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Original Article

miRNA-15b-5p promotes expression of osteoblast differentiation-associated markers via targeting SMAD7

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Abstract: Recent studies have recognized the involvement of microRNAs (miRNAs) in the development of osteoporosis, which regulates the balance between osteogenesis and osteoclasis. The present study was to investigate the regulation of miRNA-15b-5p on the osteoblast differentiation-associated markers in the mouse osteoblast-like MC3T3-E1 cells, and to identify the targeting regulation by miRNA-15b-5p on the 3' untranslated region (UTR) of *SMAD7*, which was a well-recognized inhibition to osteoblast differentiation. Firstly, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to examine the expression of Osteocalcin, Procollagen type I N-terminal propeptide (P1NP) and Runt-related transcription factor 2 (RUNX2) in the MC3T3-E1 cells, which were transfected with 30 nM miR-15b-5p mimics or scramble miRNA (as control). Then the extracellular matrix (ECM) mineralization and the Alkaline Phosphatase (ALP) activity were also examined. Finally, we investigated the regulation by miRNA-15b-5p on the expression of SMAD7, with luciferase reporter. Results demonstrated that the transfection with miRNA-15b-5p mimics significantly promoted the miRNA-15b-5p level, and then upregulated the expression of Osteocalcin, P1NP and RUNX2 in MC3T3-E1 cells; promoted the ECM mineralization and the ALP activity. The alignment analysis indicated that miRNA-15b-5p was homologous with the 3'UTR of *SMAD7*. Moreover, the luciferase reporter assay demonstrated that miRNA-15b-5p targeted the 3'UTR of SMAD7, and inhibited the expression of SMAD7 in both mRNA and protein levels. In conclusion, we found the promotive effects of miRNA-15b-5p on the expression of osteoblast differentiation-associated markers, the ECM mineralization and the ALP activity in mouse osteoblast-like MC3T3-E1 cells, via targeting the 3'UTR of SMAD7. Our study suggests that miRNA-15b-5p might be an important target to promote osteoblast differentiation and to prevent osteoporosis.

Keywords: Differentiation, miRNA-15b-5p, osteoblast, SMAD7

Introduction

Osteoporosis is a common disease, which could increase bone weakness and the risk of fracture, especially at hip, forearm, spine and wrist [1-3]. The main cause of osteoporosis is bone loss and bone fragility, and the incidence of osteoporosis is high in elderly people, especially in females [4]. In addition, osteoporosis is typically with no clinical symptoms, it has a long latent period until a bone broken. A person with fracture, more often than not, suffering from severe osteoporosis [5, 6]. Therefore, the prediction and diagnosis of osteoporosis is even more important. The balance between osteogenesis and osteoclasis affects the bone formation and resorption, which is associated with many factors, such as SMAD4, CACNG1 and TRIM63 [7, 8]. It also has reported that microRNAs (miRNAs) play important roles in osteoporosis development [4].

As we all know, microRNAs (miRNAs) are comprised of a class of small, non-coding, single stranded RNAs (containing 18-25 nucleotides) that could modulate gene expression by binding the 3'UTR of target genes at post-transcriptional level [9-12]. Pri-miRNAs (longer primary transcripts) are the precursor of miRNAs, they are transcribed from independent miRNA genes or mature from the non-coding regions (introns) of protein-coding mRNAs [13, 14]. It is well known that miRNAs take part in many physiological processes, modulate the development and pro-

gression of many diseases, such as carcinoma [15, 16], chronic hepatitis B [17] and Alzheimer's disease [18]. Recently, miRNAs have been recognized to involve in osteoporosis development, by regulating the balance between osteogenesis and osteoclasis [19, 20]. The expression level of miRNA-133a was up-regulated in the patients with postmenopausal osteoporosis, negatively correlating with CXCL11, CXCR3 and SLC39A1 genes [21]. In addition, miRNA-422a and miRNA-502 are potential biomarker for osteoblast differentiation [22, 23]. MiRNA-15 family is a highly conserved miRNA family containing six highly conserved miRNAs (miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, miRNA-497). miRNA-15 family could modulate cell apoptosis, differentiation, vascular remodeling and insulin synthesis [15, 24]. miRNA-15b-5p is processed from the 5' of the pri-miRNA-15b. The function role of miRNA-15b-5p in osteoblast differentiation still remains to be elucidated.

In this study, we investigated the function role of miRNA-15b-5p in osteoblast differentiation in the osteoblast-like MC3T3-E1 cells. miRNA-15b-5p mimics was utilized to upregulate miRNA-15b-5p level, and then the mRNA expression level of the osteoblast-differentiation markers, such as osteocalcin, procollagen type I N-terminal propeptide (P1NP) and runt-related transcription factor 2 (RUNX2) were examined. In addition, the extracellular matrix (ECM) mineralization and the alkaline phosphatase (ALP) activity were also examined in the miR-15b-5p-manipulated MC3T3-E1 cells. We confirmed the regulatory role of miRNA-15b-5p on osteoblast differentiation in MC3T3-E1 cells. Taken together, miRNA-15b-5p might be a rational biomarker for diagnostic and therapeutic applications in osteoporosis.

Materials and methods

Cell culture and transfection

The MC3T3-E1 cells (mouse embryo osteoblast precursor cells) were used in this study, which were obtained from ATCC (Rockville, MD, USA). The base medium for MC3T3-E1 cells is formulated Dulbecco's Modified Eagle's Medium (Gibco, Rockville, MD, USA). The MC3T3-E1 cells were cultured in the complete growth medium supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) based on the base medium. The culture condition of the cells was 37°C 5% CO_2 in an incubator. The MC3T3-E1 cells were seeded into a 12-well (3.8 cm^2) plate and cultured for 24 h, then the cells were transfected with miRNA-15b-5p mimics or recombinant plasmid by Lipofectamine® 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

mRNA extraction and quantitative real-time polymerase chain reaction assay

The relative mRNA level of the osteoblast differentiation-associated markers (osteocalcin, P1-NP, RUNX2, SMAD7) was utilized to represent the change of the expression level of each marker. The total RNA was extracted from the MC3T3-E1 cells transfected with miRNA-15b-5p mimics or scramble by the Tri-Reagent (Ambion, Huntingdon UK) following the user's specification. The RNase-free water was used to dissolve the total RNA. The quantitative analysis of mRNA was determined by qRT-PCR. The mRNA sequence of each marker was obtained from NCBI, and the primers for qRT-PCR were designed by Primer premier 5. One Step SYBR PrimeScript PLUS RT-PCT Kit (Takara, Tokyo, Japan) was used to examine the relative level of each sample according to the manufacturer's manual. After the reaction, the mRNA level of each marker was calculated and presented as mean ± SEM for triple independently-performed experiments, with β-actin as internal control.

Construction of the recombinant plasmid and the luciferase reporter assay

The sequence of mRNA-15b-5p and 3'UTR of SMAD7 mRNA were aligned by Geneious software. The sequence of the mutant 3'UTR of SMAD7 mRNA was synthesize by SangonBio (Shanghai, China). The luciferase gene and the 3'UTR of SMAD7 were amplified by PCR (polymerase chain reaction) with Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The gene of 3'UTR of SMAD7 or mutant 3'UTR of SMAD7 were cloned into the pLcu vector just at the downstream of the luciferase reporter. The target gene was inserted into the vector with restriction endonuclease and DNA ligase (NEB, USA), and finally received the recombinant plasmid pLuc-SMAD7 3'UTR and pLuc-SMAD7 3'UTR^{mut}. The recombinant plasmid and the miRNA-15b-5p mimics were co-transfected into the MC3T3-E1 cells. After co-transfecting, collected the cells and exam-

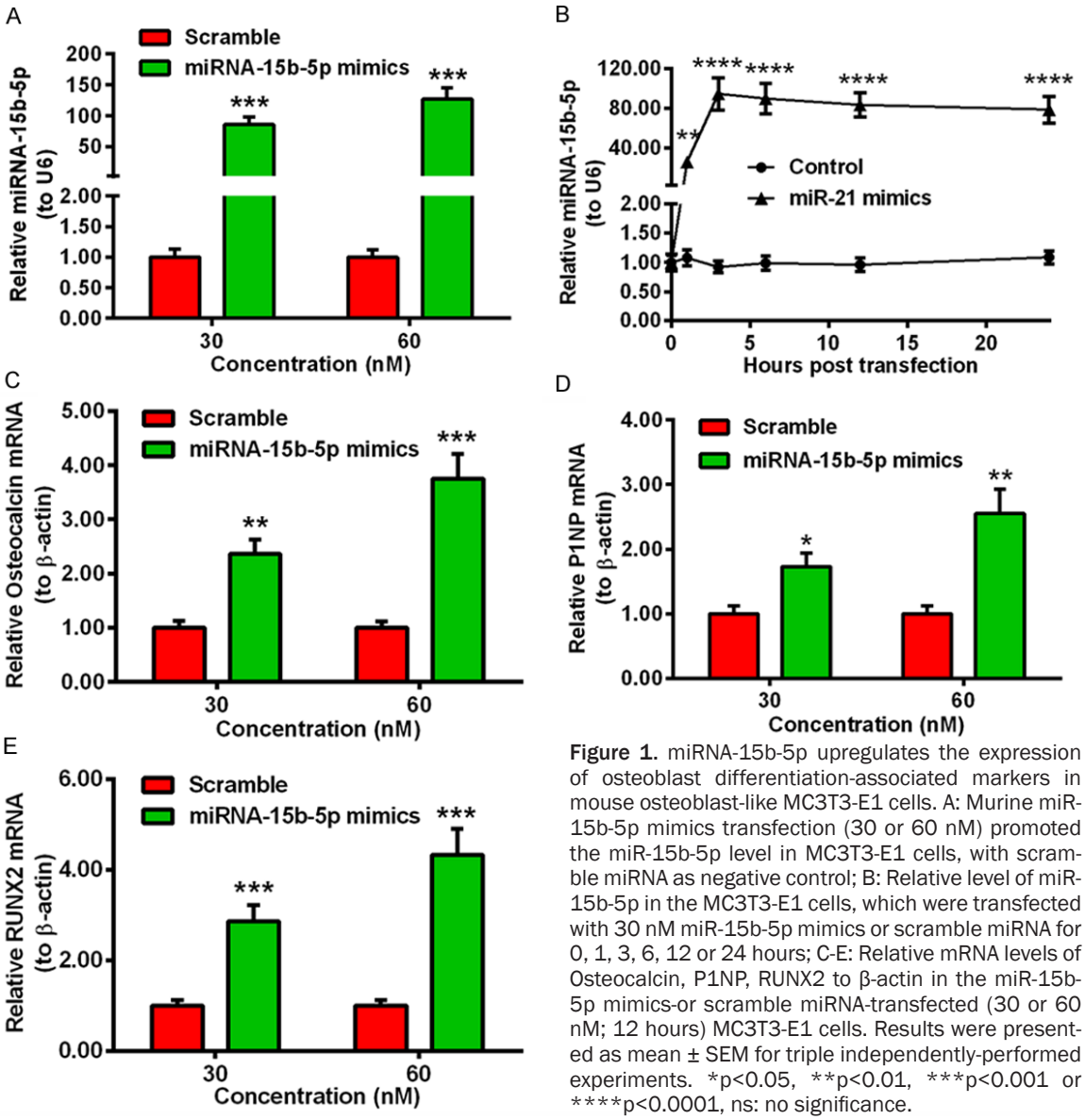

Figure 1. miRNA-15b-5p upregulates the expression of osteoblast differentiation-associated markers in mouse osteoblast-like MC3T3-E1 cells. A: Murine miR-15b-5p mimics transfection (30 or 60 nM) promoted the miR-15b-5p level in MC3T3-E1 cells, with scramble miRNA as negative control; B: Relative level of miR-15b-5p in the MC3T3-E1 cells, which were transfected with 30 nM miR-15b-5p mimics or scramble miRNA for 0, 1, 3, 6, 12 or 24 hours; C-E: Relative mRNA levels of Osteocalcin, P1NP, RUNX2 to β-actin in the miR-15b-5p mimics-or scramble miRNA-transfected (30 or 60 nM; 12 hours) MC3T3-E1 cells. Results were presented as mean ± SEM for triple independently-performed experiments. *p<0.05, **p<0.01, ***p<0.001 or ****p<0.0001, ns: no significance.

ined the relative luciferase activity assayed with the Dual-Luciferase Assay kit (Promega, Madison, WI, USA) by a GLOMAX (Promega, Madison, WI, USA).

ECM mineralization assay and alkaline phosphatase activity

The ECM mineralization assay was performed after 24 h transfection. The transfected cells were collected and washed with PBS (phosphate-buffered saline) for three times. Then the cells were fixed with 70% ethanol on ice for 60 min. After fixation and several washes in PBS, the cell layers were stained for 10 min at room temperature with 2% (w/v) Alizarin red solution (Sigma-Aldrich, St. Louis, MO, USA) at pH 4.2. After staining, the cell layers were washed to remove the unbound stain. The distribution of mineral staining was observed from image of the culture well. To further investigate the relative level of the ECM mineralization, therefore, we solubilized the bound stain in 10% cetylpyridinium chloride (Sigma-Aldrich, St. Louis, MO, USA) and the optical density value of the resultant solution determined at 450 nm by spectrophotometry (Crystaleye, Olympus, Tokyo, Japan) [25]. The relative ALP activity in the miR-15b-5p mimics or scramble miRNA-transfected (30 or 60 nM; 24 hours) MC3T3-E1 cells, was performed by the Alkaline Phosphatase Diethanolamine Activity Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. And the ALP activity was quantified at 450 nm by spectrophotometry.

Western blot assay

The expression of SMAD7 on protein level were determined by Western blotting. To harveste the MC3T3-E1 cells transfected with or without miRNA-15b-5p mimics, the cells were lysed into cell lysis buffer (Bio-Rad, Hercules, CA, USA). The protein supernatant was extracted from the cell lysis after centrifuging at 12,000×g at 4°C for 15 min. the protein extract was separated by SDS-PAGE gel. Then the protein bands were transferred onto the PVDF membranes (Millipore, Bedford, MA, USA), and the membrane was blocked with 5% skimmed milk powder at 4°C overnight. The membrane was incubated with SMAD-specific antibody (1:1000, mouse monoclonal antibody, Abcam, Cambridge, UK) or β-actin-specific antibody (1:3000, mouse monoclonal antibody, Abcam, Cambridge, UK) in TBST at 37°C for 1 h, washed the membrane with TBST, subsequently incubated with the secondary antibody, anti-mouse IgG conjugated with HRP (1:1000, New England Biolab, Ipswich, UK) at 37°C for 30 min. After washing with TBST, the membrane was treated with the ECL kit (Thermo Scientific, Rockford, IL, USA) and scanned by a Smart ChemiTM lamp Analysis System (Life Technologies, Grand Island, NY, USA). The protein level of the SMAD7 was quantified according to the band density by Quantity One software based on the western blot assay, with β-actin as loading control. Each value was averaged for triple independent results.

Statistical analysis

Data were expressed as mean ± SEM for triple independently-performed experiments. The difference between two groups was analyzed by Student's t test using the GraphPad Prism (GraphPad Software, La Jolla, CA, USA). A p value <0.05 or less was considered to be significant (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: no significance).

Results

The osteoblast differentiation-associated markers were up-regulated in mouse osteoblast-like MC3T3-E1 cells post transfection of miRNA-15b-5p

To investigate the function of miRNA-15b-5p in osteoblast, we examined the osteoblast differentiation-associated markers in MV3T3-E1 cells with or without the transfection of miRNA-15b-5p. The miRNA-15b-5p mimics was used to increase the level of miRNA-15b-5p in vitro. The level of miRNA-15b-5p was significantly upregulated in MC3T3-E1 cells transfected with 30 or 60 nM miRNA-15b-5p mimics (**Figure 1A**, ***p<0.001) by qRT-PCR with U6 as internal standard template. The relative level of miRNA-15b-5p in MC3T3-E1 cells post transfection of 30 nM miRNA-15b-5p mimics or scramble miRNA for 0, 1, 3, 6, 12 or 24 hours, the trend of the change of miRNA-15b-5p was depicted in **Figure 1B**, which showed that the level of miRNA-15b-5p was significantly increased at 3 h post transfection, moreover the level was stable within 3 h to 24 h, compared to the control group (**p<0.01, ****p<0.0001). Then we investigate the mRNA level of the differentiation-associated markers in MC3T3-E1 cells, such like osteocalcin, P1NP and RUNX2, with β-actin as internal control. The results was depicted in **Figure 1**, the mRNA level of osteocalcin was significantly increased in the MC3T3-E1 cells post transfection with miRNA-15b-5p mimics for 12 hours, compared to the scramble groups. Moreover, the mRNA level of osteocalcin was higher in the cells transfected with 60 nM miRNA-15b-5p mimics than those transfected with 30 nM miRNA-15b-5p mimics (**Figure 1C**, **p<0.01, ***p<0.001). Beyond that, **Figure 1D** and **1E** also showed that, transfected with miRNA-15b-5p mimics also increased the mRNA level of P1NP and RUNX2 in the MC3T3-E1 cells (*p<0.05, **p<0.01), especially the mRNA level of RUNX2 (***p<0.001). All the above data implied that the level of the differentiation-associated markers (osteocalcin, P1NP, RUNX2) was positive correlated with the miRNA-15b-5p in MC3T3-E1 cells.

miRNA-15b-5p upregulates the extracellular matrix (ECM) mineralization in MC3T3-E1 cells

To further confirm the facilitating role of miRNA-15b-5p in the MC3T3-E1 cells, we examined the extracellular matrix (ECM) mineralization by Alizarin Red S (ARS) staining and quantified by OD450. The MC3T3-E1 cells were transfected with 30 or 60 nM miR-15b-5p mimics or scramble miRNA for 12 hours, the image of the staining cells was depicted in **Figure 2A**. The mineralized nodes were stained into dark red, and which showed us that the formation of the mineralized node in the MC3T3-E1 cells transfect-

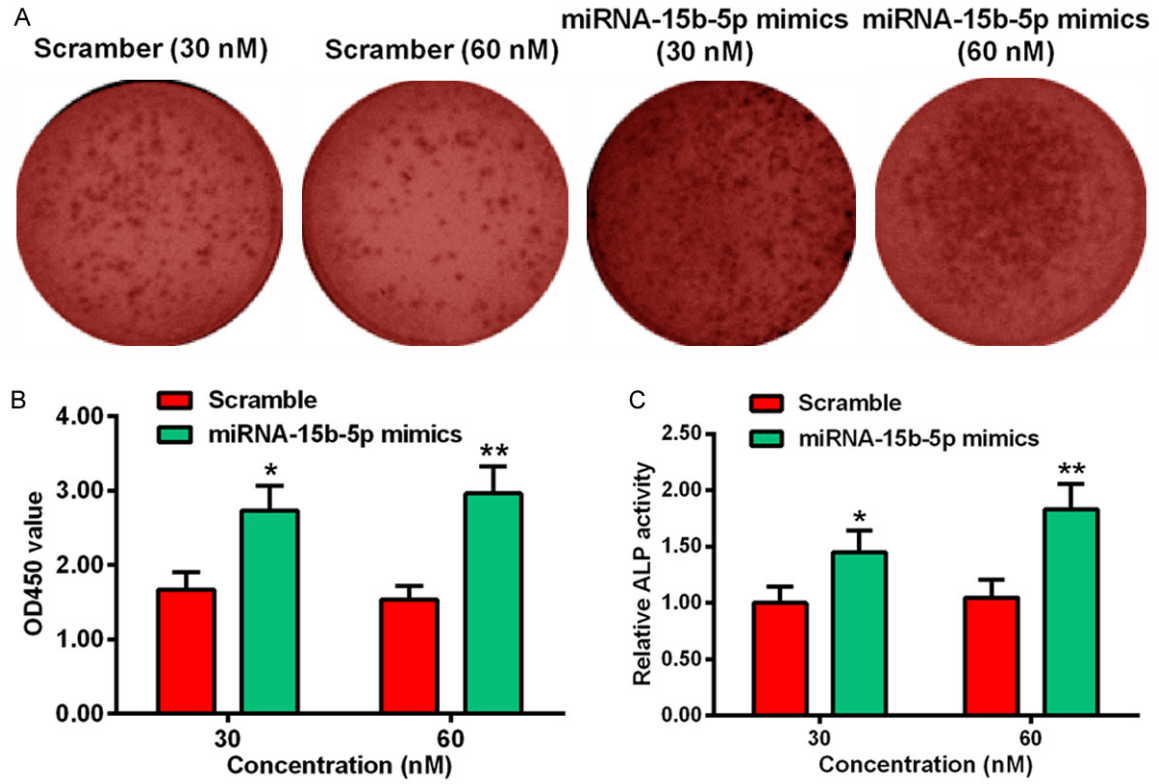

Figure 2. miRNA-15b-5p upregulates the extracellular matrix (ECM) mineralization in MC3T3-E1 cells. (A) and (B) Representative images (A) and quantification (B) of extracellular matrix mineralization in the MC3T3-E1 cells, which were transfected with 30 or 60 Nm miR-15b-5p mimics or scramble miRNA. The extracellular matrix mineralization was assessed by Alizarin Red S (ARS) staining. (C) Relative ALP activity in the miR-15b-5p mimics-or scramble miRNA-transfected (30 or 60 nM; 24 hours) MC3T3-E1 cells. All experiments were independently performed in triplicate. *p<0.05 or **p<0.01.

ed with 30 or 60 nM miR-15b-5p mimics was much increased than the cells transfected with 30 or 60 nM scramble. Besides the image, we also quantified the OD450 value of the cells. The OD450 value was increased in the cells transfected with 30 nM (*p<0.05) or 60 nM (**p<0.01) miRNA-15b-5p mimics, as compared with the scramble group (**Figure 2B**). Alkaline Phosphatase (ALP) is a marker in the early stage of osteogenesis, the relative ALP activity in the miR-15b-5p mimics-transfected MC3T3-E1 cells was much higher than the scramble cells, especially at the concentration of 60 nM miRNA-15b-5p mimics (**Figure 2C**, *p<0.05, **p<0.01).

miRNA-15b-5p targets the 3'UTR of SMAD7 in the MC3T3-E1cells

Based on the above finding, miRNA-15b-5p could increase the level of osteoblast differentiation-associated markers and promote the osteogenesis in the mouse osteoblast-like MC3T3-E1 cells. But the mechanism was still unclear. Thus, SMAD7 is a well-recognized inhibited-maker to osteoblast differentiation, so we suspected that miRNA-15b-5p may inhibit the expression of SMAD7 by some way. In order to verify this hypothesis, we aligned the sequence of miRNA-15b-5p and 3'UTR of SMAD7 mRNA, and found that 8 bases were exactly complementary (**Figure 3A**). A recombinant plasmid with a luciferase reporter and the 3'UTR of SMAD7 (or with the mutant 3'UTR of SMAD7) were designed to investigate the function of this consensus sequence (**Figure 3B**). The recombinant plasmid and the miRNA-15b-5p mimics were co-transfected into the MC3T3-E1 cells. The relative luciferase activity was significantly decreased in the cells post transfecting with pLuc-SMAD7 3'UTR and miRNA-15b-5p mimics (30 nM or 60 nM), compared to the cells transfected with pLuc-SMAD7 3'UTR and scramble (**Figure 3C**, *p<0.05, **p<0.01). However, the relative luciferase

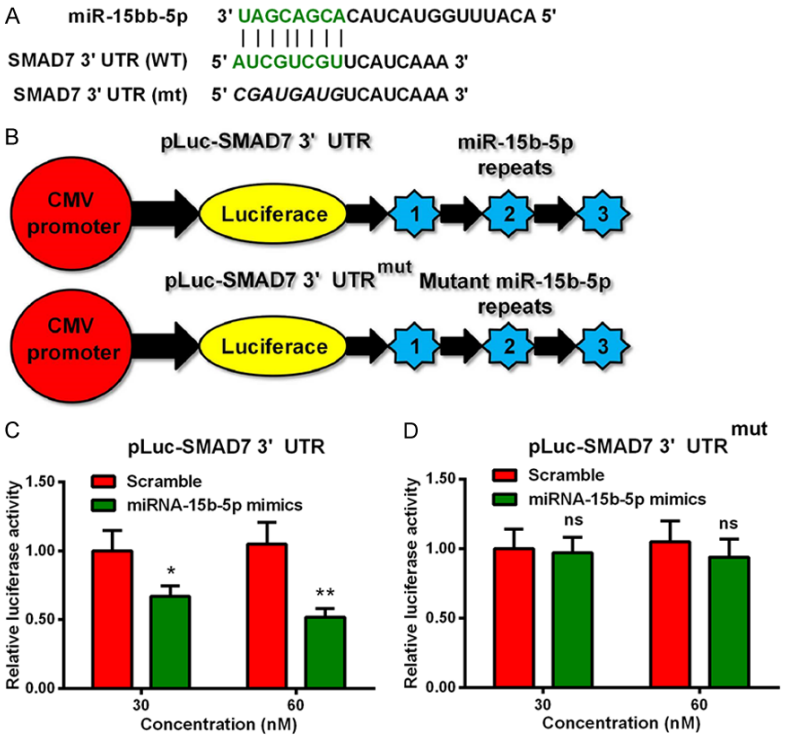

Figure 3. miRNA-15b-5p targets the 3'UTR of *SMAD7* in MC3T3-E1 cells. (A) Alignment of murine mature miRNA-15b-5p and the 3'UTR of *SMAD7* sequences; (B) Sketch of the luciferase reporter plasmid with the wild type or mutant type 3'UTR of *SMAD7*; (C) and (D) Relative luciferase activity in the MC3T3-E1 cells, which were transfected with miR-15b-5p mimics or scramble miRNA, by the reporter plasmid with the 3'UTR of *SMAD7* (C) or with the mutant 3'UTR of *SMAD7* (D). All experiments were independently performed in triplicate. *p<0.05, **p<0.01 or ns: no significance.

activity in the pLuc-SMAD7 3'UTR^{mut}-transfected MC3T3-E1 cells, there was no significant difference between the cells co-transfected with 30 nM or 60 nM miRNA-15b-5p mimics and scramble (**Figure 3D**, ns: no significance). The results demonstrated that the miRNA-15b-5p mimics reduced the relative luciferase activity by targeted the 3'UTR of SMAD7.

The expression level of SMAD7 was down-regulated by miRNA-15b-5p

The relative luciferase activity was reduced by the targeting of the miRNA-15b-5p and 3'UTR SMAD7 in the luciferase-miRNA-15b-5p-mimics co-transfection system, therefore, miRNA-15b-5p may play a role in modulating the expression level of SMAD7 in the MC3T3-E1 cells. Then, the relative mRNA level of SMAD7 in MC3T3-E1 cells transfected with or without miRNA-15b-5p mimics were examined by qRT-PCR assay. **Figure 4A** showed that transfecting with miRNA-15b-5p mimics reduced the mRNA level of SMAD7 in MC3T3-E1 cells to the scramble groups, with β-actin as internal control (*p<0.05). In addition, the protein level of SMAD7 was also determined by Western Blot assay. The MC3T3-E1 cells were transfected with 30 nM (or 60 nM) miRNA-15b-5p mimics or 30 nM (or 60 nM) scramble. The representative image of WB assay was showed in **Figure 4B**, the protein level of β-actin was stable in the four groups. But the expression level of SMAD7 in the cells transfected with 30 nM or 60 nM miRNA-15b-5p mimics was significant decreased than the cells transfected with 30 nM or 60 nM scramble. And the ratio of SMAD7 and β-actin was also calculated based on the WB assay. The data depicted that the ratio of SMAD7 and β-actin was remarkably reduced in the miRNA-15b-5p mimics-transfecting groups. Furthermore, the higher concentration of miRNA-15b-5p mimics led to the lower expression of SMAD7 (**Figure 4C**, **p<0.01, ***p<0.0001).

Discussion

miRNAs have been shown to regulate osteoblast differentiation. And miRNA-15 family has been showed to participate in various cellular processes especially the differentiation process. But there is no report on the regulator role of miRNA-15b-5p in osteoblast differentiation. In the present study, the miRNA-15b-5p mimics was used to increase the level of miRNA-15b-5p in the MC3T3-E1 cells by cell transfection. To investigate the regulation role of the miRNA-15b-5p in osteoblast differentiation, the typical osteoblast-differentiation markers were examined in this parts. Osteocalcin is an extracellular matrix protein, which is expressed in the later stage of osteogenic induction. The

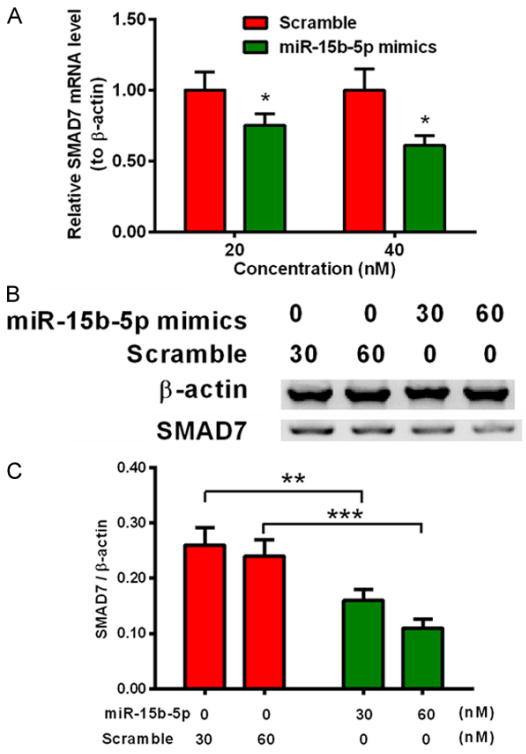

Figure 4. miRNA-15b-5p downregulates the expression of SMAD7 in MC3T3-E1 cells. A: Relative mRNA levels of SMAD7 to β-actin in the MC3T3-E1 cells, which were transfected with 30 or 60 nM miR-15b-5p mimics or scramble miRNA for 12 hours; B and C: Representative image of western blotting assay for SMAD7 in the miR-15b-5p mimics-or scramble miRNA-transfected MC3T3-E1 cells. Each value was averaged for triple independent results. Statistical significance was showed as **p<0.01, ***p<0.001.

expression level of osteocalcin is positively correlated with the mineralized deposition, it is a key index to indicate the osteoblast activity [26]. Procollagen type I N-terminal propeptide is an osteoblast-derived protein [27, 28]. The osteoblast-related gene RUNX2 is a bone transcription factor, which could regulate the differentiation of mesenchymal stem cells into osteoblasts. RUNX2 plays an important role in the differentiation and function of osteoblasts, it could promote the bone matrix deposition rate [29]. These markers are indexes of the osteoblast, the expression level of these markers are positively related with the osteoblast differentiation. The effect of miRNA-15b-5p on osteoblast differentiation was represented by these markers indirectly. We found that the mRNA level of osteocalcin, P1NP, RUNX2 were both up-regulated in the MC3T3-E1 cells transfected with miRNA-15b-5p mimics, compared to the scramble groups. The results demonstrated that the miRNA-15b-5p promoted the expression of the osteoblast-differentiation markers.

ALP activity is an early marker of bone formation, the activity is the precondition of cells (or tissues) mineralization, and affecting the formation of mineralized tissues [30]. The MC3T3-E1 cells transfected with miRNA-15b-5p mimics showed a higher activity than the control group. In addition, from the staining of the extracellular matrix (ECM) mineralization, the stained mineralized nodes was more in the cells transfected with 30 nM or 60 nM miRNA-15b-5p mimics than the cells transfected with same concentration scramble. The relative level of mineralization represented by OD450 nm was also showed the enhancement of mineralization. As is known to all, the mineralized nodes is the index for the formation of mineralized matrix, it is the final signal of the osteogenic phenotype in vitro, which means the maturity of the osteoblast [31-34]. The differentiation makers and the mineralization assay implied that miRNA-15b-5p could promote the osteoblast differentiation in MC3T3-E1 cells.

The mechanism of miRNA-15b-5p in modulating the differentiation of osteoblast was the next issue that we were going to solve. SMAD7 is a well-recognized inhibitor to osteoblast differentiation. Many studies had reported that SMAD7 plays an important role in regulating cell cycle, it also modulates cancer growth and progression [35-37]. SMAD7 promotes and enhances skeletal muscle differentiation by inhibiting TGF-b/activin signaling and bone morphogenetic protein (BMP) pathways [38]. Therefore, we suspected that miRNA-15b-5p may target the mRNA of SMAD7 to modulate the cell differentiation. The sequence of miRNA-15b-5p and the 3'UTR of SMAD7 sequences was complementary. To verify this hypothesis, we designed a recombinant plasmid contained the luciferase reporter and the wild type 3'UTR of SMAD7 (or mutant type 3'UTR of SMAD7). The relative luciferase activity was decreased in the miRNA-15b-5p-transfected cells, which demonstrated that the miRNA-15b-5p could target the wild type 3'UTR of SMAD7, and further terminate the expression of luciferase reporter. On the other hand, the protein level of SMAD7 was also reduced in the

cells transfected with miRNA-15b-5p by western blot.

In conclusion, there are two major outcomes in this study. Firstly, miRNA-15b-5p improved the expression level of the osteoblast-differentiation markers (osteocalcin, P1NP, RUNX2, ALP activity), and promoted the formation of ECM mineralization. Therefore, the miRNA-15b-5p could promote the osteoblast differentiation. Secondly, miRNA-15b-5p could modulate the cell differentiation via targeting the 3'UTR of SMAD7. Our study suggests that miRNA-15b-5p might be an important target to promote osteoblast differentiation and to prevent osteoporosis.

Disclosure of conflict of interest

None.

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