FEBS *Letters*

miR-145 suppresses osteogenic differentiation by targeting Sp7

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A B S T R A C T

Osteogenesis depends on a coordinated network of transcription factors including Sp7. Emerging evidence indicates that microRNAs (miRNAs) act as pivotal regulators in various biological processes including osteoblast proliferation and differentiation. Here, we investigated the effect of miR-145 on osteogenic differentiation. miR-145 was decreased during osteogenic differentiation, which could suppress the osteogenic differentiation of C_2C_{12} and MC3T3-E1 cells confirmed by gain- and loss-of-function experiments. Moreover, bioinformatic analysis combined with luciferase reporter assay, and Western blot validated that miR-145 negatively regulated Sp7 expression. Inhibition of Sp7 showed similar effect with miR-145 on osteogenic differentiation, whereas overexpression of Sp7 attenuated this effect. Collectively, these data indicate that miR-145 is a novel regulator of Sp7, and it suppresses the osteogenic differentiation of C_2C_{12} and MC3T3-E1 cells.

1. Introduction

Skeletal development and homeostasis depend on the activity of osteoblasts derived from mesenchymal stem cells. In addition, bone development is constantly remodeled throughout life required rigid control of a variety of gene expression and repression in response to physiological signals. Cohorts of tissue-specific transcription factors mediate the expression of genes required for osteoblast differentiation [1]. Three osteoblast-specific or highly expressed transcription factors, Runt-related transcription factor 2 (RUNX2), Sp7 (also named as Osterix), and activating transcription factor 4 (ATF4) act sequentially to regulate the differentiation from an osteochondral progenitor into a fully differentiated and functional osteoblast [2–4]. Especially, Sp7-null mice could not form mature osteoblasts [5].

MicroRNAs (miRNAs) are a large family of small non-coding (18–25 nucleotides) single-stranded RNAs, which mediate gene suppression by binding to the 3′ untranslated regions (3′ UTRs) of target mRNAs via either promoting degradation of target mRNAs or inhibiting the translation [6]. MiRNAs play essential roles in diverse biological processes, including cell proliferation, differentiation, apoptosis, and tumor oncogenesis [7–9]. Recently, numerous studies have revealed that miRNAs play critical roles in osteoblast differentiation. For example, miR-34s was reported to inhibit osteoblast differentiation by targeting Special AT-rich

sequence-binding protein 2 (SATB2) [10]. MiR-100 suppressed osteoblast differentiation by inhibiting bone morphogenetic protein receptor type II (BMPR2) [11]. MiR-133 and miR-135 functionally inhibit osteoprogenitor differentiation by abolishing Runx2 and SMAD family member 5 (Smad5) pathways which contributed to bone formation synergistically [12]. Sp7 has been recently identified as a key regulator of osteoblast differentiation, it was reported to be regulated by miR-93, miR-125b, miR-214, and miR-637 [13–16]. However, miRNAs that regulate Sp7 expression still require further investigation.

In this study, we characterized miR-145, a novel down-regulated miRNA [17], and investigated its effects on osteoblast differentiation. We identified Sp7 as its target, proposing a regulatory mechanism in which miR-145/Sp7 controls osteoblast differentiation. Our data shed new light on the roles of miRNAs in osteoblast differentiation.

2. Materials and methods

2.1. Reagents, antibodies, plasmids

Recombinant bone morphogenetic protein 2 (BMP2) was purchased from Invitrogen (CA, USA), Sp7-specific antibody was purchased from Abcam (Cambridge, UK), and anti-GAPDH antibody was purchased from Abcam. MiR-145 mimic/inhibitor and non-specific control were obtained from RiboBio (Guangzhou, China). Sp7 siRNA was purchased from Santa Cruz (CA, USA). Sp7-pcDNA3 plasmid was kindly provided by Prof. K. Watanabe. Lipofectamine 2000 was purchased from Invitrogen.

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2.2. Cell culture

C_2C_{12} and MC3T3-E1 cells were obtained from ATCC. C_2C_{12} cells were maintained in DMEM supplemented with 15% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin. MC3T3-E1 cells were maintained in α-modified Eagle's medium supplemented with 10% FBS. Cultured cells were incubated in a humid chamber containing 5% CO_2 at 37 °C.

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 µg total RNA using a Reverse Transcription Kit (TOYOBO, Tokyo, Japan) according to the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) was carried out using an ABI Stepone plus Real-Time PCR System. The following primers were used: GAPDH, forward: 5′-ACCACAGTCCATGCCATCAC-3′, reverse: 5′-TCCACCCTGTTGCTGTA-3′; alkaline phosphatase (ALP), forward: 5′-GACAAGAAGCCCTTCACTGC-3′, reverse: 5′-AGA-CTGCGCCTGGTAGTTGT-3′; collagen type I (COL1A1), forward: 5′-GAGAGCATGACCGATGGATT-3′, reverse: 5-ATGTAGGC-CACGCTGTTCTT-3′; osteocalcin (OC), forward: 5′-TGCTTGTGAC-GAGCTATCAG-3′, reverse: 5′-GAGGACAG GGAGGATCAAGT-3′; Sp7, forward: 5′-AGCGACCACTTGAGCAAACAT-3′, reverse: 5′-GCGGCTGATTGGCTTCTTCT-3′. MicroRNA was extracted with All-in-One microRNA extraction kit from GeneCopoeia (MD, USA). Primers for miR-145 and the endogenous control U6 were purchased from GeneCopoeia.

2.4. Western blot

Cells were lysed in RIPA buffer plus protease inhibitors (Roche, IN, USA). Equal amount of proteins were loaded, and separated on 10% SDS–PAGE and then transferred to a PVDF membrane (Millipore, MA, USA), blocked by incubation with 3% fat-free milk in TBS buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.6) at 25 °C for 1 h. The membranes were incubated with primary antibodies at 4 °C overnight, and then were incubated with horseradish peroxide-conjugated secondary antibodies at 25 °C for 1 h. The blots were developed with ECL reagent (Pierce, IL, USA).

2.5. Alkaline phosphatase and Alizarin Red stainings

C_2C_{12} cells seeded in 12-well plate were transfected with miR-145 inhibitor or the control using Lipofectamine 2000. 30 h after transfection, the cells were treated with BMP2 (5 ng/ml) for 4 d, and were fixed in 10% paraformaldehyde for 10 min at 25 °C. The cells were stained using 300 µg/ml BCIP/NBT buffer (Thermo, IL, USA) for 20 min at 25 °C. ALP-positive cells were stained blue/purple. For Alizarin Red staining, cells were stained with 2% Alizarin Red (pH 7.2) for 15 min. Orange and red bodies were identified as calcium nodules.

2.6. Luciferase assays

The 3′ UTR of Sp7 was amplified using the following primers: forward: 5′-CCCTCGAGATGATGACGGGTCAGGTA-3′, reverse: 5′-TTGCGGCCGCCCATTCTTCAGGAGGTGC-3′. The sequence was inserted into psiCHECK2 within XhoI and NotI restriction sites. Mutation in the miR-145 binding-sites module of Sp7 was introduced by whole-plasmid amplification in the seed region of miR-145 (NEB, Ipswich, Canada). C_2C_{12} cells were co-transfected with 0.5 µg of the reporter vector (psiCHECK-2, psiCHECK-2-Sp7-WT-3′ UTR or psiCHECK-2-Sp7-WT-3′ UTR) and 1 µg of miR-145 expression plasmid or the control vector. 48 h after transfection,

cells were harvested and luciferase activity was detected using a dual-luciferase reporter assay system (Promega, WI, USA).

2.7. Statistical analysis

Data are showed as mean ± S.D. Comparisons between groups were analyzed via Student t test using SPSS 16.0. Statistical significance was defined as P value <0.05.

3. Results

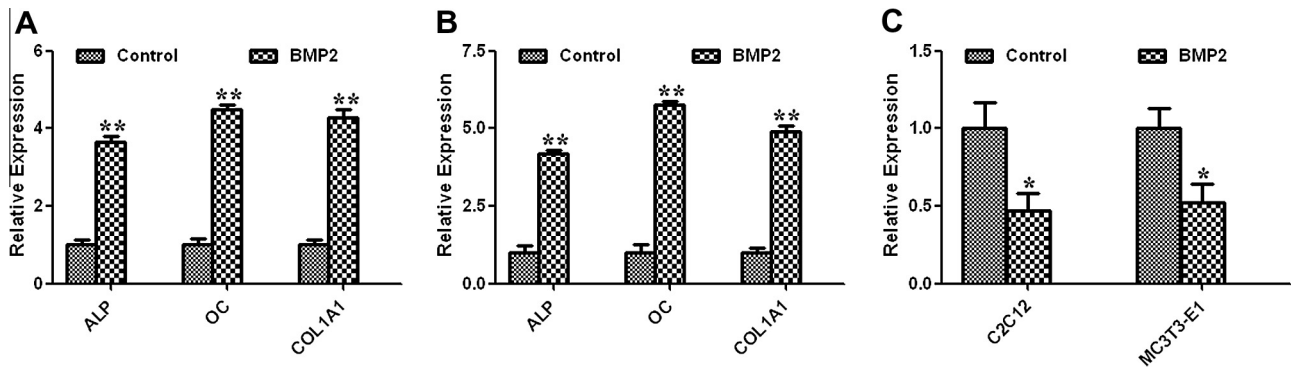## 3.1. miR-145 was decreased during osteogenic differentiation

We firstly examined the changes of miR-145 expression during osteogenic differentiation using qRT-PCR. C_2C_{12} and MC3T3-E1 cells were used as a cell model, and osteogenic differentiation was induced [18]. To induce the differentiation of C_2C_{12} cells, the medium wasreplaced with DMEM without serum, and treated with 2 nM BMP2. To induce the differentiation of MC3T3-E1 cells, the medium was replaced with α-modified Eagle's medium containing 10% FBS, 5 mM β-glycerophosphate, and 50 µM ascorbic acid. Several osteogenic factors, such as ALP, OC, and COL1A1 were used as phenotypic markers of osteogenic differentiation. As shown in Fig. 1A, there was a remarkable increase in ALP, OC, and COL1A1 mRNAs in C_2C_{12} cells following the BMP2 treatment, and similar results were observed in MC3T3-E1 cells (Fig. 1B), suggesting the successful induction of osteogenic differentiation. MiR-145 was remarkably decreased compared to the non-induced cells during osteogenic differentiation (Fig. 1C). These data suggest that miR-145 might play a role during osteogenic differentiation.
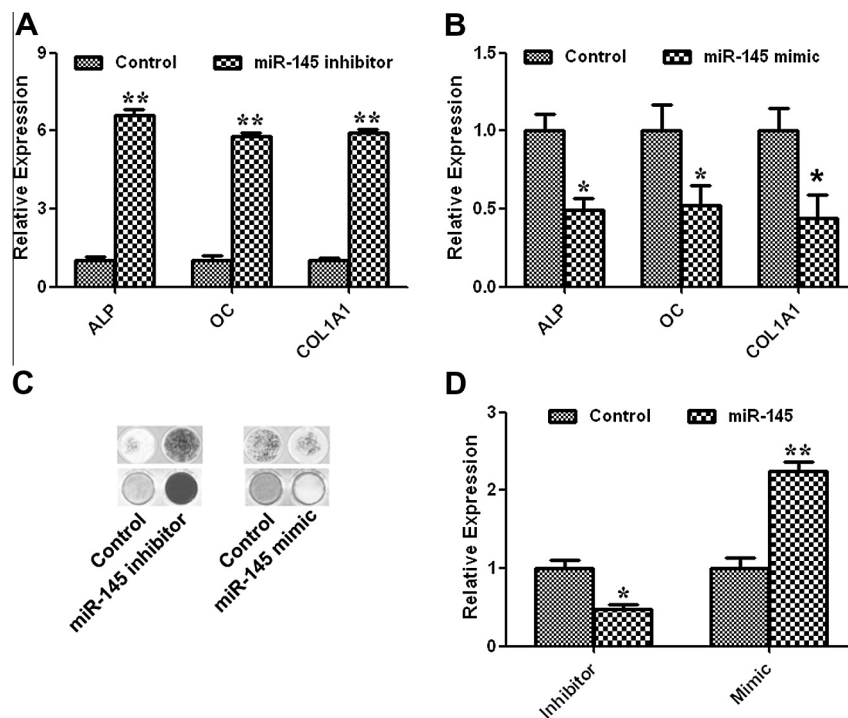
3.2. miR-145 suppressed osteogenic differentiation

We further investigated the role of miR-145 on osteogenic differentiation by gain- and loss- of function experiments. C_2C_{12} cells were transfected with miR-145 inhibitor or the control. 36 h after transfection, cells were incubated without serum for 24 h, and expression of ALP, OC, and COL1A1 was detected using qRT-PCR. All of the osteogenic differentiation markers measured were markedly increased following transfection of the miR-145 inhibitor (Fig. 2A). In contrast, C_2C_{12} cells transfected with miR-145 mimic showed decreased expression of osteogenic differentiation markers compared with those transfected with the control (Fig. 2B). Similarly, at 6 d of differentiation, ALP staining showed a significant lessened difference in miR-145 group compared with the control group, whereas, after inhibition of miR-145, the outcome was totally inversed (Fig. 2C upper). The Alizarin Red staining (ARS) (Fig. 2C lower) at 15 d showed the similar tendency at the matrix mineralization level. Efficiency of mi-145 mimic/inhibitor was determined by qRT-PCR (Fig. 2D). These results indicate that miR-145 could suppress osteogenic differentiation.

3.3. miR-145 inhibited Sp7 expression

miRNA target analysis tools TargetScan 6.2 and microrna.org were used to explore potential target of miR-145. Sp7 was predicted to be a target of miR-145 (Fig. 3A). To validate targeting of Sp7 by miR-145, we performed luciferase activity assay. The wild type Sp7 3′ UTR luciferase reporter plasmid (WT) or the mutant (Mut) was co-transfected with miR-145 mimic or the control into C_2C_{12} cells. The co-transfection of miR-145 with WT in C_2C_{12} cells showed significant inhibited luciferase activity compared with the control group, while Mut luciferase activity did not changed (Fig. 3B). Overexpression of miR-145 in C_2C_{12} cells significantly suppressed the protein level of Sp7, while inhibition of miR-145

Fig. 1. miR-145 was decreased during osteogenic differentiation. (A) C_2C_{12} cells were treated with BMP2 (2 nM) or the control for 24 h. Expression of ALP, OC, and COL1A1 was determined by qRT-PCR. (B) MC3T3-E1 cells were treated with BMP2 (2 nM) or the control for 24 h. Expression of ALP, OC, and COL1A1 was determined by qRT-PCR. GAPDH was used as the internal control. (C) The levels of miR-145 in BMP2-induced and non-induced (control) C_2C_{12} and MC3T3-E1 cells were measured using qRT-PCR. U6 was used as the internal control. The data were from three independent experiments ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$ compared with the control group.

Fig. 2. miR-145 suppressed osteogenic differentiation. (A) C_2C_{12} cells were transfected with miR-145 inhibitor (100 nM) or the control for 48 h. Expression of ALP, OC, and COL1A1 was determined by qRT-PCR. (B) C_2C_{12} cells were transfected with miR-145 mimic (100 nM) or the control for 48 h. Expression of ALP, OC, and COL1A1 was determined by qRT-PCR. GAPDH was used as the internal control. (C) ALP staining (upper) at 6 d and Alizarin Red staining (lower) at 15 d showed ALP activity and calcification of osteogenic differentiation after transfection with miR-145 mimic or miR-145 inhibitor compared with their separate negative control. (D) C_2C_{12} cells were transfected with miR-145 mimic/inhibitor (100 nM) or the control for 48 h. Expression of miR-145 was determined by qRT-PCR. U6 was used as the internal control. The data were from three independent experiments ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$ compared with the control group.

elevated the protein level of Sp7 (Fig. 3C). These results suggested that miR-145 could negatively regulate Sp7 expression.

3.4. miR-145 suppressed osteogenic differentiation by targeting Sp7

Since miR-145 could suppress osteogenic differentiation and inhibit Sp7 expression, we further explore whether inhibition of Sp7 by siRNA could also perform similar effect with miR-145 overexpression on osteogenic differentiation of C_2C_{12} cells. Effect of Sp7 siRNA was confirmed by qRT-PCR (Fig. 4A) and Western blot (Fig. 4B). As expected, C_2C_{12} cells transfected with Sp7 siRNA showed similar effect C_2C_{12} cells transfected with miR-145 mimic as showed in Fig. 2B (Fig. 4C). Furthermore, co-transfection of miR-

145 mimic with Sp7 overexpression plasmid could attenuate the effect of miR-145 on osteogenic differentiation of C_2C_{12} cells (Fig. 4D). These results suggested that miR-145 suppressed osteogenic differentiation of C_2C_{12} cells partially by inhibiting Sp7 expression.

4. Discussion

Bone homeostasis is balanced between bone formation and bone absorption by osteoblasts and osteoclasts respectively [19]. A growing body of evidence has revealed that miRNAs play critical roles in both normal biological processes and the pathogenesis of human diseases by post-transcriptionally regulating gene
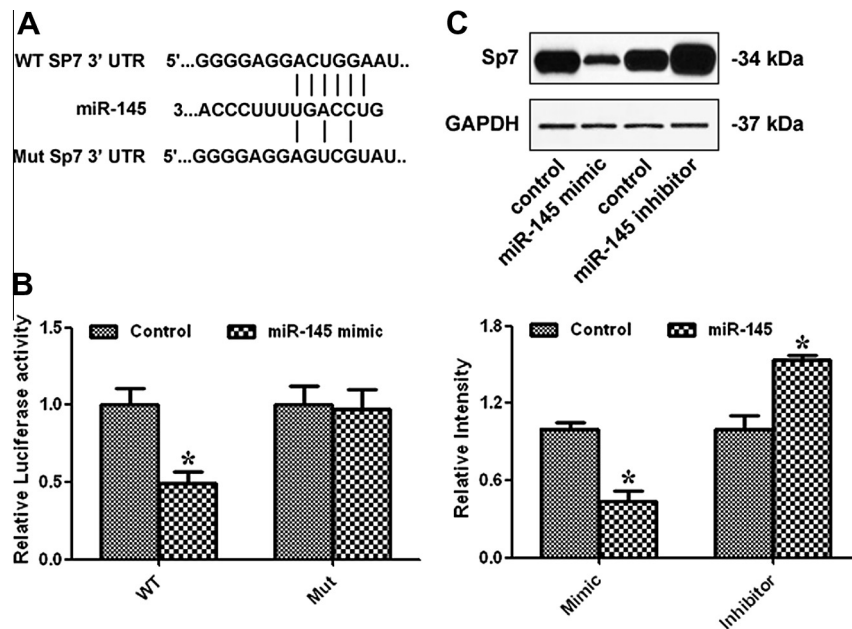
Fig. 3. miR-145 inhibited Sp7 expression. (A) Schematic diagram of miR-145 target site in the 3′ UTR of Sp7 mRNA and its mutated version. (B) The reporter construct containing WT 3′ UTR or Mut 3′ UTR was co-transfected with the miR-145 mimic or the control in C_2C_{12} for 24 h, and luciferase activity assay was performed. Relative Renilla luciferase activity was normalized to that of firefly luciferase. (C) C_2C_{12} cells were transfected with miR-145 mimic/inhibitor or the respective control for 48 h. Western blot was used to detect the protein level of Sp7. The data were from three independent experiments ($n = 4$). $^*P < 0.05$ compared with the control group.
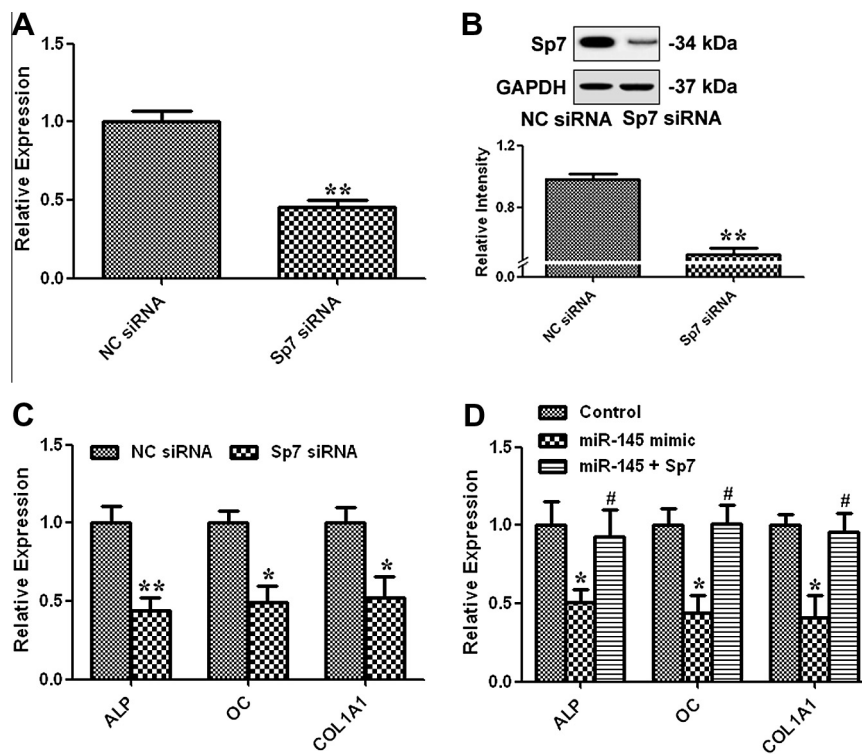
Fig. 4. miR-145 suppressed osteogenic differentiation by targeting Sp7. (A) C_2C_{12} cells were transfected with Sp7 siRNA or the control for 72 h. Expression of Sp7 was determined by qRT-PCR. (B) C_2C_{12} cells were transfected with Sp7 siRNA or the control for 72 h. Western blot was used to detect the protein level of Sp7. (C) C_2C_{12} cells were transfected with Sp7 siRNA or the control for 72 h. Expression of ALP, OC, and COL1A1 was determined by qRT-PCR. (D) C_2C_{12} cells were co-transfected with miR-145 mimic (100 nM) or the control with Sp7-pcDNA3 for 48 h. Expression of ALP, OC, and COL1A1 was determined by qRT-PCR. GAPDH was used as the internal control. The data were from three independent experiments ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$ compared with miR-145 mimic transfected group.

expression [20]. Dozens of miRNAs are considered essential for osteogenesis [21]. In the present study, we identified miR-145 as a negative regulator of osteogenic differentiation. Our data revealed that miR-145 was down-regulated during osteogenic differentiation of C_2C_{12} and MC3T3-E1 cells. Overexpression of

miR-145 inhibited osteogenic differentiation, whereas inhibition of miR-145 enhanced the osteogenic potential.

It has been reported that miR-145 was aberrantly expressed in several tumors and served as a tumor suppressor, and it suppresses cell growth and invasion by targeting a number of important genes

[22–24]. Here we found out a new role of miR-145 in the osteogenesis of C_2C_{12} and MC3T3-E1 cells. To study the molecular mechanism by which miR-145 regulates osteogenic differentiation, Targetscan and microrna.org predicted that Sp7 might be a possible target with 6 nt consecutive match site complementary to miR-145 in the 3′ UTR of Sp7. We showed that miR-145 overexpression resulted in suppression of Sp7 at the protein level, whereas functional inhibition of miR-145 led to elevation of Sp7, strongly suggesting that Sp7 was regulated by miR-145. Meanwhile, dual luciferase reporter assay also identified Sp7 as a direct target of miR-145. Sp7 supplement by Sp-pcDNA3, a Sp7 overexpression plasmid, could attenuate the effect of miR-145 on osteogenic differentiation, suggesting that miR-145 negatively regulated osteogenic differentiation might by inhibiting Sp7.

C_2C_{12}, a typical pluripotent mesenchymal precursor cell, has the potential to differentiate into osteoblasts, chondroblasts, and myoblasts [25,26]. BMP2 or other molecules direct the differentiation of C_2C_{12} cells into the osteoblast. Moreover, ALP, OC, and COL1A1 are typical osteoblast differentiation markers [18]. In this study, we used C_2C_{12} and MC3T3-E1cells to induce osteoblast differentiation, and the successful induction was revealed by the dramatic elevation of the osteoblast markers ALP, OC, and COL1A1. We found that the expression of miR-145 was decreased in osteogenic differentiation. Moreover, the transfection of miR-145 inhibitor led to an elevation in osteoblast markers. These data suggest that miR-145 might be involved in osteoblast differentiation negatively. Furthermore, ALP staining and ARS assays determined that miR-145 negatively regulated osteogenic differentiation of C_2C_{12} cells, suggesting that miR-145 might act as a suppressor in osteogenic differentiation.

Sp7 is a C2H2 zinc finger transcription factor that is essential for proliferation, osteoblast differentiation, and bone formation. Sp7 regulates the expression of several osteogenic factors, including ALP, OC, osteonectin, and osteopontin by binding to specific GC-rich sequences [5]. Osx is specifically expressed in osteoblasts and regulated by a number of miRNAs. Here, we demonstrated that miR-145 negatively target Sp7 and suppressed osteogenic differentiation.

In conclusion, our data indicate that miR-145 was a novel regulator of Sp7 and that it acted as a suppressor of osteogenic differentiation. Our findings shed new light on the roles of miRNAs in osteoblastdifferentiation.

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