Crosstalk between microRNA-21−5p and the transcription factor Dec1 maintains osteoblast function

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ARTICLE INFO

ABSTRACT

MicroRNAs are associated with pivotal post-transcriptional gene regulation in bone formation. Human differentiated embryonic chondrocyte expressed gene 1 (Dec1) is also involved in regulating osteoblastogenesis. In the present study, we aimed to investigate the distinctive role of miR-21−5p and Dec1 in osteoblast function and to determine their biological functions. MC3T3-E1 pre-osteoblastic cells were used for in vitro analyses. miR-21−5p knockout (KO) mice, Dec1KO mice and age-matched wild-type (WT) mice were used to characterize the influence of miR-21−5p and Dec1 deficiencies on bone formation. Morphological analyses [micro-computed tomography (micro-CT)] were performed, and measurements were collected to validate miR-21-5pKO mice. Histopathological changes in mouse femur tissues were assessed by H-E staining, Azan staining, Masson's Trichrome staining, and Toluidine Blue staining. Quantitative real-time RT-PCR, western blotting and immunohistochemical staining were used to characterize the expression levels of Alkaline Phosphatase, Runx2, Osterix, Osteopontin, Dec1 and miR-21−5p. Bioinformatics analyses and dual-luciferase reporter assays were performed to confirm Dec1 as a target of miR-21−5p. Dec1 expression was gradually increased from day 7 of osteoblast induction, while miR-21−5p showed a peak at day 21. In non-induced osteoblasts, a mechanistically gain-of-function transfection study with a miR-21−5p mimic enhanced Runx2 and Osterix expression but suppressed Dec1. miR-21-5pKO mice had reduced bone growth. Dec1-deficient mice showed advanced bone formation at the age of 12 weeks compared to WT mice. The Dec1 deficiency upregulated Runx2 and Osterix expression in Dec1KO mouse femurs. Those changes, however, were reversed in miR-21-5pKO mouse femurs compared to WT mouse femurs. Dual-luciferase reporter assays showed that Dec1 is a possible downstream target of miR-21−5p. These findings showed that the reduced osteogenic potential due to a miR-21−5p deficiency is achieved by enhanced Dec1 expression and that the miR-21−5p/Dec1 axis is involved in regulating osteoblast function.

1. Introduction

MicroRNAs (miRNAs) target the 3′-untranslated regions of mRNAs to regulate gene expression at the post-transcriptional level in skeletal biology [1]. Several studies have clearly demonstrated the involvement of miRNAs in regulating bone remodeling and osteoblastogenesis [2]. Among key miRNAs, miR-21−5p, which is perceived as a multifaceted miRNA, regulates diverse physiological and pathological functions, such as hypoxia and oxidative stress [3,4]. miR-21−5p has a substantial impact on ameliorating cognitive deterioration [5]. miR-21−5p expedites the osteogenic potential in murine cells with bone morphogenetic proteins or by inhibiting Smad7 [6,7]. miR-21−5p considerably increases the osteogenic effect of human, mouse and rat bone marrow-derived mesenchymal stem cells (BMSCs) [8−10]. In our previous study, the lack of miR-21−5p resulted in decreased osteoblast differentiation, including the reduced expression of runt-related transcription

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factor 2 (Runx2) in BMSCs [8]. The function of miR-21—5p in osteoblastogenesis has been mostly studied at the cellular level. We previously reported delayed wound healing in miR-21-5p-knockout (KO) mice and demonstrated impaired calvarial bone formation in those mice [8]. miR-21—5p is now considered to be one of the most extensively studied miRNAs for its ubiquitous regulation of numerous physiological processes.

The crosstalk between miRNAs and transcription factors can trigger various biological processes to maintain cellular homeostasis. The basic helix-loop-helix (bHLH) transcription factor differentiated embryonic chondrocyte expressed gene 1 (Dec1) regulates multiple cellular processes, including differentiation, bone formation and remodeling [11,12]. The overexpression of Dec1 repressed NIH3T3 cell growth, which suggested its involvement in cellular growth arrest [13]. Dec1 overexpression also restrained the adipogenic differentiation of mouse 3T3-L1 cells [14]. Several recent reports have disclosed advances of miRNAs in stem cell differentiation through post-transcriptional regulation of gene expression involved in cell renewal and maintenance [15]. Runx2 and Osterix (Osx), two bone-specific transcription factors, regulate osteoblast differentiation and miRNAs govern the expression of Runx2 mRNA [16,17]. A growing number of reports have proposed that miRNAs can facilitate osteogenic differentiation and bone formation [2].

Bioinformatics-based prediction methods have established *PIK3R1* as a direct target gene of miR-21—5p [18]. Recently, *KLF3* has also been identified as another target of miR-21—5p [19]. Because transcription factors regulate the abundance of miRNAs, we hypothesize that application of the transcription factor Dec1 in osteoblast differentiation and osteoblast function might have functional effects by potentially regulating miR-21—5p expression.

The present study used 2 strains of knockout (KO) mice (miR-21-5pKO and *Dec1*KO) to characterize differences in osteoblast function. The results revealed that miR-21—5p is critical in Runx2 and Osx regulation by targeting Dec1 and that the miR-21—5p/Dec1 axis might play a vital function in osteogenesis. Additionally, the underlying mechanism of miR-21—5p regulation in osteogenesis was investigated to provide powerful evidence for potential clinical treatments. Our study uncovers new biological properties of miR-21—5p and expands its potential pharmaceutical values.

2. Materials and methods

2.1. Cells

MC3T3-E1 pre-osteoblast cells were purchased from the RIKEN BRC CELL BANK (Tokyo, Japan). MC3T3-E1 cells were cultured in alpha-MEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Waltham, MA, USA) with 5% CO_2 in a humid environment. Osteogenic induction (MK430, Takara, Tokyo, Japan) was started when MC3T3-E1 cells reached 80—90% confluence and was continued for 21 days.

2.2. Animals

miR-21—5p knockout (miR-21-5pKO) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) [8,20]. Dec1 knockout (*Dec1*KO) mice were generated as previously described [21]. miR-21-5pKO mice (n = 6), *Dec1*KO mice (n = 6) and wild-type (WT) mice (n = 6) were housed in pathogen-free conditions and did not differ significantly in body weight. Mice were sacrificed by anesthesia followed by cervical dislocation. All animal experiments were performed after approval of the Institute's Ethics Committee (KQYY-202206-007); Nihon University School of Dentistry at Matsudo (AP17MD020).

2.3. Quantitative reverse transcription-PCR (qRT-PCR)

A miRNeasy Mini Kit (Qiagen KK, Tokyo, Japan) was used to purify total RNA from MC3T3-E1 cells and from mouse femur tissues. One μg of each RNA was transcribed to cDNA using a high-capacity cDNA Archive Kit (Thermo Fisher Scientific, Waltham, MA, USA). TaqMan® Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for miRNA cDNA transcription. TaqMan probes used for gene expression assays were miR-21—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) and Actb (Assay ID Hs01060665_g1) (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Western blot analysis

Lysis Buffer was used to extract total proteins and 20 μg protein was used for each western blot using standard protocols. The primary antibodies used were anti-Dec1 (1:500, a gift from Prof. Yukio Kato, Hiroshima University, Hiroshima, Japan), anti-β-actin (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-Runx2 (1:500, Abcam, Tokyo, Japan), anti-Osx (1:500, Abcam, Tokyo, Japan) and anti-Gapdh (1:1000, Cell Signaling Technology, Danvers, MA, USA). The membranes were incubated with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (1:2000; Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. An ECL Plus Western Blotting Detection System (GE Healthcare, Tokyo, Japan) was used to visualize and capture images of bound antibodies.

2.5. miR-21—5p mimic transfection

Eight x 10^4 MC3T3-E1 cells were cultured in antibiotic-free medium. Twenty nM negative control or miR-21—5p mimic were transfected to MC3T3-E1 cells using the Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and cell lysates were collected for further analysis 48 h after transfection.

2.6. Histological staining

The femur tissues of WT, miR-21-5pKO and *Dec1*KO mice were collected and fixed with 4% paraformaldehyde for 24 h. 10% EDTA 2Na Solution (pH: 7.0, Muto Pure Chemicals Co., Ltd., Tokyo, Japan) was used to decalcify tissues. Hematoxylin-Eosin (H-E), Azan (Muto Pure Chemicals, Tokyo, Japan), Masson's Trichrome (ab150686, Abcam, Tokyo, Japan) and Toluidine blue (Wako, Tokyo, Japan) were used to observe histological changes. Images were captured using a microscope (OLYMPUS, Tokyo, Japan).

2.7. Immunohistochemistry (IHC)

Femur tissues were embedded in paraffin and 4 μm sections were subjected to antigen retrieval (pH 6.0, Abcam, Tokyo, Japan) and peroxidase blocking (DAKO, Santa Clara, CA, USA). Primary antibodies used for immunohistochemistry were anti-Alp (1:100, a gift from Prof. Norio Amizuka, Hokkaido University, Hokkaido, Japan), anti-Runx2 (1:125, Abcam, Tokyo, Japan) and anti-Osx (1:75, Abcam, Tokyo, Japan). Images were captured using a microscope (OLYMPUS, Tokyo, Japan).

2.8. Luciferase activity

TargetScan and miRDB were used to predict binding sites between miR-21—5p and Dec1. The mutant vector of Dec1 3′-UTR on the

seeding sequence was constructed according to specified base-pairing rules. HEK 293T cells (1.0×10^4 cells/well) were treated in 96-well plates with Lipofectamine™ 6000 (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions for transient transfection. The cells were co-transfected with the non-target control or the miR-21—5p mimics. Reporter assays used a Dual-Glo Luciferase Reporter Assay system (E1910; Promega Corp., Madison, WI, USA) at 48 h post-transfection. All experiments were performed in triplicate and means, and standard deviations were calculated.

2.9. Statistical analysis

Independent two-tailed Student's t-test or analysis of variance (ANOVA) was performed using SPSS 16.0 for statistical analysis. p values < 0.05 are considered statistically significant.

3. Results

3.1. The expression of Dec1 is inhibited during osteoblast differentiation

To detect the expression level of Dec1, MC3T3-E1 pre-

osteoblastic cells were cultured under osteogenic differentiation inducing conditions. RT-PCR and WB assays showed that the expression of Alp (Fig. 1A-C, D7: p > 0.05; D14: p < 0.001; D21: p < 0.001), Runx2 (Fig. 1A-D, D7: p > 0.05; D14: p < 0.05; D21: p < 0.01), Opn (Fig. 1A-C, D7: p < 0.01; D14: p < 0.001; D21: p < 0.001) and Osx (Fig. 1A-D, D7: p < 0.01; D14: p < 0.01; D21: p < 0.001) were gradually upregulated during osteoblast differentiation until 21 days. Further, the expression of miR-21—5p was gradually induced during osteogenesis (Fig. 1E, D7: p < 0.05; D14: p > 0.05; D21: p < 0.01). However, a significantly suppressed expression of Dec1 (Fig. 1F, D7: p < 0.001; D14: p > 0.001; D21: p < 0.01) was detected during osteoblast differentiation, which indicated a negative role for Dec1 in osteogenesis.

3.2. The overexpression of miR-21—5p suppresses Dec1 expression

The inverse expression patterns of miR-21—5p and Dec1 were found as described above, thus, to examine the effect of miR-21—5p on the Dec1 expression level, transfection of a miR-21—5p mimic was used. miR-21—5p was significantly upregulated after transfection of the miR-21—5p mimic (Fig. 1G, p < 0.001). In contrast, RT-PCR and WB assays confirmed that Dec1 was suppressed
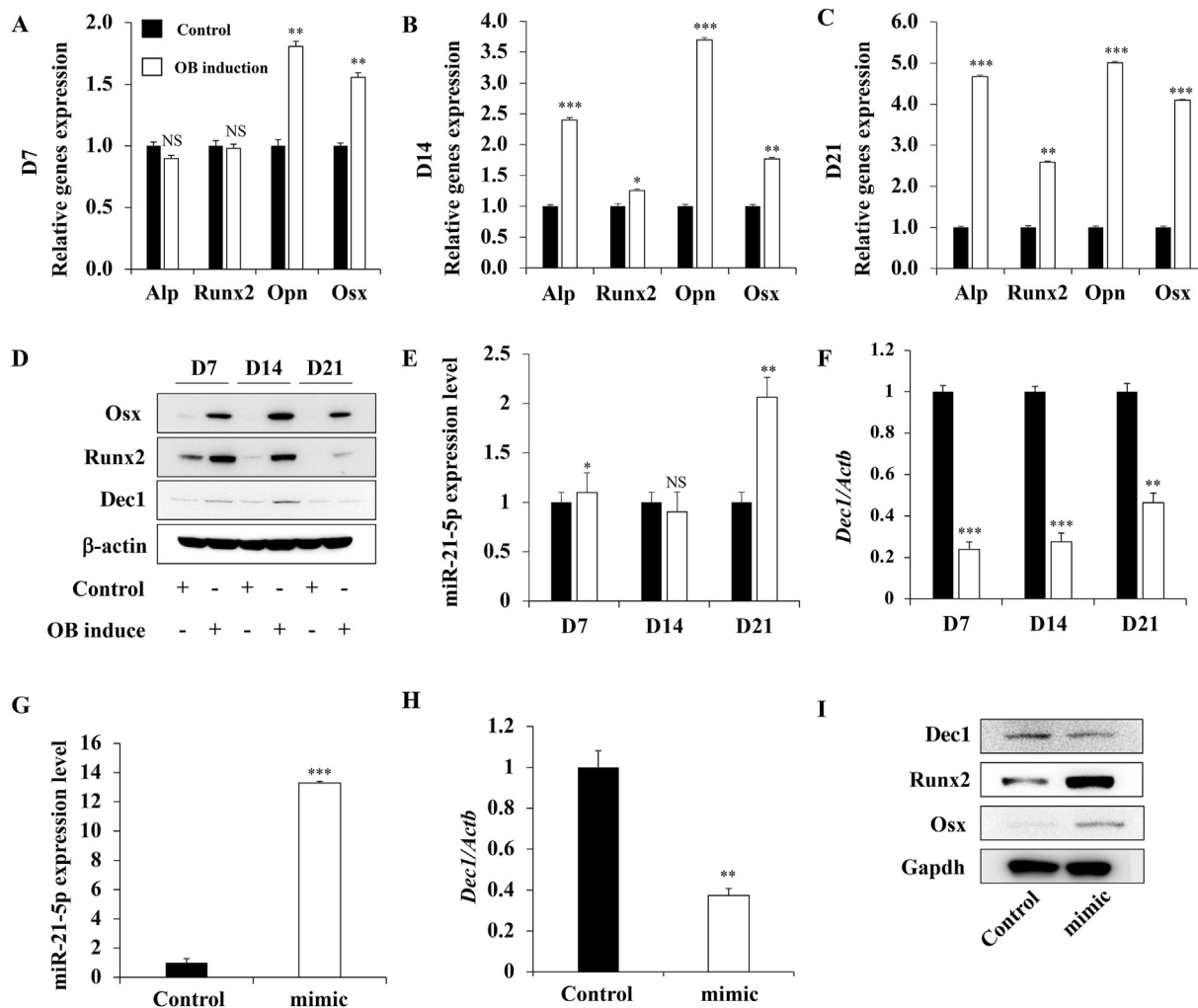

Fig. 1. miR-21—5p suppresses Dec1 expression during osteoblast differentiation. (A—D) The mRNA and protein expression levels of ALP, Runx2, Opn and Osx were gradually upregulated during osteoblast differentiation until 21 days. (E) The expression of miR-21—5p was also induced with osteogenesis. (F) The suppression of Dec1 expression was detected during osteoblast differentiation. (G) miR-21—5p was significantly upregulated after transfection with the miR-21—5p mimic. (H, I) RT-PCR and WB results confirmed that Dec1 was suppressed by miR-21—5p overexpression, followed by an increase of Runx2 and Osx. Data shown represent means ± SD; *p < 0.05, **p < 0.01, ***p < 0.001. All results are representative of at least three independent experiments.

significantly by the overexpression of miR-21—5p (Fig. 1H, p < 0.01), followed by increased levels of Runx2 and Osx (Fig. 1I).

3.3. Converse phenotypes of miR-21-5pKO and Dec1KO mice in the osteogenesis of femurs

To understand the osteogenesis of femur tissues in the absence of miR-21—5p and Dec1, miR-21-5pKO and *Dec1*KO mice were employed. Micro-CT showed that the miR-21—5p deficiency resulted in a low bone density compared with WT mice (Fig. 2A-G, **p < 0.01, ***p < 0.001). H-E staining, Masson's Trichome staining, Azan staining and Toluidine blue staining (Fig. 2H-J) revealed that miR-21-5pKO mice had reduced bone formation, whereas *Dec1*KO mice had enhanced osteogenesis of their femurs. IHC and RT-PCR analyses both confirmed that the expression level of osteoblast markers, which included Alp (Fig. 3A-C, Fig. 3D, *p < 0.05, **p < 0.01), Runx2 (Fig. 3A-C, Fig. 3E, *p < 0.05, **p < 0.01) and Osx (Fig. 3A-C, Fig. 3F, *p < 0.05, **p < 0.01), were all decreased in miR-21-5pKO mice but were induced in *Dec1*KO mice. Coincidently, the expression of miR-21—5p was significantly upregulated in *Dec1*KO mice (Fig. 3G, **p < 0.01), which implied an inverse regulation between miR-21—5p and Dec1.

3.4. Dec1 is a target of miR-21—5p

To investigate the potential direct interaction between miR-21—5p and Dec1, Targetscan and miRBD analyses were used to predict potential binding sites between miR-21—5p and the 3′UTR region of Dec1. A paired target region was identified that contained seven conserved target sites (Fig. 4A). A mutant Dec1 vector was constructed and after transfection, we found that miR-21—5p significantly decreased the luciferase activity of Dec1-WT (Fig. 4B, p < 0.05). These results demonstrated that the direct interaction between miR-21—5p and the 3'-UTR region of Dec1 inhibited the expression of Dec1.

4. Discussion

miRNAs are involved with a series of pathophysiological changes that regulate skeletal development and bone homeostasis [1,22]. Disruption of miRNA functions is closely related to disorders of the skeletal system and degenerative bone diseases [2,23], thus the crucial roles and regulation of individual miRNAs in osteogenesis are important to elucidate [24—26]. Multiple in vitro and in vivo studies have reported the functions of miRNAs in
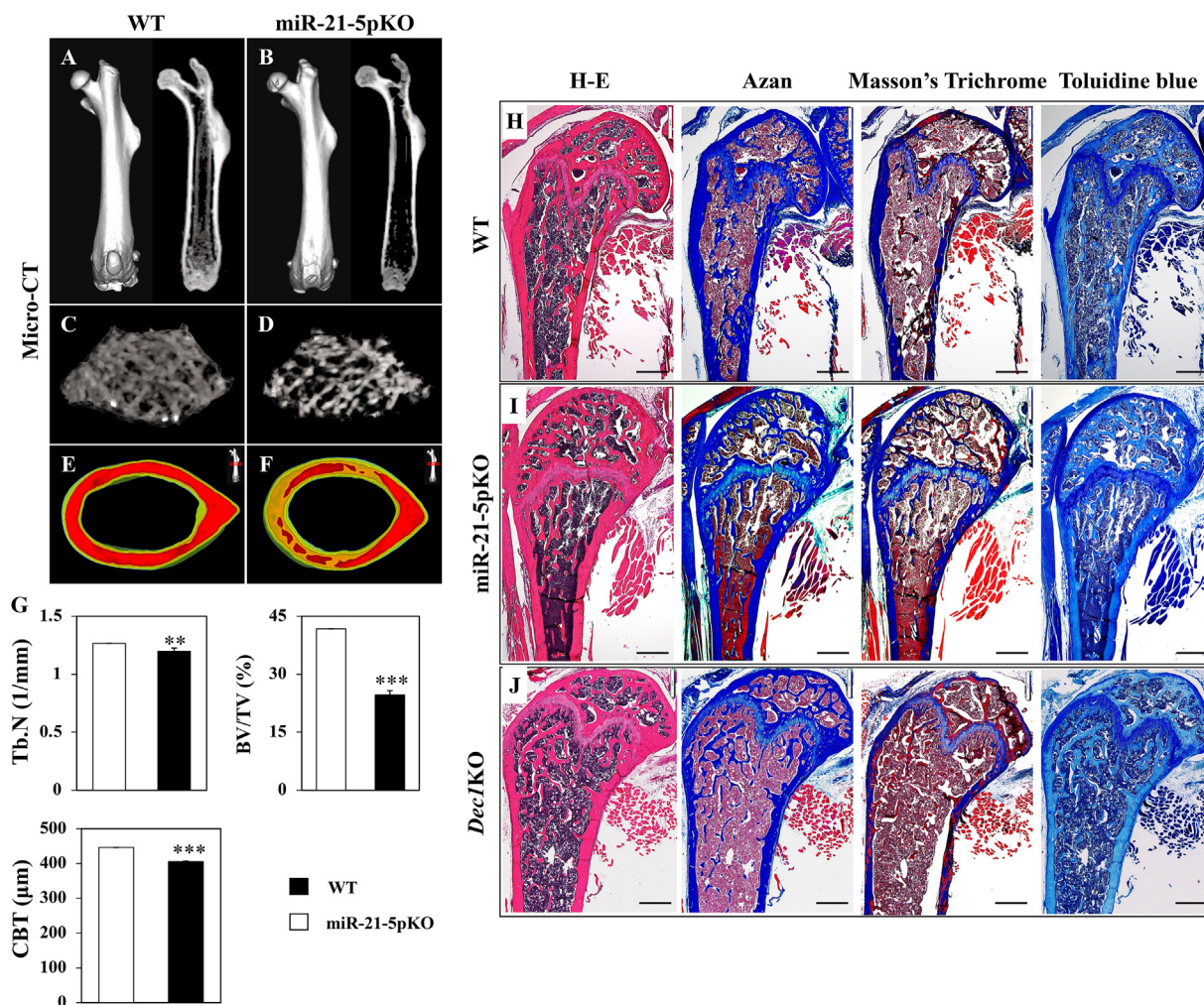

Fig. 2. Converse phenotypes of miR-21-5pKO and *Dec1*KO mice in the osteogenesis of femurs. (A—G) Micro-CT showing that a miR-21—5p deficiency in miR-21-5pKO mice causes a low bone density compared with WT mice. **(H—J)** H-E staining, Masson's Trichome staining, Azan staining and Toluidine blue staining reveal that miR-21-5pKO mice have reduced bone formation, whereas *Dec1*KO mice have enhanced osteogenesis of their femurs. Scale bar: 500 μm. Data shown represent means ± SD; **p < 0.01, ***p < 0.001. All results are representative of at least three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
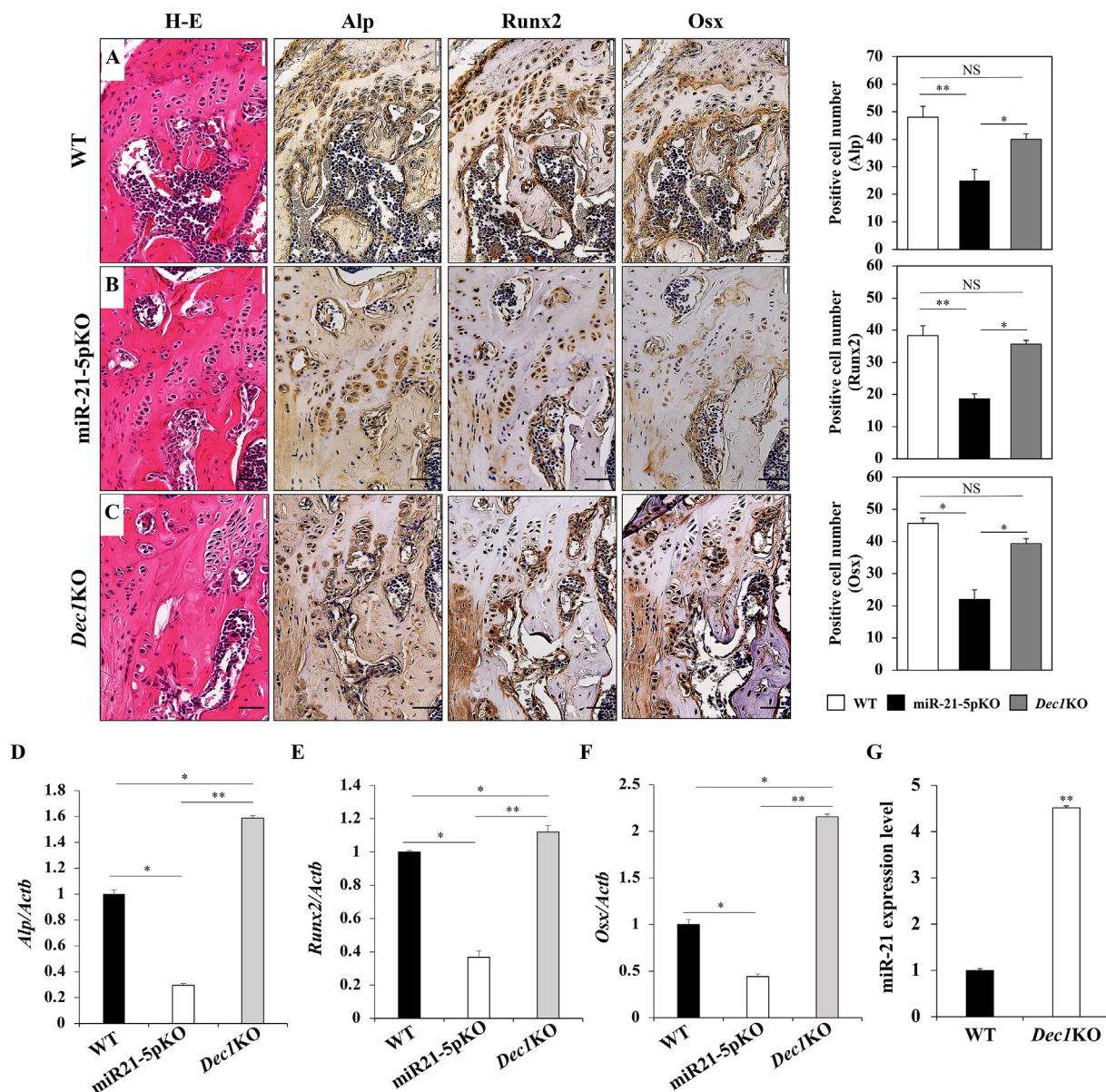
Fig. 3. Different patterns of osteogenic activity in miR-21-5pKO mice and in *Dec1*KO mice. (A−C) IHC results showing that a miR−21−5p deficiency reduces the osteoblast number, whereas a Dec1 deficiency promotes osteoblast differentiation. **(D−G)** mRNA expression levels of osteoblast markers, including Alp, Runx2 and Osx, were all decreased in miR-21-5pKO mice and were increased in *Dec1*KO mice. Scale bar: 50 μm. Positive cells were calculated, and data shown represent means ± SD; *p < 0.05, **p < 0.01. All results are representative of at least three independent experiments.

osteoblastogenesis [2,24,26,27]. miRNAs are mechanosensitive [28] and are altered to pivotal posttranscriptional regulators in the process of bone remodeling [22], thus revealing their potential values for basic research.

Here, we examined miR–21−5p, one of the most compelling miRNAs involved in the regulation of osteogenic properties. A miR-21−5p mimic was shown to prevent expression of the transcription factor Dec1, and induced levels of Runx2 and Osx proteins, which suggests that miR–21−5p functions upstream of osteogenesis proteins. A deficiency of miR–21−5p decreased the expression of osteogenesis-related proteins, resulting in reduced osteogenesis in *in vivo* studies. In addition, bioinformatics and dual luciferase reporter assays verified that Dec1 is a direct target gene of miR-21−5p, and that miR–21−5p negatively regulates Dec1. These findings, coupled with our in vivo experiments, explicitly suggest that miR–21−5p is an upstream target of Dec1 and acts as an

intermediary for osteoblastogenesis. To the best of our knowledge, this is the first study to define the physiological phenomenon of miR–21−5p and Dec1 controlling osteogenesis. These results elucidate the biological changes of miR–21−5p and deliver the first in vivo indication of a pro-osteoblastic miRNA that modulates the transcription factor Dec1 and ameliorates the regulation of osteoblasts.

The multifaceted miR–21−5p is engaged in a wide spectrum of pathophysiological regulatory activities [29,30]. *In vitro* findings revealed that miR–21−5p regulates the osteogenesis of stem cells [28,31]. Consequently, the influence of miR–21−5p regulates osteogenesis at different stages (D7, D14 and D21). The current findings are in accordance with several reports signifying the expanding role of miR–21−5p in bone formation.

Overexpression of miR–21−5p can elevate osteogenesis in vitro and can also suspend it in miR-21-5pKO mice [9]. Furthermore,
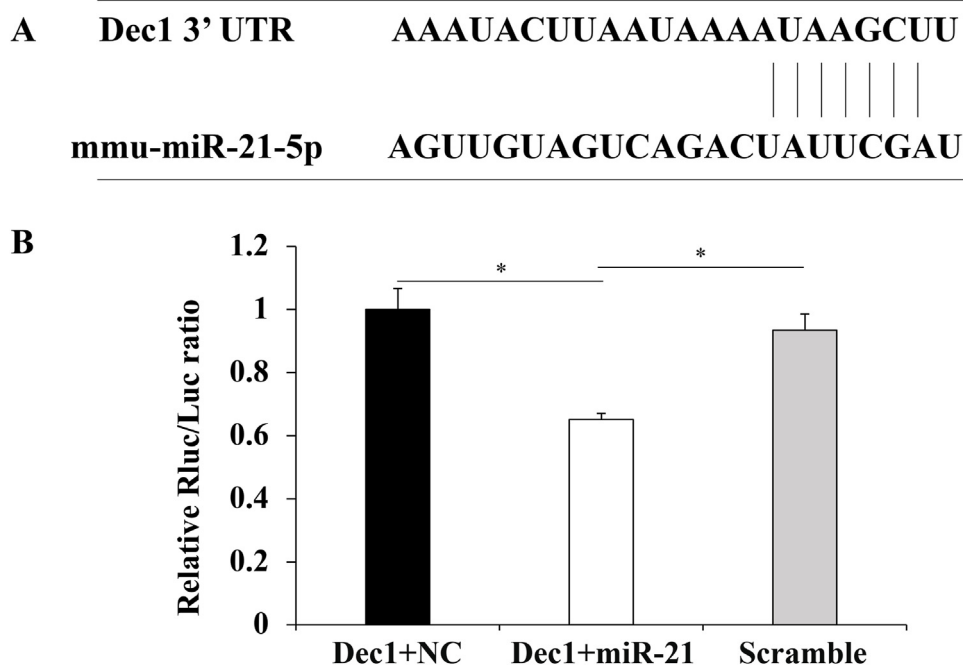
Fig. 4. Dec1 is a target of miR-21-5p. (A) Targetscan and miRBD were used to analyze the predicted binding sites between miR-21–5p and the 3′UTR region of Dec1. A paired target region was identified that contained seven conserved target sites. **(B)** A mutant Dec1 vector was constructed and after transfection, we found that miR-21–5p significantly decreased the luciferase activity of Dec1-WT. Data shown represent means ± SD; *p < 0.05. All results are representative of at least three independent experiments.

reduced bone formation was evident in miR-21-5pKO mice with calvaria bone defects in our previous study [8]. Bone marrow stromal cells of miR-21-5pKO mice have diminished osteogenic signals related to decreased Runx2 mRNA levels [8]. Further, miR-21–5p facilitates the osteogenesis of human umbilical cord mesenchymal stromal cells, thus activating Runx2 transcription [32]. Runx2 is instrumental in osteoblast differentiation [33]. In nonosteoblastic cells, the expression of Osteopontin, Osteocalcin and Bone sialoprotein were upregulated by the forced expression of Runx2 [34]. Osteopontin acts as a bone cell chemoattractant and is involved with the attachment of cellular and extracellular matrix of bone [35]. Osx is specifically expressed in osteoblasts and osteocytes, and conditional disruption of the Osx gene resulted in osteocyte dysfunction and altered the expression of Osx target genes [35]. In this study, western blot analysis demonstrated that Runx2 and Osx expression was apparently increased coupled with the miR-21–5p mimic in osteoblasts. Further, the experiments using miR-21-5pKO mice were highly informative and demonstrated that femurs of miR-21-5pKO mice had lower levels of Alp, Runx2 and Osx than the femurs of WT mice, which also emphasizes the importance of miR-21–5p to maintain osteogenesis. The present findings are in accordance with a recent study demonstrating that a miR-21–5p inhibitor transfected into BMSCs reduced Runx2 expression [8]. Our results open new avenues for additional mechanistic studies on osteoblast-specific loss or gain of miR-21–5p function.

Clarifying the miRNA pathways that lead to the up- or down-regulation of Dec1 expression in osteoblasts as well as identifying miRNAs regulated by Dec1, should contribute important insights into the molecular mechanism of osteoblast differentiation. Earlier investigations demonstrated Dec1 mRNA expression in multiple tissues [36]. Dec1 overexpression suppressed the growth of NIH3T3 cells [13,37]. In this study, osteoblasts in the differentiation group had higher levels of miR-21–5p expression compared to Dec1 (Fig. 1E–F), and crucially, those results were corroborated by the

miR-21–5p mimic treated osteoblasts (Fig. 1H-I). The in vitro and in vivo studies, along with the gain-of-function (miR-21–5p mimic) and loss-of-function (miR-21-5pKO) experiments have yielded mechanistic links between miR-21–5p and bone formation. Furthermore, the overexpression of miR-21–5p in uninduced osteoblasts downregulated Dec1 expression and upregulated the expression of several bone-related proteins, including Runx2 and Osx. To further demonstrate the roles of Dec1 in osteoblast regulation, we generated *Dec1*KO mice and interestingly observed that the in vivo deficiency of Dec1 in their femurs significantly improved the expression level of ALP, Runx2 and Osx, which provides the first evidence that Dec1 is important in osteoblast homeostasis. Dec1 may directly regulate the expression of Runx2. Previous studies demonstrated the involvement of Dec1 in Opn or Runx2 expression [12]. The transcriptional repressor Dec1 binds to the E-box or interacts with Sp1 of the target gene [38,39]. In fact, Sp1 targets the Runx2 promoter and provokes its transcription [40]. Taken together, these observations lead to the hypothesis that miR-21–5p and Dec1 constitute a regulatory axis in osteoblasts (Graphical figure, created in the https://app.biorender.com). Disturbance of that axis may be the mechanism involved in attention deficit and hyperactivity.

The present study has several limitations. First, although we originally intended to include as many time points as possible, only three time points were examined in this study. However, to minimize inconsistent and contradictory results, pre-osteoblast MC3T3-E1 cells were used for osteoblast differentiation in this study. Second, due to difficulties in obtaining appropriate specimens, only femur specimens from young mice (12 weeks old) were used in the animal experiments and levels of miR-21–5p and Dec1 expression in aged mouse femurs need to be evaluated. Third, young male mice were used in this study. Variable findings could have been observed if female animals respond differently, thus clarification of the comprehensive functions of miR-21–5p requires additional research. Fourth, conditional gene knockout mice must be used to

fully understand the diverse biological functions of miR-21–5p and Dec1. Finally, although the associations of miR-21–5p and Dec1 in bone modifications intriguingly suggest their possible implications in osteogenesis, our results did not identify those biological factors to unambiguously show they are only involved in osteogenesis, thus representing a limitation of our study. Our subsequent studies will focus on obtaining direct evidence of miR-21–5p and Dec1 using miR-21-5pKO and *Dec1*KO mice.

In conclusion, the present study recognized and experimentally established Dec1 as a potent target of miR-21–5p, implying that the former might be mechanistically involved in osteoblastogenesis. Furthermore, in miR-21-5pKO mice in vivo, the miR-21–5p deficiency reduced trabecular number, Bone volume/Tissue Volume, and attenuated osteoblast homeostasis, pointing to a mechanistic involvement of miR-21–5p in Dec1 function. The present findings extend our prior knowledge on miR-21–5p functions in osteogenesis, suggesting that it is a possible candidate for regulating bone formation. Additional experiments on the functional and physiological roles of miR-21–5p and its putative interactions with Dec1 are needed to understand the pathogenic mechanism of osteogenesis.

Author contributions

Conceptualization, T.K., X.L. and U.K.B.; writing - original draft preparation, X.L. and U.K.B.; writing - review and editing, T.K., X.L. and U.K.B.; visualization, T.K., X.L. and U.K.B.; supervision, X.L. and U.K.B.; project administration, X.L. and U.K.B. All authors have read and agreed to the published version of the manuscript.

Funding

Data availability statement

The datasets used and analyzed in this study are available from the corresponding authors (bhawal.ujjal.kumar@nihonu.ac.jp) on reasonable request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: UJJAL K. BHAWAL reports financial support was provided by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan. UJJAL K. BHAWAL reports financial support was provided by Nihon University Multidisciplinary Research Grant for 2018.

Acknowledgments

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