The Expression of miR-153-3p Regulates the Osteogenic Differentiation of Bone Marrow Derived Mesenchymal Stem Cells by Targeting RUNX2

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Research Article

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Abstract

Background

Runt-related transcription factor 2 (RUNX2) plays a key role in the osteogenic differentiation. The study aimed to explore the effect of miR-153-3p on the osteogenic differentiation of human bone marrow derived mesenchymal stem cells (hBMSCs) and confirm its targeting effect on RUNX2.

Results

During the osteogenic differentiation of hBMSCs, the expression of miR-153-3p decreased at 1 d and reached a low peak at 3 d. After miR-153-3p mimic or inhibitor transfection, miRNA-153-3p expression level of hBMSCs dramatically increased or decreased, but the cell viability and proliferation ability were not notably changed. After miR-153-3p inhibitor transfection, the osteogenic differentiation was notably promoted which was confirmed with Alizarin red staining, alkaline phosphatase (ALP) activity and Western blot for RUNX2 and Collagen I, while miR-153-3p mimic transfection, the osteogenic differentiation was notably suppressed. The result of luciferase reporter gene experiment verified that RUNX2 was the target gene of miR-153-3p.

Conclusion

The results of this study indicate that the expression of miR-153-3p regulates the osteogenic differentiation of hBMSCs by targeting RUNX2.

Background

Bone defect is a common problem in surgery, usually caused by trauma, tumor and infection. The clinical treatment is mainly autologous bone transplantation, but due to the limited source of autologous bone, secondary injury and postoperative complications of the bone donor site, the wide application of autologous bone transplantation is limited(1). The development of bone tissue engineering has opened up a new direction for solving the problem of bone transplantation. Bone tissue engineering consists of stem cells, biological scaffolds and growth factors, among which stem cells and biological scaffold materials are the core of bone tissue engineering(2). Human bone marrow derived mesenchymal stem cells (hBMSCs) have the potential to differentiate into bone, cartilage, fat, tendons, ligaments, muscles, nerves and other tissues. It has the characteristics of rich sources, easy access, simple culture, low immunogenicity, easy transfection and long-term expression of foreign genes, and excellent osteogenic differentiation ability, which is widely used in bone tissue engineering(3–5). The low osteogenic differentiation of BMSCs is still one of the greatest challenges for bone tissue engineering.

Osteogenic differentiation of hBMSCs involves many factors such as growth factors, signal pathways and regulatory genes(6). MicroRNAs (miRNAs, miRs) are important biological regulators that occur at the post-transcriptional level, which have complex regulatory effects on the body's physiological/pathological activities, including the process of osteogenic differentiation(7). Mature miRNAs, as post-transcriptional inhibitors, recognize and bind to the 3'untranslated region (3'UTR) of the target gene through complete or incomplete sequence complementary pairing, thereby inhibiting the translation of the target gene protein. Studies have shown that Runt-related transcription factor 2 (RUNX2) plays a key role in the differentiation of pluripotent mesenchymal stem cells(8–10). Its expression promotes the differentiation of osteoblasts and inhibits the differentiation of adipocytes and chondrocytes(11). We used the software targetscan (http://www.targetscan.org) to analyze which miRNAs maybe target the RUNX2, and the result showed RUNX2 maybe target by miR-153-3p. Therefore, we speculated that the low expression of miR-153-3p promoted the osteogenic differentiation of hBMSCs by targeting RUNX2. The study aimed to explore the effect of miR-153-3p on the osteogenic differentiation of hBMSCs and confirm its targeting effect on RUNX2.

Results

miR-153-3p expression level

The expression of miR-153-3p was detected by qRT-PCR. During the osteogenic differentiation of hBMSCs, the expression of miR-153-3p was detected at 0, 1, 3, 7, 14 d, and the results showed that miR-153-3p expression level decreased at 1 d and reached a low peak at 3 d, and miR-153-3p expression level at 7, 14 d was similar to that at 3 d (Fig. 1A). Compared with control and NC groups, the miR-153-3p expression level of hBMSCs transfected with miR-153-3p inhibitor dramatically decreased and the miR-153-3p expression level of hBMSCs transfected with miR-153-3p mimic dramatically increased (Fig. 1B).

Effect of miRNA-140-5p expression on the viability and proliferation ability of hBMSCs

Compared with control and NC groups, cell viability and the percentage of Ki67-positive cells of hBMSCs transfected with miR-153-3p mimic or inhibitor were not notably changed (Fig. 2). The expression changes of miR-153-3p had no effect on cell viability and proliferation ability.

Effect of miRNA-140-5p expression on osteogenic differentiation of hBMSCs

The osteogenic differentiation of hBMSCs was observed by ALP activity assay, Alizarin red staining and Western blot for RUNX2 and Collagen I. The result of ALP activity assay showed the OD value of the inhibitor group was higher than that of control, mimic and NC groups, and the OD value of the mimic group was the lowest among the groups (Fig. 3A). The result of Alizarin red staining showed that mineralized bone matrix in the inhibitor group was enhanced comparison with the control, mimic and NC groups, and the OD value of the inhibitor group was higher than that of control, mimic and NC groups,

and the mineralized bone matrix and OD value of the mimic group was the lowest among the groups (Fig. 3B). The result of Western blot showed the expression levels of RUNX2 and Collagen I in the inhibitor group were more than that of control, mimic and NC groups, and the expression levels of RUNX2 and Collagen I in the mimic group were the lowest among the groups (Fig. 3C).

RUNX2 is a target of miR-153-3p

Luciferase reporter gene experiment was used to verify that RUNX2 is a target of miR-153-3p. The result showed that when the core sequence of 3'UTR of RUNX2 was mutated, luciferase relative activity of mimic group has no change comparison with NC group. However, in the RUNX2 WT-3'UTR reporter gene system, the relative activity of luciferase in mimic group significantly decreased comparison with NC group (Fig. 4).

Discussion

miRNA is a small non-coding RNA with a length of 20 to 22 bases. It is considered to be an important epigenetic modification molecule that can mediate cell differentiation by the post-transcriptional regulation of target genes(12). The role of miRNAs in the differentiation of osteoclasts and osteoblasts has been extensively studied(13). The differentiation of osteoblasts is an important process of bone homeostasis, and miRNAs can regulate the biological process of osteoblast differentiation. Studies have shown miR-27a-3p inhibits osteogenic differentiation by targeting Osterix(14), and miR-135-5p promotes osteoblast differentiation by inhibiting HIF1AN in the osteogenic differentiation procession(15). RUNX2 is one of the members of the Runt family which contain a common DNA binding runt domain and can form a heterodimer with core binding factor beta (CBF-β)(16). Study showed that the osteogenesis process of RUNX2-deficient mice was completely interrupted, and the final manifestation was complete loss of bone(17). RUNX2 plays a vital role in the early stage of osteogenic differentiation of pluripotent mesenchymal stem cells. Its expression promotes the differentiation of osteoblasts and inhibits the differentiation of adipocytes and chondrocytes(11). RUNX2 is expressed in preosteoblasts cells, the expression is highest in the immature osteoblasts, and the expression decreases during osteoblasts mature(18). Studies show that miR-23a-5p(19), miR-365a-3p(20), miR-505(21), miR-488, miR-375(22) inhibit osteogenic differentiation by targeting RUNX2, and at the same time reduces the activity of some key osteoblast markers. We used the software targetscan (http://www.targetscan.org) to analyze which miRNAs maybe target the RUNX2, and the result showed RUNX2 maybe target by miR-153-3p. Jiang et al found that miR-153-3p inhibited osteogenic differentiation of periodontal ligament stem cells by targeting KDM6A(23). Therefore, we speculated that the low expression of miR-153-3p promote the osteogenic differentiation of hBMSCs by targeting RUNX2.

In this study, the expression level of miR-153-3p in osteogenic differentiation of hBMSCs was detected, and the results showed that miR-153-3p expression level decreased at 1 d and reached a low peak at 3 d, and then the expression level was kept at a low level. The results indicate miR-153-3p is involved in the osteogenic differentiation process of hBMSCs and plays a negative regulatory role. Therefore, we used the miR-153-3p mimic or inhibitor to up or down-regulate the miR-153-3 expression level of hBMSCs, and

its viability and proliferation ability were analyzed with CCK-8 and Ki67 immunofluorescence. The OD value in CCK-8 experiments and the percentage of Ki67-positive cells of hBMSCs transfected with miR-153-3p mimic or inhibitor were not notably changed, which indicate the expression changes of miR-153-3p has no effect on cell viability and proliferation ability. Then the transfected hBMSCs were induced to osteogenic differentiation, after 14 d culture, the osteogenic differentiation was observed by Alizarin red staining, ALP activity assay and Western blot for RUNX2 and Collagen I. The results showed that mineralized bone matrix, ALP activity, RUNX2 and Collagen I level in the inhibitor group were enhanced, and that in the mimic group decreased. ALP, RUNX2 and Collagen I are the osteogenic differentiation specific transcription factor which are the early osteogenic differentiation marker(24, 25). These results in this study indicate that the low expression of miR-153-3p promotes the osteogenic differentiation of hBMSCs, and overexpression of miR-153-3p inhibits the osteogenic differentiation of hBMSCs. Whether RUNX2 is the target gene of miR-153-3p is still unclear. Therefore, in the Luciferase reporter gene experiment, the core sequence of 3'UTR of RUNX2 was mutated, and the miR-153-3p had no effect on the luciferase relative activity. On the contrary, when the core sequence of 3'UTR of RUNX2 was wild type, miR-153-3p significantly decreased the relative activity of luciferase. The results confirm that RUNX2 is the target gene of miR-153-3p.

Conclusion

In summary, our study firstly demonstrate that the low expression of miR-153-3p promotes the osteogenic differentiation of hBMSCs by targeting RUNX2, which may provide a novel potential regulatory target for osteogenic differentiation.

Methods

Cell line and osteogenic differentiation induction medium

The hBMSCs were purchased from Fuyuanbio Co., Ltd (Shanghai, China). The cells were passaged and the third generation of hBMSCs was taken for the following experiment. The osteogenic differentiation induction medium: DMEM (Invitrogen, USA) containing 10 μM dexamethasone, 50 μM vitamin C, 10 mM β-glycerol sodium phosphate, and 10% FBS.

Cells transfection

The hBMSCs was cultured in a 6-well culture plate at a concentration of 5×10^3/ml. After 24 h, they were transfected with miRNA-153-3p mimic, inhibitor and the corresponding negative control (NC) (ThemoFisher, USA) with lipofectamine 2000 (ThemoFisher, USA) according to the transfection instructions, the cells were incubated in 5% CO_2, 37°C incubator and a blank control group was set up. After 5 h, the transfection medium was replaced with DMEM, and the cells were cultured for another 8 h, and then the transfected cells were taken for the following experiment.

Cells viability assay

The cell counting kit-8 (CCK-8; Beyotime Biotechnology, China) was used to detect the viability of the transfected hBMSCs according to the manufacturer instructions, and the OD value was read at 450 nm. The untransfected hBMSCs were as the control group.

Cells proliferation ability assay

After fixing the cell sample with 4% paraformaldehyde, washed it with PBS solution 3 times, then blocked it with 5% BSA at room temperature for 1 h, then incubated it with rabbit anti-Ki67 primary antibody (1:800; Abcam, UK) at room temperature for 6 h. Then the cell sample were washed 3 times with PBS solution and incubated with Alexa Fluor® 488 goat anti-rabbit secondary antibody (1:1000; Abcam, UK) at room temperature for 3 h. After washing 3 times with PBS solution, the cells were counterstained the cell nucleus with Hoechst 33342 dye at room temperature for 30 min. The positive cells were observed under a fluorescence microscope, the positive cells number was calculated under 10 randomly different fields, and the percentage of Ki67-positive cells = Ki67-positive cell number/Hoechst-positive cell number.

Osteogenic differentiation culture

The hBMSCs transfected with miRNA-153-3p mimic, inhibitor, mimic NC or inhibitor NC were cultured in a 24-well culture plate at a concentration of 5×10^5/ml with DMEM medium in a 37°C, 5% CO_2 incubator. When about 80% confluence, the culture medium was replaced with the osteogenic differentiation induction medium for 14 days. The untransfected hBMSCs were cultured as the control group.

miRNA-153-3p expression level assay

The total RNA in cells was extracted by TaqMan miRNA isolation kit (Applied Biosystems, USA), then the expression of mature miR-153-3p was measured with the TaqMan miRNA assay and TaqMan Universal PCR Master Mix (Applied Biosystems, USA). Reaction conditions: 94°C 4 min→94°C 30 s→56°C 30 s→72°C 30 s, cycle 40 times. U6 was used as the internal reference. The qRT-PCR relative quantitative method was used to analyze the experimental result. The miRNA-153-3p expression level was calculated with $2^{-\triangle\triangle Ct}$ method.

Alizarin red staining

After 14 days of osteogenic differentiation, the cell samples were fixed with 4% paraformaldehyde. After washing 3 times with PBS, the cell were incubated with 2% alizarin red staining solution (Beyotime, China) for 10 min at room temperature. Some of cells in each group were observed under an inverted microscope, the cell mineralization of other cells in each group were quantified with alizarin red extracted with 100 mM cetylpyridinium chloride solution (Sigma, USA), and the OD value was read at 570 nm.

Alkaline phosphatase (ALP) activity assay

After 14 days of osteogenic differentiation, the cell samples were washed with PBS. The cells were lysed with 1% Triton X-100 for 15 min, centrifuged at 10,000 g for 5 min, and the supernatant were collected for ALP activity assay which was detected with ALP Assay Kit (Beyotime Biotechnology, China) according to the manufacturer instructions, and the OD value was read at 405 nm.

Western blot analysis

After 14 days of osteogenic differentiation, the total protein of cells was extracted using RIPA buffer (Beyotime, China). Briefly, 10 μg of total protein from each sample was electrophoresed and transferred to PVDF membrane, and the PVDF membrane was blocked with 5% BSA. Then the PVDF membrane was successively incubated with rabbit anti-RUNX2 primary antibody (1:800, Abcam, UK), mouse anti-Collagen I primary antibody (1:800, Abcam, UK) or mouse anti-β-actin primary antibody (1:1000, Abcam, UK) and IRDye 800-conjugated affinity-purified goat anti-rabbit second antibody (1:4000, Rockland Immunochemicals, USA) or IRDye 700-conjugated affinity-purified goat anti-mouse second antibody (1:4000, Rockland Immunochemicals, USA). The quantity of protein bands were analyzed with Odyssey laser scanning system (LI-COR Inc., USA).

Luciferase reporter gene experiment

The QuickChange site-directed mutagenesis kit (Stratagene, USA) was used to mutate the core sequence of 3'UTR of RUNX2. The wild-type (WT) and mutated (MU) sequence were cloned into pMIR-Report luciferase vector to obtain RUNX2 WT-luc and RUNX2 Mu-luc. The RUNX2 WT-luc, RUNX2 Mu-luc, miR-153-3p mimic (ThemoFisher, USA) and the corresponding NC were transfected into HEK293 cells. The cells were incubated in a saturated humidity, 37°C, 5% CO_2 incubator for 48 h, and then were lysed and analyzed with a dual-luciferase reporter assay system (Promega Corporation, USA) according to the manufacturer instructions.

Statistical analysis

The data obtained in this research were statistically analyzed with SPSS 22.0 software. The data obtained in this study were all measurement data and conform to the normal distribution, which were presented as mean ± standard deviation (M ± SD). Comparison among groups was tested by independent t test or one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

List Of Abbreviations

Abbreviations	Full Name
ALP	alkaline phosphatase
CBF-β	core binding factor beta
CCK-8	cell counting kit-8
hBMSCs	human bone marrow derived mesenchymal stem cells
NC	negative control
RUNX2	Runt-related transcription factor 2
WT	wild-type
MU	mutated
3'UTR	3'untranslated region

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Nantong University.

Consent for publication

N/A

Availability of data and materials

The data that support the findings of this study are available in the “pan.baidu” [https://pan.ntu.edu.cn/l/RJJskG, Code: isyf].

Competing interests

The authors declare that they have no conflict of interest.

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None.

Authors' contributions

XY and XZ conceived and designed the experiments. RM, JM, SF, and MJ performed the experiments. RM and XY analyzed the data. RM and XZ wrote the paper. All authors read and approved the final manuscript.

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Figures

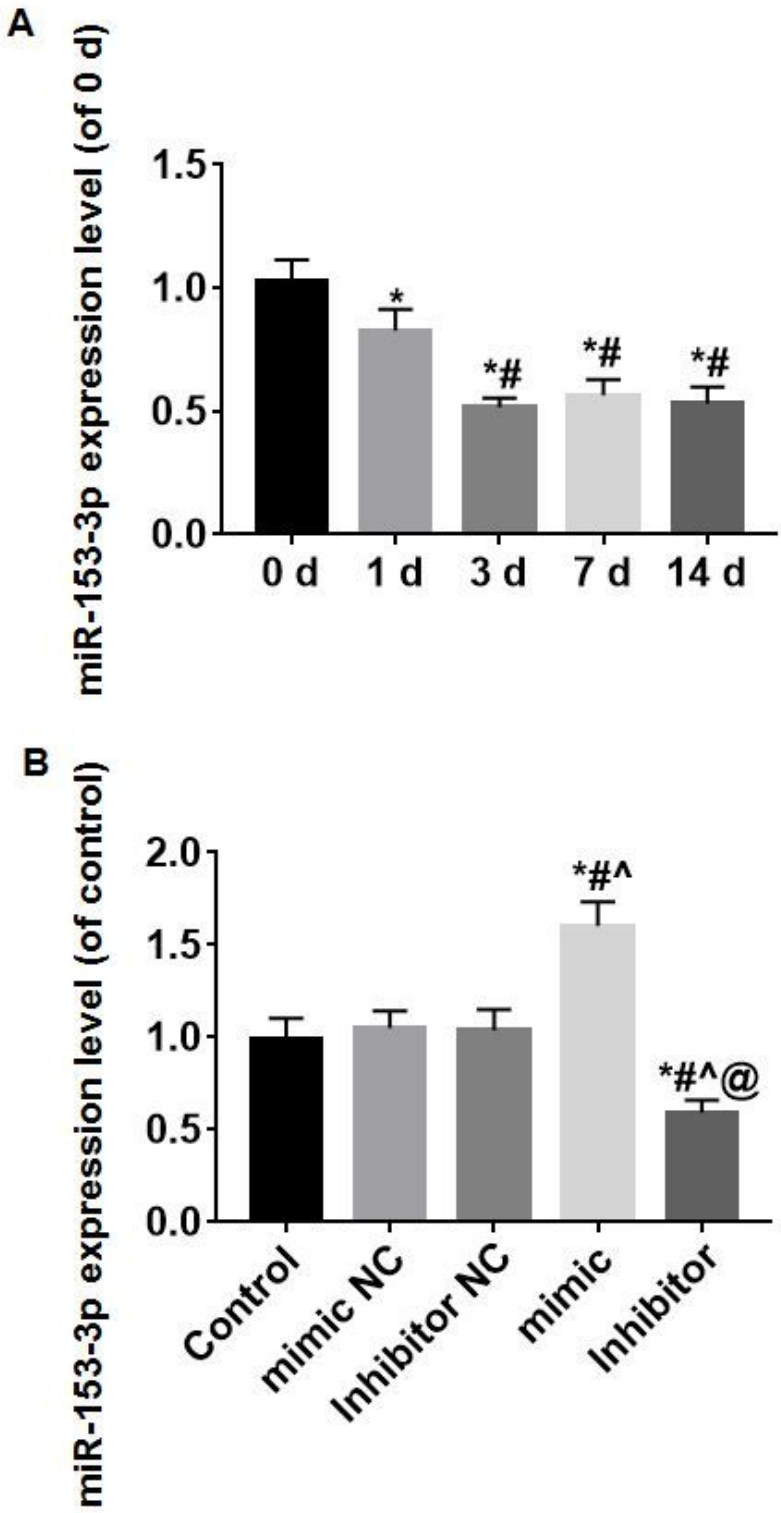

Figure 1

The expression of miR-153-3p was detected by qRT-PCR. (A) During the osteogenic differentiation of hBMSCs, the miR-153-3p expression level decreased at 1 d and reached a low peak at 3 d, and miR-153-3p expression level at 7, 14 d was similar to that at 3 d. * vs. 0 d, $P < 0.05$; # vs. 1 d, $P < 0.05$. (B) Compared with control and NC groups, miR-153-3p expression level of hBMSCs dramatically decreased in

the inhibitor group and increased in the mimic group. * vs. control group, $P < 0.05$; # vs. mimic NC group, $P < 0.05$; ^ vs. inhibitor NC group, $P < 0.05$; @ vs. mimic group, $P < 0.05$.

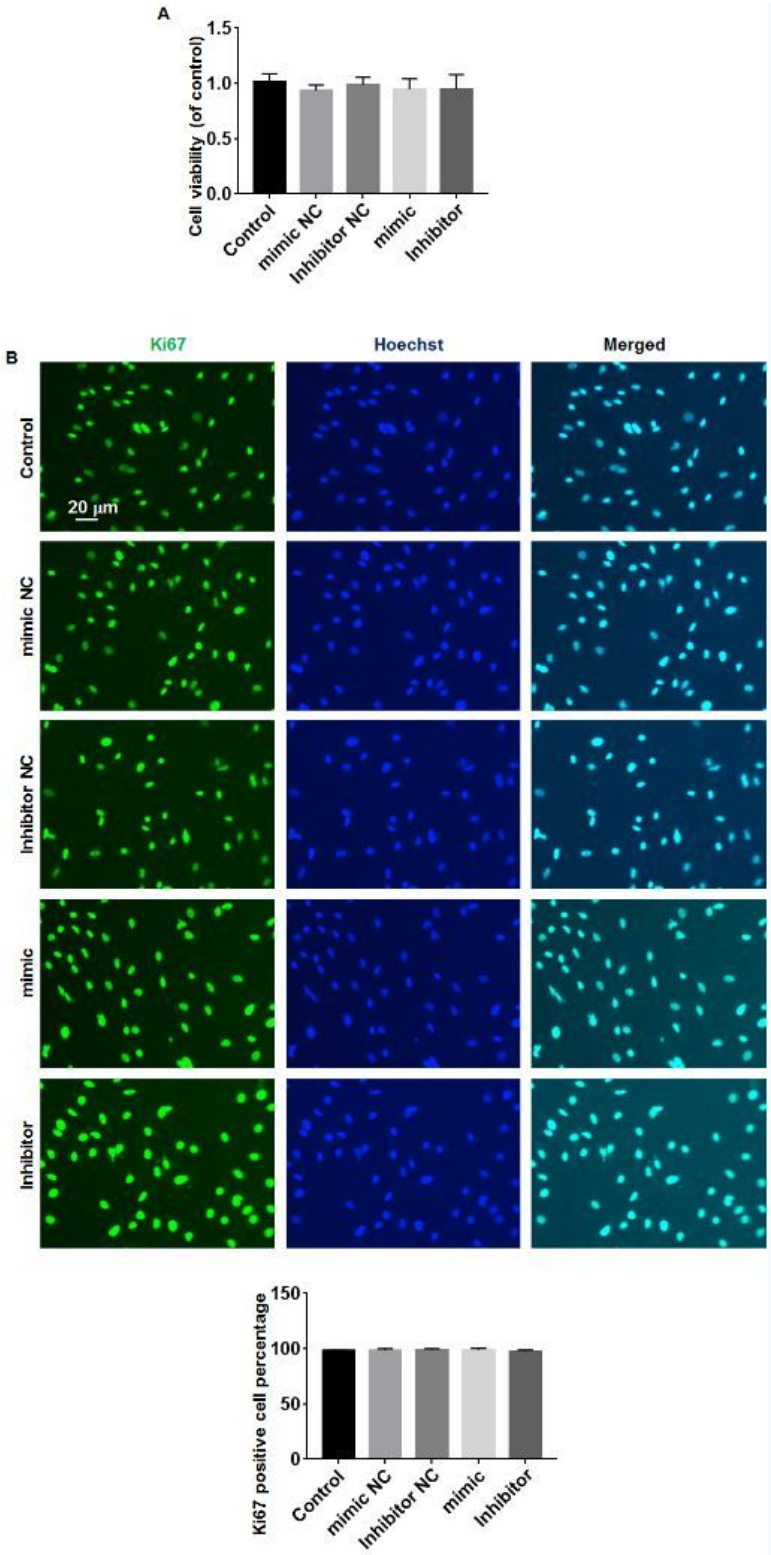

Figure 2

The cell viability and proliferation ability of hBMSCs. (A) The cell viability of hBMSCs was detected by CCK-8. Compared with control and NC groups, the cell viability of hBMSCs transfected with miR-153-3p

inhibitor was not notably changed. (B) The proliferation ability of hBMSCs was detected by Ki67 immunofluorescence. Many Ki67-positive cells were found in all three groups. The Ki67-positive cells percentage in mimic and inhibitor group was not notably changed comparison with control and NC groups. Bar = 20 mm.

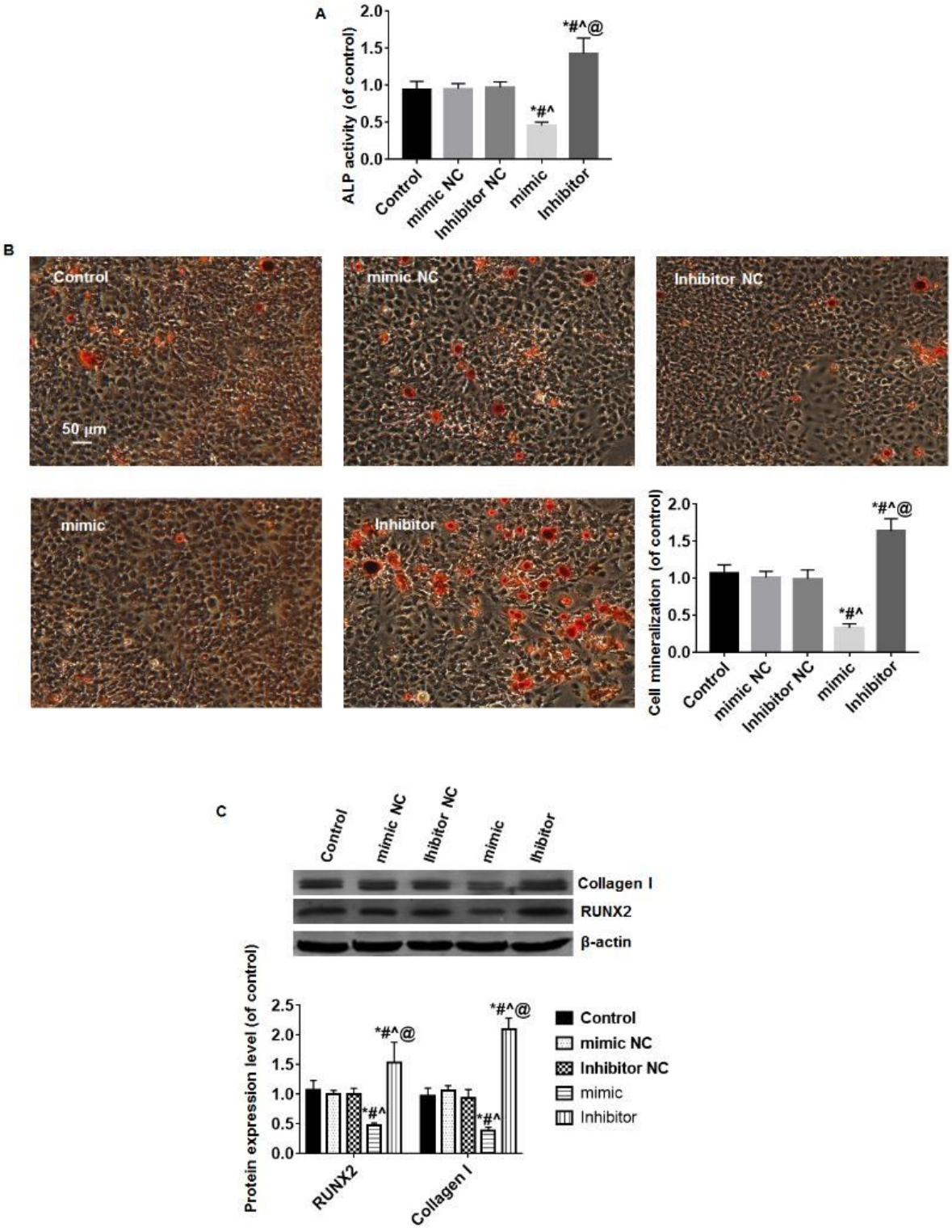

Figure 3

Effect of miRNA-140-5p low expression on osteogenic differentiation of hBMSCs. (A) The result of ALP activity assay showed the OD_{405} value of the inhibitor group was higher than that of control, mimic and NC groups, and that in the mimic group was the lowest. (B) The result of Alizarin red staining showed that mineralized bone matrix in the inhibitor group was enhanced comparison with the control, mimic and NC groups, and the OD_{570} value of the inhibitor group was higher than that of control, mimic and NC groups, the mineralized bone matrix and OD_{570} value of the mimic group were the lowest. (C) The result of Western blot showed the expression levels of RUNX2 and Collagen I of inhibitor group were more than that of control, mimic and NC groups, and that of mimic group were the lowest. Bar = 50 mm. * vs. control group, $P < 0.05$; # vs. mimic NC group, $P < 0.05$; ^ vs. inhibitor NC group, $P < 0.05$; @ vs. mimic group, $P < 0.05$. Full-length blots/gels are presented in Supplementary Figure 1.

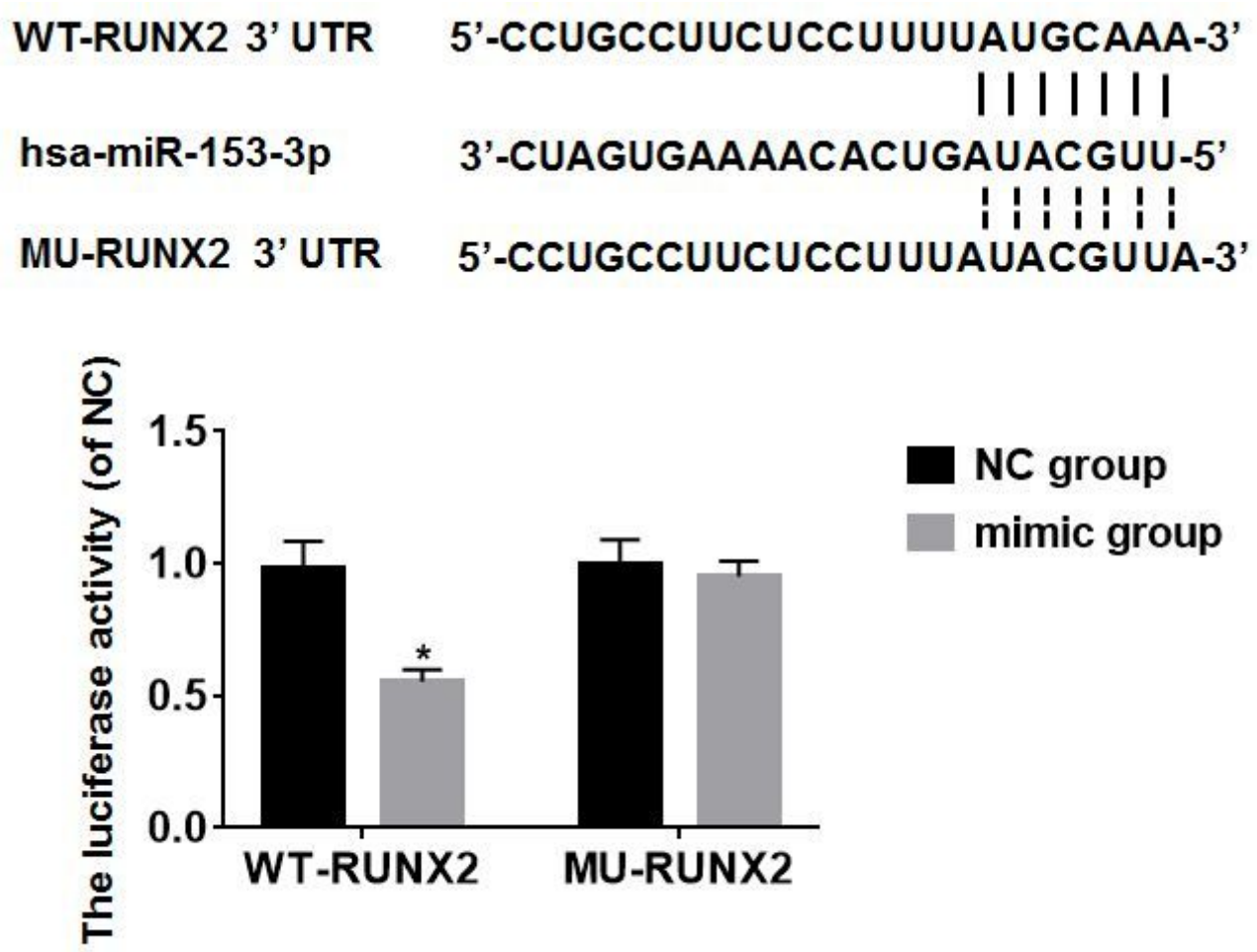

Figure 4

RUNX2 is a target of miR-153-3p. Luciferase reporter gene experiment was used to verify that RUNX2 is a target of miR-153-3p. The result showed that when the core sequence of 3'UTR of RUNX2 was mutated, luciferase relative activity of mimic group has no change comparison with NC group. However, in the RUNX2 WT-3'UTR reporter gene system, the relative activity of luciferase in mimic group significantly decreased comparison with NC group. * vs. NC group, $P < 0.05$.

Supplementary Files

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