MicroRNA-223 promotes osteoblast differentiation of MC3T3-E1 cells by targeting histone deacetylase 2

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Abstract. MicroRNAs (miRNAs) have emerged as pivotal regulators in various physiological and pathological processes at the post-transcriptional level, and may serve important roles in osteogenic differentiation. However, their roles and functions are not fully understood. In the present study, miR-223-5p was identified as a modulator of osteoblastic differentiation in MC3T3-E1 cells. Reverse transcription-quantitative polymerase chain reaction and western blotting demonstrated that miR-223-5p was significantly upregulated in MC3T3-E1 cells following the induction of osteoblast differentiation. Overexpression of miR-223-5p promoted osteogenic differentiation both *in vitro* and *vivo*. Expression of histone deacetylase 2 (HDAC2), which acts as a negative regulator of osteogenesis, was regulated by miR-223-5p. Collectively, the results of the present study revealed a novel miR-223-5p/HDAC2 axis that regulates osteoblast differentiation, and may serve as a potential target for enhancing bone formation *in vivo*.

Introduction

Osteoblasts are responsible osteogenesis and bone regeneration. The balance between osteoblasts and osteoclasts is crucial for the maintenance of normal morphology and bone tissue strength, in which osteogenic differentiation from mesenchymal stem cells serves an important role (1,2). A previous study demonstrated that disturbances in osteogenic differentiation leads to bone loss (3). This may result in health problems such as osteoporosis (OP) and osteoarthritis (4). Furthermore, bone defects following trauma due to the inhibition of osteogenic differentiation severely compromise patient quality of life (5). Therefore, understanding the molecular mechanisms associated with osteogenic differentiation is essential for the treatment of osteogenic disorders.

Non-protein coding RNAs encoded by the human genome are known to be important regulatory transcripts associated with multiple biological processes and pathologies (6). Among them, microRNAs (miRNAs/miRs) are small non-coding RNAs composed of 1-25 nucleotides. miRNAs regulate the expression of multiple genes by disrupting mRNA stability and/or inhibiting translation in conjunction with the RNA-induced silencing complex (6). An increasing number of miRNAs have emerged as pivotal regulators of osteoblast differentiation. miR-34a has been identified as an inhibitor of human stromal cell differentiation through targeting Jagged 1 and downregulating Notch signaling, resulting in bone formation abnormalities (7). miR-628-3p has been shown to regulate osteoblast differentiation by targeting runt-related transcription factor 2 (RUNX2), the master osteoblast transcription factor (8). miR-375 serves as a regulator of osteogenic differentiation in human adipose-derived stem cells, and overexpression of miR-375 promotes osteogenic differentiation via the Yes-associated protein 1/DEP domain containing mTOR-interacting protein/protein kinase B regulatory network (9). These findings highlight the essential role of miRNAs in the process of osteoblast differentiation. However, the number of miRNAs regulating osteoblast differentiation is predicted to be large, and further research focusing on the role of miRNAs in the regulatory mechanisms of osteogenesis is required.

miR-223 participates in multiple biological processes, including myocardial infarction, inflammation and cancer development (10-14). miR-223 may protect myocardial cells from hypoxia-induced apoptosis and excessive autophagy by targeting poly(ADP-ribose) polymerase 1 (10). In addition, the miR-223-5p/-3p duplex has been verified to cooperatively inhibit ischemia/reperfusion-induced cardiac necroptosis (11). miR-223 is involved in the regulation of neutrophil wound response and nuclear factor-κB activation by directly targeting Cullin1a/b, tumor necrosis factor receptor-associated factor 6, and transforming growth factor-β-activated kinase 1

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(MAP3K7)-binding protein 1 (12). miR-223-5p inhibits the migration and invasion of bladder cancer cells by regulating anillin actin-binding protein (13). Decreased miR-223 levels abate the osteogenic differentiation potential of bone marrow mesenchymal stromal cells derived from patients with multiple myeloma (14), suggesting that that miR-223 may serve as a regulator of osteogenic differentiation.

The present study demonstrated that miR-223-5p has critical function in osteoblast differentiation. miR-223-5p was upregulated during consecutive osteogenic induction, and promoted osteogenic differentiation. Furthermore, histone deacetylase 2 (HDAC2) was identified as a target of miR-223-5p, and downregulation of HDAC2 by miR-223-5p induced osteoblast differentiation. Therefore, miR-223-5p may represent a potential therapeutic target for bone regeneration-associated diseases.

Materials and methods

Cell culture and osteogenic differentiation. MC3T3-E1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and routinely maintained in growth medium (GM) consisting of α-modified Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37˚C in a humidified atmosphere with 5% CO_2. To induce osteogenic differentiation, the cells were incubated with osteogenic medium (OM), containing 10 ng/ml β-glycerophosphate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 10^{-7} mmol/l dexamethasone (Sigma-Aldrich; Merck KGaA), and 50 μg/ml vitamin C (Sigma-Aldrich; Merck KGaA) for 7 or 14 days. Next, the induced cells were digested for further detection. For the CAY10683 treatment, CAY10683 (Selleck Chemicals, Houston, TX USA) was added to the OM at concentrations of 0.01, 0.1 and 1 μM for 7 days. CAY10683 was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA).

Cell transfection. miR-223-5p mimics, inhibitor and their corresponding negative controls (NCs) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences were as follows: miR-223-5p mimic, 5'-CGUGUA UUUGACAAGCUGAGUUG-3'; miR-223-5p inhibitor, 5'-CAA CUCAGCUUGUCAAAUACACG-3'; mimics-NC, 5'-UUU GUACUACACAAAAGUACUG-3'; and inhibitor-NC, 5'-UUU GUACUACACAAAAGUACUG-3'. Small interfering (si)RNAs against HDAC2 (5'-AAGCCUCAUAGAAUCCGCAUG-3') and plasmid overexpressing HDAC2 (HDAC2-pcDNA3.1) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). MC3T3-E1 cells were cultured in six-well plates at a concentration of $2x10^5$ cells/well. When cells reached 70-80% confluence, mimics (50 nM), inhibitor (25 nM), siRNAs (25 nM) or plasmids (2.5 μg/ml) were transfected into cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h incubation, cells were subjected to further experiments.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the reverse transcription reaction was performed with 1 μg total RNA using a PrimeScript™ RT reagent kit according to the manufacturer's protocol. RT-qPCR was conducted with SYBR-Green I (both Takara Bio, Inc., Otsu, Japan) on the LightCycler 480 II system (Roche Diagnostics, Basel, Switzerland). The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 36 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 15 sec and extension at 72˚C for 15 sec. Primer sequences were as follows: miR-223-5p, 5'-CGCGCGTGTATTTGACA AGC-3', and 5'-AGTGCAGGGTCCGAGGTATT-3'; U6, 5'-CTCGCTTCGGCAGCACA-3', and 5'-AACGCTTCACGA ATTTGCGT-3'; Alkaline phosphatase (ALP), 5'-TGGCTC TGCCTTTATTCCCTAGT-3', and 5'-AAATAAGGTGCT TTGGGAATCTGT-3'; Osteocalcin (OCN), 5'-GCCATCA CCCTGTCTCCTAA-3', and 5'-GCTGTGGAGAAGACACA CGA-3'; RUNX2, 5'-GCCGGGAATGATGAGAACTA-3', and 5'-GGTGAAACTCTTGCCTCGTC-3'; HDAC2, and 5'-GCTATTCCAGAAGATGCTGTTC-3', 5'-GTTGCTGAG CTGTTCTGATTTG-3'; β-actin, 5'-TCACCCACACTGTGCC CAT-3', and 5'-CTCTTGCTCGAAGTCCAGGG-3'. The $2^{-\Delta\Delta Cq}$ method was used to quantify mRNA and miRNA expression. Data were normalized to β-actin (mRNA) or U6 (miRNA).

Measurement of alkaline phosphatase (ALP) activity. Cultured cells were rinsed in PBS three times, and total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich; Merck KGaA) and quantified with a bicinchoninic acid (BCA) protein assay. ALP activity was measured with the Alkaline Phosphatase, Diethanolamine Detection kit (cat. no. AP0100; Sigma-Aldrich; Merck KGaA). Equal volumes of cell lysate (50 μl) were added to each well of the 96-well plates, and incubated with an ALP staining solution at 37˚C for 1 h. Following the addition of stop solution, ALP activity was measured spectrophotometrically at 405 nm, and normalized to total protein concentration.

Alizarin Red S staining. Mineralization was determined by Alizarin red S staining. Cells were plated in six-well plates ($5x10^5$ cells/well), fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 0.1% Alizarin red staining solution (pH 4.2; Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. The cells were washed with PBS, and images were captured under a light microscope (x200).

Western blotting. The cells were harvested and lysed with RIPA lysis buffer containing phenylmethane sulfonyl fluoride and protease inhibitors (both Sigma-Aldrich; Merck KGaA). The concentration of each sample was determined by a BCA protein assay. Equal amounts of protein (30 μg/lane) were loaded and separated by 10% SDS-PAGE, followed by transfer onto 0.4 μm polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 1 h, and incubated with different primary antibodies overnight at 4˚C. The primary antibodies included anti-ALP rabbit polyclonal antibody (1:1,000; cat. no. 11187-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), anti-OCN rabbit polyclonal antibody (1:500; cat. no. 23418-1-AP; ProteinTech Group, Inc.), anti-RUNX2 rabbit polyclonal antibody (1:800;

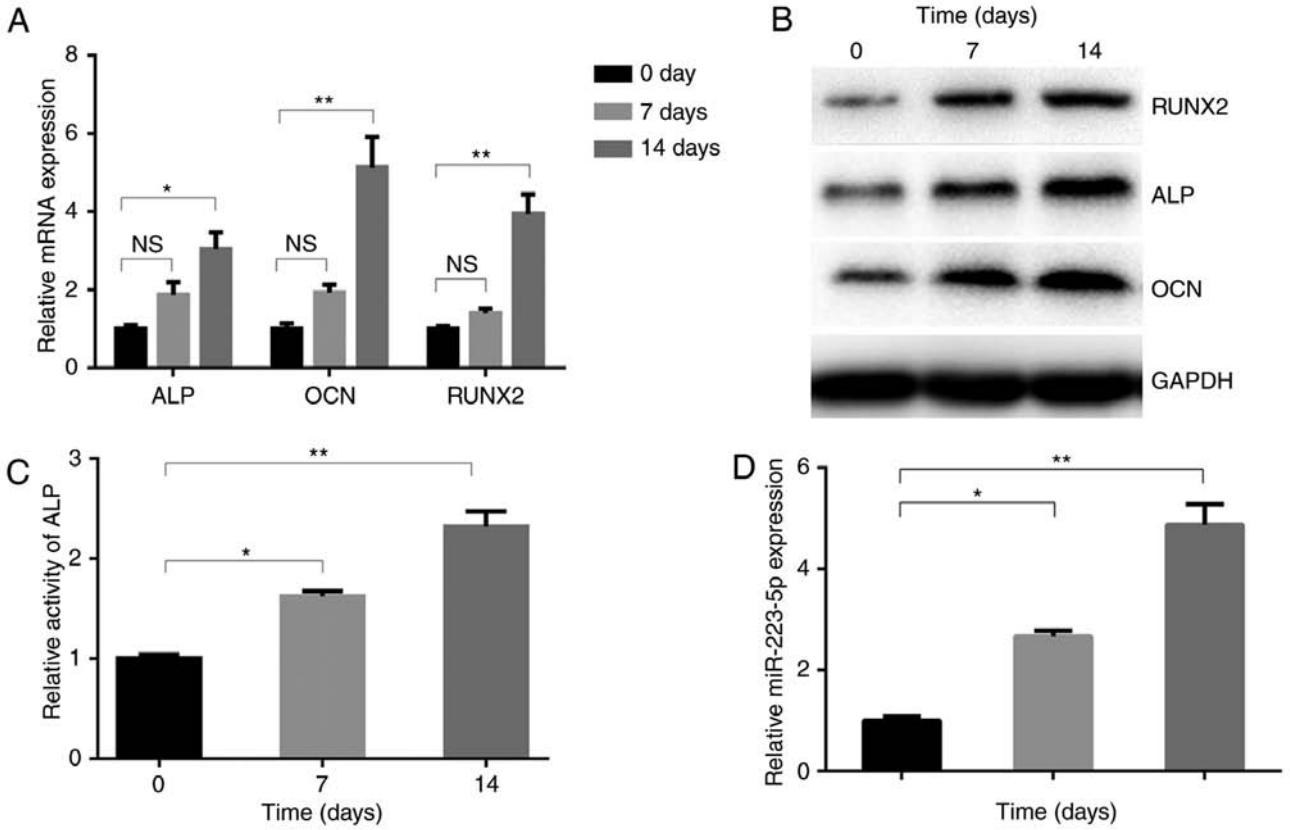

Figure 1. miR-223-5p is upregulated in osteogenic differentiation. (A) Osteogenesis-associated gene and (B) protein expression, including ALP, OCN and RUNX2, in MC3T3-E1 cells following treatment with OM for 0, 7 and 14 days. (C) ALP activity and (D) the expression of miR-223-5p was assayed by RT-qPCR at 0, 7 and 14 days after OM treatment. *P<0.05, **P<0.01. OM, osteogenic medium; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ALP, alkaline phosphatase; OCN, osteocalcin; RUNX2, runt-related transcription factor 2; NS, not significant.

cat. no. 12556; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-HDAC2 rabbit polyclonal antibody (1:1,000; cat. no. 12922-3-AP; ProteinTech Group, Inc.), and anti-β-actin rabbit polyclonal antibody (1:2,000; cat. no. 20536-1-AP; ProteinTech Group, Inc.). Membranes were next probed with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.), and protein signals were obtained with enhanced chemiluminescence plus substrate (EMD Millipore).

In vivo transplantation. All animal procedures were approved by the Animal Care Committee of Southern Medical University. Healthy female NOD/SCID mice (5 weeks old; ~20 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China) were randomly divided into two groups, with six mice per group. Transfected cells ($5x10^6$) were loaded onto 20 mg hydroxyapatite-tricalcium phosphate scaffold (HA-TCP; Sigma-Aldrich; Merck KGaA) and subcutaneously implanted into the dorsal region of NOD/SCID mice under anesthesia. After 4 weeks, xenografts were removed, fixed with 4% paraformaldehyde for 2 days at room temperature and decalcified in 10% EDTA (pH 6.0) for another 7 days at room temperature. Xenografts were then embedded in paraffin, sectioned at 4 μm thickness and stained with hematoxylin and eosin (H&E; Beyotime Institute of Biotechnology, Shanghai, China), or Masson's Trichrome stain (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocols.

Statistical analysis. All data were presented as the mean ± standard deviation. Statistical analyses were performed with SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA). The significance of mean values between two groups was analyzed using a two-tailed unpaired Student's t-test. Differences in multiple groups were determined by one-way analysis of variance with subsequent Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-223-5p is upregulated in osteogenic differentiation. To investigate the role of miR-223-5p in osteogenic differentiation, the present study detected its dynamic expression profiles in MC3E3T1 cells incubated with osteogenic inducers. The expression levels of osteogenesis-associated genes, including ALP, OCN and RUNX2 were significantly upregulated under differentiation-inducing conditions. On day 14, they had increased by 3 (ALP), 5.1 (OCN) and 3.9 (RUNX2) fold, compared with day 0 (Fig. 1A). The upregulation of RUNX2, ALP and OCN protein was also confirmed by western blotting (Fig. 1B). Additionally, the activity of ALP, an indicator of mineralization induced by osteogenic differentiation, was promoted following induction (Fig. 1C). Furthermore, miR-223-5p expression in MC3T3-E1 cells gradually increased during osteogenic differentiation, reaching a >4 fold increase at day 14, compared with day 0 (Fig. 1D), indicating the potential involvement of miR-223-5p in osteogenic induction.

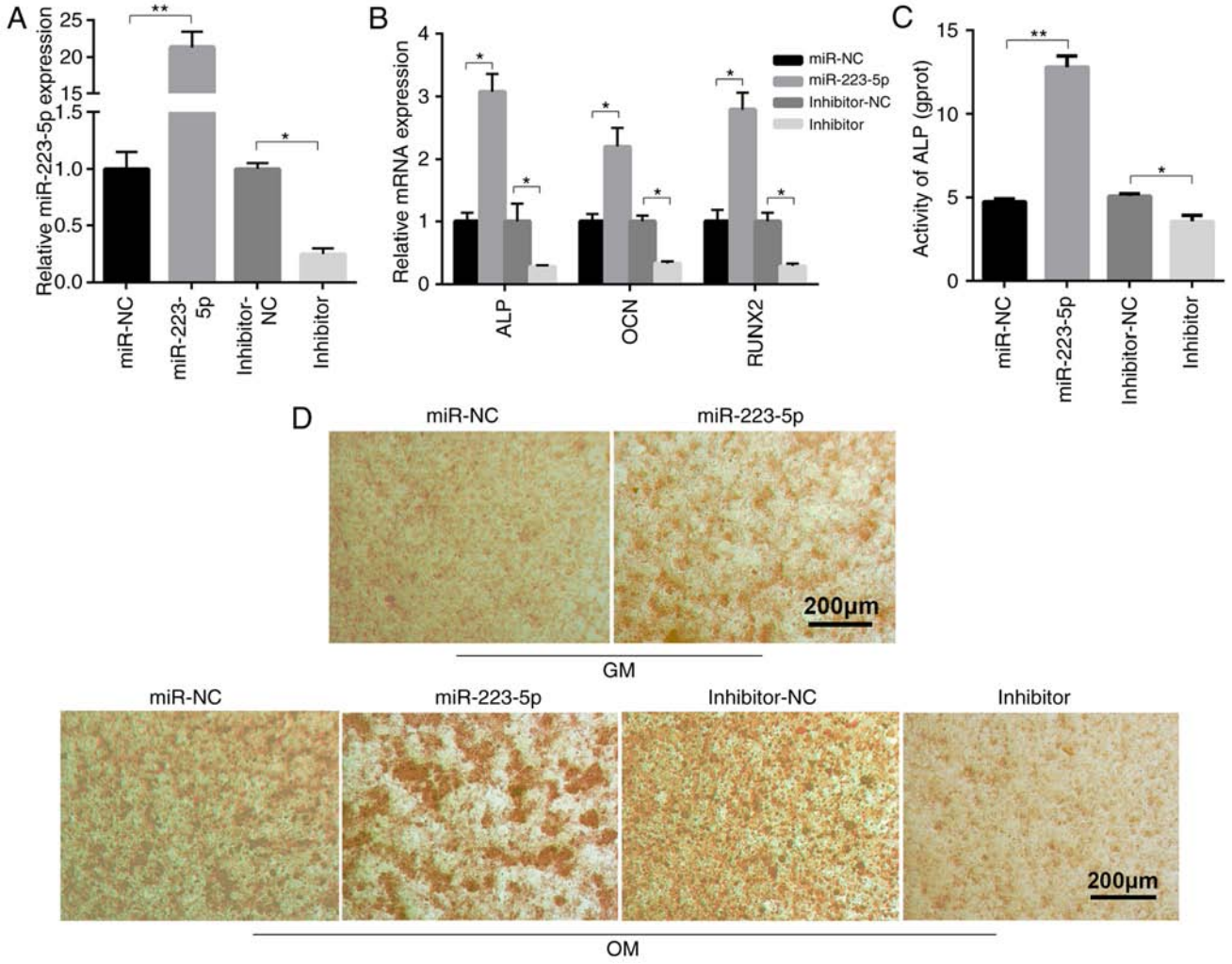

Figure 2. miR-223-5p promotes osteogenic differentiation. (A) miR-223-5p expression was validated in MC3T3-E1 cells transfected with miR-223-5p mimics or inhibitor and their corresponding controls by RT-qPCR. (B) ALP, OCN and RUNX2 expression and (C) ALP activity was measured in transfected MC3T3-E1 cells after 7 days of OM treatment. (D) Alizarin red S staining was also performed after 7 days of OM treatment. *P<0.05, **P<0.01. miR, microRNA; ALP, alkaline phosphatase; OCN, osteocalcin; RUNX2, runt-related transcription factor 2; OM, osteogenic medium; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

miR-223-5p contributes to osteogenic differentiation and mineralization. To clarify the biological roles of miR-223-5p in osteogenic differentiation, miR-233-5p overexpression and inhibition was induced in MC3T3-E1 cells. The effect of miR-223-5p and HDAC2 on osteogenic differentiation can be detected in 7 days OM treatment. The expression of ALP, OCN and RUNX2 increased in 7 days OM treatment although they had no statistical significance. While miR-223-5p treatment promoted the expression of ALP, OCN and RUNX2 after 7 days of OM treatment, which was confirmed by RT-qPCR. miR-223-5p overexpression and silencing was shown to be successful by RT-qPCR (Fig. 2A). miR-223-5p mimic transfection markedly increased the expression of ossification-associated genes, compared to transfection with negative control (NC) miRNA mimics. In contrast, inhibiting miR-223-5p expression significantly reduced ossification-associated gene expression (Fig. 2B). Further results revealed that ALP activity in the differentiated MC3T3-E1 cells was inhibited by transfection with miR-223-5p mimics, but enhanced by the miR-223-5p inhibitor, compared with the corresponding NC group (Fig. 2C). The degree of cell mineralization, as determined by Alizarin red S staining, increased following miR-223-5p mimic transfection compared with the control group in MC3T3-E1 cells cultured with either GM or OM medium. miR-223-5p inhibitor reduced osteogenic differentiation, as shown by the notable reduction in mineralization nodules (Fig. 2D). Together, these results indicated that miR-223-5p served an important role in osteogenic differentiation and mineralization.

HDAC2 is a negative target of miR-223-5p and is involved in osteogenic differentiation. It has previously been reported that HDAC2 is a downstream target of miR-223-5p in chronic obstructive pulmonary disease (15), but their relationship in osteogenic differentiation remains to be elucidated. In the present study, it was investigated whether miR-223-5p regulated HDAC2 expression. RT-qPCR and western blotting demonstrated that HDAC2 gene expression was reduced by 70% of the NC group in the presence of miR-223-5p mimics, and increased >3 fold when transfected with miR-223-5p inhibitor, compared with the inhibitor NC group (Fig. 3A). HDAC2

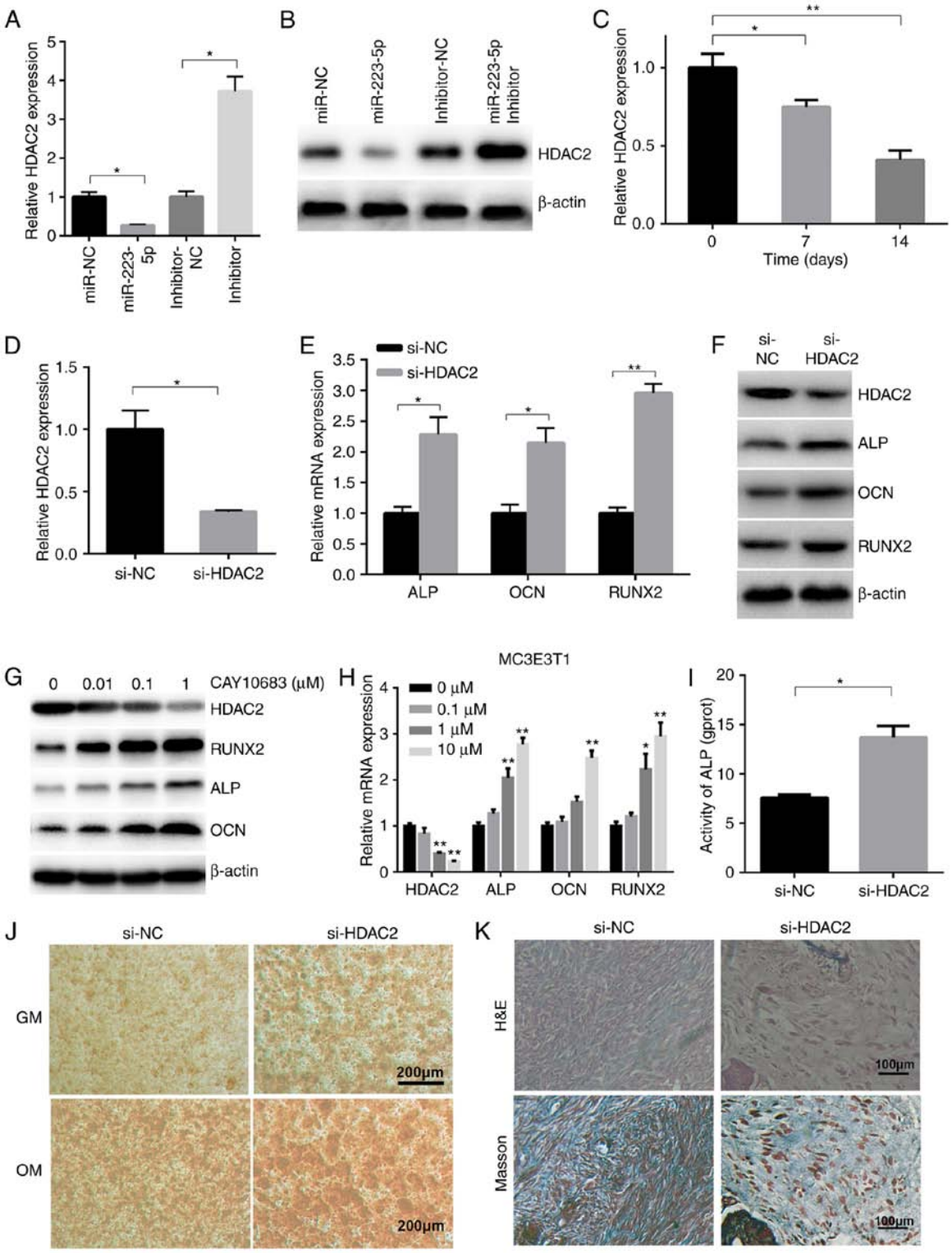

Figure 3. HDAC2 is a target of miR-223-5p and is involved in osteogenic differentiation. (A) The gene and (B) protein expression of HDAC2 was detected in transfected MC3T3-E1 cells. (C) After 0, 7 and 14 days of OM treatment, HDAC2 expression was determined by RT-qPCR. (D) Silencing of HDAC2 expression was validated in MC3T3-E1 cells transfected with si-HDAC2 or NC by RT-qPCR. (E) ALP, OCN and RUNX2 mRNA, as well as (F) ALP, OCN, RUNX2 and HDAC2 protein expression, was detected in MC3T3-E1 cells transfected with si-HDAC2, 7 days after osteogenic induction. *P<0.05, **P<0.01. (G) ALP, OCN, HDAC2 and RUNX2 mRNA and (H) protein expression was also detected in MC3T3-E1 cells treated with OM and CAY10683 at the indicated concentrations for 7 days. **P<0.01 vs. 0 μM group. (I) ALP activity in si-HDAC2-transfected MC3T3-E1 cells after 7 days of OM treatment. (J) The osteogenic differentiation of MC3T3-E1 cells transfected with si-HDAC2 was determined by Alizarin Red S staining. (K) Osteogenic differentiation of xenografts was determined by H&E and Masson's Trichrome staining. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HDAC2, histone deacetylase 2; ALP, alkaline phosphatase; OCN, osteocalcin; RUNX2, runt-related transcription factor 2; OM, osteogenic medium; H&E, hematoxylin and eosin.

protein expression was also decreased in miR-223-5p-overexpressing MC3T3-E1 cells, and increased in MC3T3-E1 cells with downregulated miR-223-5p (Fig. 3B). RT-qPCR revealed that HDAC2 mRNA expression markedly decreased during

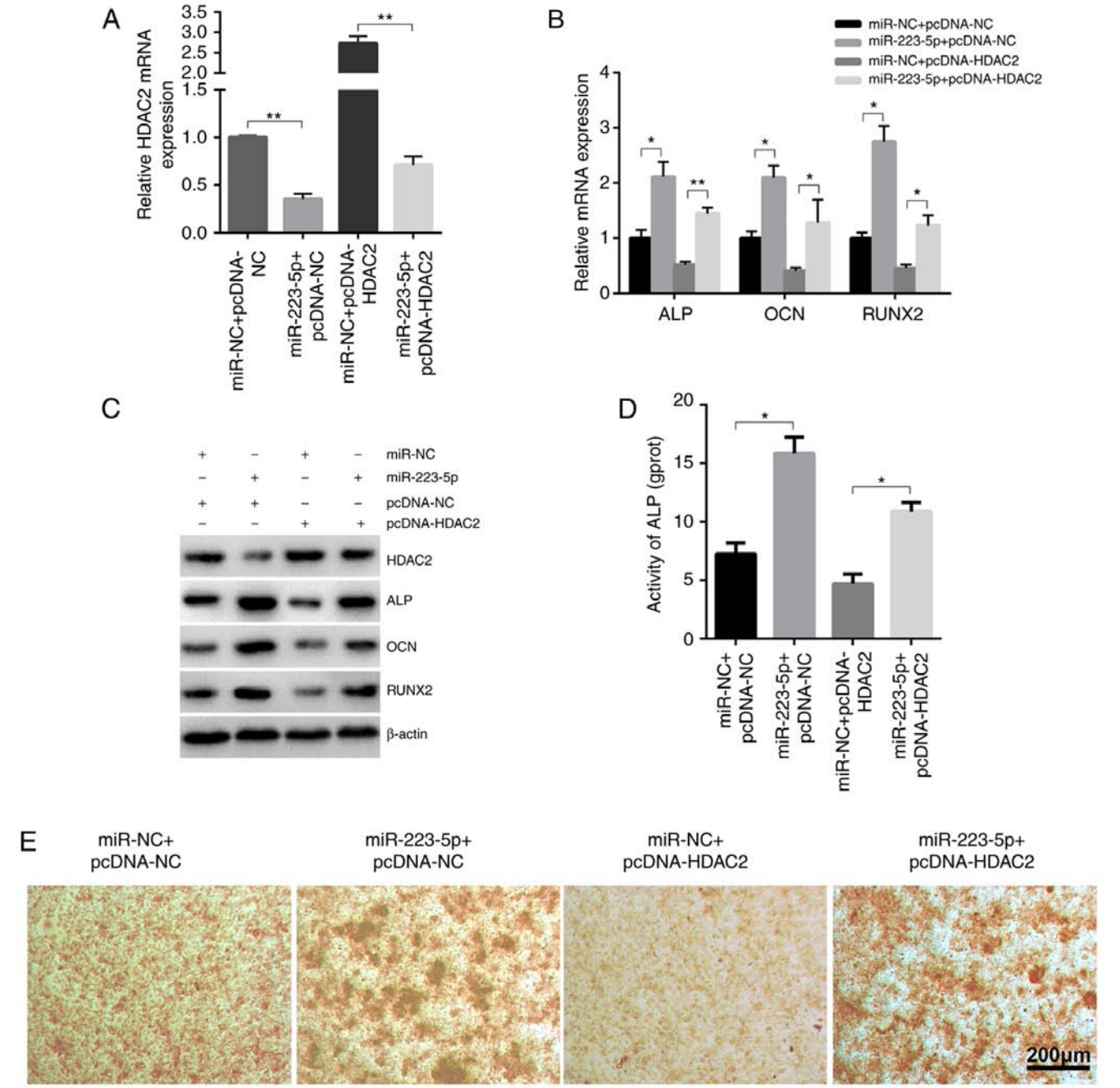

Figure 4. HDAC2 overexpression attenuates the effects of miR-223-5p in osteogenic differentiation. (A) mRNA expression of HDAC2 in MC3T3-E1 cells co-transfected with miR-223-5p mimics and/or pcDNA-HDAC2. (B) The mRNA and (C) protein expression of ALP, OCN and RUNX2 was assessed in MC3T3-E1 cells co-transfected with miR-223-5p mimics and/or pcDNA-HDAC2. (D) ALP activity assay and (E) Alizarin Red S staining of MC3T3-E1 cells co-transfected with miR-223-5p mimics and/or pcDNA-HDAC2. *P<0.05, **P<0.01. miR, microRNA; HDAC2, histone deacetylase 2; ALP, alkaline phosphatase; OCN, osteocalcin; RUNX2, runt-related transcription factor 2.

osteoblast differentiation (Fig. 3C). To evaluate the role of HDAC2 in osteoblast differentiation, the present study transfected MC3T3-E1 cells with si-HDAC2 (Fig. 3D). ALP, OCN and RUNX2 mRNA (Fig. 3E) and protein (Fig. 3F) expression was increased in cells transfected with si-HDAC2. Consistently, CAY10683, a HDAC2 inhibitor, induced a dose-dependent decrease in HDAC2 expression, and a consequent increase in osteogenic-associated gene and protein expression (Fig. 3G and H). Furthermore, si-HDAC2 transfection increased ALP activity, compared with the NC group (Fig. 3I). Alizarin red S staining also revealed that HDAC2 silencing increased mineralized bone matrix formation (Fig. 3J). Next, MC3E3T1 from control groups and si-HDAC2 groups were loaded onto HA-TCP and then implanted into NOD/SCID mice. The results showed that the MC3E3T1 in the si-HDAC2 groups group formed more osteoids than those in the negative control groups (Fig. 3K).

To further determine the role of HDAC2 in miR-223-5p-mediated osteogenic differentiation, HDAC2-overexpressing and control groups were established by transfecting pcDNA3.1-HDAC2 plasmid and pcDNA3.1-NC, which were then co-transfected with miR-223-5p mimics or

mimics-NC (Fig. 4A). The upregulating effects of miR-223-5p on the expression of ossification-associated genes were markedly inhibited by HDAC2, as confirmed by RT-qPCR (Fig. 4B), western blot analysis (Fig. 4C), ALP activity (Fig. 4D) and Alizarin red S staining (Fig. 4E). Collectively, these findings suggested that HDAC2 may be a negative regulator of osteogenic differentiation.

Discussion

OP is a common disease, with an incidence of 13.2% in China (16). The serious complications of OP, including osteoporotic fractures, pain and misshapen bones, are associated with a high cost of treatment and prevention, and pose a major socioeconomic burden (17). Abnormal stimulation of osteoclasts generally results in bone loss, which leads to OP, and this disease is primarily characterized by decreased bone mass and disorders of the bone microstructure (18-20). Current therapies for OP, such as bone resorption inhibitors and bisphosphonate, are mainly focused on the balance of bone remodeling, which is critical during maintenance and regeneration of bone tissue. However, effective methods for promoting bone formation are still under development (21,22). Therefore, novel effective methods for treating OP and promoting bone synthesis are urgently needed.

miRNAs belong to a small RNA family and have important regulatory properties (23). miRNAs play key roles in the physiological processes and in the pathogenic mechanism of numerous diseases (24,25). An increasing number of studies have confirmed the involvement of several miRNAs in the regulation of bone biology; their potential role in osteogenesis has been reported, but the relative functional significance is not yet completely understood (5,7,26-28). A recent study using miRNA microarray chip technology demonstrated that miR-223 is abnormally expressed in patients with diffuse idiopathic skeletal hyperostosis (DISH), which is associated with sclerostin metabolism (29). DISH is characterized by new bone formation, constitutional abnormalities and metabolic abnormalities, leading to the biomechanical alterations in the musculoskeletal system and/or the formation of obstructive cervical masses (30,31). However, the function of miR-223 in osteoblasts remains unknown.

To the best of our knowledge, the present study is the first to reveal that miR-223-5p was upregulated during osteoblast differentiation. miR-223-5p expression increased gradually in MC3T3-E1 cells over a 14 day period. Accordingly, the expression levels of ALP, OCN and RUNX2 were all significantly upregulated in a time-dependent manner. ALP (32), OCN (33) and RUNX2 (27) are classic biomarkers, that reflect the functional status of osteoblast cells. The capacity of each factor in regulating osteogenic differentiation is highly associated with the bone formation process due to of the dynamic feedback system in the human body. The expression of miR-223-5p exhibited a similar tendency in the present study, suggesting that miR-223-5p may be used as a biomarker for diagnostic purposes for osteogenic differentiation. However, the mechanisms underlying the role of miR-223-5p in this process has not yet been fully elucidated.

The present study demonstrated that miR-223-5p was a positive regulator of osteogenic differentiation, as its overexpression led to the enhancement of osteogenic differentiation, and its silencing had to the opposite effect. Following transfection of miR-233 mimics, biomarkers of osteogenic differentiation (ALP, OCN and RUNX2) were upregulated at 7 days, and the Alizarin S red staining results revealed a higher number of mineralized nodules compared with the control group. The transfection of miR-233 inhibitor exerted the opposite effects. In the animal experiment, the newly formed bone tissue stained red with H&E and stained blue with Masson's Trichrome. The area of new bone tissue was larger in implants containing si-HDAC2 cells, compared with negative controls, indicating that HDAC2 silencing increased mineralized bone matrix formation. These results indicated that miR-223-5p may be a target for the treatment of bone loss and the optimization of fracture healing. Several miRNAs including miR-5100 (34), miR-192 (22) and miR-10a (5), have been proven to act as regulatory factors, as their expression markedly affect osteogenic differentiation. Accumulating evidence highlights the crucial role of miRNAs during osteogenic differentiation, and these may exert their effects through targeting their downstream genes. miR-590, miR-9 and miR-5100 control osteogenic differentiation by targeting mothers against decapentaplegic homolog 7, Dickkopf WNT Signaling Pathway Inhibitor 1 and various other genes (27,35,36). However, the target genes of miR-223-5p in osteogenic differentiation remain unknown.

The present study demonstrated that miR-223-5p promoted osteogenic differentiation, at least in part by targeting HDAC2. The experimental data demonstrated that miR-223-5p downregulated HDAC2 gene expression, which has been described as an anti-proliferative gene involved in cell cycle regulation (37,38). A study of chronic obstructive pulmonary disease (COPD) demonstrated that miR-223-5p overexpression decreased HDAC2 expression in human pulmonary artery endothelial cells, whereas HDAC2 expression was preserved when miR-223-5p was silenced (15). These findings suggest that miR-223-5p controls the expression of HDAC2 in COPD (15). Thus, it is probable that miR-223-5p controlled the expression of HDAC2 in osteoblasts, which is a novel regulatory axis.

In conclusion, HDAC2 expression at the mRNA and protein level was altered following transfection with miR-223-5p mimics or inhibitor in the present study. Therefore, it was demonstrated that miR-223-5p regulated HDAC2 expression to promote osteogenesis. Taken together, the results of the present study indicated a novel potential therapeutic approach to the treatment of osteogenic conditions.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Authors' contributions

JC contributed the central idea, analyzed the majority of the data and wrote the initial draft of the manuscript. GH and YW performed the *in vivo* experiments. DC contributed to study design, revised and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The research protocols associated with the experimental mice were approved by the Experimental Animal Ethics Committee of Southern Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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