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miR-137 Targets Runt-related Transcription Factor 2 to Regulate Osteogenic Differentiation of MC3T3-E1 Cells

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KEYWORDS Expression. MC3T3-E1 Cell. Micro Ribonucleic Acid-137. Osteogenic Differentiation. Runt-related Transcription Factor 2

ABSTRACT The present study sought to clarify the regulatory roles of miR-137 and runt-related transcription factor 2 (RUNX2) in the osteogenic differentiation of MC3T3-E1 cells. Specifically, MC3T3-E1 cells fell into control, negative control (NC) mimic and miR-137 mimic groups. According to the online database TargetScanHuman, a binding site was detectable between miR-137 and RUNX2 in the 3' untranslated region. It was unveiled that miR-137 caused a plummet in the expression of wild-type RUNX2 ($P<0.05$), but had no obvious effect on mutant RUNX2. Relative to control and NC mimic groups, miR-137 mimic group had significantly lower activity of alkaline phosphatase, concentration of alizarin red S, and mRNA and protein expression levels of RUNX2, osteopontin and osteocalcin, but higher expression levels of lipoprotein lipase, adipocyte binding protein-2 and leptin ($P<0.05$). To sum up, miR-137 affects the osteogenic differentiation of MC3T3-E1 cells *via* negatively regulating the RUNX2 expression.

INTRODUCTION

The metabolic imbalance between osteoblasts and osteoclasts reduces the bone mineral density, and the abnormalities in bone resorption and remodeling during *in vivo* metabolism contribute to osteoporosis (Li et al. 2020). Osteoblasts, with the main function of forming new bone tissues, are adapted for the role in the synthesis, secretion, and mineralization of bone matrix, and play vital roles in bone remodeling (Fratzl et al. 2021). The synthesis and expressions of such extracellular matrix proteins as alkaline phosphatase (ALP) and osteocalcin (OCN) decrease when the function of osteocytes is damaged, thus causing bone formation disorders (Ruiz et al. 2020). If the bone resorption rate exceeds the formation rate, the bone mass drops, thereby inducing osteoporosis (Appelman-Dijkstra et al. 2022). Osteoblast differentiation involves the maturation and mineralization of extracellular matrix, as an important stage of bone formation (Wang et al. 2019). ALP hydrolyzes phosphates in the osteogenic process and provides essential phosphoric acids for hydroxyapatite deposition, thus initiating and promoting osteogenesis. Its activity is a functional index of osteoblast differentiation. Specifically, a higher ALP activity indicates higher differentiation degree of osteoblasts (Tüysüz et al. 2012; Alp et al. 2017). Besides, as the most typical marker of osteoblast differentiation, OCN is a crucial player in bone mineralization, and a non-collagen protein synthesized and secreted by osteoblasts (Komori 2020). In the presence of calcium, OCN can bind hydroxyapatite and stabilize its conformation (Chen et al. 2019). Both ALP and OCN are key indices for osteoblast differentiation *in vitro*.

MiRNAs are a type of small non-coding RNAs. There is a binding relationship between miRNAs and the 3' untranslated regions (UTRs) of mRNAs, based on which miRNAs function in suppressing target gene expression, foreboding that miRNAs are critical players in the onset and progression of osteoporosis (Yang et al. 2020). It is well-documented that miRNAs exert significant regulatory effects on osteoblasts and other cells (Wijnen et al. 2013; Guo et al. 2015; Zhang et al. 2017). MC3T3-E1 cells can direct osteoblast differentiation, so they are ideal for studying the biological behaviors of osteoblasts, including proliferation and differentiation

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(Lu et al. 2020). Hence, the influences of miR-137 on cell osteogenic differentiation and related gene expressions in MC3T3-E1 cells were uncovered.

MATERIAL AND METHODS

Cells, Reagents and Instruments

Research objects included MC3T3-E1 cells (ATCC), and negative control (NC) mimic and miR-137 mimic (Guangzhou RiboBio Co., Ltd., China). Reagents used in the experiment included α-MEM, ALP staining kit and alizarin red S (ARS) staining kit (Shanghai Beyotime Biotechnology Co., Ltd., China). Anti-runt-related transcription factor 2 (anti-RUNX2), anti-OCN and anti-osteopontin (anti-OPN) primary antibodies (CST, USA), horseradish peroxidase-labeled secondary antibodies (Beijing Bersee Science and Technology Co., Ltd., China), and antibodies against lipoprotein lipase (LPL), adipocyte binding protein-2 (AP-2) and leptin (Otwo Biotech (Shenzhen) Inc., China). Moreover, instruments employed in the experiment encompassed bicinchoninic acid (BCA) protein assay kit (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., China), a refrigerated centrifuge (Beckman, USA), electrophoresis apparatus (Bio-Rad Laboratories, Inc., USA) and an electron microscope (Olympus Corporation, Japan).

Transfection and Grouping

Following routine culture of MC3T3-E1 cells in 10 percent fetal bovine serum-based RPMI-1640 medium under conditions of 5 percent CO_2 and 37°C, the medium was changed every other day. Upon arriving at 80 percent confluence, the cells underwent trypsinization and subculturing. Then the cells fell into control, NC mimic and miR-137 mimic groups. Control group received no transfection, while the other two groups underwent miR-137 mimic and NC mimic transfection into MC3T3-E1 cells as specified in the guideline of Lipofectamine™ 2000. Finally, the medium was replaced 4 h post transfection. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting (WB) were conducted 24 h later to examine whether the transfection was successful.

MC3T3-E1 cells were selected for osteogenic induction and differentiation using osteogenic induction medium. After 3 weeks, ARS staining was implemented, and mineralized nodules were observed under an optical microscope (×100).

MC3T3-E1 cells were subjected to adipogenic induction and differentiation by adipogenic induction medium. Oil red O staining was carried out when the lipid droplets were large and round enough, and adipogenic staining results were observed under the optical microscope (×100).

Detection of Target Binding of miR-137 to RUNX2 by Luciferase Reporter Gene Assay (LRGA)

TargetScanHuman (http://www.targetscan.org/vert_72/) was employed for the prediction of the target binding site between miR-137 and RUNX2, and it appeared that RUNX2 is probably a target gene of miR-137. Afterwards, the wild-type 3'UTR vector (RUNX2-WT) and mutant 3'UTR luciferase reporter vector of RUNX2 (RUNX2-MUT) were constructed, which were transfected into MC3T3-E1 cells with miR-137 mimic and NC mimic, respectively for 48 h in accordance with the protocol of Lipofectamine™ 2000 reagent. Subsequently, the cells were collected to prepare lysates and to determine luciferase activity as instructed by the kit. Finally, the binding of miR-137 to 3'UTR of RUNX2 was confirmed.

ALP Staining

Seeded into 24-well plates, the transfected MC3T3-E1 cells underwent 7 days of culture in a medium comprising calcium salt, and rinsed by phosphate-buffered saline (PBS) thrice. Subsequent to 20-min fixation in 4 percent paraformaldehyde (PFA) at room temperature, the cells were stained by ALP using NBT/BCIP solution in dark for 30 min after PFA was discarded, and observed. According to the ALP staining results, the blue contours in the cytoplasm indicated the conjugation of ALP with the dye in cells. The amount of ALP needed to produce 1 μM paranitrophenol by hydrolyzing p-nitrophenyl phosphate chromogenic substrate in diethanolamine buffer (pH 9.8) at 37°C per min was defined as

an activity unit. Furthermore, the ALP activity in samples was assessed *as per* the definition of enzyme activity.

ARS Staining

Subsequent to inoculation into 24-well plates, the transfected MC3T3-E1 cells cultured with medium containing calcium salt for 14 days, and rinsed by PBS thrice. Later, the cells underwent 20-min fixation in 4 percent PFA at room temperature and staining by 0.4 percent ARS staining solution following the discarding of PFA. Next, the reaction was terminated using deionized water, and calcium salt deposition was observed under a microscope. Calcium was generated when dark red calcified nodules appeared outside the ARS-stained cells. Subsequently, a microplate reader was employed to read the absorbance at 405 nm by in keeping with the guidance of the kit. Finally, standard curves were plotted, and the concentration of ARS in samples was calculated.

Detection of mRNA Expressions of miR-137 and RUNX2, Osteogenic Markers OCN and OPN as well as Adipogenic Markers LPL, AP-2 and Leptin in MC3T3-E1 Cells by qRT-PCR

Isolation of total RNAs from MC3T3-E1 cells was implemented by use of the TRIzol method, and the obtained RNAs were reversely transcribed into cDNA. Later, SYBR Green method was adopted for amplification under the conditions as follows: 5-min pre-denaturation at 95°C and 40 cycles (95°C for 10 + 62°C for 30), in which glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serve as an endogenous control. In the end, the $2^{-\Delta\Delta Ct}$ method was employed for the calculation of the relative mRNA expression levels of RUNX2, OPN and OCN. Primer sequences of PCR: miR-137-F: 5'-ACGAACTCTGCTCCTTAG-3', miR-137-R: 5'-GAGCGTCCTCTGTTTG-3'; RUNX2-F: 5'-GCACTACCCAGCCACCTTTA-3', RUNX2-R: 5'-TATGGAGTGCTGCTGGTCTG-3'; OPN-F: 5'-GAGCAAACAGACGATGTGGA-3', OPN-R: 5'-GACCAGCTCATCGGATTCAT-3'; OCN-F: 5'-TCACACTGCTTGCCCTACTG-3', OCN-R: 5'-TGCCATAGAAGCGCCGATAG-3'; LPL-F: 5'-TTGCTATTCCAGGGTATCCA-3', LPL-R: 5'-TGTTAGTTGTTGTCCCATCG-3'; AP-2-F: 5'-TCCTGCACCACCAACTGCTTAG-3', AP-2-R: 5'-AGTGGCAGTGATGGCATGGACT-3'; leptin-F: 5'-CCAGGATGACACCAAAACCC-3', leptin-R: 5'-TATCTGCAGCACGTTTTGGG-3'; GAPDH-F: 5'-GGTGAAGGTCGGAGTGAACG-3', GAPDH-R: 5'-CGTGGGTGGAATCATACTGGA-3'.

Detection of Protein Expressions of RUNX2, Osteogenic Markers OCN and OPN as well as Adipogenic Markers LPL, AP-2 and Leptin in MC3T3-E1 Cells by WB

An appropriate amount of RIPA lysate was added into MC3T3-E1 cells for total protein isolation, and then measurement of the protein concentration was taken with the aid of BCA kit. After dilution with buffer, the proteins were set apart by way of SDS-PAGE, and transfer of proteins was quickly conducted from the gel to a PVDF membrane. Afterwards, the membrane underwent 2-h blocking by Tris-buffered saline-Tween 20 (TBST) comprising 5 percent skim milk at room temperature and incubation with primary antibodies against RUNX2, OPN, OCN, LPL, AP-2 and leptin (1:2,000) at 4°C throughout the night. Then the membrane was subjected to TBST rinsing thrice and 2 h of secondary antibody (1:10,000) incubation at room temperature, followed by color development with ECL reagent in dark. Next, target protein bands were photographed by a Bio-Rad gel imager. With GAPDH as the endogenous control, the gray values of the bands were measured to assess the relative expression of proteins.

Statistical Processing

Statistical processing was implemented with the aid of SPSS16.0 software. Measurement data were described by mean ± standard deviation ($\chi \pm s$) and compared *via* *t*-test between groups. The significance level α was set at 0.05.

RESULTS

Construction of MC3T3-E1 Cell Lines with miR-137 Overexpression

MiR-137 mimic group displayed a strikingly higher miR-137 expression relative to control and NC mimic groups ($P<0.001$) (Table 1), indicating

the successful construction of cell lines with stable miR-137 overexpression.

Table 1: MiR-137 expression in cells

Group	*miR-137 expression*
Control	0.23±0.02
NC mimic	0.21±0.04
miR-137 mimic	3.78±0.21*,#

* P<0.05 *vs.* control group; #P<0.05 *vs.* NC mimic group.

Targeted Regulatory Effects of miR-137 on RUNX2

It was ascertained that miR-137 bound to its target gene RUNX2 in the 3'UTR. As unveiled by LRGA, miR-137 could cause a notable decline in the expression of wild-type RUNX2 (P<0.05), but had no notable effect on mutant RUNX2 (Fig. 1 and Table 2), suggesting the regulatory roles of miR-137specific to RUNX2.

Function of miR-137 in Osteogenic Differentiation of MC3T3-E1 Cells

In order to evaluate the influence of miR-137 on the osteogenic differentiation of MC3T3-E1 cells, osteogenic markers were determined by ALP staining, ARS staining, qRT-PCR and WB. The results of ALP staining (Fig. 2A) exhibited that compared with control and NC mimic groups, miR-137 mimic group exhibited a decline in ALP (P<0.05). ARS staining results (Fig. 2B) yielded that miR-137 mimic group displayed a strikingly lower concentration of ARS versus control and NC mimic groups (P<0.05). The results of qRT-PCR (Fig. 2C) and WB (Fig. 2D) exhibited that miR-137 mimic group had pronouncedly lower mRNA and protein expression levels of RUNX2, OPN and OCN than control and NC mimic groups (P<0.05) (Table 3). Taken together, miR-137 acted as an inhibitor for osteogenic marker expressions in MC3T3-E1 cells, thus suppressing their osteogenic differentiation.

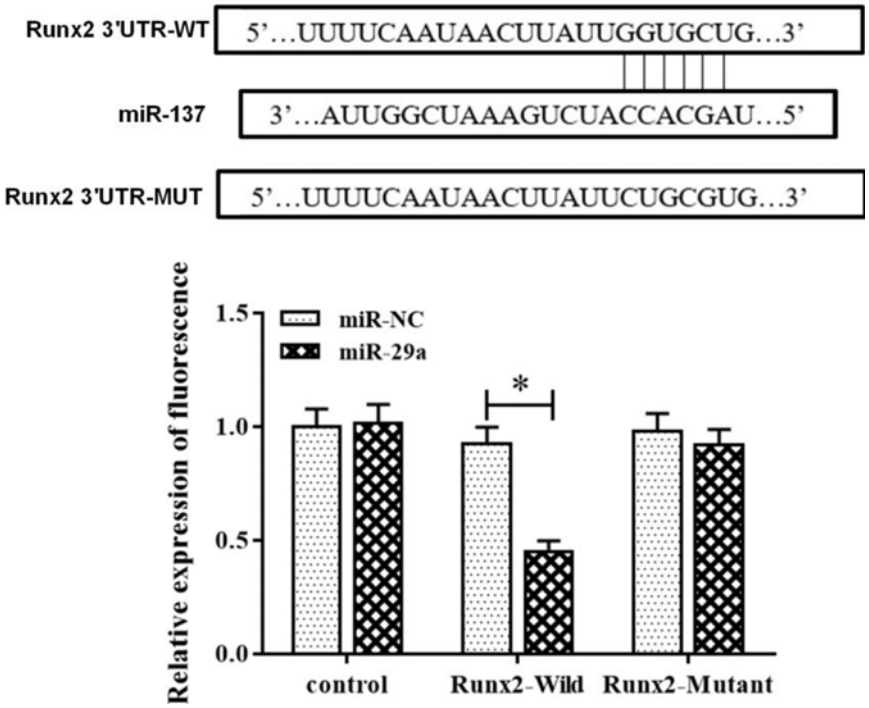

Fig. 1. Targeted regulatory effects of miR-137 on RUNX2 ascertained *via* LRGA

Table 2: Luciferase activity

Group		*miR-137 expression*	*P*
Control	miR-NC	1.02±0.22	>0.05
	miR-137 mimic	1.03±0.24	
Runx2-Wild	miR-NC	0.92±0.21*ŷ#	<0.05
	miR-137 mimic	0.46±0.13	
Runx2-Mutant	miR-NC	1.08±0.26	<0.05
	miR-137 mimic	0.98±0.23	

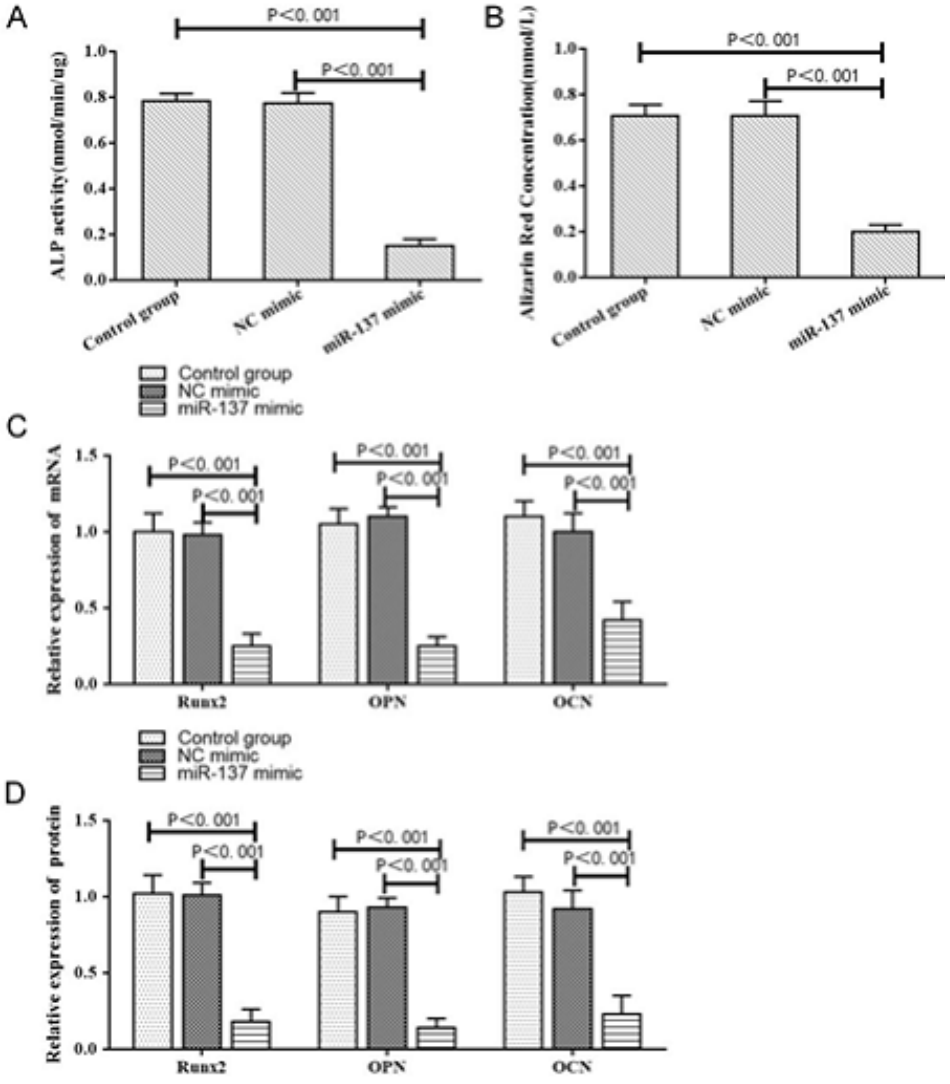

Fig. 2. Osteogenic differentiation of MC3T3-E1 cells. A: ALP staining results, B: ARS staining results, C: MRNA levels of osteogenic markers measured with the aid of qRT-PCR, D: Protein levels of osteogenic markers examined *via* WB

Table 3: ALP activity, alizarin red concentration, and mRNA and protein expression levels of osteogenic markers

Group	*ALP (nmol/min/ìg)*	*Alizarin red concentration (mmol/L)*	*Runx2 mRNA*	*OPN mRNA*	*OCN mRNA*	*Runx2*	*OPN*	*OCN*
Control	0.78±0.12	0.76±0.11	1.01±0.15	1.03±0.12	1.05±0.14	1.07±0.13	0.92±0.13	1.07±0.13
NC mimic	0.79±0.14	0.77±0.15	0.99±0.13	1.06±0.09	0.98±0.15	1.06±0.11	0.94±0.11	0.98±0.16
miR-137 mimic	0.18±0.05*,#	0.21±0.05*,#	0.28±0.03*,#	0.24±0.05*,#	0.41±0.05*,#	0.24±0.05*,#	0.19±0.05*,#	0.26±0.05*,#

*P<0.05 *vs.* control group; #P<0.05 *vs.* NC mimic group.

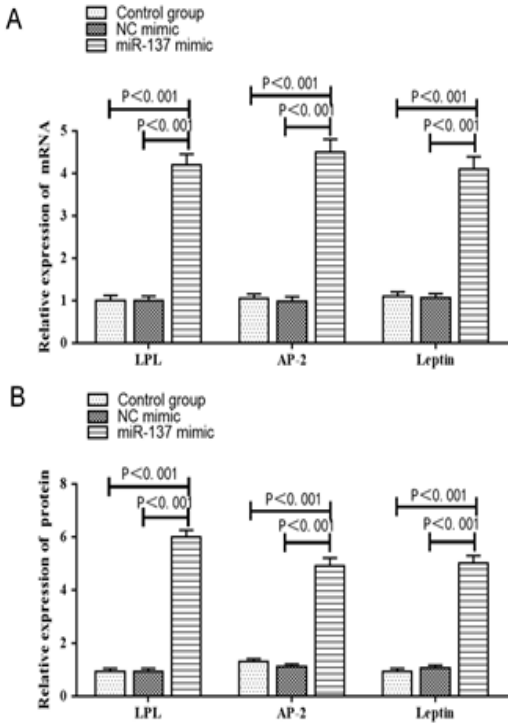

Fig. 3. Adipogenic differentiation of MC3T3-E1 cells. A:MRNA levels of adipogenic markers measured with the aid of qRT-PCR, B: Protein levels of adipogenic markers examined *via* WB

Function of miR-137 in Adipogenic Differentiation of MC3T3-E1 Cells

In order to evaluate the effect of miR-137 on the adipogenic differentiation of MC3T3-E1 cells, qRT-PCR and WB were adopted for measurement of adipogenic markers. As disclosed by qRT-PCR (Fig. 3A) and WB results (Fig. 3B), miR-137 mimic group had pronouncedly higher mRNA and protein expression levels of LPL, AP-2 and leptin versus control and NC mimic groups (P<0.05) (Table 4). Collectively, miR-137 increased the levels of adipogenic markers, thus facilitating the adipogenic differentiation of MC3T3-E1 cells.

Osteoporosis results from the imbalance between osteoblasts and osteoclasts (Wu et al. 2020). However, there have been few studies on the roles of miRNA in bone cell activities and osteogenic signaling pathways. Hence, miRNAs specifically expressed in the case of osteoporosis should be further explored from osteoblast proliferation and differentiation or osteogenesis-associated signal pathways. Given that osteoblasts differentiate to generate new bone and osteoclasts can digest and absorb old bones, a dynamic balance of bone metabolism exists in normal adults (Ooi and Sahrir 2018). In case this balance is disrupted, osteogenesis deficiency or excessive bone resorption may occur, which reduces bone mass and leads to osteoporosis

Table 4:MRNA and protein levels of adipogenic markers

Group	*LPL mRNA*	*AP-2 mRNA*	*Leptin mRNA*	*LPL*	*AP-2*	*Leptin*
Control	1.01±0.08	1.04±0.06	1.06±0.07	1.02±0.04	1.12±0.08	1.01±0.04
NC mimic	1.00±0.05	1.01±0.05	1.03±0.06	1.03±0.03	1.04±0.05	1.08±0.06
miR-137 mimic	4.18±0.15*,#	4.24±0.06*,#	4.13±0.15*,#	6.04±0.06*,#	5.43±0.07*,#	5.66±0.06*,#

*P<0.05 *vs.* control group; #P<0.05 *vs.* NC mimic group.

(Lu et al. 2021; Shah et al. 2021). The abnormal expressions of multiple genes have been verified to induce osteoporosis. As the most pivotal transcription factor for osteoblast differentiation and skeletal morphogenesis, RUNX2 controls bone metabolism (Wang et al. 2020a; Wang et al. 2020b; Xiong et al. 2020). However, its mechanism of action remains elusive.

According to reports, miRNAs are crucial players in osteoblast and osteoclast differentiation (Fu et al. 2019). For instance, miR-29a is capable of promoting the osteogenic differentiation of mesenchymal stem cells *via* its effect specific to histone deacetylase 4 (Tan et al. 2018). Furthermore, miR-29a facilitates osteoclast differentiation through targeting receptor activator of nuclear factor-κB ligand, thus protecting bone tissue from osteoporosis. Located on the chromosome 1p22, miR-137 has been studied mostly using tumor cells (Luo et al. 2013; Han et al. 2015; Zeng et al. 2016; Du et al. 2019; Wang et al. 2020c). Tumors are regulated by miR-137 mainly through CpG island methylation.

In osteoporosis cases, the bone mass of cancellous bone is inversely proportional to adipocytes, and excessive differentiation toward adipocytes is one of the principal causes for osteoporosis (Herrmann 2019). ALP is a common indicator for evaluating bone formation and turnover. Additionally, RUNX2, OPN and OCN are crucial regulators for bone metabolism and multiple bone metabolic disorders, including the progression of osteoporosis (Zhao et al. 2015; Lin et al. 2019). LPL, AP-2 and leptin can promote adipogenesis and suppress osteogenic differentiation. Herein, it came to a prediction that miR-137 targets and binds to RUNX2 gene sequence, and RUNX2 expression showed a downtrend in MC3T3 E1 cells transfected with miR-137 mimics. As revealed by the LRGA, RUNX2 was the target gene of miR-137. Besides, the overexpression of miR-137 suppressed the expressions of osteogenic markers ALP, RUNX2, OPN and OCN, and promoted those of adipogenic markers LPL, AP-2 and leptin, indicating that miR-137 targets RUNX2 to suppress the osteogenic differentiation of MC3T3-E1.

CONCLUSION

In summary, miR-137 affects the osteogenic differentiation of MC3T3-E1 cells by negatively modulating RUNX2 expression. MiR-137, a suppressor of osteogenic differentiation, may be an eligible target for the intervention of abnormal osteogenic differentiation-related diseases such as osteoporosis.

RECOMMENDATIONS

The roles of miR-137 in osteogenic differentiation and osteoporosis should be further elucidated by using animal experiments.

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ABBREVIATIONS

ALP: Alkaline phosphatase;
AP-2: adipocyte binding protein-2;
BCA: bicinchoninic acid;
GAPDH: glyceraldehyde-3-phosphate dehydrogenase;
LPL: lipoprotein lipase;
NC: negative control;
OCN: osteocalcin;
OPN: osteopontin;
PBS: phosphate-buffered saline;
qRT-PCR: quantitative reverse transcription-polymerase chain reaction;
RUNX2: runt-related transcription factor 2;
TBST: Tris-buffered saline-Tween 20;
UTR: untranslated region.

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