miR-216a-3p inhibits osteogenic differentiation of human adipose-derived stem cells via Wnt3a in the Wnt/β-catenin signaling pathway

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Abstract. The current study aimed to investigate the potential function and mechanism of microRNA (miR)-216a-3p in the osteogenic differentiation of human adipose-derived stem cells (hADSCs). Dynamic expression changes of miR-216a-3p in the osteogenic differentiation of hADSCs were examined by reverse transcription-quantitative PCR (RT-qPCR). Regulatory effects of miR-216a-3p on the relative levels of osteogenesis-associated genes were also detected by RT-qPCR and western blotting. The relationship between miR-216a-3p and Wnt3a was verified through a dual-luciferase reporter assay. Furthermore, the influence of miR-216a-3p on the Wnt/β-catenin signaling pathway during the osteogenic differentiation of hADSCs was investigated by western blotting. The results revealed that during the osteogenic differentiation process of hADSCs, miR-216a-3p was downregulated and Wnt3a was upregulated. It was further verified that Wnt3a was the target of miR-216a-3p. Through inactivation of the Wnt/β-catenin signaling pathway, miR-216a-3p was able to mediate osteogenic differentiation of hADSCs. In conclusion, by targeting Wnt3a, miR-216a-3p mediated the osteogenic differentiation of hADSCs, which negatively regulated the Wnt/β-catenin signaling pathway.

Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that present self-renewal and differentiation potential to bone cells, chondrocytes, and adipocytes (1-3). MSCs can be isolated from various tissues or organs such as the blood, bone marrow, adipose tissue, umbilical cord, placenta, lung, liver, and skin (1,4). An abundant source of MSCs is adipose tissue, from which MSCs can be easily isolated. Human adipose-derived stem cells (hADSCs) are a widely used source in tissue engineering repair and regeneration because of their adipogenic, chondrogenic, and osteogenic potential (5-7). Therefore, it is very important to clarify the molecular mechanism of the osteogenic differentiation of hADSCs.

The regeneration of bones is essential for bone development, continuous bone remodeling in adults, and bone damage repair (8,9). Bone regeneration consists of a cascade of precisely regulated biological processes involving various cell types, and intracellular and extracellular signaling pathways (9). The Wnt signaling pathway is capable of mediating multiple biological activities during bone damage repair and bone regeneration, including osteoblast differentiation and bone formation (10,11). It constitutes a typical β-catenin-dependent pathway and two atypical β-catenin-independent pathways (the atypical Wnt/PCP pathway and the Wnt/Ca^{2+} pathway) (12). The typical Wnt/β-catenin pathway exerts a dominant role in regulating osteoblast differentiation during bone fracture repair (11). It inhibits the differentiation of multifunctional MSCs into adipocytes and promotes their differentiation into osteoblasts (13,14). Previous studies have shown that the expression of Runx2, ALP, OCN and OPN changes significantly during osteogenic differentiation, which could be the markers of osteogenic differentiation (15,16).

MicroRNAs (MiRNAs) are small-chain, single-stranded non-coding RNAs (approximately 22 nucleotides long). They incompletely or completely bind to the 3' untranslated region (3'-UTR) of target mRNAs, thus regulating cell signaling pathways through negatively mediating post-transcriptional gene expression or degrading mRNAs (17,18). Studies have shown that miR-216a-3p promotes cancer (19-21), and many reports showed that miR-216a-3p significantly influenced the activity of Wnt/β-catenin signaling pathway. Song *et al* (22). reported that knockdown of miR-216a-3p induces differentiation of BMMSCs into ACE II cells through the Wnt/β-catenin pathway, thereby alleviating NRDS. It was also demonstrated that the BRD4/miR-216a-3p/Wnt/β-catenin pathway regulates

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the stemness of gastric cancer cells (23). Its potential function in bone formation, however, is largely unclear.

This article aimed to investigate the potential function and mechanism of miR-216a-3p in the osteogenic differentiation of hADSCs.

Materials and methods

Cell culture. hADSCs (Cat. No. 7510, hADSCs) were obtained from ScienCell Company (Carlsbad, CA, USA), and the 293T cells were purchased from Mingzhou Bio (Cat. No. MZ-0266, Ningbo, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C.

In vitro osteogenic differentiation. At 80-90% cell confluence, the medium was replaced with osteogenesis induction medium (DMEM containing 10% FBS, 10 mM β-glycerophosphate, 0.1 μM dexamethasone, and 0.2 mM ascorbic acid). Cells were collected before osteogenic differentiation (day 0) and 3, 7 and 14 days after osteogenic differentiation.

RT-qPCR. Cells were digested in EDTA containing 0.25% trypsin, washed in phosphate-buffered saline (PBS) 2-3 times, and lysed in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to isolate cellular RNAs. After erasing gDNAs using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.), RNAs were reversely transcribed to cDNAs at 37˚C for 15 min and 85˚C for 5 sec, and were maintained at 4˚C. Using the SYBR Premix Ex Taq™ II Kit (Takara Bio, Inc.), cDNAs were subjected to thermal cycles at 95˚C for 15 min, followed by 40 cycles at 95˚C for 5 sec, 60˚C for 30 sec, 72˚C for 60 sec, and extension at 72˚C for 10 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Relative levels of Runx2, ALP, OCN, OPN, COL1A1, Wnt3a, and miR-216a-3p were calculated by $2^{-\Delta\Delta Cq}$ for three independent measurements (24). The sequences of the primers used in RT-qPCR are listed in Table I.

Western blotting. Cells were washed in PBS and lysed in cell lysis buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 1% SDS, 1% NP-40, 1% Triton X-100; pH 7.4) on ice. Protein concentrations were measured using a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Jiangsu, China). The proteins were loaded at an amount of 10 μg, and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Following incubation for 2 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) with 5% skim milk, immunoblotting with primary antibodies (anti-Runx2, anti-ALP, anti-Wnt3a, anti-OPN, anti-β-catenin, and anti-GAPDH; 1:1,000, ABclonal Biotech Co., Ltd.) at 4˚C overnight and secondary antibodies (1:1,000, ABclonal Biotech Co., Ltd.) for 1 h was performed. All of the antibodies used in this manuscript were purchased from Abclonal, Wuhan, China, and the catalog numbers were as listed: Runx2 (A11753), ALP (A0514), OPN (A19092), OCN (A6205), Wnt3a (A0642), β-catenin (A19657), GAPDH (A19056), and the secondary antibody (AS014). Band exposure was achieved by the enhanced chemiluminescence (ECL) method, followed by measurement of grey value analysis using the Quantity One® 1-D Analysis Software (Bio-Rad, Hercules, CA, USA).

Lentivirus synthesis and transfection. Lentivirus overexpression vectors GV287-miR-NC, GV287-miR-216a-3p, GV287-anti-miR-NC, GV287-anti-miR-216a-3p, GV287-Wnt3a and the control group GV287 were purchased from GenePharma (Shanghai, China), and a 2nd generation system was used to the package of lentivirus. The lentiviral plasmid, packaging vector and envelope vector were mixed at a 4:3:2 ratio for a total DNA mass of 20 μg. The mixture was firstly incubated with 1 ml Lenti-Easy Packaging Mix (Shanghai GeneChem Co., Ltd.) for 15 min, then incubated for another 20 min incubation with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and then added into 293T cell culture medium for 6 h at 37˚C. In brief, the 293T cells were seeded at a density of 2.5 * 10^5 cells/plate in a 10-cm plate and cultured to 80% confluence, then incubated in Opti-MEM for 4 h, and then incubated with the transfection mixture as described. The supernatant of the transfected 293T cells was collected after three days by filtering through a 0.45-μm filter, and the viral particles were concentrated by ultracentrifugation at 70,000 g for 2 h at 4˚C. hADSCs cells were infected with the lentivirus at a multiplicity of infection of 5 and with polybrene (Sigma-Aldrich; Merck KGaA) at a final concentration of 8 μg/ml at 37˚C with 5% CO_2 for 24 h. Fresh culture medium was then used to replace the old medium. Fluorescence was measured 72 h post-infection when the achieved infection efficiency was 80%. Screening of stable cells using green fluorescent protein.

Dual-luciferase reporter assay. It was predicted using TargetScan 7.2 that Wnt3a is the direct target of miR-216a-3p. Wnt3a 3' UTR or mutant sequences were cloned into the pmirGLO vector (Promega, Beijing, China) for the synthesis of pmirGLO-Wnt3a-WT and pmirGLO-Wnt3a-Mut. pRL-TK vector (Takara, Dalian, Liaoning, China) was used as the negative control. The cells seeded in a 6-well plate with higher than 80% density were co-transfected with luciferase vector/pRL-TK vector and miR-216a-5p mimic/negative control. After transfection for 48 h, relative firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China).

Statistical analysis. GraphPad PRISM 8.01 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analyses. The data were obtained from three independent experiments and are expressed as the mean ± standard deviation (SD). Differences between groups were compared by the *t*-test, and those among groups were analyzed by one-way analysis of variance (ANOVA), and followed by Tukey's or Bonferroni's post hoc test. All experiments were performed in triplicate and repeated three times. $P<0.05$ was considered as statistically significant. All experiments were performed in triplicate and repeated three times.

Table I. Sequence of the forward and reverse primers used for RT-qPCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
miR-216a-3p	TAATCTCAGCTGGCAACTGTGA	TCACAGTTGCCAGCTGAGATTA
Runx2	CAAGGACAGAGTCAGATTAC	GTGGTAGAGTGGATGGAC
ALP	TAAGGACATCGCCTACCAGC	TGGCTTTCTCGTCACTCTCA
OCN	GGTGCAGCCTTTGTGTCCAAGC	GTCAGCCAACTCGTCACAGTCC
OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT
COL1A1	CAATGCTGCCCTTTCTGCTCCTTT	ATTGCCTTTGATTGCTGGGCAGAC
Wnt3a	CCATCCTCTGCCTCAAATTC	TGGACAGTGGATATAGCAGCA
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

ALP, alkaline phosphatase; COL1A1, collagen, type I, alpha 1; miR, microRNA; OCN, osteocalcin; OPN, osteopontin; Runx2, runt-related transcription factor 2.

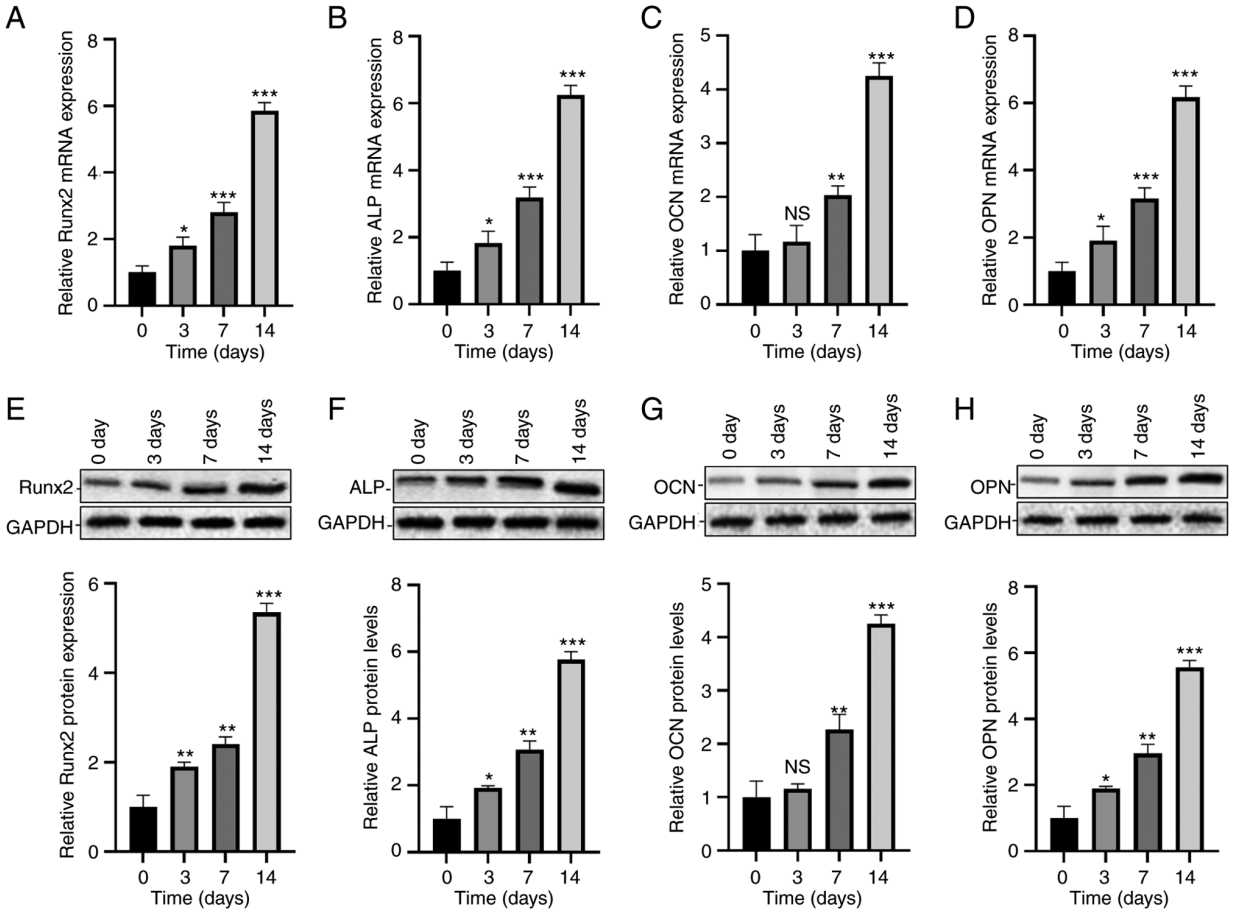

Figure 1. Osteogenic differentiation of hADSCs. Relative mRNA expression levels of (A) Runx2, (B) ALP, (C) OCN and (D) OPN in hADSCs on day 0, 3, 7, 14. Protein expression levels of (E) Runx2, (F) ALP, (G) OCN and (H) OPN in hADSCs on day 0, 3, 7 and 14. *P<0.05, **P<0.01 and ***P<0.001 vs. day 0. ALP, alkaline phosphatase; hADSCs, human adipose-derived stem cells; NS, no significance; OCN, osteocalcin; OPN, osteopontin; Runx2, runt-related transcription factor 2.

Results

Osteogenic differentiation of hADSCs. The osteogenic differentiation potential of hADSCs was determined by detecting relative levels of osteogenesis markers at day 0, 3, 7 and 14, respectively. The mRNA levels of Runx2, ALP and OPN were significantly upregulated at day 3, 7 and 14, and that of OCN increased at day 7 and 14 (Fig. 1A-D). In addition, protein levels of Runx2, ALP, OCN and OPN in hADSCs undergoing 0, 3, 7 and 14-day osteogenic differentiation were detected by Western blot, and were significantly upregulated (Fig. 1E-H).

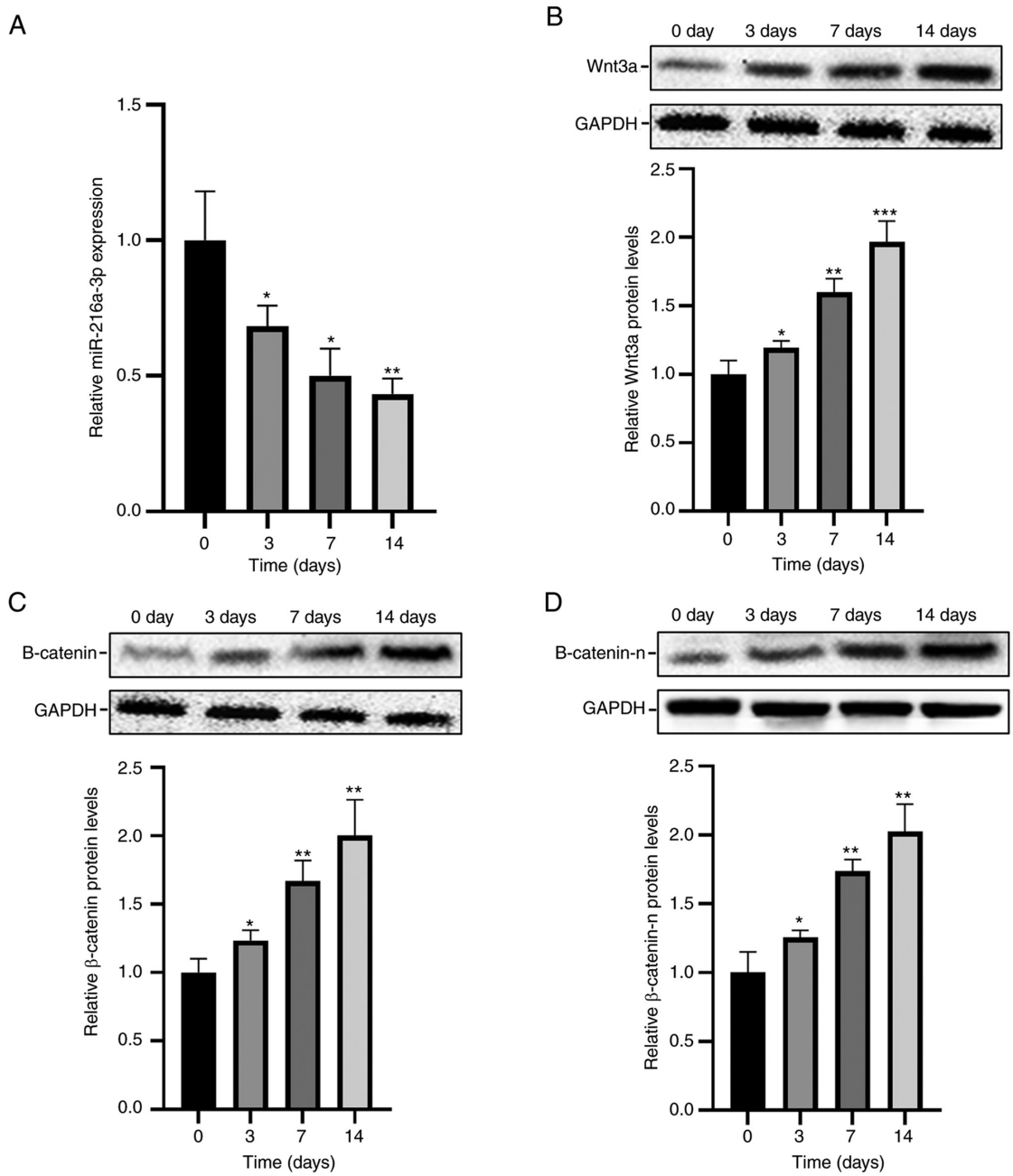

Figure 2. Dynamic expression changes of miR-216a-3p and Wnt3a in osteogenic differentiation of hADSCs. (A) Relative miR-216a-3p expression in hADSCs on day 0, 3, 7 and 14. Relative protein expression of (B) Wnt3a, (C) β-catenin and (C) β-catenin-n on days 0, 3, 7 and 14. *P<0.05, **P<0.01 and ***P<0.001 vs. day 0. hADSCs, human adipose-derived stem cells; miR, microRNA; β-catenin-n, β-catenin-nucleus.

Dynamic expression changes of miR-216a-3p and Wnt3a in osteogenic differentiation of hADSCs. Dynamic expression changes of miR-216a-3p during the osteogenic differentiation of hADSCs were examined. Endogenous miR-216a-3p was gradually downregulated, and showed a continuous downward trend (Fig. 2A). Conversely, the protein levels of Wnt3a, β-catenin, and β-catenin-n (nuclear β-catenin) were gradually elevated, and the trend was continuously upregulated (Fig. 2B-D).

Regulatory effect of miR-216a-3p on osteogenic differentiation of hADSCs. The lentiviral vector carrying miR-216-3p affects the miR-216-3p level, and the transfection efficacy was tested by RT-qPCR (Fig. 3A and B). On the 3rd day of transfection, the miR-216a-3p level in the miR-216a-3p overexpression group was 8-10 times higher than that of the control group, and it remained at a high level until the 14th day (Fig. 3C). On the contrary, it was significantly downregulated in cells transfected with anti-miR-216a-3p, at levels approximately 4.5 times lower than that of the controls, and it remained at a low level on the 14th day (Fig. 3D). Hence, the transfection efficacy of lentiviruses was verified.

Furthermore, the regulatory effects of miR-216a-3p on the expression levels of osteogenesis markers at day 7 of osteogenic differentiation of hADSCs were determined. In hADSCs overexpressing miR-216a-3p, mRNA levels of OCN, OPN, Runx2, and ALP were significantly downregulated, and were upregulated in cells with miR-216a-3p knockdown

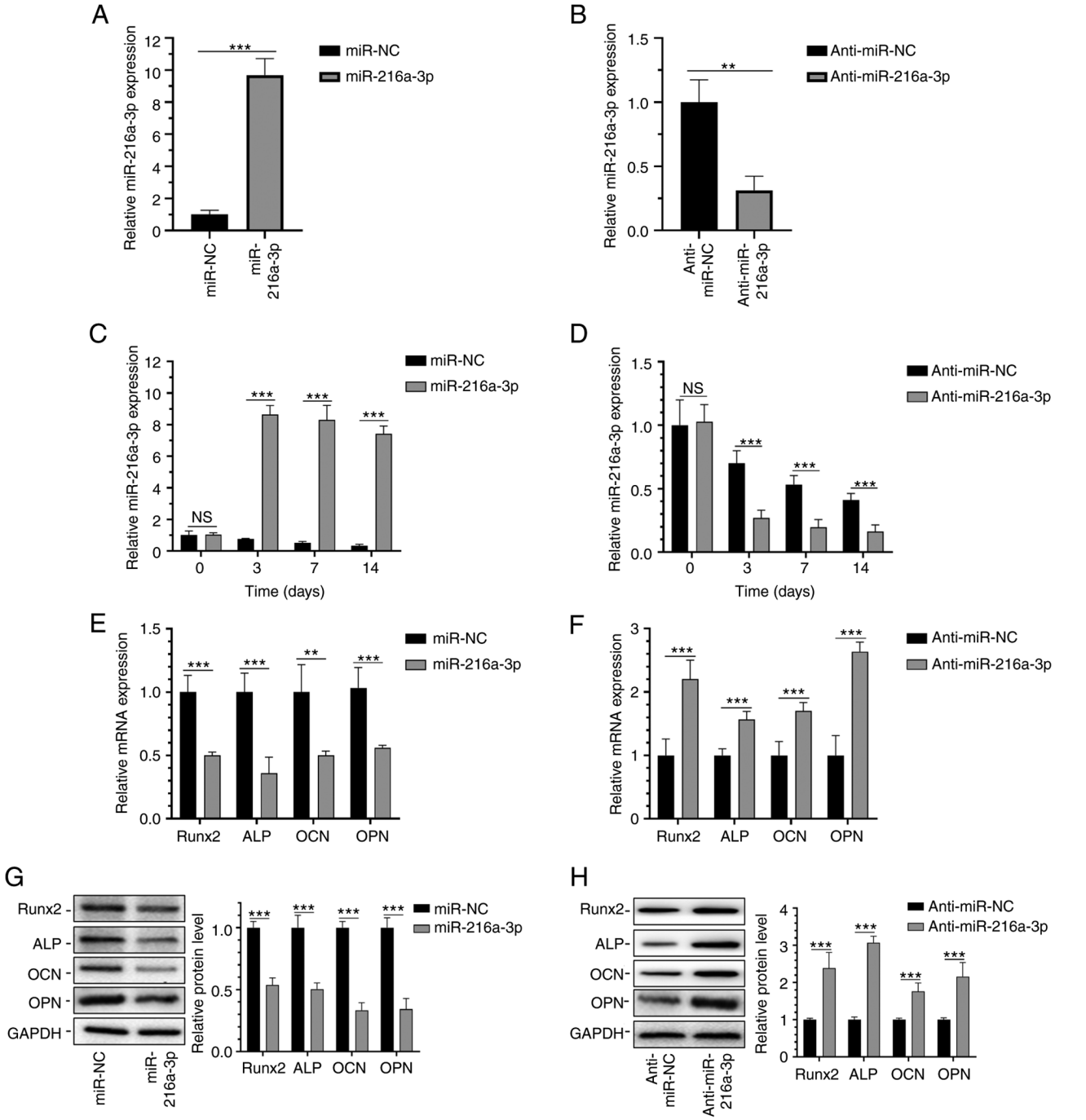

Figure 3. Regulatory effect of miR-216a-3p on the osteogenic differentiation of hADSCs. Transfection efficiency of (A) miR-216a-3p and (B) anti-miR-216a-3p plasmids. (C) Expression level of miR-216a-3p in hADSCs transfected with (C) miR-216a-3p and (D) anti-miR-216a-3p plasmids. mRNA expression of Runx2, ALP, OCN and OPN in hADSCs on the 14th day after transfection with (E) miR-216a-3p and (F) anti-miR-216a-3p plasmids. Protein expression of Runx2, ALP, OCN and OPN in hADSCs on the 14th day after transfection with (G) miR-216a-3p and (H) anti-miR-216a-3p plasmids. **P<0.01 and ***P<0.001 as indicated. ALP, alkaline phosphatase; hADSCs, human adipose-derived stem cells; miR, microRNA; NC, negative control; NS, no significance; OCN, osteocalcin; OPN, osteopontin; Runx2, runt-related transcription factor 2.

(Fig. 3E and G). As expected, the protein levels of OCN, OPN, Runx2, and ALP were similarly regulated by miR-216a-3p (Fig. 3G and H).

MiR-216a-3p directly targets Wnt3a. Using TargetScan 7.1, it was predicted that miR-216a-3p could target Wnt3a, and highest score (Fig. 4A). We thereafter performed dual-luciferase reporter assays, and the data showed that compared with the negative control, the luciferase activity in cells co-transfected with pmirGLO-Wnt3a-WT and miR-216a-3p mimics was reduced by 45%. However, no significant difference in luciferase activity was detected between cells co-transfected with pmirGLO-Wnt3a-Mut and miR-216a-3p mimic or Anti-miR-216a-3p, and those of the controls (Fig. 4B); overexpression of miR-216b-3p downregulated the level of Wnt3a, β-catenin and β-catenin-n, while anti-miR-216b-3p upregulated the level of Wnt3a, β-catenin and β-catenin-n (Fig. 4C and D). Therefore, it was proven that Wnt3a was the target of miR-216a-3p.

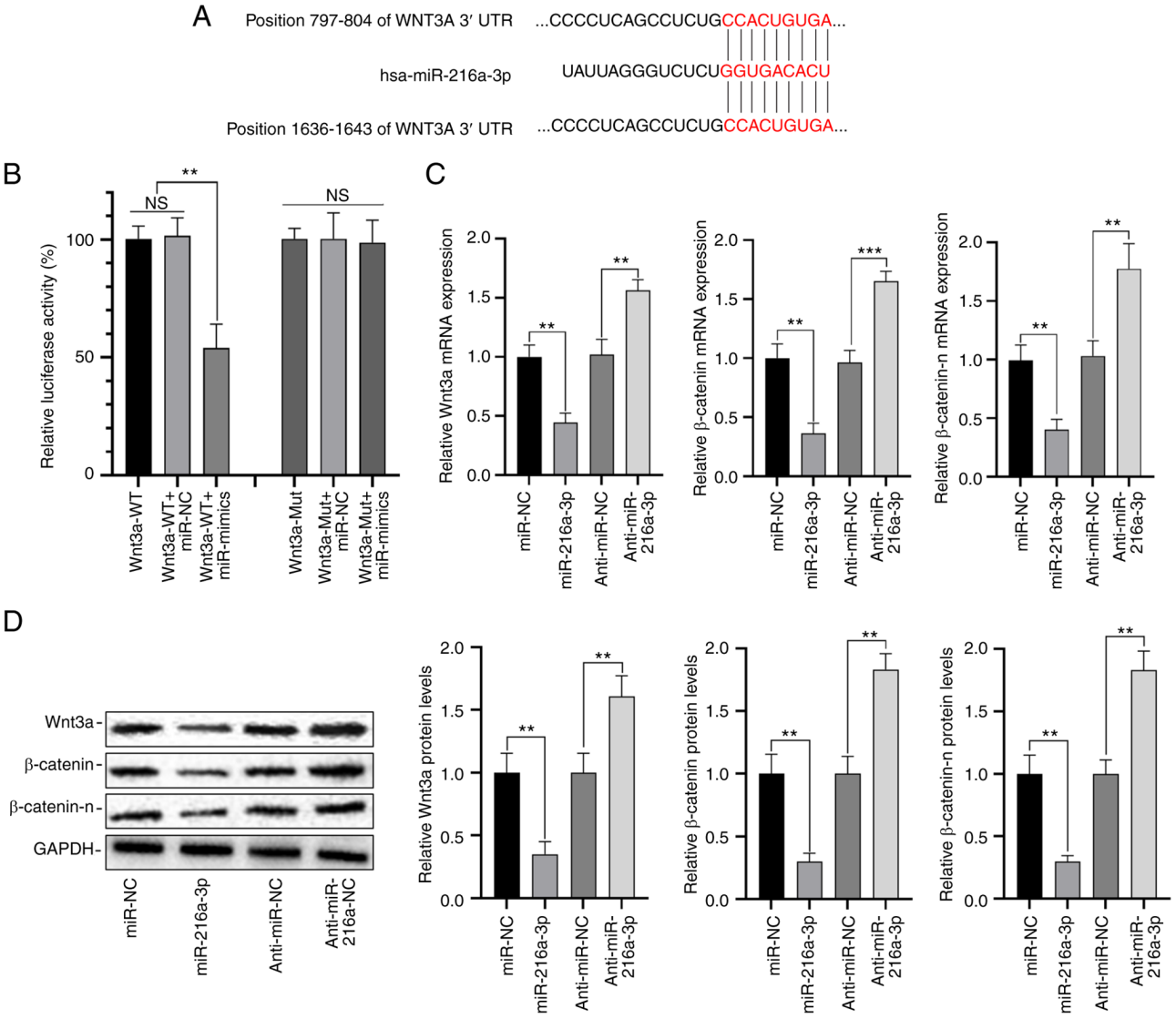

Figure 4. MiR-216a-3p directly targets Wnt3a. (A) Targetscan predicted that miR-216a-3p directly targeted the Wnt3a 3'-UTR. (B) The relative luciferase activity of transfected hADSCs was assessed. Relative (C) mRNA and (D) protein expression of Wnt3a, β-catenin and β-catenin-n was evaluated in hADSCs. **P<0.01 and ***P<0.001 as indicated. hADSCs, human adipose-derived stem cells; miR, microRNA; Mut, mutant; NC, negative control; NS, no significance; UTR, untranslated region; β-catenin-n, β-catenin-nucleus.

MiR-216a-3p mediated the osteogenic differentiation of hADSCs through the Wnt3a/β-catenin signaling pathway. Wnt3a was overexpressed by the transfection of lentiviral vector, and the overexpression level was detected by RT-qPCR (Fig. 5A); then the miR-216a-3p overexpression plasmid was transfected and the transfection level was tested (Fig. 5B); the results showed that overexpression of Wnt3a could significantly reverse the effect of miR-216a-3p on regulation of β-catenin and β-catenin-n (Fig. 5C and D); moreover, miR-216a-3p could reverse the effects of Runx2, ALP, OCN and OPN (Fig. 5E and F). These results indicate that Wnt3a is a direct target of miR-216a-3p, and overexpression of Wnt3a can effectively reverse the inhibition of miR-216a-3p on osteogenic differentiation.

Discussion

As a type of MSC, characteristics of ADSCs are high proliferation and strong lineage-specific differentiation (24). ADSCs are easily and abundantly isolated with relatively low risk. Additionally, they are optimally applied in bone regeneration and osteogenic tissue engineering because they can be *in vitro* differentiated into lipocytes, osteoblasts, chondrocytes, nerve cells, or myogenic cells under certain conditions (24,25). In the present study, hADSCs were cultivated in osteogenesis medium for in vitro osteogenic differentiation. By examining the relative levels of osteogenesis markers (Runx2, ALP, and OCN), we have proven that osteogenic differentiation of hADSCs could be induced via inhibition of miR-216a-3p.

MiRNAs are widely involved in the osteogenic differentiation of ADSCs. Zhang *et al* (26) showed that through the k-Ras/MEK/ERK signaling pathway, knockdown of miR-143 triggers osteogenic differentiation of hADSCs. In Fan's study (27), miR-450b is critically important for accelerating *in vitro* osteogenic differentiation of hADSCs and *in vivo* bone formation by targeting BMP3. Ai *et al* (28) found that the upregulation of microRNA-196a promoted

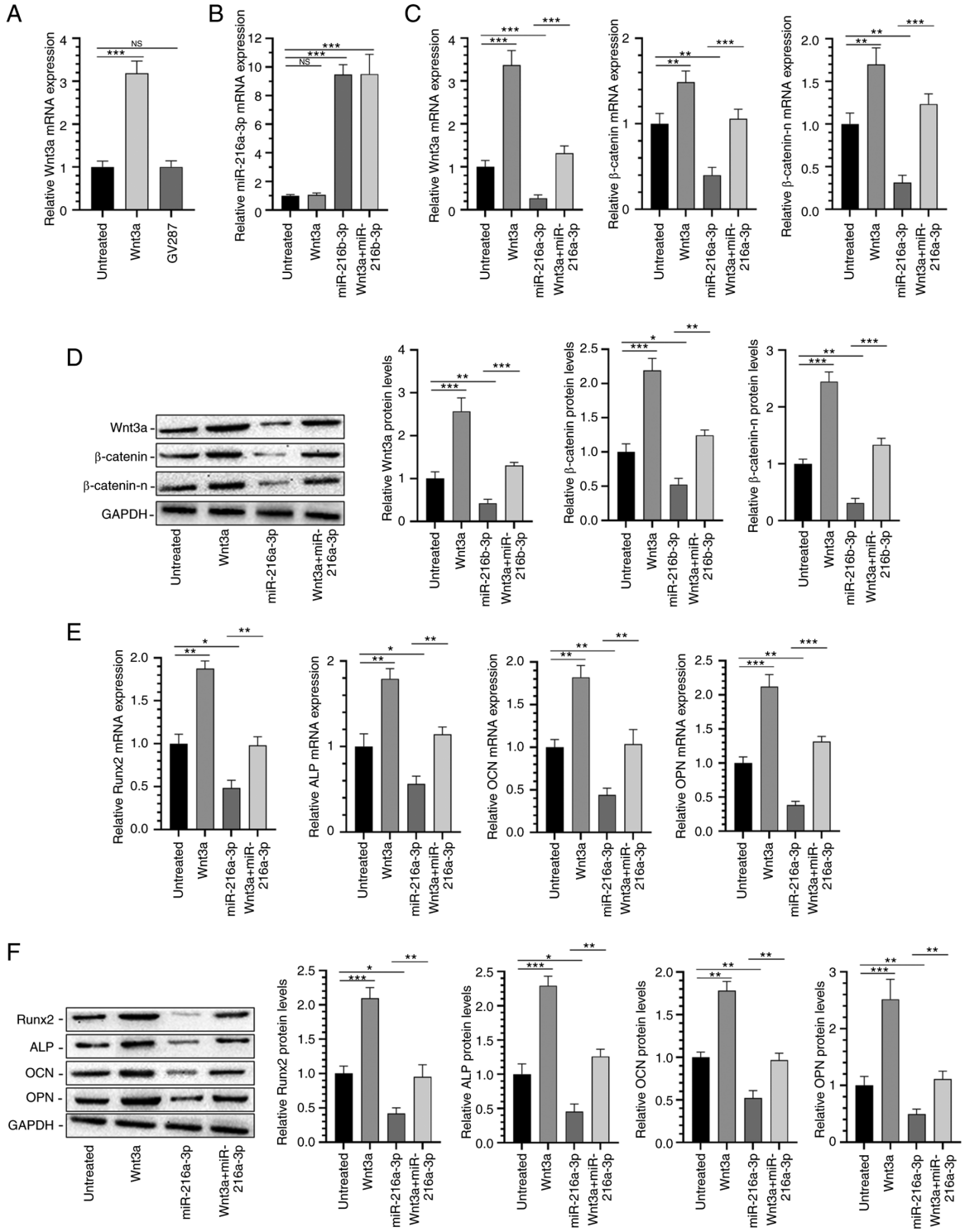

Figure 5. MiR-216a-3p mediates the osteogenic differentiation of hADSCs through the Wnt3a/β-catenin signaling pathway. Transfection efficiency of (A) Wnt3a and (B) miR-216a-3p plasmids. Relative (C) mRNA and (D) protein expression levels of Wnt3a, β-catenin and β-catenin-n in hADSCs. (E) Relative (E) mRNA and (F) protein expression levels of Runx2, ALP, OCN and OPN in hADSCs. *P<0.05, **P<0.01 and ***P<0.001 as indicated. NS, No significance. ALP, alkaline phosphatase; hADSCs, human adipose-derived stem cells; miR, microRNA; NS, no significance; OCN, osteocalcin; OPN, osteopontin; Runx2, runt-related transcription factor 2; β-catenin-n, β-catenin-nucleus.

the osteogenic differentiation of ACS, but the downregulation of microRNA-196a induced adipogenic differentiation, promoted the osteogenic differentiation of adipose stem cells, and inhibited adipogenic differentiation by regulating the β-catenin pathway. The results of Yang *et al* (29) showed that MiR-100-5p is upregulated in NONFH exosomes, and by targeting BMPR2 and inhibiting the BMPR2/SMAD1/5/9 signaling pathway, it leads to NONFH-like damage, thereby inhibiting the osteogenesis of hBMSCs.

Our results clarified that there is significant downregulation of miR-216a-3p during osteogenic differentiation of hADSCs. Overexpression of miR-216a-3p markedly downregulated

osteogenesis markers, including OCN, OPN, COL1A1, Runx2, and ALP, which were upregulated following miR-216a-3p knockdown. We concluded that miR-216a-3p plays an important role in the osteogenic differentiation of hADSCs.

There is a clear correlation between the Wnt/β-catenin signaling pathway, bone development, and osteogenic differentiation of MSCs (8,11). The study by Fan *et al* (27) also supported the fact that the Wnt/β-catenin signaling pathway is responsible for senescence and osteogenesis of hADSCs mediated by miR-1292. In the present research, Wnt3a was upregulated during the process of osteogenic differentiation of hADSCs. Wnt3a is a classic ligand and stimulator of the Wnt/β-catenin signaling pathway (22,30). Consistent with previously reported findings, miR-216a-3p possessed the ability to mediate the Wnt/β-catenin signaling pathway by targeting Wnt3a (22,23). However, since the current research does not include any experiments or data of cellular immunohistochemistry, more in-depth research remains to be carried out, which is a major limitation of this study.

MiR-216a-3p plays an important role in the osteogenic differentiation of hADSCs and is downregulated during the osteogenesis process. By targeting Wnt3a, it was determined that miR-216a-3p exerts an active role in the osteogenic differentiation of hADSCs through mediating the Wnt/β-catenin signaling pathway. Collectively, miR-216a-3p mediates the osteogenic differentiation of hADSCs by targeting Wnt3a, thus negatively regulating the Wnt/β-catenin signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL, GS and ZZ conceived and designed the study, and acquired, analyzed and interpreted the data. All of the authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved. All authors confirm the authenticity of all the raw data, and read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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