem cells (MSCs) [2]. MSCs is an important member of stem cell family and derives from mesoderm and ectoderm at early developmental stage. As a pluripotent stem cell, MSCs have pluripotent differentiation potency, and can differentiate into multiple tissues including adipocytes, bone, cartilage, muscle, tendon, ligament, nerve, liver, heart muscle and endothelium [3, 4]. In addition, MSCs can self-regulate differentiation under effects of differentiated factors. For example, RUNX2 can induce MSCs to differentiate into osteoblast [5, 6]. The study of MSCs osteogenesis process is of critical importance for the knowledge of bone development, bone cell differentiation and bone injury repair.

MicroRNA is a family of non-coding small RNA molecule with highly conserved evolution, and can regulate gene expression at post-transcriptional level via RNA interference mechanism [7]. Previous study revealed important roles of microRNA in cell behavior regulation, such as the close correlation between miR-21 and proliferation of various cancer cells [8]. MiR-363 might participate in CD4 + T cell induced inflammatory process [9], whilst miR-124 and miR-137 also exert important roles in neuron differ-

Table 1. Nucleic acid sequence for cell transfection

Name	Sequence
miR-106a-F	5'TGCTTACAGTGCAGGTAG3'
miR-106a-R	5'GTAAGAAGTGCTTACATTGC3'
U6-F	5'CTCGCTTCGGCAGCACA3'
U6-R	5'AACGCTTCACGAATTTGCGT3'

entiation [10, 11]. Recent studies showed that various miRNA including miR-106a is closely correlated with MSCs osteogenesis differentiation [12]. This study investigated the relationship between miR-106a and bone morphogenesis related proteins, and its role in MSCs osteogenesis differentiation.

Materials and methods

MSCs separation

MSCs were cultured in low glucose DMEM medium (Hyclone, US). Bone marrow was obtained from healthy volunteer donors from our hospital. Bone marrow was diluted 5-fold in DMEM medium, followed by removal of supernatant and lipid layer after centrifugation. Low layer cell was mixed with DMEM medium containing 5% fetal bovine serum (FBS) for re-suspending cells. MSCs were separated by Percoll method. In brief, Percoll reagent (Sigma, US) was paved on the tube bottom. Cell suspensions were then slowly added at 1:1 ratio. The tube was centrifuged at 400 g for 20 min. The middle layer was carefully collected and rinsed twice in PBS. Cells were counted under microscope, and inoculated in MSCs culture medium (low glucose DMEM medium containing 15% FBS (Hyclone, US), 100 u/L ampicillin (Hyclone, US), 100 mg/L streptomycin (Hyclone, US) and 250 mg/L amphotericin (Hyclone, US) in a 37°C incubator with 5% CO_2.

Flow cytometry analysis

Cells were cultured as abovementioned. Culture medium was removed and rinsed twice in PBS. Cells were then digested with trypsin and EDTA. Non-specific binding sites were blocked by BSA. Mouse anti-human CD29, CD34, CD44 and CD45 antibody were added for incubation, followed by FITC labelled secondary antibody. Cells were centrifuged and collected. After resuspension, flow cytometry was performed to analyze cell phenotype.

Induction of MSCs osteogenesis differentiation

MSCs were cultured to third generation, and inoculated into 6-well plate, which was inserted with sterile coverslips for further incubation. Experimental group utilized high glucose DMEM medium, with replenishment of 10% FBS, 10 mM β-glycerolphosphatide, 10 mM dexamethasone, and 0.2 mM vitamin C. In control group, high glucose DMEM complete medium was used for changing fresh medium at day 3. After 14 day culture, coverslips were removed for further analysis.

qRT-PCR

Those cells with differentiation induction were extracted for total RNA using RNAprep pure Tissue Kit (QIAGEN, Germany). Using total RNA extracted from control group as the control, qRT-PCR was performed to measure miR-106a expression. Firstly, primers were designed based on miR-106a sequence (GeneBank access # NR_029523) for RT-PCR amplification. Primer sequences were shown in **Table 1**. qRT-PCR amplification was performed using mirVanat qRT-PCR miRNA test kit (Ambion, US). Reverse transcription was firstly performed in a system containing 10 μL RNA, 5 μL RT Buffer, 1 μL RNase Mix, 2 μL cDNA and 10 μL qPCR Mix, plus 2 μL forward/reverse primers, and 4 μL ddH_2O. Reaction conditions were 95°C 3 min, followed by 40 cycles each containing 95°C 15 s and 60°C 30 s. Using U6-RNA sequence as internal reference, results were analyzed by $2^{-\Delta\Delta Ct}$ method.

MiR-106a functional prediction

Bioinformatics software TargetScan Release 5.1 (www.targetscan.org) was used to predict miR-106a function. Luciferase reporter gene assay confirmed possible targets. Based on 3'UTR sequence of BMP2 mRNA (Genebank access number: NM_006474), primers were designed as 5'-AAAAA GCACG TATCG GCGAG GATGA TCTCT ATC-3' and 5'-AGAAA GCTGG AC CAA CACAG GTGTG ACCAA GAA-3'. PCR amplification was performed to obtain 3'UTR sequence of BMP2 mRNA, and was inserted into downstream region of firefly luciferase gene coding region in pmirGLO plasmid to construct pmirGLO-BMP2 vector. HEK293 cells were transfected with pmirGLO-BMP2 or pmirGLO plasmid. Those cells with successful transfec-

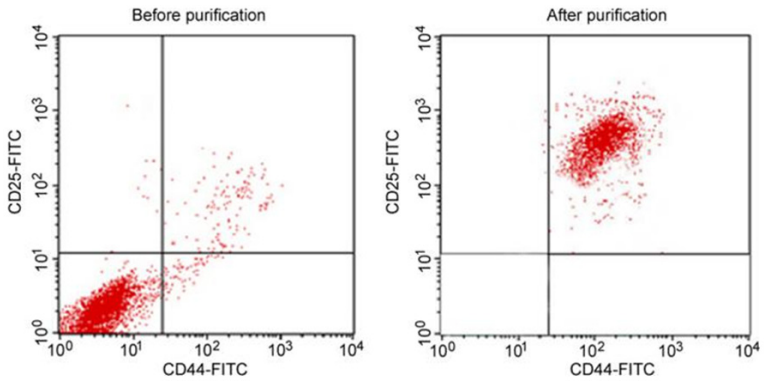

Figure 1. Flow cytometry for measuring separated MSCs. Cultured cells were collected and non-specific binding sites were blocked by BSA followed by addition of mouse anti-human CD29, CD34, CD44 and CD45 antibody and subsequent addition of FITC labelled secondary antibody. Cells were centrifuged and collected. After re-suspension, flow cytometry was performed to analyze cell phenotype.

tion were transfected with miR-106a mimic to elevate miR-106a activity. Cell transfection was performed using liposome INTERFERin™ transfection reagent kit (Polyplus transfection, France). HEK293 cell line was purchased from Cell Bank, Chinese Academy of Science. Cryopreserved cells were resuscitated and cultured till log-growth phase, and were digested in trypsin, counted, diluted in fresh culture medium, and inoculated into 96-well plate. After 24 h incubation, transfection was performed following manual instruction of test kit. With successful transfection, cells were cultured for 48 h for fluorescent intensity analysis. Dual luciferase reporter gene analysis system (Promega, US) was used for analyzing fluorescent intensity on MicroLumatPlus LB96V spectrometry (Berthold, Germany) [13].

Cell transfection

To study the effect of miR-106a expression on MSCs, miR-106a mimics and miR-106a inhibitor were designed based on miR-106a sequence, and were transfected into MSCs using methods described in previous sections.

Meanwhile, BMP2 expression was manipulated using overexpression vector pcDNA3.1-BMP2 and RNA interference (downregulation of BMP2) in which, the anti-BMP2 sequence was F: 5'-GACGAGGTCCTGAGCGAG-3'; R: 5'-GACTGCTCCAGGACTCGC-3' and the scramble sequence was F: 5'-CATCTTCAGCACCATATAC-3'; R: 5'-GAGTAGAAGTCGTGGTATA-3'. After BMP2 expression was manipulated, they were induced into cells with transfected with miR-106a inhibitor (BMP2 downregulation) or mimic (BMP2 upregulation).

Western blot assay

MSCs were cultured for 48 h, and lysed to extract total proteins, which were separated by SDS-PAGE, followed by transferring to a PVDF membrane. After blocking in 5% defatted milk powder, the membrane was washed in TBST and incubated with primary antibody (rabbit anti-human BMP2, rabbit anti-human RUNX2, rabbit anti-human OCN, rabbit anti-human ALP, rabbit anti-human osteocalcin, and anti-human β-actin antibody at 1:1000 dilution) at 4°C overnight. With TBST rinsing, HERP labelled mouse anti-rabbit IgG secondary antibody (1:200) was added for 1 h room temperature incubation. All primary antibody used in Western Blot were purchased from Sanying Biotech (China). After TBST rinsing, freshly prepared DAB was added for 10 min staining. Western-Blot images were analyzed for gray integrity value for calculating relative expression level as previously described [14].

Statistical analysis

All data were presented as mean ± standard deviation (SD). SPSS20.0 was used for statistical analysis. Comparison among multiple groups was performed using one way analysis of variance (ANOVA). Student t-test was used for comparing difference between two groups. A statistical significance was defined when $p<0.05$.

Experimental results

MSCs separation

Percoll method was used to separate MSCs from bone marrow. Those cells were cultured in high glucose DMEM medium, followed by flow cytometry analysis of the phenotype of MSCs. As shown in **Figure 1**, all MSCs showed positive expression of CD44 and CD25.

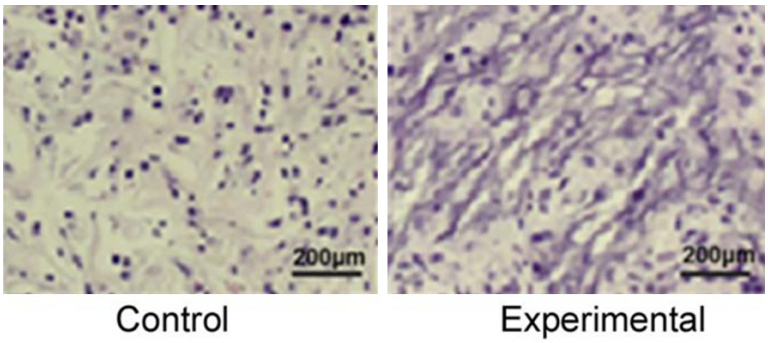

Figure 2. Osteogenesis differentiation of MSCs. MSCs were cultured to third generation, and inoculated into 6-well plate. Experimental group utilized high glucose DMEM medium. In control group, high glucose DMEM complete medium was used for changing fresh medium at day 3. After 14 day culture, coverslips were removed for analysis of osteogenesis differentiation.

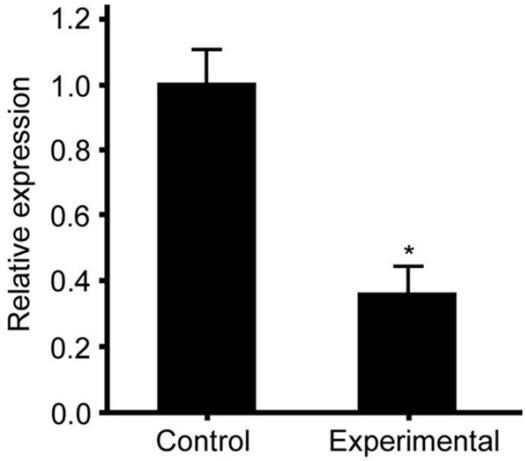

Figure 3. MiR-106a expression in MSCs. *, p<0.05 compared to control group. Total RNA was isolated from MSCs followed by measuring miR-106a expression by qRT-PCR.

MSCs induction for osteogenesis differentiation and miR-106a expression

Separated MSCs were cultured *in vitro* till third generation for induction of osteogenesis differentiation. Cell morphology was observed under a microscope. Compared to control group, experimental group cells displayed features of osteoblast, as shown by spindle or pyramidal shape with cell-to-cell connection. Nucleus locates on one side of cell with clearly visible nucleolus (**Figure 2**).

Separated cells were cultured and extracted for total RNA. qRT-PCR was used to measure miR-106a expression. We found that, compared to control group, miR-106a expression was significantly decreased in induced osteogenesis MSCs ($p<0.05$, **Figure 3**).

Relationship between miR-106a and BMP2 gene expression

Bioinformatics software TargetScan Release 5.1 was used to predict target genes of miR-106a. We found homology between miR-106a and 3'UTR sequence of BMP2 (**Figure 4A**). We thus speculated that BMP2 might be the target gene of miR-106a. To test that, we constructed luciferase reporter gene expression system for confirmation. As shown in **Figure 4B**, experimental group cells with miR-106a mimic transfection had significantly lower fluorescent intensity, whilst transfection of miR-106a inhibitor significantly elevated cell fluorescent intensity. These results showed that 3'UTR of BMP2 was the functional target of miR-106a.

BMP2 expression

Western Blot was performed to measure BMP2 protein expression in those MSCs with successful transfection. Gel imaging analysis system was used to calculate BMP2 expression between control and experimental group. As shown in **Figure 5**, transfection of miR-106a mimic significantly decreased BMP2 expression in MSCs ($p<0.05$), whilst transfection of miR-106a inhibitor remarkably elevated BMP2 expression ($p<0.05$), indicating successful modulation of BMP2 protein expression by cell transfection.

Effects of miR-106a and BMP2 expression on osteogenesis differentiation

MSCs with successful transfection were continuously cultured. After 7 d, microscope was used to observe cell morphology changes. As shown in **Figure 6**, we found that by inhibiting miR-106a expression in MSCs with cell transfection, MSCs showed features of osteogenesis differentiation.

To evaluate whether miR-106a exerts its effect on osteogenesis differentiation through BMP2, we manipulated the expression of BMP2 using

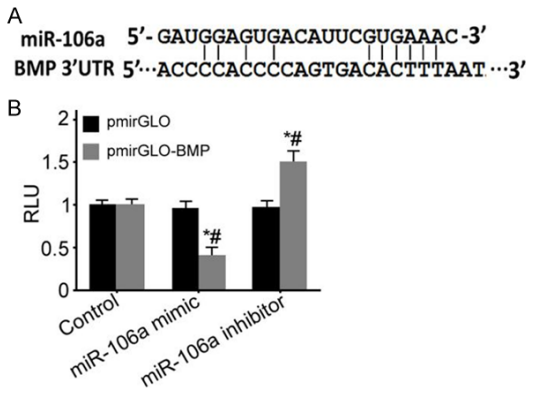

Figure 4. Relationship between miR-106a and BMP2 gene. A. Sequence homology between miR-106a and 3'UTR of BMP2. B. Effects of miR-106a on BMP2 gene expression. *, p<0.05 compared to control group; #, p<0.05 compared to pmir-GLO plasmid in the same group.

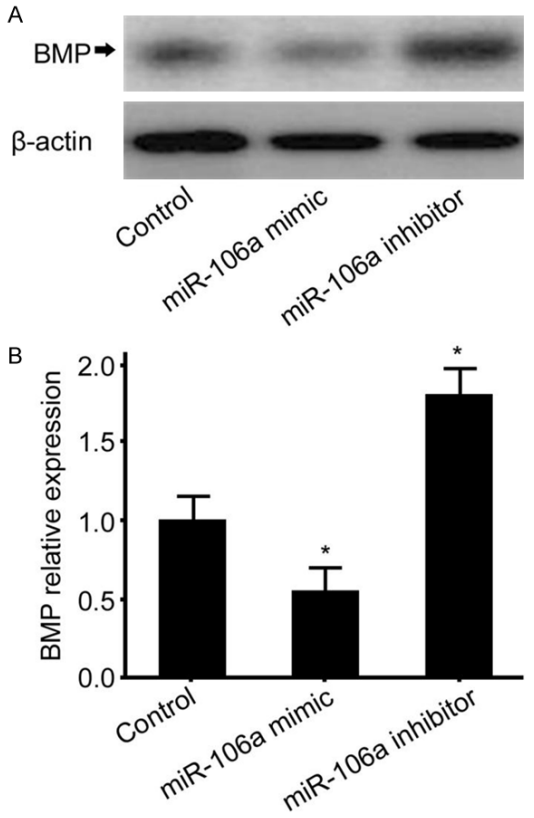

Figure 5. BMP2 expression in MSCs. A. BMP2 expression by Western Blot; B. Relative expression of BMP2. *, p<0.05 compared to control group. Total protein and RNA was isolated from cells transfected with miR-106a mimic or inhibitor for measuring BMP2 protein expression by western blot and RNA expression by qRT-PCR.

overexpression vector or RNA interference. As seen in **Figure 6**, overexpression of BMP2 induced osteogenesis differentiation even when miR-106a expression was upregulated by transfection of mimic. Consistently, inhibition of BMP2 expression by RNA interference abolished the effect of miR-106a inhibition on osteogenesis differentiation. Taken together, these data demonstrated that miR-106a exerts its function depending BMP2 expression.

Western Blot for osteogenesis differentiation related protein expression

Those MSCs with successful transfection were cultured for 7 days, and were tested for RUNX2, OCN and ALP protein expression. Using β-actin as the control, gel imaging analysis system was used to calculate relative expression level of two inflammation related proteins. As shown in **Figure 7**, compared to control group, transfection of miR-106a inhibitor in MSCs had elevated RUNX2, OCN and ALP levels to different extents ($p<0.05$). Whilst transfection of miR-106a mimic into MSCs suppressed RUNX2, OCN and ALP expression levels ($p<0.05$). In addition, cells with transfection of miR-106a mimic + pcDNA3.1-BMP2 displayed significantly higher expression of RUNX2, OCN and ALP, whereas, cells with transfection of miR-106a inhibitor + anti-BMP2 showed reduced expression of RUNX2, OCN and ALP.

Discussion

MSCs are important member of stem cell family and are one group of pluripotent stem cells that can differentiate into various cells including bone, muscle and neural tissues [13]. Osteogenesis differentiation is critical for maintaining normal human bone density and quality [14]. The study of MSCs osteogenesis differentiation process and regulatory mechanism are important for the knowledge of bone development, bone cell differentiation and bone injury repair [15]. This study firstly separated MSCs from healthy adult bone marrows. By passage culture and osteogenesis differentiation induction, we found that miR-106a expression was significantly down-regulated in MSCs, suggesting that miR-106a might participate in osteogenesis differentiation process of MSCs. By analyzing and prediction, miR-106a might use BMP2 gene as the target to exert its effect via suppressing gene expression.

MicroRNA is a type of small molecular non-coding RNA with conserved evolution. It can bind to

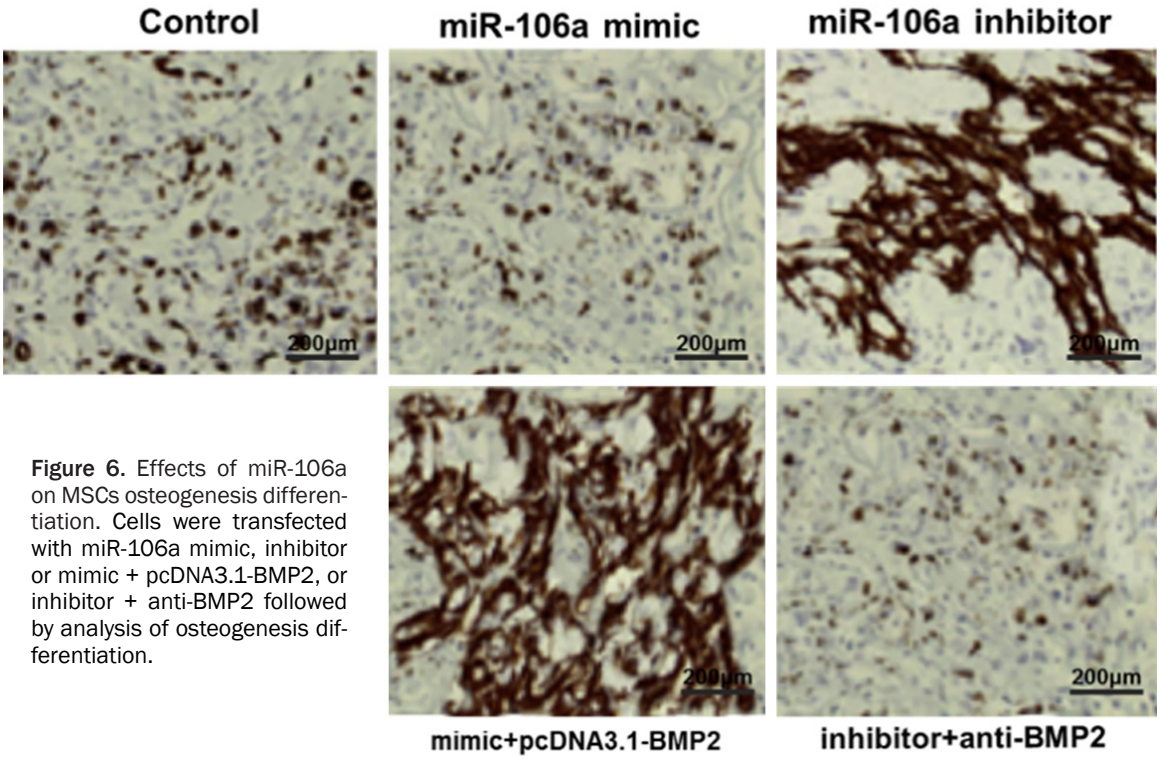

Figure 6. Effects of miR-106a on MSCs osteogenesis differentiation. Cells were transfected with miR-106a mimic, inhibitor or mimic + pcDNA3.1-BMP2, or inhibitor + anti-BMP2 followed by analysis of osteogenesis differentiation.

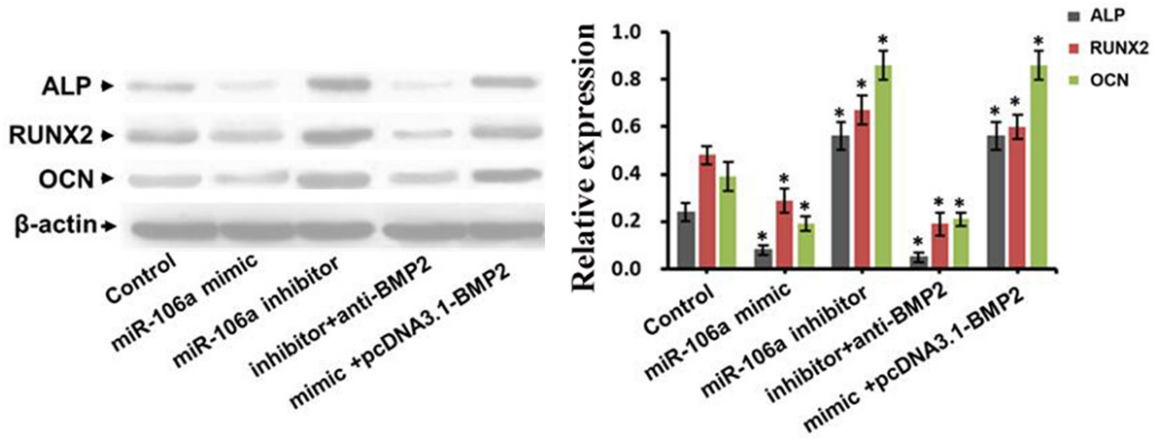

Figure 7. Osteogenesis differentiation related protein expression in MSCs. *, $p<0.05$ compared to the same protein in control group. Total protein was isolated from cells transfected with miR-106a mimic, inhibitor or mimic + pcDNA3.1-BMP2, or inhibitor + anti-BMP2 followed by measuring protein expression of ALP, RUNX2 or OCN by western blot.

the 3'UTR of target gene to modulate gene expression at RNA level, eventually participating in the modulation of various cell behaviors and body metabolic process [7]. Previous study showed that miR-21 could also participate in cancer cell proliferation and metabolism via affecting PTEN gene expression [16]. Moreover, miR-124 can also use PTBP1 mRNA as the target to regulate its expression level, and PTBP1 can mediate alternative splicing pattern of various mRNA precursors in neural cells, eventually modulating neural cell differentiation [10]. These studies showed important roles of miRNA in cell differentiation modulation.

Bone morphogenesis protein BMP2 was initially discovered as a protein for ectopic induction of bone and cartilage formation [17]. This protein participates in embryonic body development, organogenesis and tissue homeostatic

regulation, as well as postnatal cartilage and bone tissue formation. Meanwhile, other studies found the participation of BMPs in pathological process of certain diseases [18, 19]. Laura et al analyzed differential gene expression in glioma and found important roles of BMP in malignant tumor growth and maintenance [20].

The study of osteogenesis differentiation process and regulatory mechanism is critical for the knowledge of bone development, bone cell differentiation and damage repair. Results of this study is of critical importance for further illustration of osteogenesis differentiation and bone tissue maintenance. Meanwhile, this study provided evidences for using molecular biology approach to facilitate repair of bone injury and to recover motility [21].

Conclusion

During osteogenesis differentiation process of MSCs, miR-106a expression level was downregulated to elevate target gene BMP2 expression level, and eventually facilitating MSCs osteogenesis differentiation.

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Disclosure of conflict of interest

None.

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