MicroRNA-21 promotes osteogenic differentiation by targeting small mothers against decapentaplegic 7

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Abstract. Previous studies have suggested that microRNAs (miRNAs/miRs) may positively or negatively control osteogenic differentiation and mineralization by targeting negative regulators of osteogenesis or important osteogenic factors. miR-21 is important in osteoblast differentiation and *Smad7* is a critical regulator of osteogenic differentiation, which inhibits proliferation, differentiation and mineralization in mouse osteoblast cells. However, the association between *Smad7* and miR-21 remain to be elucidated. In the present study, miR-21 was found to promote the level of osteogenic differentiation and increase matrix mineralization in MC3T3-E1 cells. Furthermore, *Smad7* was identified as a direct target of miR-21 in the MC3T3-E1 cells. The overexpression of miR-21 affected the protein levels of SMAD7, but not the mRNA levels, which suggested that miR-21 regulates the levels of SMAD7 by inhibiting translation, rather than by promoting mRNA decay. Forced expression of miR-21 promoted osteogenic differentiation and mineralization, while inhibition of miR-21 suppressed these processes. The present study also identified for the first time, to the best of our knowledge, the promotion of osteogenic differentiation and mineralization by miR-21, by repressing the expression of *Smad7*.

Introduction

MicroRNAs (miRNAs/miRs) are short, noncoding RNAs, which are critical in numerous biological processes due to their regulation of gene expression. miRNAs suppress target gene expression by sequence-specific binding to 3'-untranslated regions (UTRs) of target mRNAs, thereby inducing mRNA degradation or translation inhibition (1).

Previous studies have suggested that miRNAs may be important in bone formation (2) and may positively or negatively control osteogenic differentiation and mineralization by targeting negative regulators of osteogenesis or important osteogenic factors, respectively. miR-542-3p inhibits osteoblast proliferation and differentiation, suppresses osteogenic differentiation and promotes osteoblast apoptosis by repressing bone morphogenetic protein (BMP)7 and its downstream signaling (3). miR-335-5p directly targets and downregulates the Wnt inhibitor, DKK1, to enhance Wnt signaling and promote osteogenesis (4). miR-15b promotes osteogenic differentiation by indirectly protecting the Runt-related transcription factor 2 (Runx2) protein from small mothers against decapentaplegic (SMAD)-specific E3 ubiquitin protein ligase 1-mediated degradation (5).

The transforming growth factor (TGF)-β family members, bone morphogenetic protein (BMP)2 and TGF-β, which signal via receptor-regulated Smads (R-SMADs), induce bone formation (6). To understand the role of miRNAs in osteoblasts, several miRNAs that are modulated by TGF-β/SMAD signaling, including miR-15b (5), miR-142-3p (7) and miR-322/503 (8), have been investigated. SMAD7 also interacts with all subfamilies of activated type I receptors and inhibits BMP and TGF-β signaling. SMAD7 is a critical regulator of osteogenic differentiation, which inhibits the proliferation, differentiation, and mineralization of mouse osteoblastic cells (9).

miR-21 regulates cell proliferation, survival and migration in numerous types of cancer and is involved in other diseases, including asthma (10), coronary plaque instability (11), and intervertebral disc degeneration (12). Yang *et al* (13) demonstrated the role of miR-21 in estrogen deficiency-induced osteoporosis, and confirmed that it promotes osteoblast differentiation. However, the association between miR-21 and *Smad7* in osteoblast differentiation remains to be elucidated. The present study aimed to investigate whether miR-21 targets *Smad7 in vitro* and is involved in osteogenic differentiation and mineralization. It also aimed to investigate whether miR-21 alters levels of SMAD7 through translation inhibition or mRNA decay.

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Table I. Primer sequences used in reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer	Length (bp)
β-actin	5'-AGATGTGGATCAGCAAGCAG-3'	5'-GCGCAAGTTAGGTTTTGTCA-3'	20
Smad7	5'-CTGCAGCGGCCAATGACCA-3'	5'-ATGAGCCTCTCAGCCGGGGG-3'	19
ALP	5'-GCAGCTTGGTGCACACCTAG-3'	5'-GAGACATTTTCCCGTTCACC-3'	20
RUNX2	5'-CCGGCAAGATGAGCGAGGTCA-3'	5'-GTGGGTTGAGAAGCGGCTCT-3'	21
OCN	5'-ATGAGGACCCTCTCTCTGCT-3'	5'-GGAGCTGCTGTGACATCCAT-3'	19
OSX	5'-AGCGACCACTTGAGCAAACAT-3'	5'-GCGGCTGATTGGCTTCTTCT-3'	21

ALP, alkaline phosphatase; *OCN*, osteocalcin; *OSX*, osterix; *RUNX2*, Runt-related transcription factor 2.

Materials and methods

Cell culture and osteogenic differentiation. The MC3T3-E1 cell line, derived from murine calvaria, was obtained from American Type Culture Collection (Manassas, VA, USA). The MC3T3-E1 cells were seeded at a density of $2x10^4$ cells/cm^2 and cultured in α-modified Eagle's minimum essential medium (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (Mediatech, Inc., Manassas, VA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C and 5% CO_2. For osteogenic differentiation, the MC3T3-E1 cells were plated in 24-well plates with medium containing 50 *μ*g/ml ascorbic acid (Sigma-Aldrich), 10 mM β-glycerol-phosphate (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich).

Transfection. The MC3T3-E1 cells were transfected with either mimic-miR-21 (RiboBio, Guangzhou, China), inhibitor-miR-21 (RiboBio, Guangzhou, China), or short interfering (si)RNA-Smad7 (Invitrogen Life Technologies) using Lipofectamine® 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from the MC3T3-E1 cells ($1x10^6$ cells) using TRIzol reagent (Invitrogen Life Technologies) and cDNA was generated via RT. The miRNAs were purified using an All-in-One microRNA extraction kit (GeneCopoeia, Rockville, MD, USA), according to the manufacturer's instructions. qPCR was performed using an ABI StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used are listed in Table I. The relative expression levels of miR-21 were calculated using the $2^{-\Delta\Delta CT}$ method (14), in which ΔCT was defined as the threshold cycle (CT) value of the U6 internal control minus the CT value of the target miRNA. The expression levels of *Smad7*, alkaline phosphatase (ALP), *Runx2*, *osteocalcin (OCN)*, and *osterix (OSX)* were normalized against *β-actin* and calculated using the $2^{-\Delta\Delta CT}$ method.

Target prediction. The Target Scan (http://www.targetscan.org), PicTar (http://pictar.bio.nyu.edu) and miRanda (http://www.microrna.org) target prediction tools were used to screen for the miR-21 target genes.

Mineralization. For the investigation of mineralization, ALP (Sigma-Aldrich) and alizarin red staining (ARS; Sigma-Aldrich) were performed. To measure the formation of bone nodules, the extracellular matrix calcium deposits were stained using 0.2% ARS for 30 min, as previously described (3-5). The mineralization values were normalized to the relative value of the control.

Western blot analysis. The cells ($1x10^6$) were lysed in lysis buffer (pH 7.5), containing 50 mM Tris, 0.1% SDS (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 250 mM NaCl, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40 and protease inhibitor cocktail). Western blotting was performed, according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Equal quantities of protein (100 *μ*g) were separated on 8-10% polyacrylamide-SDS gels. The proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). The activation of SMAD7 was detected using a mouse monoclonal anti-phospho-SMAD7 IgG antibody (1:500; sc-365846; Santa Cruz Biotechnology, Inc.).

Luciferase assays. The 3'-UTR of *Smad7* was amplified using the following primers from Invitrogen Life Technologies: Sense 5'-TTTTTCTAGACCGCGTGCGGAGGGGACAGA-3' and antisense 5'-TTTTTCTAGAGGAGTCCTTTCTCTCTC AAAGC-3'. The fragment was inserted into the *Xho*I and *Not*I restriction sites of psiCHECK2 (Promega, Madison, WI, USA). Mutations in the miR-21 binding site module of *Smad7* were introduced by whole-plasmid amplification in the seed region of miR-21 (New England Biolabs, Ipswich, MA, USA). The MC3T3-E1 cells were transfected with either the wild-type (WT) *Smad7* 3'-UTR or the mutant *Smad7* 3'-UTR (Mut), in combination with either the miR-21 mimic or the control mimic-negative control (NC). The cells were collected 48 h after transfection, and luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega).

Statistical analysis. Data are presented as the mean ± standard deviation. Comparisons between groups were analyzed with a paired sample t-test using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

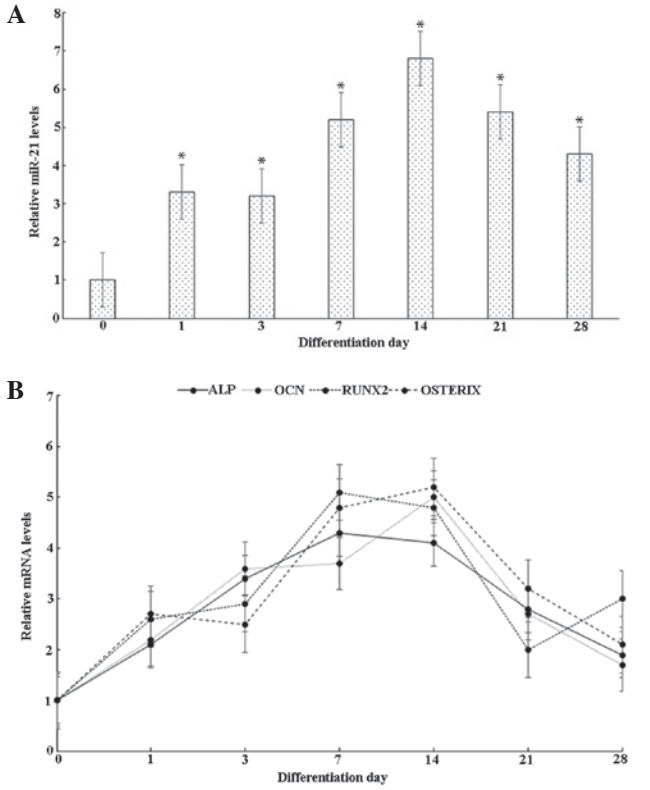

Figure 1. (A) miR-21 expression increased during osteoblast differentiation in MC3T3-E1 cells. miR-21 was prepared at the indicated time points. (B) ALP, OCN, RUNX2, and OSX expression increased during osteoblast differentiation in MC3T3-E1 cells. *P<0.01 vs. day 0. miR, microRNA; ALP, alkaline phosphatase; OCN, osteocalcin; OSX, osterix; RUNX2, Runt-related transcription factor 2.

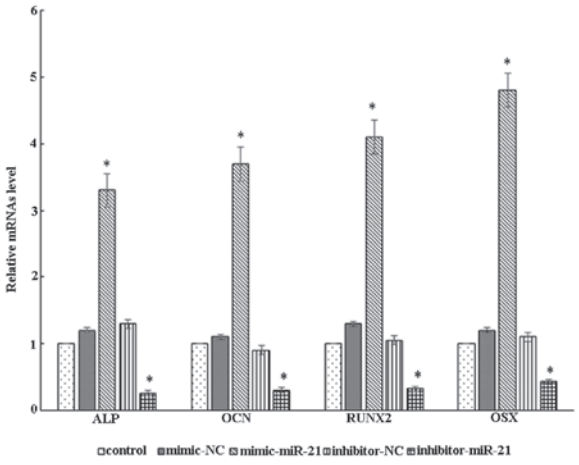

Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis of the mRNA levels of *ALP*, *OCN, RUNX2* and *OSX* in MC3T3-E1 cells following treatment with 100 μM mimic-NC, mimic-miR-21, inhibitor-NC or inhibitor-miR-21 for 48 h. Data are presented as the mean ± standard deviation; *P<0.01 vs. control group. miR, microRNA; NC, negative control; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; *OSX*, osterix; *RUNX2*, Runt-related transcription factor 2.

Results

miR-21 promotes osteogenic differentiation and mineralization. To investigate the role of miR-21 in osteogenic differentiation, the MC3T3-E1 cells were differentiated and the expression of miR-21 was measured at different time-points using RT-qPCR (Fig. 1A). The expression of miR-21 was upregulated following 1 day of differentiation and peaked after 14 days. Subsequently, the levels of expression gradually decreased, but remained upregulated for up to 28 days. The mRNA levels of the *ALP*, *OCN*, *Runx2* and *OSX* osteogenic differentiation marker genes were significantly increased at different time-points, indicating that the induction of osteogenic differentiation was successful (Fig. 1B). These data suggested that miR-21 may be involved in osteogenic differentiation.

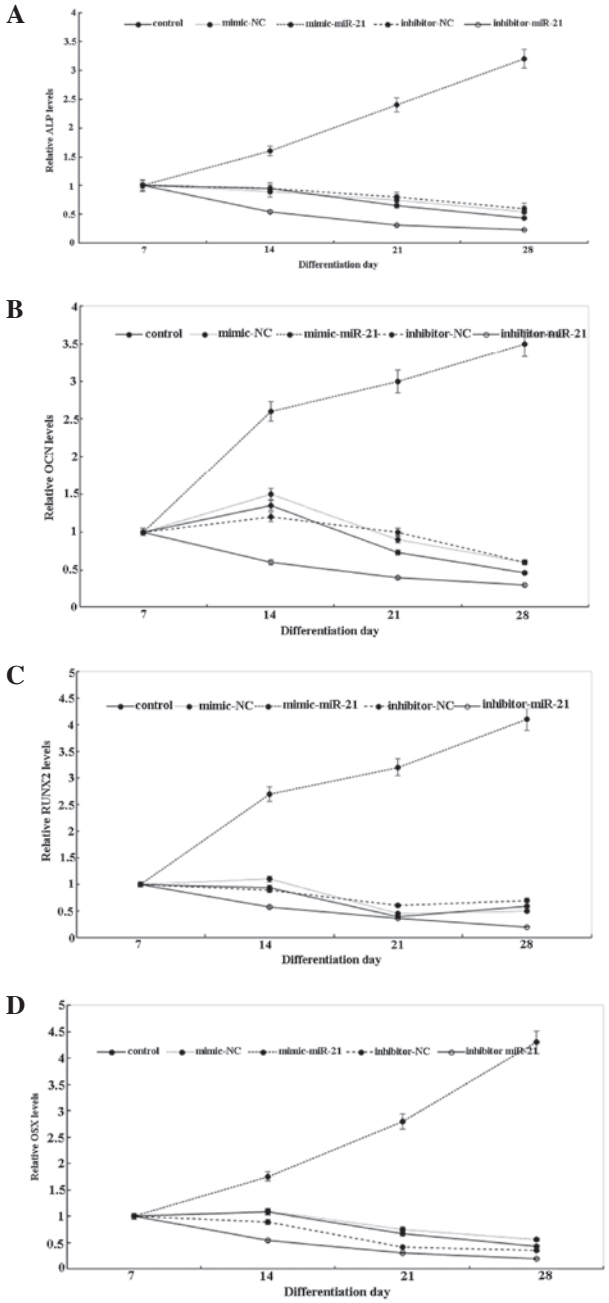

Figure 3. Reverse transcription-quantitative polymerase chain reaction analysis of changes in the mRNA expression levels of (A) alkaline phosphatase, (B) osteocalcin, (C) Runt-related transcription factor 2 and (D) osterix in MC3T3-E1 cells over time, following treatment with 100 μM mimic-NC, mimic-miR-21, inhibitor-NC, or inhibitor-miR-21 in osteoblast medium for 28 days. Data are presented as the mean ± standard deviation. miR, microRNA; NC, negative control; *ALP*, alkaline phosphatase, *OCN*, osteocalcin, *RUNX2*, Runt-related transcription factor 2; *OSX*, osterix.

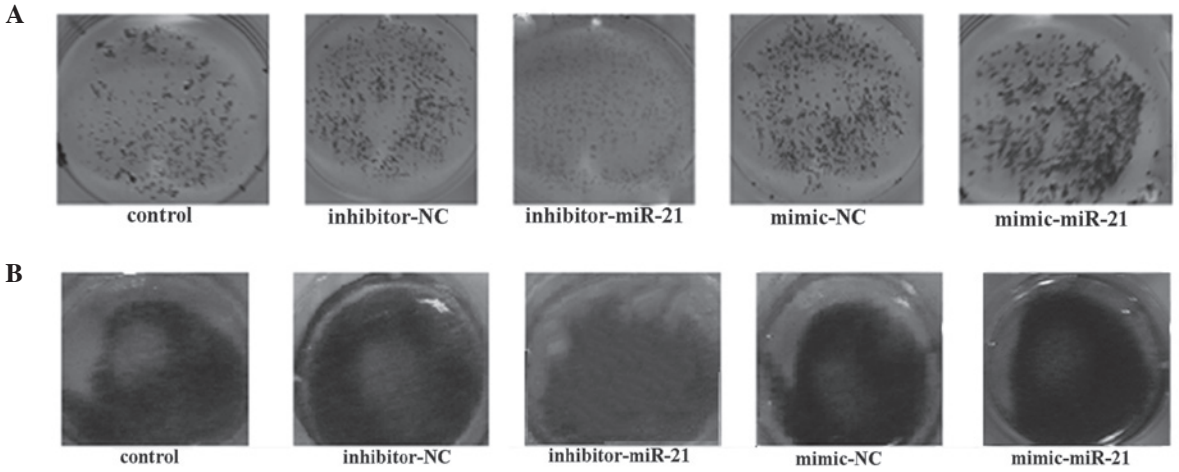

Figure 4. (A) Calcium deposition staining using alizarin red in MC3T3-E1 cells following treatment with 100 μM mimic-NC, mimic-miR-21, inhibitor-NC, or inhibitor-miR-21 for 48 h. (B) Alkaline phosphatase staining of MC3T3-E1 cells following treatment with 100 μM mimic-NC, mimic-miR-21, inhibitor-NC, or inhibitor-miR-21 for 48 h. miR, microRNA; NC, negative control.

To investigate the role of miR-21 in regulating osteoblast activity, the MC3T3-E1 cells were transfected with mimic-NC, mimic-miR-21, inhibitor-NC or inhibitor-miR-21. The mRNA expression levels of *ALP*, *OCN*, *Runx2*, and *OSX* were significantly upregulated 48 h after mimic-miR-21 transfection and were downregulated at 48 h after inhibitor-miR-21 transfection compared with the controls, mimic-NC or inhibitor-NC transfected cells (Fig. 2). The mRNA expression levels of *ALP*, *OCN*, *Runx2*, and *OSX* were consistently higher in the mimic-miR-21 group, and consistently lower in the inhibitor-miR-21 treatment group at different time-points during osteogenic differentiation, compared with the corresponding control groups (Fig. 3).

The ARS revealed that transfection with the mimic-miR-21 and inhibitor-miR-21 markedly increased and decreased matrix mineralization, respectively (Fig. 4A). Transfection with the mimic-miR-21 enhanced ALP staining, whereas inhibitor-miR-21 weakened ALP staining (Fig. 4B). These data suggested that miR-21 promoted osteogenic differentiation and mineralization.

Smad7 is a target of miR-21 in MC3T3-E1 cells. To further elucidate the mechanism by which miR-21 regulates osteoblast activity, the potential targets of miR-21 were predicted using miRNA target analysis tools. Among the candidate target genes, *Smad7* was predicted as a target of miR-21 (Fig. 5A). To assess whether miR-21 directly targets *Smad7*, luciferase reporters were constructed with either a wild-type (WT) *Smad7* 3'-UTR or a mutant (Mut) *Smad7* 3'-UTR, which contained a mutant miR-21 binding site sequence. The results of the luciferase activity assays revealed that miR-21 significantly suppressed the activity of the WT reporters, but not the Mut reporters, in the MC3T3-E1 cells (Fig. 5B).

During osteogenic differentiation, the levels of miR-21 gradually increased (Fig. 6A) and the protein levels of SMAD7 decreased over time (Fig. 6B). The overexpression of miR-21 significantly suppressed the protein expression levels of SMAD7, while inhibition of miR-21 elevated their levels in the MC3T3-E1 cells (Fig. 7B). By contrast, no differences were observed in the mRNA levels of *Smad7* between the groups

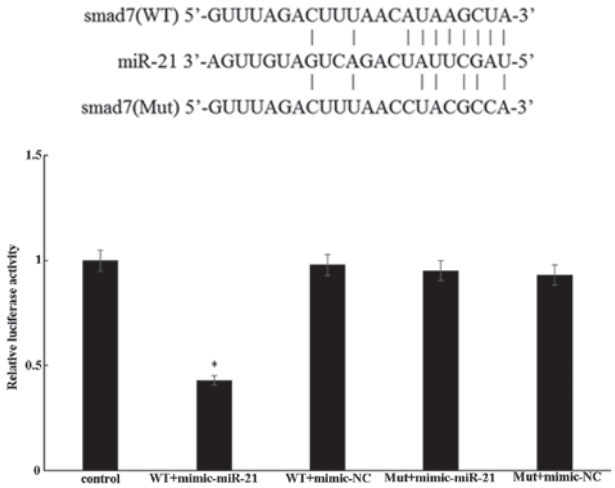

Figure 5. Luciferase assay indicating that microRNA-21 only acts on the wild-type Smad7 3'-untranslated region, but not the mutant Smad7 3'-untranslated region. Data are presented as the mean ± standard deviation; *P<0.01 vs. control group. WT, wild-type; NC, negative control; Mut, mutant; miR, microRNA.

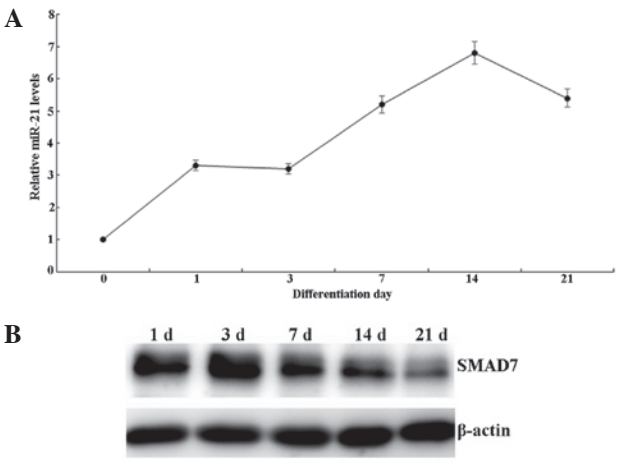

Figure 6. Association between the levels of (A) microRNA-21 and (B) SMAD7 protein during osteoblast differentiation and mineralization in MC3T3-E1 cells. Data are presented as the mean ± standard deviation. SMAD, small mothers against decapentaplegic.

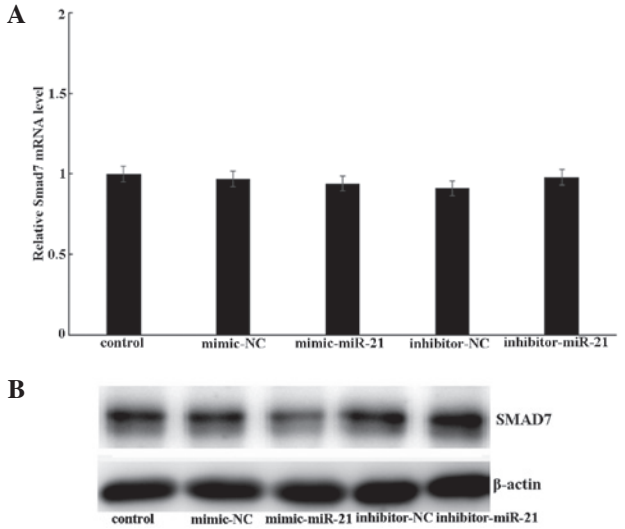

Figure 7. (A) mRNA levels of *Smad7* and (B) protein levels of SMAD7 in MC3T3-E1 cells transfected with mimic-NC, mimic-miR-21, inhibitor-NC, or inhibitor-miR-21 for 48 h. miR, microRNA; NC, negative control; SMAD, small mothers against decapentaplegic.

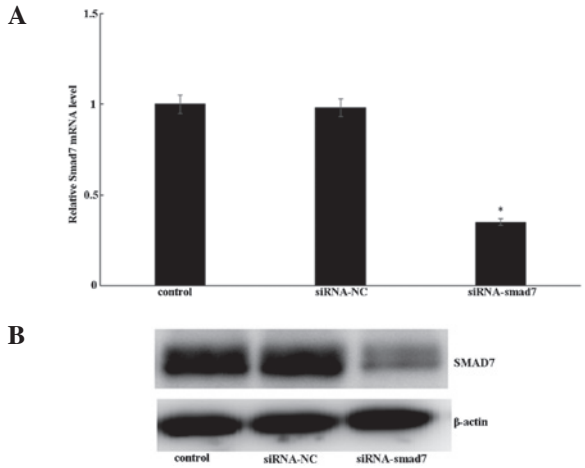

Figure 8. (A) mRNA levels of *SMAD7*. Data are presented as the mean ± standard deviation. (B) Protein levels of SMAD in the MC3T3-E1 cells transfected with siRNA-Smad7 orsiRNA-NC for 48 h. siRNA, short interfering RNA; SMAD, small mothers against decapentaplegic; NC, negative control.

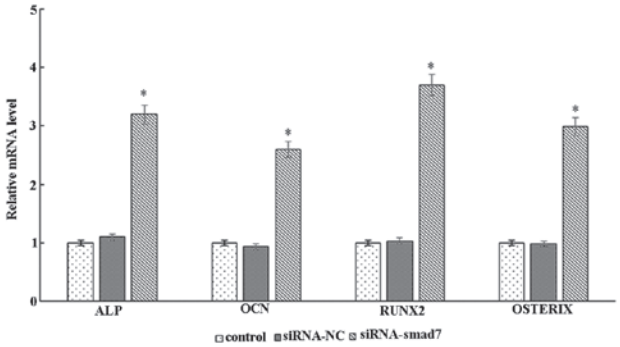

Figure 9. Reverse transcription-quantitative polymerase chain reaction analysis of *ALP*, *OCN*, *RUNX2*, and *OSX* levels in MC3T3-E1 cells following transfection with siRNA-Smad7 or siRNA-NC for 48 h. Data are presented as the mean ± standard deviation; *P<0.01 vs. control group. siRNA, short interfering RNA; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; *RUNX2*, Runt-related transcription factor 2; *OSX*, osterix; SMAD, small mothers against decapentaplegic; NC, negative control.

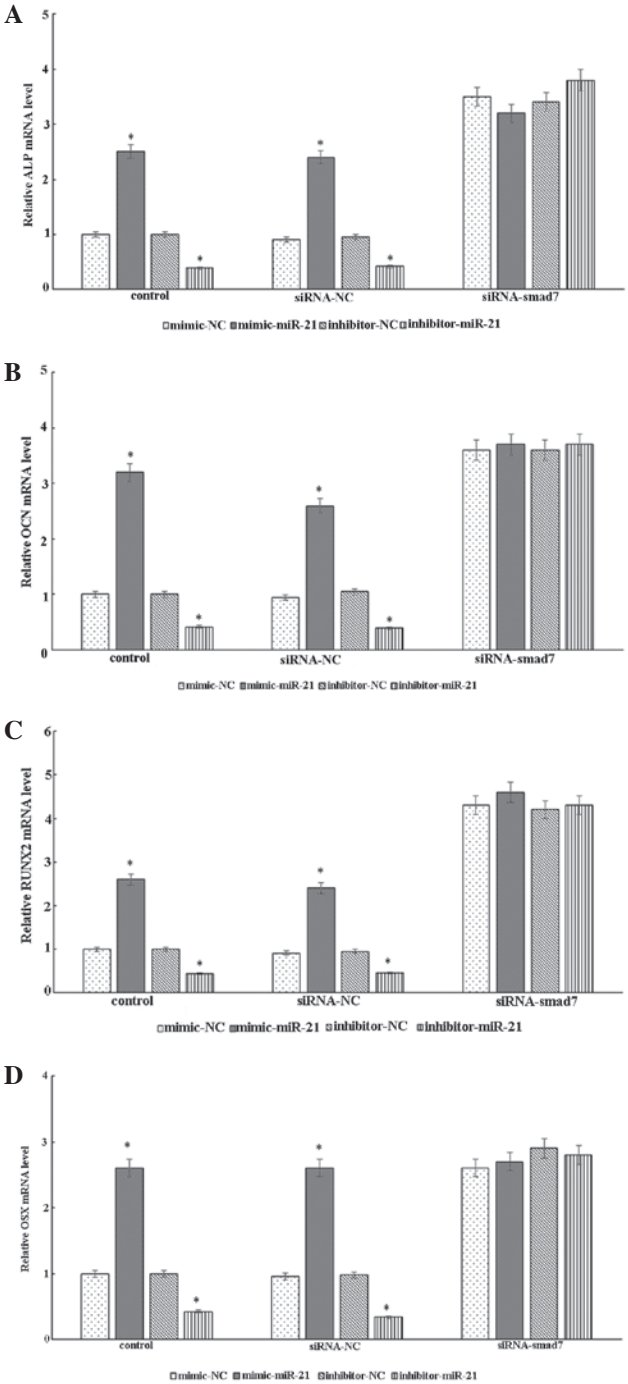

Figure 10. Reverse transcription-quantitative polymerase chain reaction analysis of the mRNA levels of (A) *ALP*, (B) *OCN*, (C) *RUNX2* and (D) *OSX* in MC3T3-E1 cells following siRNA-Smad7 transfection with mimic-NC, mimic-miR-21, inhibitor-NC or inhibitor-miR-21 for 48 h. Data are presented as the mean ± standard deviation; *P<0.01 vs. control group. siRNA, short interfering RNA; NC, negative control; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; *RUNX2*, Runt-related transcription factor 2; *OSX*, osterix; *SMAD*, small mothers against decapentaplegic.

(Fig. 7A). These results suggested that miR-21 negatively regulated the protein expression of SMAD7.

miR-21 promotes osteogenic differentiation and mineralization by targeting SMAD7. As miR-21 promoted osteogenic differentiation and mineralization and downregulated levels of SMAD7, the present study investigated whether the inhibition

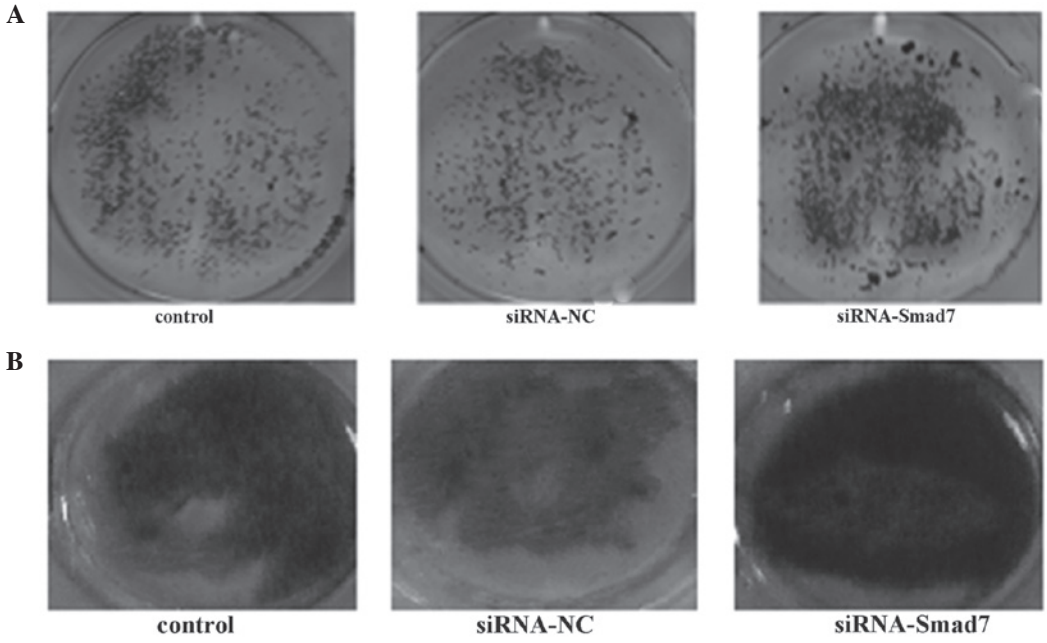

Figure 11. Calcium deposition, visualized using (A) alizarin red and (B) alkaline phosphatase staining in MC3T3-E1 cells following treatment with siRNA-Smad7 or siRNA-NC for 48 h. siRNA, short interfering RNA; NC, negative control.

of *Smad7* by siRNA produced a similar effect in the MC3T3-E1 cells.

The effects of siRNA on the mRNA and protein levels of *Smad7* were confirmed using RT-qPCR (Fig. 8A) and western blot analysis (Fig. 8B). The effects in the MC3T3-E1 cells transfected with *Smad7* siRNA were similar to those observed in the cells transfected with mimic-miR-21 (Fig. 9). Following transfection of the MC3T3-E1 cells with siRNA-Smad7, the mRNA levels of *ALP*, *OCN*, *RUNX2*, and *OSX* remained higher compared with the cells transfected with mimic-NC, mimic-miR-21, inhibitor-NC or inhibitor-miR-21 (Fig. 10).

The ARS revealed that siRNA-Smad7 markedly increased matrix mineralization (Fig. 11A) and enhanced ALP staining (Fig. 11B).

These results suggested that miR-21 promoted osteogenic differentiation and mineralization in the MC3T3-E1 cells, in part, by inhibiting the mRNA expression of *Smad7*.

Discussion

Previous studies have revealed that miRNAs are essential in the regulation of osteogenic differentiation (2,15). In the present study, the expression of miR-21 was significantly increased during osteogenic differentiation in the MC3T3-E1 cells. Furthermore, miR-21 was observed to be an active regulator of osteogenic differentiation and mineralization, as the forced expression of miR-21 promoted osteogenic differentiation and mineralization, while inhibition of miR-21 suppressed these processes. The present study also identified for the first time, to the best of our knowledge, that miR-21 promoted osteogenic differentiation and mineralization by repressing the expression of *Smad7*.

miR-21 was the first miRNA to be identified as an onco-microRNA. It is aberrantly expressed in several types of tumor and acts as a tumor suppressor (16). It targets a number of essential genes in certain types of cancer, including tongue squamous cell carcinoma (17), thymic lymphoma (18) and ovarian cancer (19), and is involved in tumor progression and metastasis, specifically cell proliferation and differentiation. Eguchi *et al* (20) hypothesized that miR-21 recognized osteogenic differentiation-, stemness-, epigenetic-, and cell cycle-associated mRNAs and was, therefore, designated as an OstemiR. Yang *et al* (13) revealed that miR-21 promotes mesenchymal stem cell osteogenesis by repressing its target gene, sprouty homolog 1 (*Spry1)*, and confirmed the function of the miR-21-Spry1 axis by demonstrating the promotion of bone formation in ovariectomized mice, in which the TNF-α signal is inhibited. However, the role of miR-21 in regulating osteogenic differentiation and mineralization remains to be elucidated. The normal progression of osteogenic differentiation is accompanied by expression of several marker genes, including *ALP*, *OCN*, *Runx2* and *OSX*. The present study revealed that miR-21 was upregulated during the osteogenic differentiation of the MC3T3-E1 cells. The osteogenic marker genes were upregulated following overexpression of miR-21 and downregulated following miR-21 inhibition. Mineralization was enhanced by the overexpression of miR-21 and reduced by miR-21 inhibition. This evidence suggested that *Smad7* is a functional target of miR-21 and may mediate its regulatory role in osteogenic differentiation and mineralization.

Smad7 is a critical regulator of TGF-β signaling and its deregulation has been associated with various diseases (21). It can potentially inhibit BMP and TGF-β signals and is able to inhibit the proliferation, differentiation and mineralization of mouse osteoblastic cells (9). Wang *et al* (22) revealed that Collagen XXIV (Col24α1) interacts with integrin β3, and that silencing Col24α1 upregulates the expression of *Smad7* during osteogenic differentiation. Several previous studies have demonstrated miR-21-mediated downregula-

tion of SMAD7 in carcinoma-associated fibroblasts (23), scleroderma (24), myelodysplastic syndromes (25) and renal fibrosis (26). In the present study, bioinformatics analysis combined with luciferase activity assays and western blot analysis confirmed that *Smad7* was a direct target of miR-21, which explains the association between miR-21 and *Smad7* in regulating osteogenesis in MC3T3-E1 cells. The overexpression of miR-21 affected the protein, but not the mRNA levels of SMAD7, which suggested that miR-21 regulated the levels of SMAD7 by inhibiting translation, rather than by promoting mRNA decay.

In conclusion, the present study demonstrated that miR-21 promoted *in vitro* osteogenic differentiation and mineralization by regulating *Smad7*. These results assist in elucidating the potential molecular mechanism underlying the regulation of bone formation. Furthermore, the association between miR-21 and *Smad7* in osteogenic differentiation and mineralization has potential value in developing therapeutic strategies to treat osteoporosis. Further *in vivo* investigations are to be performed in ovariectomized mice.

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