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MicroRNA-21 facilitates osteoblast activity

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ABSTRACT

MicroRNAs are emerging as critical post-transcriptional modulators in bone remodeling, regulating the functions of osteoblasts and osteoclasts. Intercellular crosstalk between osteoblasts and osteoclasts is mediated by miR-21 that controls the bone homeostasis response, providing potential targets for the maintenance of osteoblast function. The aim of this study was to investigate the effects of miR-21 on osteoblast function, and to explore the underlying mechanism. Increased alkaline phosphatase (ALP) activity and accelerated matrix mineralization was observed in mouse pre-osteoblast MC3T3-E1 cells compared with the non-induction (control) group. MiR-21 positively regulates osteogenic differentiation and mineralization by facilitating the expression of key osteogenic factors (ALP, Runx2, Osteopontin (OPN), Osterix (OSX) and Mef2c) in MC3T3-E1 cells. Furthermore, a deficiency of miR-21 suppresses the expression of those factors at both the mRNA and protein levels, indicating that miR-21 is a positive regulator of osteoblastic differentiation. H-E staining, Azan staining, Masson's Trichrome staining and Toluidine blue staining were performed in jaw and femur tissues of miR-21 knockout (miR-21KO) and wild-type (WT) mice. Immunohistochemical staining revealed substantially lower levels of ALP, Runx2 and OSX expression in jaw and femur tissues of miR-21KO mice. A similar trend was observed in femur tissues using quantitative real-time (RT) PCR. A total of 17 osteogenesis-related mRNAs were found to be differentially expressed in miR-21KO femur tissues using Mouse Gene Expression Microarray analysis. GeneSpring and Ingenuity Pathway Analysis revealed several potential target genes that are involved in bone remodeling, such as IL-1β and HIF-1α. Several important pathways were determined to be facilitators of miR-21, which provides a reliable reference for future studies to elucidate the biological mechanisms of osteoblast function. Taken together, these results lead us to hypothesize a potential role for miR-21 in regulating osteoblast function, thus representing a potential biomarker of osteogenesis.

1. Introduction

The dynamic nature of bone as a special mineralized connective tissue has been well established. Even in a disease-free state, bone undergoes the processes of formation and resorption, creating homeostasis. Osteocytes mature from osteoblast progenitors and regulate the balance of osteoblast and osteoclast functions. Thus, osteocytes act as sensory cells regulating the mineralized tissue catabolism and anabolism in response to various stimuli [1]. Osteoblast differentiation is associated with an increase in alkaline phosphatase (ALP) activity and mineralization. At the molecular level, Runt-related transcription factor 2 (Runx2) acts as one of the predominant factors required for the

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differentiation of osteoblasts [2,3]. The master transcription factor, Sp7/Osterix (OSX), is another important transcription factor that regulates the differentiation of osteoblasts to osteocytes [4].

With the increased understanding of the role of microRNAs (miRNAs) in recent years, their regulatory roles in several molecular pathways of cell biology have been elucidated [5,6]. Since miRNAs have been found to critically influence the regulation of several cellular processes and post-transcriptional gene expression [7], abnormalities in miRNAs can be key factors in the initiation and progression of disease [8,9]. A similar essential role of miRNAs in bone metabolism through the regulation of osteoblastogenesis and osteoclastogenesis has also been well established.

Alterations in the expression of specific miRNAs have been associated with an increased risk of fracture and metabolic disorders of bone [10]. miR-33-5p positively regulates osteoblast differentiation [11] while miR-503-5p inhibits the osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMMSCs) [12]. miRNAs associated with specific diseases have emerged as diagnostic markers and therapeutic targets. MiR-21 participates in the regulation of a multitude of cellular functions for homeostasis maintenance. *In vitro* studies have revealed a dual role of miR-21 in promoting the pro-osteogenic differentiation of BMMSCs [13] and the differentiation of osteoclasts via the receptor activator of nuclear factor κB ligand (RANKL) pathway [14]. Chen et al. have also found similar regulatory roles of miR-21 in alveolar bone remodeling, including the osteogenic differentiation of PDLSCs and the differentiation of osteoclasts [15]. Inhibition of miR-21 results in the decreased osteogenic ability of BMMSCs [16]. Accumulating evidence has revealed the stimulatory effect of miR-21 on osteoblastic differentiation [17,18].

Another critical regulatory protein in bone metabolism is Mef2c, which controls the expression of sclerostin (encoded by SOST) through the modulation of Wnt signaling [19,20]. SOST has been shown to inhibit the differentiation of osteoblasts *in vitro* and the over-expression of SOST has been reported in mice with an osteoporotic phenotype [21].

In this study, we aimed to evaluate the mechanistic role of miR-21 in the osteogenic differentiation of MC3T3-E1 mouse pre-osteoblast cells, identify the regulatory targets and analyze the loss of function under physiologic conditions. The results show that osteogenic differentiation upregulates miR-21 biogenesis, leading to the increased expression of key osteogenic markers, Runx2 and OSX, and a concomitant increase in the expression of an osteoblast regulator, Mef2c. Treatment of MC3T3-E1 cells with a miR-21 inhibitor increased SOST expression by down-regulating key osteogenic markers. Furthermore, the deletion of miR-21 induced osteogenic defects *in vivo*, and alterations in the mRNA expression signature associated with osteoblast maturation were screened using Mouse Gene Expression Microarray, GeneSpring and Ingenuity Pathway Analyses and verified by quantitative RT-PCR. Our study sheds light on novel insights of the role of miR-21 in applications related to bone regeneration.

2. Material and methods

2.1. Cell culture

MC3T3-E1 mouse pre-osteoblast cells were obtained from the RIKEN BRC CELL BANK (Tokyo, Japan) and were cultured in alpha-MEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Waltham, MA, USA) under 5% CO_2 in a humid environment. MC3T3-E1 cells were seeded in 6-well plates and their osteogenic induction (MK430, Takara, Tokyo, Japan) was started when the cells were 80–90% confluent and was continued for 21 days. Mineralization was detected by fixing the cells with 4% paraformaldehyde and incubating them with 2% Alizarin Red S stain solution (WAKO, Tokyo, Japan) for 30 min. Images of calcium nodules were captured using a microscope (OLYMPUS, Tokyo, Japan).

2.2. Animals

The miR-21 knockout (miR-21KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). MiR-21KO mice (n = 6) and wild-type (WT) mice (n = 6) were maintained at Capital Medical University, Beijing, China. The mice were housed in 12 h light: 12 h dark cycle in pathogen-free conditions. Their nutrition and hydration were maintained as per the best practices of handling the animals. All animal experiments were performed after approval of the Institute's Ethics Committee (Capital Medical University #2012-x-53).

2.3. Quantitative RT-PCR

Total RNA of MC3T3-E1 cells was extracted using a RNeasy Mini Kit (Qiagen KK, Tokyo, Japan). One μg RNA was transcribed to cDNA using a high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Ten ng RNA was transcribed to miRNA cDNA using TaqMan® Advanced miRNA cDNA Synthesis Kit (Qiagen KK, Tokyo, Japan). TaqMan gene Expression Assays were used to detect the expression of miR-21a-5p (Assay ID mmu482709_mir), ALP (Assay ID Mm00475834_m1), Runx2 (Assay ID Mm00501583_m1), OSX (Assay ID Mm04933803_m1), Osteopontin (OPN) (Assay ID Mm00436767_m1), Mef2c (Assay ID Mm01340842_m1), SOST (Assay ID Mm00470479_m1), IL-1β (Assay ID Mm00434228_m1) and HIF-1α (Assay ID Mm00468869_m1).

2.4. Western blot (WB)

Lysis Buffer was used to extract total proteins of MC3T3-E1 cells, and 20 μg protein of each sample were separated on polyacrylamide-SDS gels (Wako, Osaka, Japan) and transferred to PVDF membranes. The membranes were incubated with anti-Runx2 (1:500, Abcam, Tokyo, Japan), anti-OSX (1:500, Abcam, Tokyo, Japan), anti-Mef2c (1:500, Abcam, Tokyo, Japan), anti-β-actin (1:1000, Cell Signaling Technology, Danvers, MA, USA) and anti-GAPDH (1:1000, Cell Signaling Technology, Danvers, MA, USA) after blocking with 5% skim milk at 4 °C overnight. The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (1:2000; Cell Signaling Technology, Danvers, MA, USA) for 1 h. Images were visualized and captured using an ECL Plus Western Blotting Detection System (GE Healthcare, Tokyo, Japan).

2.5. miR-21 inhibitor transfection

0.8×10^5 MC3T3-E1 cells were cultured in 6-well plates in antibiotic-free medium. The cells were transfected with 30 nM negative control or miR-21 inhibitor with Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher, Tokyo, Japan) after 16 h, and then collected 48 h later.

2.6. Histological staining

The jaw and femur tissues were fixed with 4% Formaldehyde for 24 h and decalcified using 10% EDTA 2Na Solution (pH: 7.0, Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The jaw and femur tissues were embedded in paraffin and 4 μm sections were stained with Hematoxylin-Eosin (H-E), Masson's Trichrome (Abcam, Tokyo, Japan), Azan (Muto Pure Chemicals, Tokyo, Japan) or Toluidine blue (Waldeck, Wako, Tokyo, Japan). Histological changes were observed by two independent and blinded pathologists using a microscope (OLYMPUS, Tokyo, Japan).

2.7. Immunohistochemical staining (IHC)

Sections of jaw and femur tissues were subjected to antigen retrieval (pH 6.0, Abcam, Tokyo, Japan), followed by peroxidase blocking (DAKO, Santa Clara, CA, USA). Anti-ALP (1:100, a gift from Hokkaido

University, Hokkaido, Japan), anti-Runx2 (1:125, Abcam, Tokyo, Japan), anti-OSX (1:75, Abcam, Tokyo, Japan), anti-Mef2c (1:500, Abcam, Tokyo, Japan), anti-SOST (1:50, Abcam, Tokyo, Japan), anti-IL-1β (1:100, Abcam, Tokyo, Japan) and anti–HIF–1α (1:100, Abcam, Tokyo, Japan) were used as primary antibodies. The specimens were exposed to the same DAB reaction conditions, and all images were captured using a microscope (OLYMPUS, Tokyo, Japan).

2.8. Mouse Gene Expression Microarray analysis

The RNA integrity from WT and miR-21KO femur tissues was determined using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Variations of mRNAs between samples were characterized by hybridization to SurePrint G3 mouse 8 × 60 K arrays (ver. 2.0, Agilent Technologies, Santa Clara, CA, USA). Data analysis was performed by Agilent Feature Extraction and Agilent GeneSpring GX software. The relevant pathways were analyzed using an Ingenuity Pathway Analysis Suite

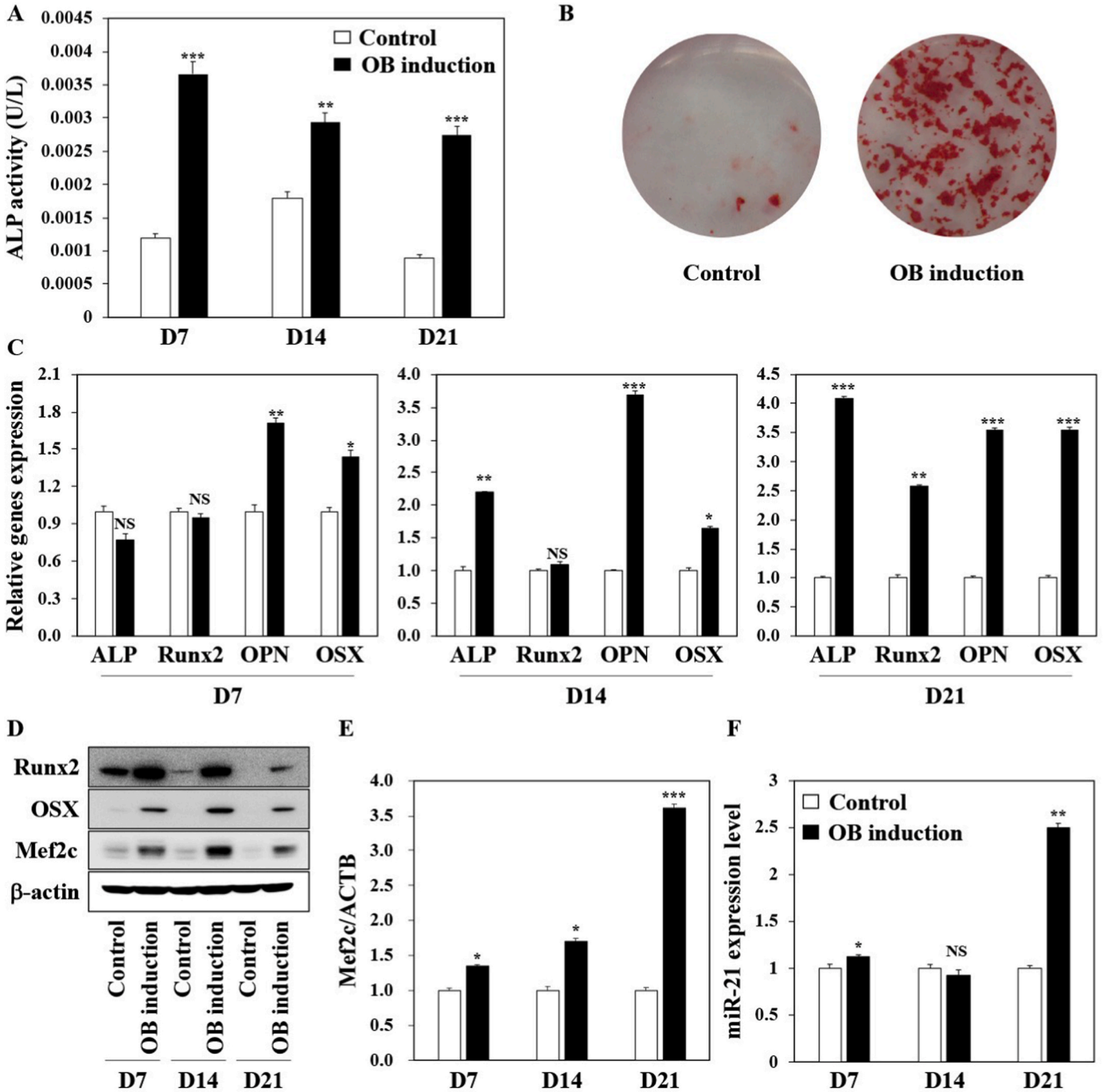

Fig. 1. The expression of Mef2c and miR-21 is induced during osteogenesis. (A) ALP activity is induced during osteoinduction. **(B)** Alizarin Red staining showing the calcified nodule formation in the OB induction group. **(C, D)** Expression of ALP, Runx2, OPN and OSX is increased during osteoblast differentiation. **(E, F)** Mef2c and miR-21 expression levels are elevated during osteoinduction. The data shown represent means ± SD; *p < 0.05; **p < 0.01; ***p < 0.001. All results are representative of at least three independent experiments. OB: osteoblast. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(IPA; Ingenuity, Redwood City, CA, USA).

2.9. Statistical analysis

SPSS 16.0 was used to perform statistical analysis through an independent two-tailed Student's t-test or analysis of variance (ANOVA). A *p value* less than 0.05 is considered to show a significant difference.

3. Results

3.1. The expression of Mef2c and miR-21 is induced during osteogenesis

To characterize the expression levels of Mef2c and miR-21, we cultured MC3T3-E1 pre-osteoblast cells in osteoinduction medium for 21 days. ALP activity was dramatically induced in the osteoinduction groups at 7, 14 and 21 days compared with the non-induction (control) group (Fig. 1A, $^{**}p < 0.01$, $^{***}p < 0.001$). Consistently, Alizarin Red staining also confirmed the formation of calcified nodules with osteoblast differentiation (Fig. 1B). Quantitative RT-PCR and WB results further confirmed that the induction of the osteoblast markers, ALP, Runx2, OPN and OSX started from day 7 (Fig. 1C–D, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) as well as Mef2c were significantly upregulated with increased induction time compared with the non-induction group (Fig. 1D–E, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). The expression level of miR-21 was dramatically induced at the day 21 of the osteoinduction (Fig. 1F, $^{*}p < 0.05$, $^{**}p < 0.01$).

3.2. The downregulation of miR-21 decreases the expression of Mef2c and osteoblast markers

To reveal the interaction between miR-21 and Mef2c, a miR-21 inhibitor was transfected into MC3T3-E1 cells. Quantitative RT-PCR and WB analysis showed that the miR-21 inhibitor had a strong inhibitory effect on the expression of osteoblast markers, ALP (Fig. 2A, $p < 0.05$), OPN (Fig. 2B, $p < 0.05$), OSX (Fig. 2C, F, $p < 0.05$) and Runx2 (Fig. 2F) of MC3T3-E1 cells. Consistently, quantitative RT-PCR and WB analysis showed that the expression of Mef2c was reduced after miR-21 inhibitor transfection (Fig. 2D, F, $p < 0.05$). For confirmation, miR-21 significantly reduced after treatment with the inhibitor. These results confirmed that miR-21 indirectly regulates the expression of Mef2c and osteoblast markers, and that the reduction of miR-21 led to the suppressed expression of Mef2c and of osteoblast markers.

3.3. Osteogenic activity is suppressed in alveolar bone and femur of miR-21KO mice

To confirm the effect of miR-21 in osteogenesis, immunohistochemical staining was used to detect the expression of osteoblast markers in alveolar bone (Fig. 3A) and femur tissues (Fig. 3B). A deficiency in miR-21 significantly reduced the expression of ALP, Runx2, OSX and Mef2c compared to WT mice, while in contrast, SOST was highly expressed in the miR-21KO group (Fig. 3A and B). Consistently, quantitative RT-PCR analysis showed that miR21-KO femur tissues had

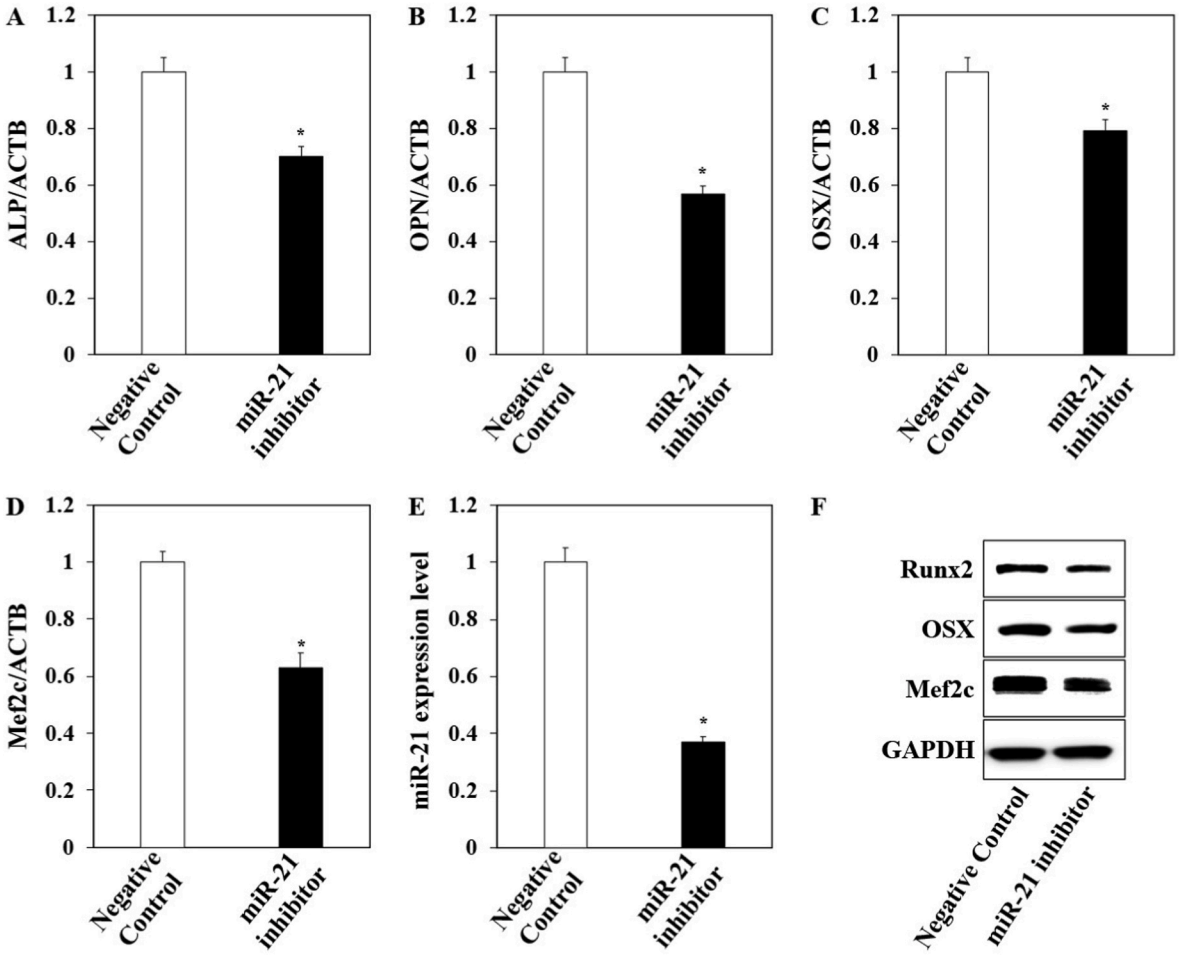

Fig. 2. The downregulation of miR-21 decreases Mef2c expression. (A–C) ALP, OPN and OSX expression are reduced after transfection with a miR-21 inhibitor. **(D)** Mef2c is downregulated with miR-21 suppression. **(E)** MiR-21 is reduced in MC3T3-E1 cells after transfection with a miR-21 inhibitor. **(F)** WB showing reduced expression of Mef2c, Runx2 and OSX following the inhibition of miR-21. The data shown represent means ± SD; $^{*}p < 0.05$. All results are representative of at least three independent experiments.

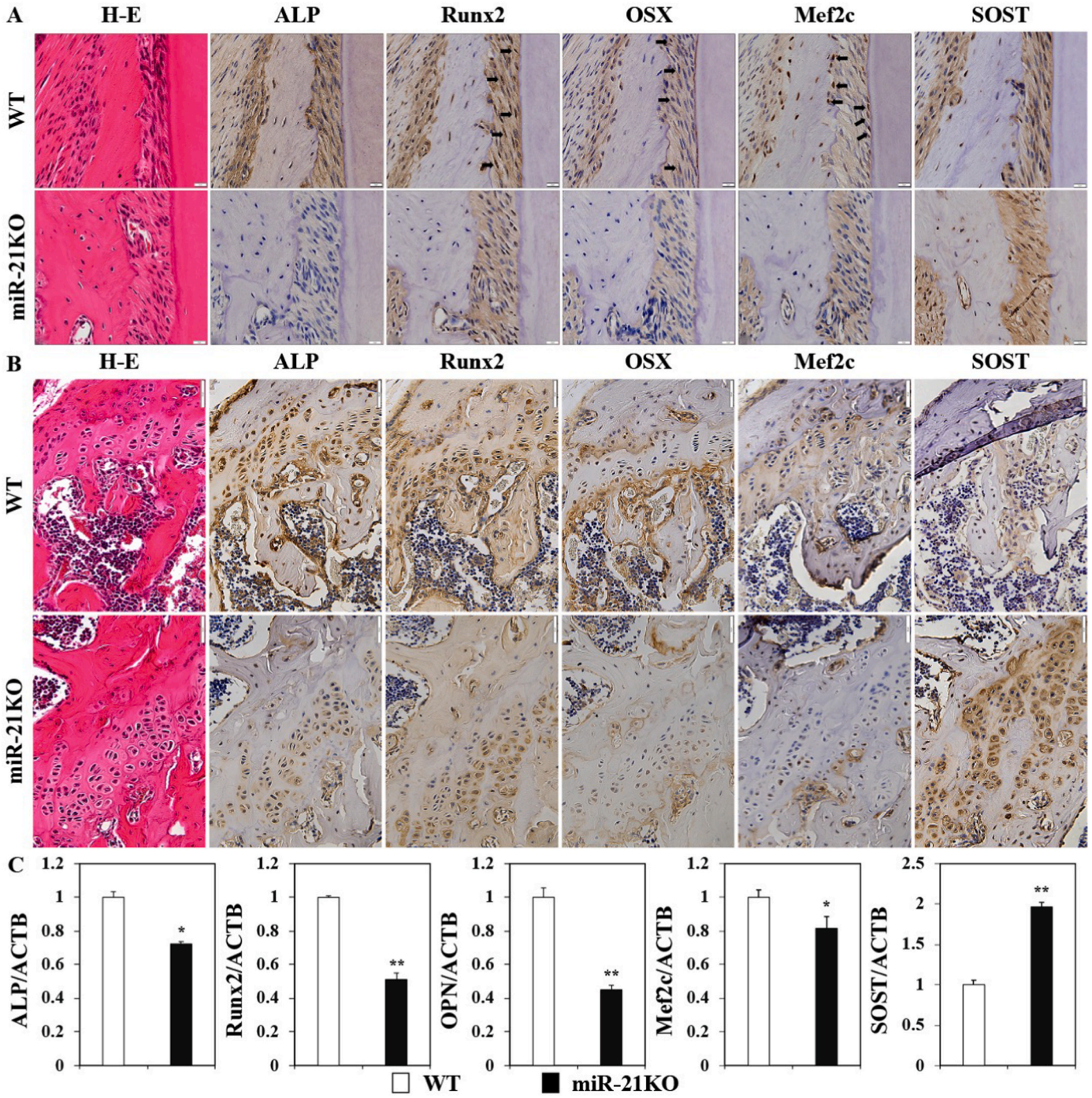

Fig. 3. Osteogenic activity is suppressed in alveolar bone and femur tissues of miR-21KO mice. (A, B) Immunohistochemical staining showing reduced expression of ALP, Runx2, OSX and Mef2c in alveolar bone and femur of miR-21KO mice, whereas the expression of SOST was induced by the miR-21 deficiency. **(C)** Quantitative RT-PCR results showing the suppressed expression of ALP, Runx2, OPN and Mef2c and the upregulated expression of SOST in miR-21KO femur tissues. All results are representative of at least three independent experiments. The data shown represent means ± SD; *$p < 0.05$; **$p < 0.01$.

decreased mRNA expression levels of ALP ($p < 0.05$), Runx2 ($p < 0.01$), OPN ($p < 0.01$) and Mef2c ($p < 0.05$) expression compared to femur tissues of WT mice, whereas SOST ($p < 0.01$) expression in osteoblasts was increased in femur tissues of miR21-KO mice compared to WT mice, indicating the suppression of osteoblast activity (Fig. 3C). The sum of those results confirmed the function of miR-21 to stimulate and enhance osteoblast differentiation and bone formation.

3.4. MiR-21KO mice have impaired bone formation

MiR21-KO mice were used to investigate the genomic and morphological changes in femur tissues with miR-21 deficiency. H-E staining showed less trabecular bone in miR-21KO mice compared with WT mice. Azan staining, Masson's Trichrome staining and Toluidine Blue staining all confirmed the detrimental changes in the cortical bone of the femurs of miR-21KO mice, namely, an increased matrix deposition and reduced mineralization (Fig. 4A). Additionally, Mouse Gene Expression Microarray analysis identified several variations in osteogenesis-related

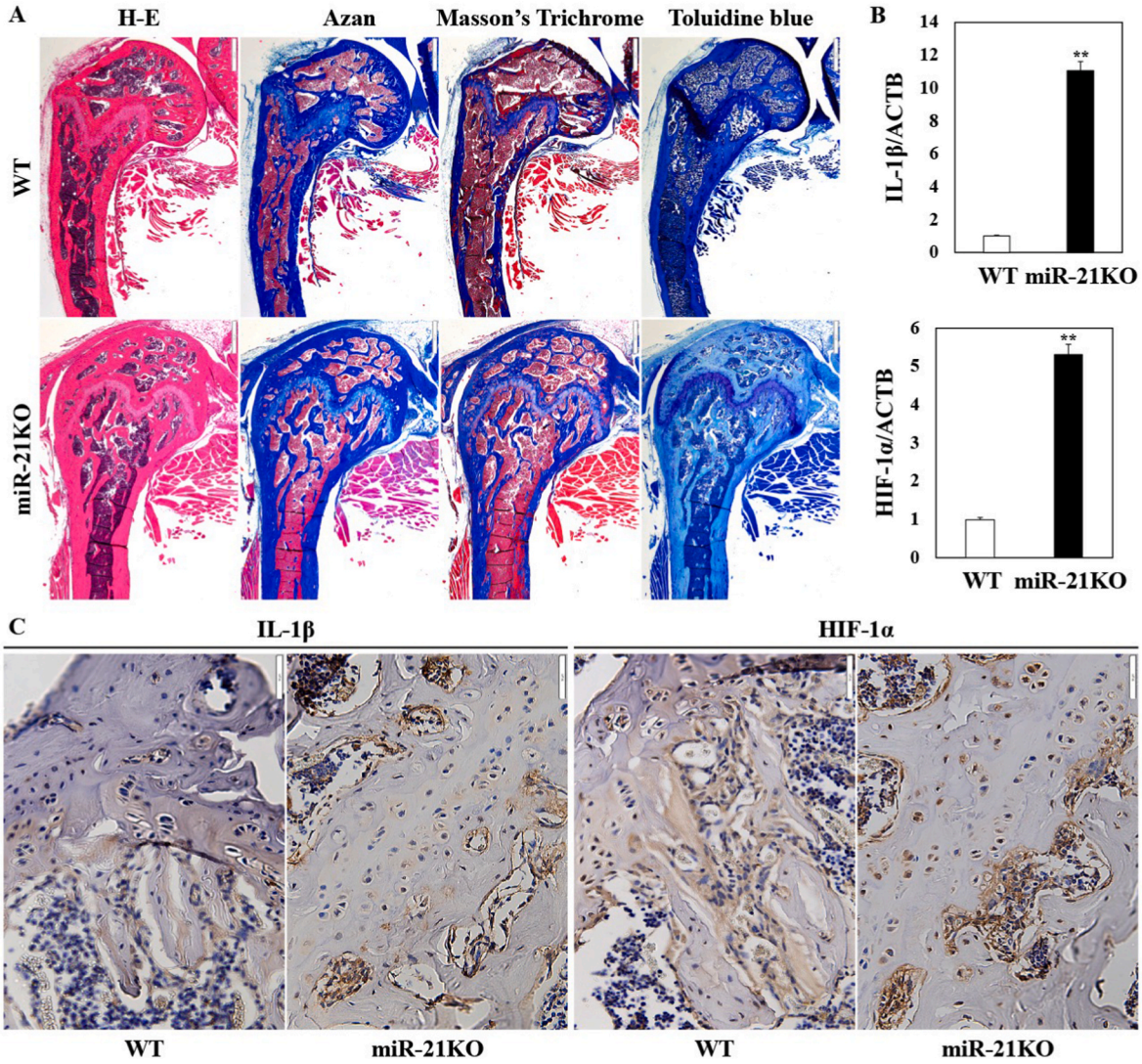

Fig. 4. MiR-21KO mice have impaired bone formation. (A) H-E staining showing less trabecular bone in miR-21KO mice compared with WT mice. Azan, Masson's Trichrome and Toluidine Blue staining all confirmed the impaired cortical bone in miR-21KO femur tissues. All results are representative of at least three independent experiments. **(B, C)** Quantitative RT-PCR and IHC results confirming that the expression levels of IL-1β and HIF-1α correlated with the DNA microarray data. All results are representative of at least three independent experiments. The data shown represent means ± SD; $^{**}p < 0.01$; $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mRNAs and signaling pathways between the femurs of miR21-KO and WT mice (Supplementary Tables 1 and 2). Among the mRNAs identified, IL-1β and HIF-1α were the most related with osteogenesis and were chosen for verification. Quantitative RT-PCR and IHC analysis confirmed that the expression levels of those target genes were correlated with the Mouse Gene Expression Microarray analysis data (Fig. 4B and C). The suppressed osteoblastic differentiation and activity led to an osteoporosis-like change in femur tissues of miR21-KO mice. The above results further reveal the indispensable regulatory role of miR-21 in the osteogenesis of bone.

4. Discussion

The functions of miRNAs are dependent on their cellular microenvironments and the presence of their target genes. In order to demonstrate the molecular mechanisms involved in the regulation of osteoblast differentiation by miR-21 and its role in bone biology, we evaluated key osteogenic factors in MC3T3-E1 cells, and also searched for potential target genes of miR-21 related to osteogenic signaling pathways. We now report that miR-21 expression is induced during osteoblast differentiation at the cellular level, as evidenced by the upregulation of ALP, Runx2, OPN and OSX in MC3T3-E1 cells. Conversely, those osteogenic marker genes were downregulated following the inhibition of miR-21 *in vitro* and *in vivo*.

An increasing body of literature has identified various miRNAs as key regulators of bone formation and osteoblast differentiation. MiR-21 is a versatile miRNA that is involved in a variety of physiological and pathological events. Levels of miR-21 increase during the differentiation of embryonic stem cells [22]. MiR-21 can promote stem cell osteogenesis via the Smad7-Smad1/5/8-Runx2 and Akt pathways [17,23]. MiR-21 is an osteogenesis promoter in bone marrow MSCs and periodontal ligament stem cells and the over-expression of miR-21 promotes osteogenesis and fracture healing [18]. Despite numerous *in vitro* studies confirming the function of miR-21 in bone homeostasis, the *in vivo* function of miR-21 in osteoblasts has not been elucidated. Here, the function of miR-21 in bone formation was investigated in miR-21 knockout mice using tissue staining, immunohistochemistry, quantitative RT-PCR, WB and DNA microarray analysis, all of which suggest that a miR-21 deficiency might affect the biological characteristics of osteoblasts. Our data support that miR-21 is a critical factor for bone healing

suggesting a plausible molecular target to enhance bone regeneration in regenerative medicine.

MiRNAs have been found to combine with transcription factors to regulate osteogenic differentiation. Runx2, an early and primary osteogenic transcription factor, is a key molecule mediating the differentiation of osteoblasts [24]. As a downstream gene of Runx2, OSX is specifically expressed during osteoblast differentiation and is critical for bone formation [25]. The selection of miR-21 as a target was made based on its crucial role as a regulator of Runx2. A miR-21 inhibitor decreased the accumulation of intracellular Runx2 at the mRNA [16] and protein levels in MSCs [17]. MiR-21 overexpression improved matrix mineralization, whereas miR-21 inhibition lowered calcium deposition [26]. MiR-21 levels are decreased in bone tissue and in the serum of patients with osteoporosis [16]. OPN expression increases in MSCs because of miR-21 upregulation [27]. Thus, miR-21 is now recognized as a potential biomarker of bone diseases.

ALP is expressed by osteoblasts and is a crucial indicator of bone mineralization. Differentiated osteoblasts exhibit enhanced ALP activity and bone mineralization. ALP participates in inorganic phosphate homeostasis and is associated with matrix vesicle mineralization [28]. Those results are in good agreement with our data, confirming that the miR-21 deficiency is associated with decreased ALP expression.

The involvement of Mef2c in bone formation has been reported in several studies [20]. Notably, the expression of Mef2c during the terminal stages of differentiation (day 21) was significantly elevated in osteoinduction medium-treated cells, which suggests the possibility that Mef2c functions as a regulator of mineralization. Conversely, the inhibition of miR-21 suppressed the expression of Mef2c in MC3T3-E1 cells. In addition, the Mef2c locus has been linked genetically to human bone mineral density [29]. Mef2c is expressed in osteoblastic cells and is a key osteoblast transcription factor, upstream of Runx2 and OSX [30]. Our *in vivo* model suggests that Mef2c functions as a key osteoblast transcription factor upstream of both Runx2 and OSX in osteoblasts. Mef2c has been demonstrated to have a role in regulating SOST. Mef2c controls SOST gene expression via binding to the SOST distal enhancer in osteoblast cells. A SOST deficiency promotes bone formation, while overexpression of SOST accelerates bone loss [31,32]. Here, we provide evidence that Mef2c is important in bone tissues for the transcriptional activation of SOST in osteoblasts. MiR-21 is robustly expressed in osteoblasts and our results show a novel role for miR-21 in controlling the expression of SOST and thereby in the regulation of adult bone mass. MiR-21KO mice have activated SOST expression leading to the secretion of SOST, which inhibits osteoblast-mediated bone formation [33,34].

Based on bioinformatics predictions, the target genes of miR-21 are significantly enriched in the Jak-STAT and MAPK signaling pathways, which are related to osteogenic differentiation [35]. MiR-21 down--regulates the expression of phosphatase and tensin (PTEN) [36], NFIB [37], etc. MiR-21 may promote the migration and osteogenic differentiation of BMSCs via the PTEN/PI3K/Akt/HIF-1α pathway [32]. Hu et al. previously identified miR-21 sequences complementary to the IL-1β 3′ UTR sequence [38]. Nevertheless, the molecular function of miR-21 in the regulation of bone remodeling *in vivo* remains elusive. To test this hypothesis, we took advantage of mice lacking expression of miR-21 to confirm whether miR-21 contributes to defective osteogenesis and/or bone formation. In this study, we used Mouse Gene Expression Microarray analysis to identify the expression profiles of mRNAs. According to our results, the expression levels of 17 osteogenesis-related mRNAs were altered in miR-21KO femur tissues and those were further validated by quantitative RT-PCR and IHC. The differences in results of other studies compared to our results may be attributed primarily to the analytical methods used. Further studies must be performed to determine whether these osteo-mRNAs are involved in regulating the osteogenic process.

In summary, we have demonstrated that miR-21 regulates osteogenic differentiation by targeting several key genes. Importantly, the functional inhibition of miR-21 deaccelerates the osteogenic function of MC3T3-E1 cells and leads to decreased bone formation *in vivo*. We first identified and characterized the expression profile of miR-21 in femur tissues using DNA microarray analysis. Furthermore, we explored the regulatory network between differentially expressed mRNAs and transcription factors to identify the potential mechanisms underlying osteogenic differentiation. Our results provide mechanistic insights into the molecular process of osteoblast differentiation. Targeting miR-21 could serve as a promising therapeutic tool for stimulating bone formation and mitigating pathological conditions associated with bone loss.

Credit authorship contribution statement

Shunichi Oka and Xiaoyan Li: Conceptualization, Investigation, Methodology, Formal analysis, Writing - Original Draft. Fengzhu Zhang and Nitesh Tewari: Investigation, Resources, Writing - Review & Editing. Ri Ma, Liangjun Zhong, and Makoto Makishima: Writing - Review & Editing. Yi Liu: Resources, Writing - Review & Editing. Ujjal K. Bhawal: Conceptualization, Resources, Writing - Review & Editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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