European Review for Medical and Pharmacological Sciences 2018; 22: 1580-1587

Upregulation of miR-132 attenuates osteoblast differentiation of UC-MSCs

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Abstract. – OBJECTIVE: Wnt/β-catenin signaling pathway plays a role in upregulating expression of osteoblast (OB) specific transcriptional factor Osterix and promoting OB differentiation. It was shown that the elevation of the miR-132 level was associated with sclerotizing inhibition. Bioinformatics analysis revealed the complementary binding site between miR-132 and 3'-UTR of β-catenin. This study investigated the influence of miR-214 in regulating β-catenin expression and differentiation of umbilical cord mesenchymal stem cells (UC-MSCs) into OB.

MATERIALS AND METHODS: UC-MSCs were induced to differentiate to OB. The expressions of miR-132, β-catenin, Osterix, and ALP, together with ALP activity were detected on day 0, 5, 10, and 15. The regulatory relationship between miR-132 and β-catenin was confirmed by dual luciferase reporter gene assay. UC-MSCs were divided into five groups, including agomir-control, miR-132 agomir, pGPH1-NC, pGPH1-β-catenin, and miR-132 agomir + pGPH1-β-catenin groups. β-catenin, Osterix, and ALP expressions, together with ALP activity were tested after induction for 15 days.

RESULTS: MiR-132 was downregulated, while β-catenin Osterix and ALP expressions, together with ALP activity were enhanced in the process of UC-MSCs differentiating into OBs. MiR-132 agomir and/or pGPH1-β-catenin transfection significantly reduced β-catenin expression, downregulated Wnt/β-catenin signaling pathway activity, declined Osterix level, weakened ALP expression and activity, and attenuated OB differentiation of UC-MSCs.

CONCLUSIONS: The level of β-catenin was enhanced, while the miR-132 level was decreased in the process of UC-MSCs differentiating into OBs. Upregulation of miR-132 inhibited the differentiation of UC-MSCs through suppressing β-catenin expression, attenuating Wnt/β-catenin signaling pathway activity, and downregulating Osterix level.

Key Words:
miR-132, Wnt/β-catenin, Osterix, UC-MSCs, Osteoblast.

Introduction

Mesenchymal stem cells (MSCs) belong to adult stem cells distributed in bone marrow, umbilical cord, fat, and placenta. It can differentiate into multiple cells, including osteoblast (OB), cartilage cells, fat cells, nerve cells, and muscle cells[1,2]. Umbilical cord-derived mesenchymal stem cells (UC-MSCs) are featured as being from extensive source, easy obtaining, and no components of ethical issues, thus exhibiting wide clinical value.

Several studies[3,4] showed that Wnt/β-catenin signaling pathway plays a key role in regulating OB differentiation, bone mineralization, and skeletal development. It was demonstrated that β-catenin reduction was associated with the transduction of Wnt/β-catenin signaling pathway, blockage of OB differentiation, skeletal development disorder, and osteoporosis, suggesting that β-catenin served as an independent factor in OB differentiation and skeletal development[5]. MiRNA is a type of endogenous single-stranded noncoding RNA at the length of 22-25 nt that widely exists in multiple cells and tissues[6]. As an important epigenetic regulatory molecule, each miRNA regulates multiple target genes, while several miRNAs also modulates the same gene. MiRNA is involved in various processes, such as cell proliferation, differentiation, tissue development, and organogenesis[7,8]. Increasing evidence revealed that miRNAs play a crucial role in OB differentiation and sclerotizing. Aberrant miRNA expression and dysfunction may cause OB differentiation obstacle and skeletal growth abnormity, and is closely associated with multiple bone diseases[9-12]. It was exhibited that miR-132 contributed to regulatory function in OB differentiation[13]. Bioinformatics analysis unraveled the complementary binding site between miR-132 and 3'-UTR of β-catenin mRNA. Osterix is a newly discovered transcrip-

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tional factor containing zinc finger that is necessary to OB differentiation and maturation[14]. This study aimed to investigate the effect of miR-132 in regulating β-catenin and Osterix expressions, and differentiation of UC-MSCs into OB.

Materials and Methods

Main Reagents and Materials

Dulbecco's Modified Eagle Medium (DMEM) and penicillin-streptomycin were bought from Lonza (Basel, Switzerland). Fetal bovine serum (FBS) and type II collagenase were purchased from Gibco (Waltham, MA, USA). OriCell™ human UC-MSCs OB differentiation medium and gelatin were got from Cyagen Biosciences (Guangzhou, Guangdong, China). PCR primers were synthetized by Sangon (Shanghai, China). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Hilden, Germany). Mouse anti-human Osterix and β-catenin antibodies were bought from Abcam (Cambridge, MA, USA). pGL3 luciferase reporter gene plasmid was obtained from Promega (Madison, WI, USA). Light Switch luciferase reporter gene detection system was derived from Active Motif (Shanghai, China). MiR-132 mimic, mimic NC, miR-132 agomir, and agomir-control were designed and synthetized by Ribobio (Guangzhou, Guangdong, China). Transfection reagent FuGENE6 was purchased from Roche (Indianapolis, IN, USA). TRIzol was got from Invitrogen (Carlsbad, CA USA). Alkaline phosphatase (ALP) activity detection kit and bicinchoninic acid (BCA) protein quantification kit were bought from Beyotime (Beijing, China). CD44 and CD90 flow cytometry antibodies were bought from Biolegend (San Diego, CA, USA). CD73 and CD105 flow cytometry antibodies were bought from eBioscience (Waltham, MA, USA). shRNA plasmid pGPH1-β-catenin and negative pGPH1-NC were synthetized by Genepharma (Shanghai, China). The study protocol was approved by the Research Ethics Committee of Shanghai Jiading Nanxiang Hospital.

UM-MSCs Isolation

The umbilical cord was collected and washed by phosphate buffered saline (PBS) for three times. After tissues were cut into pieces by scissors, the umbilical cord was digested in 0.1% type II collagenase at 37°C for 3 h. Then the cells were centrifuged and resuspended in DMEM medium containing 10% FBS and 1% penicillin-streptomycin. At last, the cells were seeded in 10 cm dish at 100000/cm^2 and passed. P2 cells were used for flow cytometry identification.

UM-MSCs Identification

P2 cells were resuspended in 400 μl loading buffer and added with 5 μl CD44, CD73, CD90, and CD105 flow cytometry antibodies. After incubated avoid of light for 30 min at 4°C, the cells were resuspended in 500 μl loading buffer and tested on flow cytometry.

OB Induction

OB induction complete medium was prepared by ascorbic acid, sodium β-glycerophosphate, human UM-MSCs FBS, penicillin-streptomycin, glutamine, and human UM-MSCs OB induction medium. The six-well plate was coated by 0.1% gelatin solution at room temperature for 40 min. Then, the UM-MSCs at P2 were resuspended in DMEM complete medium and seeded in the plate at 5000/cm^2. After the cell fusion reached 60%, the medium was changed to osteogenic differentiation medium for 0, 5, 10, and 15 days, respectively. Next, the cells were collected for protein and ALP activity detection.

ALP Activity Detection

Chromogenic substrate solution and 0.5 mM *p*-nitrophenol standard substance working solution were prepared. The cells were cracked by RIPA (radioimmunoprecipitation assay) and protein was quantified by BCA method. The sample was added to the plate and incubated at 37°C for 10 min. After the reaction was stopped by 100 μl stop buffer, the plate was read at 405 nm. ALP activity was presented as OD/mg total protein after standardization.

Dual Luciferase Reporter Gene Assay

The PCR products containing the full length of β-catenin gene 3'-UTR or mutant segment were digested and connected to pGL3, namely pGL3-β-catenin-wt and pGL3-β-catenin-mut, respectively. Then, pGL3-β-catenin-wt (or pGL3-β-catenin-mut) was co-transfected to HEK293T cells using FuGENE6 together with miR-132 mimic (or miR-NC). The luciferase activity was detected after cultured for 48 h using Light Switch.

Cell Transfection

UM-MSCs were divided into five groups, including agomir-control, miR-132 agomir, pGPH1-NC, pGPH1-β-catenin, and miR-132 agomir + pGPH1-β-catenin groups. The cells were collected after 15 days' induction.

qRT-PCR

Total RNA was extracted using TRIzol and amplified using QuantiTect SYBR Green RT-PCR Kit. The primers used were as follows. β-catenin: forward, 5′-CATCTACACAGTTTGATGCTGCT-3′, reverse, 5′-GCAGTTTTGTCAGTTCAGGGA-3′; ALP: forward, 5′-ACGTGGCTAAGAATGTCATC-3′, reverse, 5′-CTGGTAGGCGATGTCCTTA-3′; Osterix: forward, 5′-CCTGCGACTGCCCTA-AT-3′, reverse, 5′-GCGAAGCCTTGCCATACA-3′; β-actin: forward, 5′-GAACCCTAAGGCCAAC-3′, reverse, 5′-TGTCACGCACGATTTCC-3′. The PCR system contained 10.0 μL 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL Primers (0.5 μm/L), 1.0 μg RNA, 0.5 μL QuantiTect RT Mix, and H_2O. The reverse transcription condition was 37°C for 15 min and 85°C for 5 s. The PCR reaction was composed of 95°C pre-denaturation for 5 min, followed by 40 cycles of 94°C denaturation for 15 s, 60°C annealing for 30 s, and 74°C elongation for 30 s. Real-time PCR was performed on ABI7500 to test the relative expression.

Western Blot

Total protein was extracted by RIPA. A total of 50 μg protein was separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to a membrane. Next, the membrane was blocked and incubated in primary antibody at 4°C overnight (β-catenin, Osterix, and β-actin at 1:300, 1:200, and 1:500, respectively). Then, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:8000) at room temperature after washed by PBST (phosphate-buffered saline and Tween 20) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc. Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by *t*-test or ANOVA. $p < 0.05$ was considered as statistical significance.

Results

UM-MSCs Isolation

Flow cytometry showed that MSC marker CD44, CD73, CD90, and CD105 were abundantly expressed in isolated P2 cells, indicating successful isolation of UC-MSCs (Figure 1A-D).

UC-MSCs Differentiated to OB

qRT-PCR detection showed that expressions of ALP and Osterix mRNA were gradually increased in the process of cell differentiation (Figure 2A and C). ALP activity was gradually elevated following differentiation and reached peak on the 15th day (Figure 2B). Western blot demonstrated that the level of Osterix protein was gradually upregulated over time (Figure 2D).

MiR-132 and β-catenin Expression Changed in the OB Differentiation Process of UC-MSCs

qRT-PCR detection exhibited that the level of β-catenin mRNA was gradually increased, while miR-132 expression was gradually declined in the process of differentiation of UC-MSCs into OB (Figure 3A and B). Western blot demonstrated that β-catenin protein was gradually enhanced over time (Figure 3C). Bioinformatics analysis revealed the complementary binding site between miR-132 and the 3'-UTR of β-catenin (Figure 3D), indicating the targeted regulatory relationship between them. Dual luciferase assay further validated that miR-132 mimic significantly declined relative luciferase activity (Figure 3E), confirming the regulatory relationship between miR-132 and β-catenin mRNA.

MiR-132 Overexpression Inhibited OB Differentiation of UC-MSCs

After OB induction for 15 days, UC-MSCs were collected for detection. It was showed that miR-132 agomir and/or β-catenin shRNA pGPH1-β-catenin transfection significantly reduced β-catenin expression, declined Osterix level (Figure 4A), and decreased ALP expression (Figure 4B) and activity (Figure 4C).

Discussion

Previous studies reported that differentiation of MSCs into OB is mainly regulated by TGF-β/BMPs[15], FGFs/FGFRs[16], and MAPK/ERK signaling pathways[17,18]. Recently, it was indicated that Wnt/β-catenin signaling pathway was involved in OB differentiation and new bone formation. The accumulation and stability of β-catenin in cytoplasm plays a crucial role in canonical Wnt/β-catenin signaling pathway[19]. β-catenin binds with the complex of axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK-3β). It is phosphorylated by GSK-3β and de-

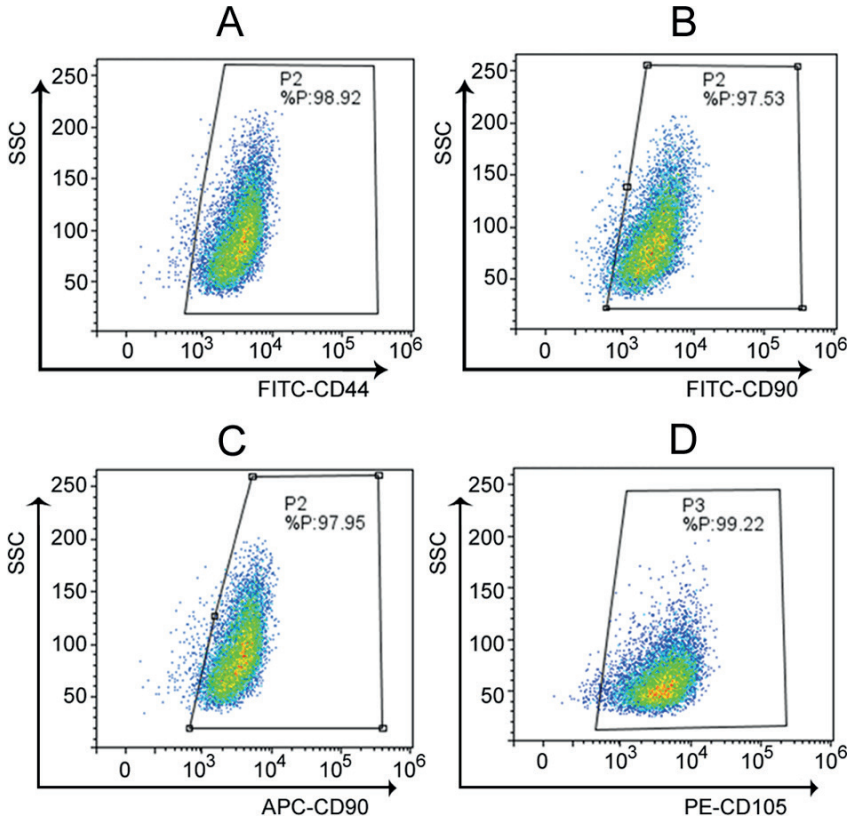

Figure 1. Flow cytometry detection of MSCs biomarker expressions. ***(A)*** CD44 expression; ***(B)*** CD73 expression; ***(C)*** CD90 expression; ***(D)*** CD105 expression.

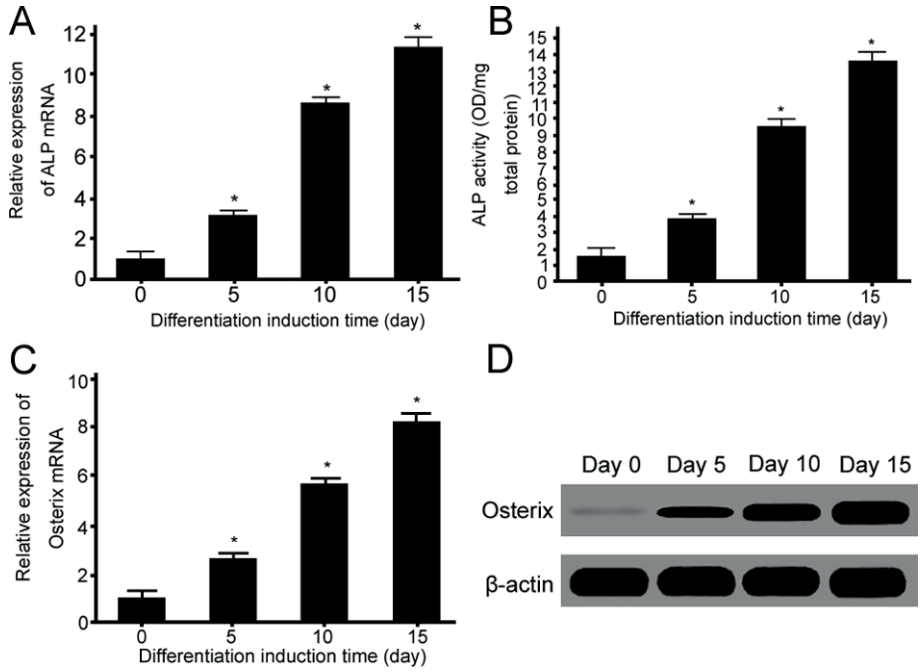

Figure 2. UC-MSCs differentiated to OB. ***(A)*** qRT-PCR detection of ALP mRNA expression; ***(B)*** ALP activity detection; ***(C)*** qRT-PCR detection of Osterix mRNA expression; ***(D)*** Western blot detection of Osterix protein expression; *$p < 0.05$, compared with day 0.

graded by ubiquitin/proteasome pathway[20]. APC-Axin-GSK-3β degradation complex dysfunction causes the increase of β-catenin in cytoplasm and its entry into the nucleus to promote target gene transcription and expression by binding with T-cell factor/lymphoid enhancing factor (TCF/LEF)[21,22]. Human Osterix gene locates in chromosome 12q13.13, which is closed to Sp1 gene and encodes a 46kD protein containing 428 amino acids[23]. It was shown that OB differentiation was inhibited in Osterix knockout mice, resulting in the suppression of bone tissue formation[23]. Osterix can suppress cartilage cell differentiation by inhibiting Sox9 and Sox 5 expressions[24,25], or can inhibit adipocyte differentiation by restraining PPARγ expression[26,27], so as to promote OB differentiation. Several researches[28,29] revealed that Osterix was one of the important target genes of Wnt/β-catenin signaling pathway.

This study demonstrated that Wnt/β-catenin signaling pathway enhancement is a promoting factor, whereas miR-132 may play an inhibitory role in UC-MSCs differentiating into OBs. Hu et al[13] showed that, compared with control, miR-132 expression was significantly increased in the bone tissue of rat model of bone loss induced by Hindlimb unloaded (HU), indicating that miR-132 elevation is a negative factor to restrain bone formation. Our results suggested that miR-132 upregulation may block OB differentiation, which was similar with Hu[13]. Zhao et al[30] reported that the expression of β-catenin was elevated during the process of MSCs differentiating into OBs, revealing that the increase of β-catenin and activation of Wnt/β-catenin signaling pathway are associated with OB differentiation, which was in accordance with our study.

Dual luciferase assay showed that miR-132 mimic significantly declined relative luciferase activity, confirming the regulatory relationship between miR-132 and β-catenin. Moreover, miR-132 agomir and/or β-catenin shRNA

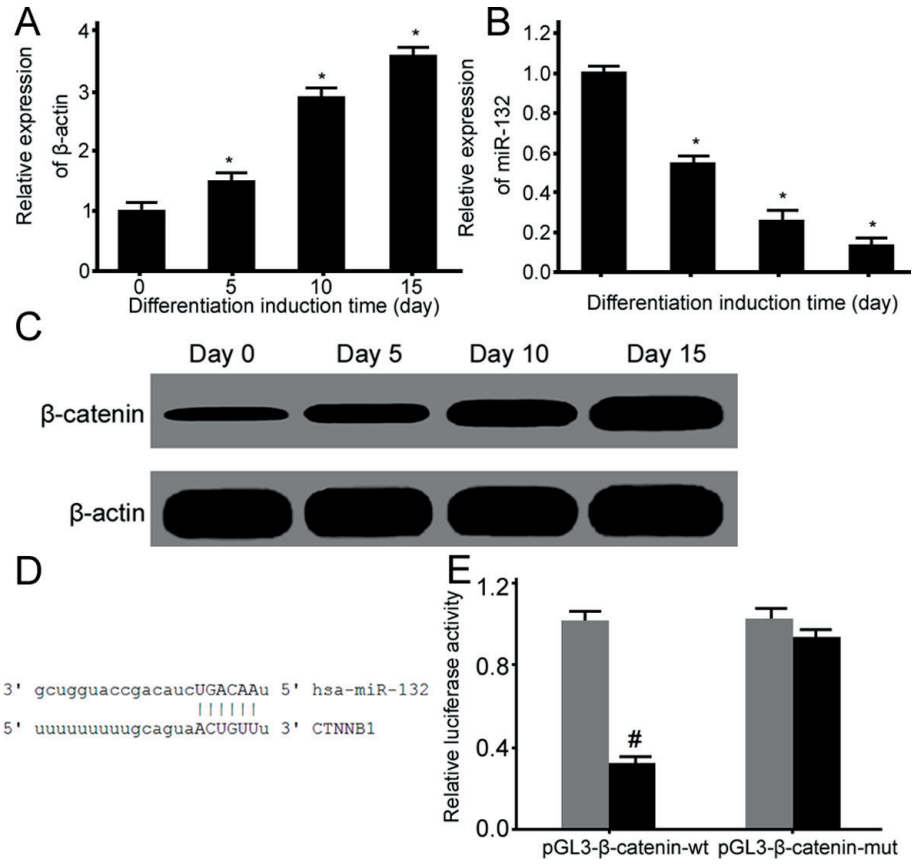

Figure 3. MiR-132 and β-catenin expression changed in the OB differentiation process of UC-MSCs. ***(A)*** qRT-PCR detection of β-catenin mRNA expression; ***(B)*** qRT-PCR detection of miR-132 expression; ***(C)*** Western blot detection of β-catenin protein expression; ***(D)*** The binding site between miR-132 the 3'-UTR of β-catenin mRNA; ***(E)*** Dual luciferase reporter gene assay; *$p < 0.05$, compared with day 0; #$p < 0.05$, compared with mimic NC.

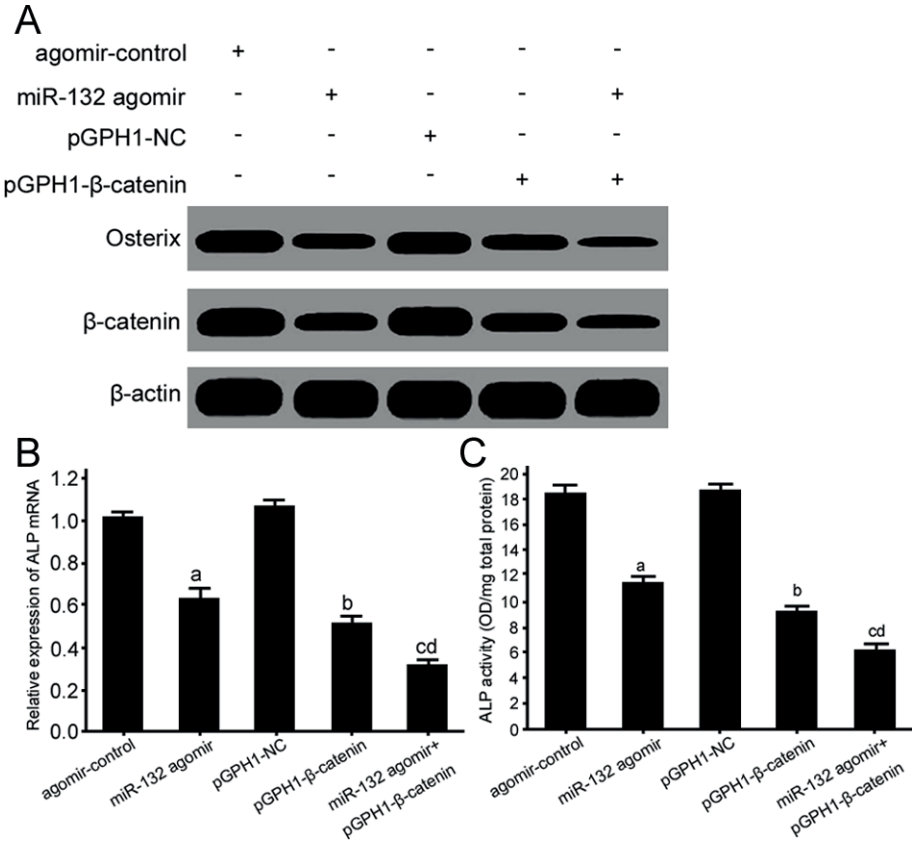

Figure 4. MiR-132 overexpression inhibited OB differentiation of UC-MSCs. ***(A)*** Western blot detection of Osterix protein expression; ***(B)*** qRT-PCR detection of ALP mRNA expression; ***(C)*** ALP activity detection; a, $p < 0.05$, compared with agomir-control; b, $p < 0.05$, compared with pGPH1-NC; c, $p < 0.05$, compared with agomir-control; d, $p < 0.05$, compared with pGPH1-NC.

pGPH1-β-catenin transfection significantly reduced β-catenin expression, declined Osterix level, and decreased ALP expression and activity, indicating that miR-132 reduced Osterix expression by inhibiting β-catenin level, so as to attenuate OB differentiation. It has been demonstrated that miR-132 restrained OB differentiation through suppressing Ep300 expression, whereas miR-132 downregulation attenuated the inhibitory effect of microgravity on osteoblastic progenitor cell prOB differentiating into OB. Qu et al[31] exhibited that miR-132 suppressed the OB differentiation process of ligamentum flavum cell by inhibiting FOXO1, GDF5, and SOX6. Gong et al[32] demonstrated that miR-132 elevation restrained the differentiation process of mouse embryonic osteoblastic progenitor cell MC3T3-E1 to mature bone cell through targeting Sirt1 gene expression, manifesting that miR-132 upregulation may be involved in pathogenesis of diabetic osteoporosis. In this study, the overexpression of miR-132 suppressed OB differentiation, which was in agreement with Hu et al[13], Qu et al[31], and Gong et al[32]. Hill et al[33] found that downregulation of β-catenin restrained the differentiating effect of bone marrow MSCs to OB, alleviated skeletal mineralization, and facilitated cartilage cell differentiation, while β-catenin upregulation led to the opposite effect, which was similar with our results. Consistent with our study, recent evidence[34] also indicated that miR-214 inhibited human mesenchymal stem cells differentiating into osteoblasts through targeting beta-catenin. However, the mechanism of involvement of Wnt/β-catenin signaling activity in reducing Osterix expression is still unclear.

Conclusions

We demonstrated that the level of β-catenin was enhanced, while miR-132 expression was decreased in the process of UC-MSCs differentiating into OBs. Upregulation of miR-132 inhibited the OB differentiation of UC-MSCs through

suppressing β-catenin expression, attenuating the activity of Wnt/β-catenin signaling pathway, and downregulating Osterix level.

Acknowledgments

This work was supported by Research project of Wei Planning Commission of Jiading District, Shanghai (No. 2014-KY-05).

Conflict of Interest

The Authors declare that they have no conflict of interest.

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