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MiRNA-128-3p induces osteogenic differentiation of bone marrow mesenchymal stem cells *via* activating the Wnt3a signaling

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Abstract. – OBJECTIVE: To clarify the biological function of miRNA-128-3p in influencing the progression of osteoporosis by inducing osteogenic differentiation of MSCs via activating the Wnt3a signaling.

PATIENTS AND METHODS: Dynamic expression levels of miRNA-128-3p in osteogenically differentiated MSCs at the different time points were detected by qRT-PCR. The binding sites in the seed sequence of miRNA-128-3p and Wnt3a were predicted using the bioinformatic tool, and their interaction was further confirmed by Dual-Luciferase reporter assay. Co-regulation of miRNA-128-3p and Wnt3a on relative levels of osteogenesis-associated genes, ALP activity and mineralization ability in glucocorticoid-induced MSCs were assessed.

RESULTS: MiRNA-128-3p was gradually upregulated with the prolongation of osteogenic differentiation of MSCs. Overexpression of miRNA-128-3p reversed the declines in glucocorticoid-induced expression levels of osteogenesis-associated genes (Bglap, RUNX2 and BMP-2), ALP activity and mineralization ability in MSCs. Wnt3a was able to bind miRNA-128-3p. Its level was positively regulated by miRNA-128-3p in MSCs. Enhanced ALP activity and mineralization ability in glucocorticoid-induced MSCs overexpressing Wnt3a were partially abolished by knockdown of miRNA-128-3p.

CONCLUSIONS: By positively regulating Wnt3a, miRNA-128-3p alleviates the progression of osteoporosis through inducing osteogenic differentiation of MSCs.

Key Words:
Osteoporosis, MiRNA-128-3p, Wnt3a, Osteogenic differentiation.

Introduction

Osteoporosis is a systemic bone metabolism disease. Imbalance of osteogenic and osteoclastic activities in the bone microenvironment leads to declined bone mass and increased bone fragility. Osteoporotic fracture is the most serious consequence caused by the imbalanced bone microenvironment[1]. It is estimated that the number of osteoporosis patients reaches 200 million in the world[2]. Clarifying the molecular mechanism of osteoporosis development is of great significance.

MicroRNAs (miRNAs) are small, noncoding RNAs involved in post-transcriptional mediation on cell phenotypes. They contain 19-22 nucleotides, and extensively distributed in the body[3,4]. A dynamic balance between osteoblasts-induced bone formation and osteoclasts-induced bone resorption maintains the health of mature bone tissues, which can be damaged by miRNAs[5]. It is believed that abnormally expressed miRNAs during bone metabolism are closely related to bone diseases[6,7]. Researches[8-10] have identified that miRNA-128-3p is able to suppress the malignant proliferation of T-lymphocytic leukemia, hepatocellular carcinoma and glioma. Lowly expressed miRNA-128-3p may predict poor prognosis in the abovementioned malignant diseases. The correlation between miRNA-128-3p and osteoporosis, however, remains unclear.

Potential influence of the Wnt signaling on MSCs functions has been highlighted in the research of bone metabolism and bone tissue engineering[11]. Wnt3a is a secreted glycoprotein enriched with cysteine that can affect surrounding cells *via* paracrine or autocrine. It is an activator of the Wnt signaling, which is responsible for triggering cell division, growth and differentiation[12,13]. Zhang et al[14] demonstrated the capacity of Wnt3a in driving osteogenic differentiation. Through targeting Wnt3a, miR-9-5p deteriorates the progression of osteoporosis by stimulating adipogenesis[15]. This study aims to explore the co-regulation of miRNA-128-3p and Wnt3a on osteogenic differentiation of MSCs, and thus the progression of osteoporosis.

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Patients and Methods

Isolation of MSCs and Cell Culture

This study was approved by the Ethics Committee of The First People's Hospital of Fuyang. Signed written informed consents were obtained from all participants before the study. 9 cases of open fracture and ilium transplantation were recruited. Patients with osteoporosis, tumors, blood diseases and other systemic diseases were excluded. During the operation, 10 mL of fresh bone marrow was collected and placed in ethylenediaminetetraacetic acid (EDTA) anticoagulation tubes. Collected bone marrow was mixed in 10 mL of serum-free, low-glucose α-modified eagle medium (α-MEM) (HyClone, South Logan, UT, USA), and centrifuged at 400 g for 10 min. The fat layer was discarded. The precipitant was re-suspended in α-MEM, and the isodose Fircoll solution was slowly added alongside the tube wall. Liquid stratification can be observed. After gradient centrifugation for 30 min, the second layer (monocytes) was washed and resuspended in α-MEM containing 10%. Cell suspension (2×10^6/mL) was cultivated in bottles. On the other day, un-adherent cells were washed by fresh medium. Cell passage was conducted every 3-5 days using 0.25% trypsin. The morphology and growth condition of MSCs were regularly observed under the microscope.

Osteogenic Differentiation

The third-generation MSCs were inoculated in a 24-well plate (2×10^4 /well) and cultivated to 80% confluence. Osteogenic differentiation was induced in H-Dulbecco's Modified Eagle's Medium (H-DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 10 nM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM ascorbic acid for 2-21 days.

Glucocorticoid Induction

100 μL of suspension per well [(5-8) × 10^4 / mL] was applied in a 96-well plate. On the next day, MSCs were cultivated in DMEM containing 1 μM glucocorticoid for 24 h.

Cell Transfection

The third-generation MSCs were inoculated in a 24-well plate (2×10^4 /well) and cultivated to 80% confluence. They were transfected with plasmids using Lipofectamine 2000 for 48 h and subjected to osteogenic differentiation for 2-21 days.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular RNAs were isolated using TRIzol and their concentrations were determined by NanoDrop 2000 (Invitrogen, Carlsbad, CA, USA). RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) and subjected to qRT-PCR. Primer sequences were as follows: MiRNA-128-3p: forward: 5′-UCACAGUGAACCGGUCUCUUU-3′, reverse: 5′-AGAGACCGGUUCACUGUGAUU-3′; U6: forward: 5′-GCTTCGGCAGCACATATACTAAAAT-3', reverse: 5′-CGCTTCACGAATTTGCGTGTCAT-3'; Wnt3a: forward: 5′-ATCGAGTTTGGTGGGATGGT-3′, reverse: 5′-CGCTGTCGTACTTGTCCTTG-3′; RUNX2: forward: 5′-GGGTAAGACTGGTCATAGGACC-3′, reverse: 5′-CCCAGTATGAGAGTAGGTGTCC-3′; BMP-2: forward: 5′-TTGGAGGAGAAACAAGGTG-3′, reverse: 5′-AACAATGGCATGATTAGTGG-3′; Bglap: forward: 5′-AAAGCCTGGTGATGCAGAGT-3′, reverse: 5′-CTAGACTGGGCCGTAGAAGC-3′; GAPDH forward: 5′-ACTGCCACCCAGA-AGACT-3′, reverse: 5'-GCTCAGTG-TAGCCCAGGAT-3′.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) on ice for 30 min. Cell lysate was centrifuged at 4°C, 1000 rpm for 10 min. Extracted protein samples were quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses of grey values were finally conducted.

Determination of ALP Activity

On the 7th day of osteogenic differentiation, MSCs were lysed for collecting the supernatant, which was incubated in the binding buffer at 37°C for 0.5 h and terminated by applying NaOH. ALP activity (Sigma-Aldrich, St. Louis, MO, USA) was determined by measuring absorbance at 405 nm. Each test was repeated in triplicate.

ALP Staining

MSCs were washed in PBS twice, fixed in 70% ethanol for 10 min, and incubated in ALP

buffer (0.15M NaCl, 0.15M Tris-HCl, 1mM $MgCl_2$, pH9.5) for 15 min. Subsequently, MSCs were incubated in NBT-BCIP solution (Sigma-Aldrich, St. Louis, MO, USA) in the dark at 37°C for 30 min. Cells were washed in deionized water, dried and captured.

ARS Staining

On the 21st day of osteogenic differentiation, MSCs were fixed in 4% paraformaldehyde for 30 min, washed in deionized water for three times and dyed in ARS-Tris-HCL solution (pH 4.1-4.3) (Sigma-Aldrich, St. Louis, MO, USA) in the dark for 45 min. Images were captured under an inverted microscope.

Dual-Luciferase Reporter Assay

The binding sites in the seed sequence of miRNA-128-3p and Wnt3a were predicted using the bioinformatic tool and amplified. The amplified fragments were inserted into Luciferase vectors for constructing wild-type and mutant-type Wnt3a vectors. They were co-transfected into cells with miRNA-128-3p mimic or miR-NC. Luciferase activity was measured using the Dual-Luciferase® Report (Promega, Madison, WI, USA).

Statistical Analysis

Data processing was conducted using Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA). Differences between groups were compared by the Student's *t*-test. A significant difference was set at $p < 0.05$.

Results

Dynamic Expression Levels of MiRNA-128-3p in MSCs During the Process of Osteogenic Differentiation

On the 1st, 3rd, 7th and 14th day of osteogenic differentiation, miRNA-128-3p level in MSCs was time-dependently upregulated, indicating a close relation between miRNA-128-3p and osteogenic differentiation of MSCs (Figure 1A). We subsequently tested the transfection efficacy of miRNA-128-3p mimic and inhibitor in MSCs, respectively, (Figure 1B). Transfection of either of them could effectively intervene miRNA-128-3p level.

Overexpression of MiRNA-128-3p Upregulated the Inhibited Expressions of Osteogenesis-Associated Genes in Glucocorticoid-Induced MSCs

After glucocorticoid induction in MSCs, mRNA levels of osteogenesis-associated genes, including Bglap, RUNX2 and BMP-2, were downregulated. However, the declined trends were abolished by overexpression of miRNA-128-3p (Figure 2A-2C). Similar results were obtained by detecting their protein levels *via* Western blot (Figure 2D, E). It is suggested that miRNA-128-3p could drive osteogenic differentiation of MSCs.

MiRNA-128-3p Triggered Osteogenic Differentiation of MSCs

We subsequently explored the role of miRNA-128-3p in regulating ALP activity and mineralization ability in MSCs. Glucocorticoid induction markedly suppressed ALP activity in MSCs, which was further enhanced by overexpressed miRNA-128-3p (Figure 3A). Consistently, gluco-

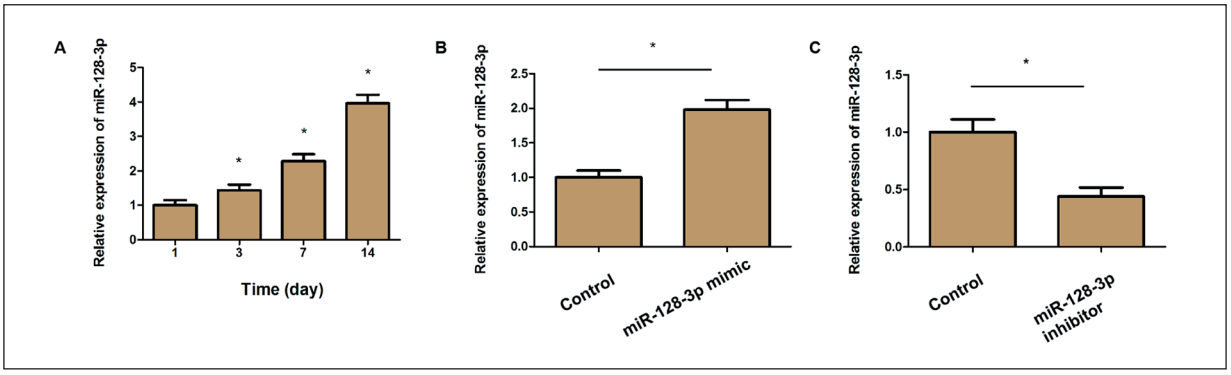

Figure 1. Dynamic expression levels of miRNA-128-3p in MSCs during the process of osteogenic differentiation. **A,** MiRNA-128-3p in osteogenically differentiated MSCs was time-dependently upregulated; **B,** MiRNA-128-3p was significantly upregulated by transfection of miRNA-128-3p mimic in MSCs; **C,** MiRNA-128-3p was significantly downregulated by transfection of miRNA-128-3p inhibitor in MSCs.

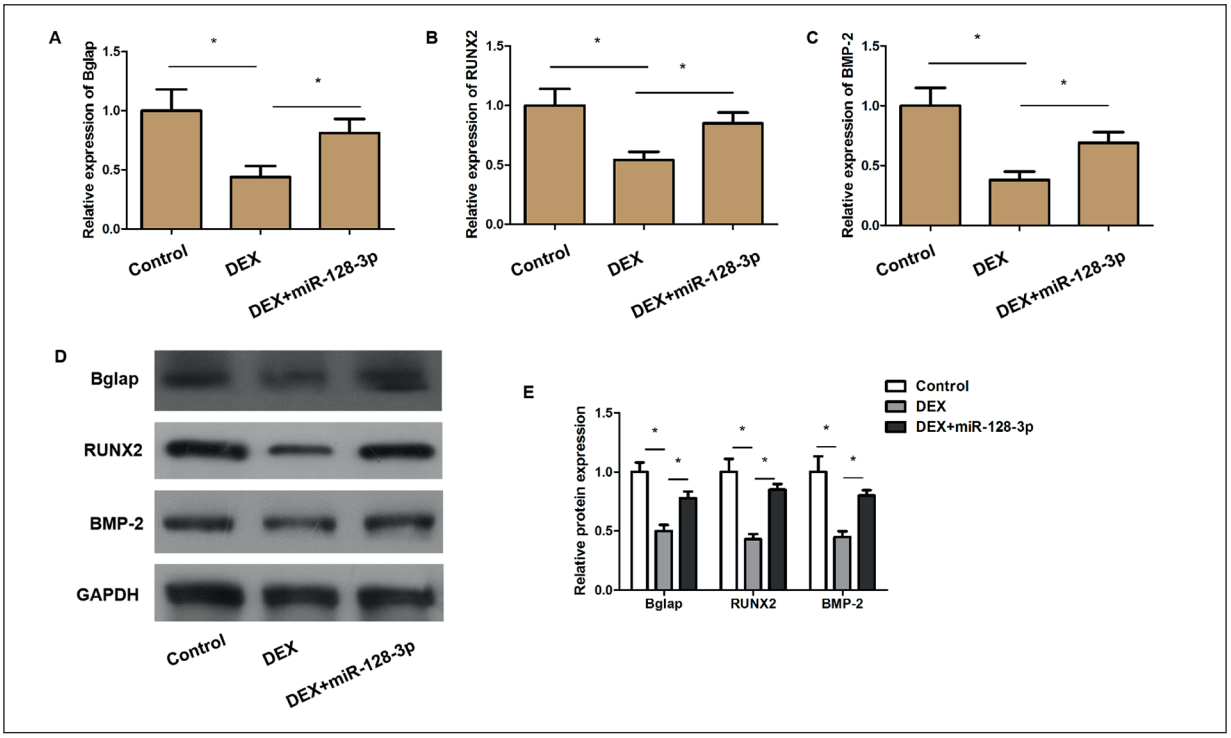

Figure 2. Overexpression of miRNA-128-3p upregulated the inhibited expressions of osteogenesis-associated genes in glucocorticoid-induced MSCs. A-C. Glucocorticoids induction downregulated mRNA levels of Bglap (**A**), RUNX2 (**B**) and BMP-2 (**C**) in MSCs, which were reversed by overexpression of miRNA-128-3p; **D, E,** Glucocorticoids induction downregulated protein levels of Bglap, RUNX2 and BMP-2 in MSCs, which were reversed by overexpression of miRNA-128-3p.

corticoid induction intervened positive staining of ALP and ARS in MSCs, and the inhibited trends were reversed by overexpression of miRNA-128-3p (Figure 3B). It is suggested that glucocorticoid inhibits osteogenic differentiation, bone mineralization and bone metabolism, which could be protected by miRNA-128-3p.

MiRNA-128-3p Upregulated Wnt3a Level

Through predicting in Starbase 3.0, binding sites in the sequence of miRNA-128-3p and Wnt3a were identified (Figure 4A). Dual-Luciferase reporter assay showed that overexpression of miRNA-128-3p remarkably quenched luciferase activity in the wild-type Wnt3a vector, verifying

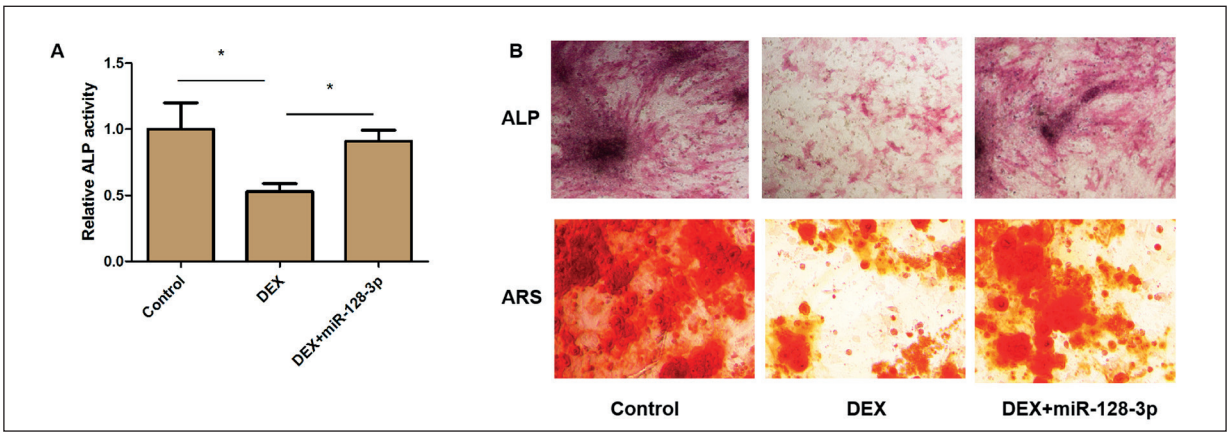

Figure 3. MiRNA-128-3p triggered osteogenic differentiation of MSCs. **A,** ALP activity was reduced by glucocorticoids induction in MSCs, which was abolished by overexpression of miRNA-128-3p; **B,** Positive staining of ALP and ARS were reduced by glucocorticoids induction in MSCs, which were abolished by overexpression of miRNA-128-3p, (magnification: 40×).

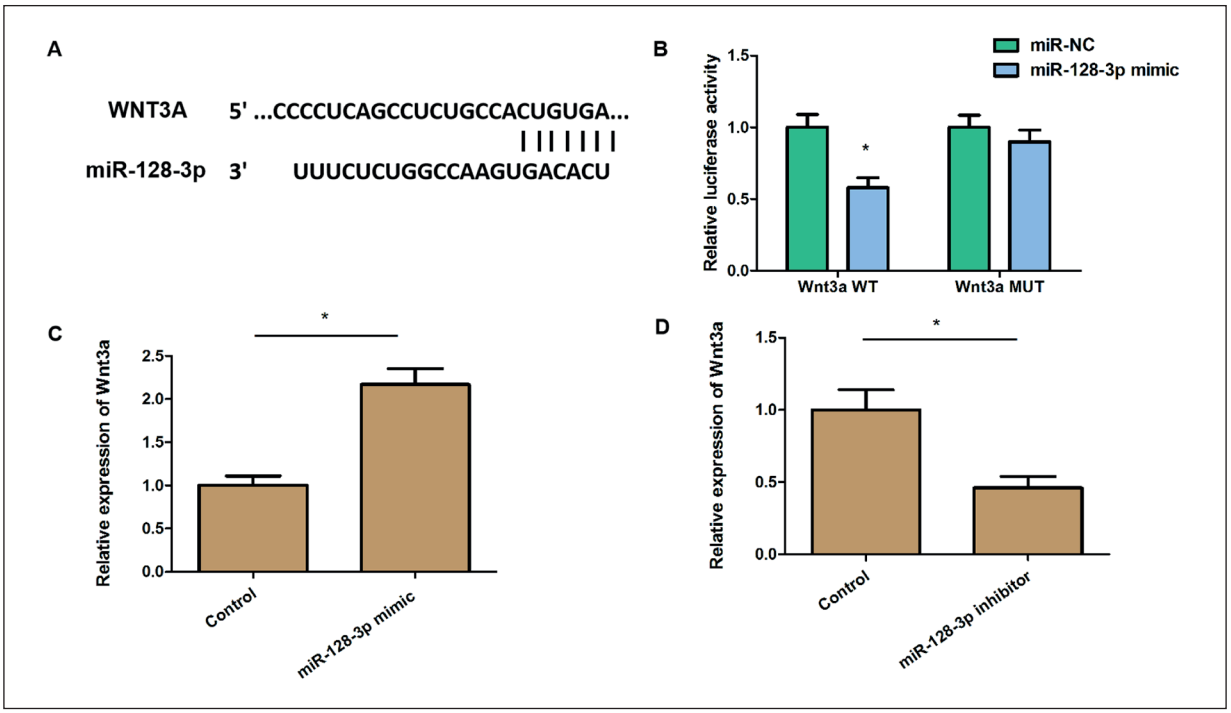

Figure 4. MiRNA-128-3p upregulated Wnt3a level. **A,** Binding sites in the seed sequence of Wnt3a and miRNA-128-3p; **B,** Dual-Luciferase reporter assay verified the binding between Wnt3a and miRNA-128-3p; **C,** Wnt3a was significantly upregulated in MSCs transfected with miRNA-128-3p mimic; **D,** Wnt3a was significantly downregulated in MSCs transfected with miRNA-128-3p inhibitor.

the binding between miRNA-128-3p and Wnt3a (Figure 4B). To verify the regulatory interaction between them, Wnt3a level in MSCs with intervened miRNA-128-3p expression was detected. It is shown that Wnt3a was positively regulated by miRNA-128-3p in MSCs (Figure 4C, 4D).

MiRNA-128-3p Regulated Osteogenic Differentiation by Targeting Wnt3a

The involvement of Wnt3a in the process of osteogenic differentiation was further explored. In glucocorticoid-induced MSCs overexpressing Wnt3a, both ALP activity and mineralization ability were enhanced, which were partially reversed by knockdown of miRNA-128-3p (Figure 5A, 5B). Collectively, miRNA-128-3p triggered osteogenic differentiation in MSCs by regulating Wnt3a, thus participating in the progression of osteoporosis.

Discussion

MSCs are featured by wide sources, easy isolation, rapid amplification and high purity. They are able to differentiate to osteoblasts, chondrocytes, adipocytes or hepatocytes under the certain circumstances[16]. Owing to the multilineage differentiation potential, bone regeneration using MSCs as seed cells is feasible, which provides novel strategies for the treatment of bone diseases. Notably, how to improve the osteogenesis ability of MSCs is a key event for triggering bone regeneration and repair[17].

MiRNAs can regulate the osteogenic differentiation of different cells[18]. In recent years, the role of miRNA modification in regulating osteogenic differentiation of MSCs has been well concerned[19]. They target different transcription factors that drive MSCs to differentiate into osteoblasts or osteoclasts[20,21]. MiRNAs can either drive osteogenic differentiation or inhibit it. It is reported that miR-26a increases expressions of fat-derived stem cell genes by activating the classic BMP-Smads signaling, thus stimulating osteogenic differentiation[22]. By regulating the target gene Cbfβ, miR-125b downregulates osteogenesis-associated genes, thereby inhibiting osteogenic differentiation[23]. This study successfully isolated MSCs from human bone marrow and induced *in vitro* osteogenic differentiation. We found that miRNA-128-3p was time-dependently upregulated during osteogenic differentiation of MSCs.

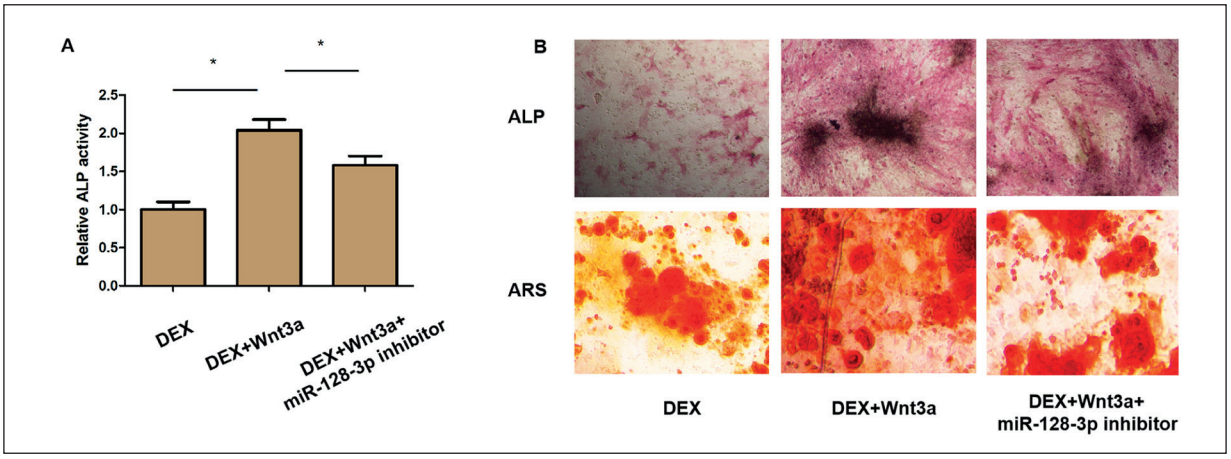

Figure 5. MiRNA-128-3p regulated osteogenic differentiation by targeting Wnt3a. **A,** ALP activity was elevated by glucocorticoids induction in MSCs overexpressing Wnt3a, which was abolished by knockdown of miRNA-128-3p; **B,** Positive staining of ALP and ARS were reduced by glucocorticoids induction in MSCs overexpressing Wnt3a, which were abolished by knockdown of miRNA-128-3p, (magnification: 40×).

During osteogenic differentiation, osteoblasts secrete some key proteins responsible for differentiation. RUNX2 is the master switch for osteoblast differentiation and it regulates expressions of multiple osteogenesis genes. Bglap is a marker of late phase of osteogenic differentiation, which exerts an important role in the maturation of bone matrix mineralization[24]. These proteins are important landmarks for osteogenic differentiation and mineralization. In addition, osteoblasts generally undergo the early, middle and late phase of bone formation, and ALP is an indicator for the former two phases[25]. Alizarin red staining (ARS) produces a dark red compound by a color reaction between alizarin red and calcium salt deposits. The formation of calcified nodule is a sign of osteoblast differentiation, maturation and mineralization[26]. Therefore, ARS contributes to revealing the condition of late phase of osteogenesis. Sun et al[27] found that knockdown of miR-145 can enhance expressions of Runx2, Osterix, β-catenin, and T cytokine 1, thereby promoting osteogenic differentiation. In the present study, miR-128-3p not only upregulated osteogenesis genes (Bglap, RUNX2, BMP-2), but also promoted ALP activity and the ability of mineralization.

The way in which the Wnt signaling regulates osteogenic differentiation has been identified[28]. The Wnt signaling is involved in induction of bone formation, suppression of adipogenesis and tumorigenesis[29]. Insertion of vesicles containing purified Wnt3a protein in the area of bone defect stimulates the rapid regeneration of bones[30]. Leucht et al[31] analyzed osteogenesis in transplanted bones in adult animals. They demonstrated that Wnt3a remarkably inhibits cell apoptosis and drives bone regeneration in transplanted bones. Besides, overexpression of Wnt3a is proven to promote proliferative rate in MSCs and inhibit chondrogenic differentiation. By silence of miR-203a-3p.1, the activated Wnt3a affects osteogenic differentiation of MSCs[32]. Our findings first revealed the binding between Wnt3a and miRNA-128-3p, and their positive interaction. Enhanced ALP activity and mineralization ability in glucocorticoid-induced MSCs overexpressing Wnt3a were partially abolished by knockdown of miRNA-128-3p. To sum up, glucocorticoids inhibit osteogenic differentiation of MSCs, and miRNA-128-3p can abolish the inhibition by targeting Wnt3a, thereby alleviating osteoporosis. Our findings provide novel ideas in clinical management of osteoporosis.

Conclusions

We showed that, by positively regulating Wnt3a, miRNA-128-3p alleviates the progression of osteoporosis through inducing osteogenic differentiation of MSCs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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