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ORIGINAL RESEARCH

Effect of miR-26a-5p on the Wnt/Ca^{2+} Pathway and Osteogenic Differentiation of Mouse Adipose-Derived Mesenchymal Stem Cells

Shasha Li[1] · Chen Hu[3] · Jianwei Li[4] · Lei Liu[1] · Wei Jing[1] · Wei Tang[1] · Weidong Tian[1] · Jie Long[1,2]

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Abstract Elucidation of the molecular mechanisms that regulate the differentiation of adipose-derived mesenchymal stem cells into osteogenic cells may lead to new methods for bone tissue engineering. We examined the role of miR-26a-5p in the regulation of osteogenic differentiation of mouse adipose-derived mesenchymal stem cells (mADSCs) by using mimics and inhibitors of this microRNA. Our results showed that over-expression of miR-26a-5p inhibited osteogenesis and that suppression of endogenous miR-26a-5p promoted osteogenesis. Four bioinformatics algorithms indicated that the 3′UTR of *Wnt5a* was a potential target of miR-26a-5p. We confirmed this prediction by use of dual-luciferase reporter assay and GFP/RFP assay. We also examined the molecular mechanisms by which miR-26a-5p regulates osteogenesis. Fura-2AM and Western blot assays after transfection indicated that miR-26a-5p repressed WNT5A, inhibited calcium flux and protein kinase C, and suppressed osteogenic differentiation of mADSCs. By contrast, miR-26a-5p inhibition activated these signal proteins and promoted osteogenic differentiation. Taken together, our results suggest that up-regulation of miR-26a-5p inhibits osteogenic differentiation of mADSCs by directly targeting the 3′UTR of *Wnt5a*, thereby down-regulating the Wnt/Ca^{2+} signaling pathway.

Keywords MicroRNA-26a-5p · Adipose-derived mesenchymal stem cell (ADSC) · Osteogenic differentiation · WNT5A · Non-canonical Wnt pathway

Abbreviations

ADSCs	Adipose-derived mesenchymal stem cells
mADSCs	Mouse adipose-derived mesenchymal stem cells
hADSCs	Human adipose-derived mesenchymal stem cells
MSCs	Mesenchymal stem cells
miRNAs	MicroRNAs
miR-26a	MicroRNA-26a
miR-NC	MicroRNA-26a-5p negative control
UTR	Untranslated regions
CDK6	Cyclin-dependent kinase 6
HDAC4	Histone deacetylase 4
USSCs	Unrestricted somatic stem cells
BMP	Bone morphogenetic protein
GSK-3β	Glycogen synthase kinase -3β
PCP	Planar cell polarity
ROCK	Rho-associated kinase
PBS	Phosphate buffer saline
FBS	Fetal calf serum
qRT-PCR	Quantitative real-time polymerase chain reaction
GFP	Green fluorescent protein
RFP	Red fluorescent protein

Shasha Li, Chen Hu, and Jianwei Li are co-first author and equally contributed to this work.

✉ Jie Long
dr.jielong@hotmail.com

[1] The State Key Laboratory of Oral Diseases, Sichuan University, Chengdu 610041, People's Republic of China

[2] Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University, Chengdu 610041, People's Republic of China

[3] Department of Oral and Maxillofacial Surgery, General Hospital of Ningxia Medical University, Yinchuan 750004, People's Republic of China

[4] Department of Oral and Maxillofacial Surgery, Binzhou Medical University Hospital, Binzhou 256603, People's Republic of China

MOI	Multiplicity of infection
WT	Wild type
Mu	Mutation
PKC	Protein kinase C
ALP	Alkaline phosphatase
OCN	Osteocalcin
OPN	Osteopontin
COL1	Collagen type 1
Runx2	Runt homology domain transcription factor 2
Osx	Osterix
CaMKII	Calmodulin-dependent protein kinase II
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ARS	Alizarin red staining
D-HBSS	D-Hanks balanced salt solution
Dsh	Dishevelled
RhoA	Ras homolog gene family, member A
JNK	c-Jun N-terminal kinase
Fz3	Frizzled receptor 3
Ror2	Receptor tyrosine kinase-like orphan receptor 2
P-PDLSCs	Periodontal ligament stem cells from chronic periodontitis patients

Introduction

There is an increasing focus on bone tissue engineering research because of the growing prevalence of bone-related diseases [1, 2]. Adipose-derived mesenchymal stem cells (ADSCs) are considered candidate seed cells for bone regeneration [3]. In recent years, there has also been increasing attention to the promotion of osteogenesis in adipose-derived stem cells. Osteogenic differentiation of mesenchymal stem cells (MSCs) is a complex process that involves many bioactive factors and signaling pathways, making study of this process more challenging [4–6].

MicroRNAs (miRNAs) are small non-coding endogenous RNAs, typically with 19–25 nucleotides, that bind to the 3′UTR of target mRNAs, alter mRNA stability or protein translation, and whereby regulate various cell signaling pathways [2, 7]. Although there is increasing interest in the effects of miRNAs on the osteogenesis of MSCs [8–11], research on the role of miR-26a in this process is limited. A review of the literature indicated there were approximately 274 studies of miR-26a, but only 8 of these studies examined its role in the osteogenic differentiation of MSCs [12–16]. Among these eight studies, Luzi et al. [12, 14] concluded that miR-26a was a negative regulator for terminal differentiation of hADSCs because it reduced the availability of active SMAD1. However, Trompeter et al. [13] found that miR-26a regulated osteo-inhibitory and osteo-promoting factors in parallel, but the osteo-inhibitory effects of CDK6 and HDAC4 likely outweighed the osteo-promoting effects of SMAD1. Thus, they concluded that miR-26a mainly promoted the osteogenic differentiation of unrestricted somatic stem cells (USSCs) [13]. Taken together, these results suggest the specific function and molecular mechanism of miR-26a are uncertain during the osteogenic differentiation of MSCs.

Previous studies have demonstrated that miR-26a can regulate osteogenic differentiation of MSCs through the BMP/SMAD1 signaling pathway. However, recent research indicated that miR-26a regulates osteogenic differentiation via inhibition of GSK-3β, a repressor of the Wnt canonical pathway [17]. The Wnt signaling pathways include the β-catenin-dependent canonical pathway and 2 β-catenin-independent non-canonical pathways (the planar cell polarity [PCP] pathway and the Wnt/calcium pathway) [18, 19]. There has been more focus on the canonical β-catenin-dependent pathway in osteogenetic differentiation [20, 21], but there is recent interest in the effect of non-canonical Wnt signaling on osteogenetic differentiation [22, 23]. Our previous study demonstrated that miR-154-5p regulated the Wnt non-canonical PCP pathway by targeting *Wnt11* under tensile stress, and this led to suppression of osteogenesis [24]. WNT5A is another activator of non-canonical Wnt signaling. Santos et al. [25] confirmed that WNT5A activated non-canonical Wnt signaling via rho-associated kinase (ROCK) and thereby inducing osteogenic differentiation of human adipose stem cells. Furthermore, it is well known that WNT5A can activate the non-canonical Wnt/Ca^{2+} pathway by promoting calcium influx, and this signaling pathway may be related to osteogenesis [18]. Although Chakravorty et al. [26] reported that miR-26a is a potential regulator of the *WNT5A* gene during the differentiation of human osteoprogenitor cells, the specific mechanism is unclear and no available research supports this hypothesis at present. Based on our preliminary bioinformatics analysis, we predicted that *Wnt5a* is a potential target of miR-26a-5p. This finding and the research mentioned above provides the basis for our hypothesis that miR-26a-5p regulates osteogenic differentiation via non-canonical Wnt signaling.

In this study, we identified miR-26a-5p as a potential regulator of osteogenesis. By modulating miR-26a-5p activity, we demonstrated that over-expression of miR-26a-5p inhibited osteogenic differentiation, whereas suppression of miR-26a-5p promoted differentiation. The dual-luciferase reporter assay and our further gain- or loss-of-function experiments confirmed that miR-26a-5p interacted with the 3′UTR of *Wnt5a* and negatively modulated the expression of genes that have important roles in osteogenesis. Our findings suggest that an increased level of miR-26a-5p inhibits the expression of WNT5A, suppresses the

Wnt/Ca^{2+} signaling pathway, and inhibits the differentiation of mADSCs into osteoblasts.

Materials and Methods

Culture and Harvest of mADSCs

Six-week-old male C57BL/6 mice obtained from the Sichuan University Animal Experimental Center were used as the source of mADSCs, and all procedures were in accordance with the guidelines of the Animal Research Committee of Sichuan University. The inguinal fat pads of the mice were isolated as previously described [24], soaked in PBS with 1 % penicillin/streptomycin, and transferred into a sterile clean bench. The tissue was washed 3 times with PBS, minced as small as possible, and then digested by 0.2 % collagenase type I in a constant-temperature water bath (37 °C) on a shaker for 50 min. Following centrifugation at 1000 rpm for 8 min, mADSCs were resuspended in 3 mL of regular growth medium consisting of α-MEM (HyClone Laboratories Inc., Logan, UT), 10 % FBS (Gibco BRL, Grand Island, NY), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Sigma-Aldrich, St. Louis, MO), and cultured in a 50 mL flask at 37 °C in a humidified incubator with 5 % CO_2. The medium was refreshed every 2 days, and cells in the third passage were used for experiments. Our previous flow cytometry analysis of cells obtained by this method showed that the isolated mADSCs had high purity based on positivity for CD29, Sca-1, and CD105, and negativity for hematopoietic stem cell markers (CD45 and CD31) [24].

Induction of Osteogenic Differentiation In Vitro

To induce osteogenic differentiation, the medium was replaced with osteoblast-inducing conditional α-MEM consisting of 10 % FBS, 10 mM β-glycerophosphate, 1×10^{-8} M dexamethasone, 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO), 0.01 μM 1,25-dihydroxyvitamin D3, 100 U/mL penicillin, and 100 μg/mL streptomycin. The extent of differentiation was assayed by alkaline phosphatase (ALP) staining at 7 days after induction and the mineralization of the matrix was evaluated by Alizarin Red staining at 14 days after induction. The expressions of osteoblast marker genes (*Alp* and *Ocn*) were also monitored by qRT-PCR at 0, 3, 7, and 14 days after induction. Cells were visualized using bright-field optics on a microscope and the images were captured using a camera.

Construction of Lentivirus Vectors and Transfection

GFP-labeled lentivirus vectors containing miR-26a-5p, miR-NC, antimiR-26a-5p, and antimiR-NC were obtained from Saier Biotechnology (Tianjin, China). The primers are listed in Table 1. Cells were seeded in 6-well plates (4×10^5 cells/well) before transfection. Transfection was conducted in Opti-MEM with 8 mg/mL of polybrene for 24 h. Each lentivirus was added to reach a multiplicity of infection (MOI) of 50. After transfection, the medium was changed into induction medium for the study of differentiation or into regular growth medium for other experiments.

Bioinformatics Analysis

A search for predicted target mRNAs was performed using four databases: TargetScan (http://www.targetscan.org), microRNA.org (http://www.microrna.org), RNA22 (https://cm.jefferson.edu/rna22v2/), and DIANAMicroT (http://diana.cslab.ece.ntua.gr/microT/).

Luciferase Reporter and Luciferase Assays

Reporter constructs which contain a miR-26a-5p-binding site (pmirGLO-*Wnt5a*-WT) or a mismatch sequence (pmirGLO-*Wnt5a*-Mu) in the 3′UTR of *Wnt5a* mRNA were synthesized by Saier Biotechnology (Tianjin, China). Table 1 shows the primer sequences for *Wnt5a* 3′UTR-wild type (WT) and *Wnt5a* 3′UTR-mutation (Mu).

Each recombinational vector, with 100 ng pmirGLO and 200 nmol/L miR-26a-5p mimics, miR-26a-5p inhibitors or their negative controls, was transfected into 293T cells using the Lipofectamine 2000 reagent (Invitrogen, USA) as previously reported [24]. Cells were harvested at 24 h after transfection and assayed for Renilla and Firefly luciferase activity using the Dual-Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China).

GFP/RFP Reporter Assay

Cells were inoculated onto 24-well plates with growth medium 1 day before transfection. When they were at 60–80 % confluence, the medium was replaced with 5 μg/mL polybrene. GFP/RFP/*Wnt5a*-UTR (GFP: internal reference, RFP: reporter) was co-transfected with miR-26a-5p, antimiR-26a-5p, or their negative controls at an MOI of 10. After 72 h, the medium was again switched to complete medium without polybrene and cells were incubated for another 72 h at 37 °C in an atmosphere of 5 % CO_2. The expression of GFP and RFP were observed by fluorescence microscopy. The constructed sequences of *Wnt5a* 3′UTR are listed in Table 1.

Quantitative Real-Time PCR (qRT-PCR) Analysis

The expression of miR-26a-5p and the mRNA levels of *Wnt5a*, *Ocn*, *Opn*, *Col1*, *Runx2*, *Alp*, and *Osx* were

Table 1 Constructed sequences used in this study

Genes	Forward (5′–3′)	Reverse(5′–3′)
miR-26a-5p	CGCGAATTCTTGAGGTGAGGCTCAGGAGG	ACGGGATCCTTGGCTACAGGCAAAGGGTT
miR-NC	AATTCTTCTCCGAACGTGTCACGTCTTCCTGTC AGAACGTGACACGTTCGGAGAATTTTTG	GATCCAAAAATTCTCCGAACGTGTCACGTTCTGAC AGGAAGACGTGACACGTTCGGAGAAG
antimiR-26a-5p	AATTCAGCCTATCCTGGATTACTTGAAG	GATCCTTCAAGTAATCCAGGATAGGCTG
antimiR-NC	AATTCGTGGATATTGTTGCCATCAG	GATCCTGATGGCAACAATATCCACG
U6	TGCGGGTGCTCGCTTCGGCAGC	CCAGTGCAGGGTCCGAGGT
pmirGLO-*Wnt5a*-WT	CTAGCTAGCGGCCGCTAGTAAACTGTTCCTAGTGTACTTGAA G	TCGACTTCAAGTACACTAGGAACAGTTTACTAGCGGCCGCTAG
pmirGLO-*Wnt5a*-Mu	CTAGCTAGCGGCCGCTAGTAAACTGTTCCTAGTGAAGTAGTAG	TCGACTACTACTTCACTAGGAACAGTTTACTAGCGGCCGCTAG
Wnt5a 3′UTR GFP/RFP	GATCCATTACCCTGCAGGTGTACCCTAAAACTG TTCCTAGTGTACTTGAACAGTTGCATTTATAAGC	GGCCGCTTATAAATGCAACTGTTCAAGTACACT AGGAACAGTTTTAGGGTACACCTGCAGGGTAATG

determined by qRT-PCR. For these experiments, total RNA was extracted from cells with Trizol (Invitrogen, Carlsbad, CA), cDNA was prepared, and qPCR was performed in triplicate as previously described [19]. The relative expression of mRNA or miRNA was evaluated by $2^{-\triangle\triangle Ct}$ method and normalized to that of GAPDH or U6. Table 2 shows the primers that were used.

Western Blotting Analysis

The protein levels of WNT5A, CaMKII, COL1, OPN, ALP, and β-catenin were determined by Western blotting. Protein lysates were generated using a radio-immunoprecipitation assay lysis buffer and the protein concentration was determined using the BCA protein assay kit (Beyotime, Jiangsu, China). Protein (32 μl) from each sample was subjected to 10 % SDS–PAGE, after which proteins were transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with a BLOTTO solution and then incubated with anti-WNT5A, anti-OPN, anti-COL1, anti-CaMKII, anti-ALP, or anti-β-catenin at 4 °C overnight. The membrane was then re-probed with appropriate secondary antibodies conjugated with horseradish peroxidase for 2 h (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Blots were processed using Western Lightning®-ECL, enhanced chemiluminescence substrate (PerkinElmer, USA), and exposed to film. The results were analyzed using the Quantity One system (Bio-Rad, Hercules, CA).

Assays for Protein Kinase C Activation and Quantitative ALP

Measurement of protein kinase C (PKC) activation was performed using the PepTag® Non-Radioactive PKC Assay kit (Promega, USA) and spectrophotometry was used to quantitate kinase activity. To measure intracellular ALP, washed cells (1×10^5) were homogenized in the assay buffer and centrifuged at $13{,}000\times g$ for 3 min to remove insoluble material. Then, 10 μL was added into each well of a 96-well plate and the total volume was brought to 80 μL by addition of assay buffer. ALP was then measured using the Alkaline Phosphatase Assay Kit (Biovision, Milpitas, CA).

Alkaline Phosphatase (ALP) Staining and Alizarin Red Staining (ARS)

After induction, transfected control and transfected mADSCs were prepared for ARS/ALP staining. ALP staining was performed with the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China). For Alizarin Red staining, the solution was

Table 2 qRT-PCR primers sequences used in this study

Genes	Accession no.	Length (bp)	Primer (5′–3′)	Primer sequence
Alp	NM_001287172.1	106	Forward	ATCTTTGGTCTGGCTCCCATG
			Reverse	TTTCCCGTTCACCGTCCAC
Runx2	NM_009820.5	137	Forward	AGGGACTATGGCGTCAAACA
			Reverse	GGCTCACGTCGCTCATCTT
Ocn	NM_007541.3	147	Forward	GCAATAAGGTAGTGAACAGACTCC
			Reverse	GTTTGTAGGCGGTCTTCAAGC
Osx	NM_130458	243	Forward	ACACCTACTCCTTGGTGGGA
			Reverse	AACCGCCTTGGGCTTATAG
Col1	NM_007742.3	183	Forward	ACTCAGCCGTCTGTGCCTCA
			Reverse	GGAGGCCTCGGTGGACATTA
Opn	NM_001204201.1	160	Forward	CACTCCAATCGTCCCTACA
			Reverse	CTTAGACTCACCGCTCTTCAT
Wnt5a	NM_009524.3	125	Forward	TTGGCCACGTTTTTCTCC
			Reverse	TGGCTGCAGAGAGGCTGT
GAPDH	NM_008084.2	172	Forward	GGTGAAGGTCGGTGTGAAC
			Reverse	GACTGTGCCGTTGAATTTG

prepared with Tris–HCl at pH 4.2. The mADSCs were first washed with PBS and fixed in 4 % paraformaldehyde for 30 min. Then, fixed cells were incubated with the staining solution for 6–10 min. Finally, the cells were washed thoroughly with distilled water before visualization.

Fura-2AM Assay

After washing cells with D-Hanks balanced salt solution (D-HBSS), cells were incubated for 45 min in a Fura-2AM working solution in darkness. Then, the Fura-2AM working solution was removed and D-HBSS was used to wash cells. Cells were incubated for another 20 min in D-HBSS to assure thorough de-esterification of the AM group. Fluorescence was measured at 380 nm (Fura-2) and 340 nm (Ca-Fura-2).

Statistical Analysis

All data are expressed as means ± SDs and all experiments were performed at least three times. Statistical analysis was performed by SPSS Statistics 20.0 software, using Student's two-tailed *t* test or a one-way ANOVA. Statistical values were illustrated using GraphPad PRISM, version 6.05. A *p* value of 0.05 was considered statistically significant.

Results

Osteogenic Differentiation of mADSCs

Our initial experiments demonstrated that mADSCs turned to osteogenic differentiation when cultured in the differentiation-inducing medium, as indicated by cell morphology (Fig. 1a) and ALP and Alizarin Red staining (Fig. 1b, c). Moreover, during differentiation, we measured the expression of two osteoblast marker genes (*Alp* and *Ocn*) at different times after induction. The results show that relative to day 0, *Alp* expression was elevated at days 3, 7, and 14 (Fig. 1d) and *Ocn* expression was elevated at days 7 and 14 ($p < 0.05$ for all comparisons) (Fig. 1e).

Expression of miR-26a-5p and *Wnt5a* During Osteogenic Differentiation of mADSCs

Our qPCR experiments showed that endogenous miR-26a-5p decreased gradually during cell differentiation; the level was significantly lower at day 7 and continued to decrease with as differentiation progressed. Compared with the undifferentiated control group, the expression of miR-26a-5p was more than 50 % lower at day 14 ($p < 0.05$) (Fig. 2a). The expression of *Wnt5a* during differentiation had the opposite pattern. In particular, the level of *Wnt5a* was significantly higher at day 7, and this elevation remained at days 10 and 14 (Fig. 2b). The expression of WNT5A protein (Fig. 2c) was similar to that of *Wnt5a* mRNA.

We examined the relationship between miR-26a-5p and *Wnt5a* by using four different miRNA target prediction databases to assess their potential interaction. All four of the computational algorithms indicated that miRNA-26a-5p targets the 3′UTR of *Wnt5a*. Moreover, the miR-26a-5p target site in the 3′UTR of *Wnt5a* is highly conserved among vertebrates (Fig. 2d). These results indicate that miR-26a-5p and WNT5A play important roles in regulating osteogenic differentiation of mADSCs and that there is a direct relationship between these molecules.

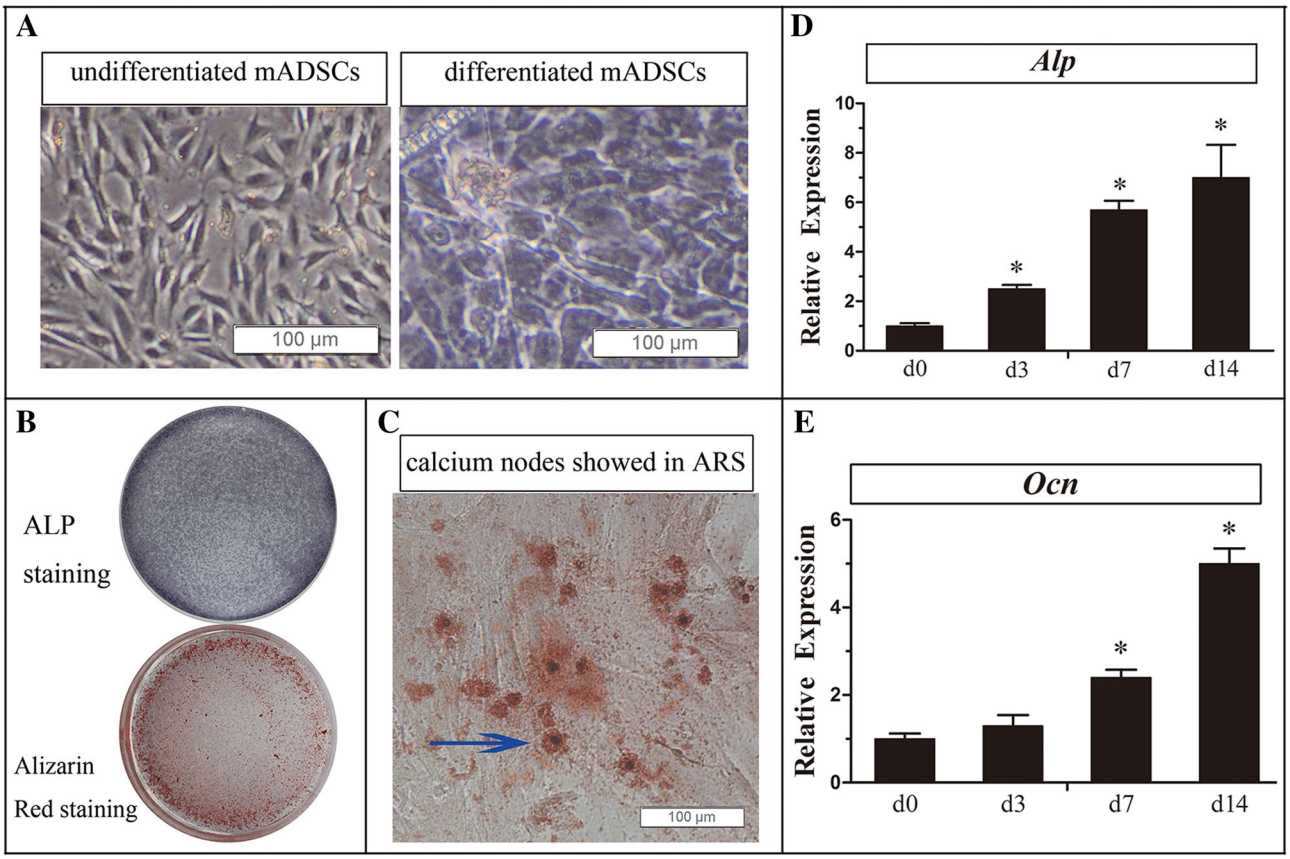

Fig. 1 Changes in cell morphology and gene expression during osteogenic differentiation of mADSCs. **a** Morphology of undifferentiated mADSCs (*left*) and differentiated mADSCs (*right*) induced by osteo-differentiation-inducing medium. **b** Osteogenic differentiation was determined by ALP staining (*top*) at day 7 and Alizarin Red staining (*bottom*) at day 14. **c** Calcium nodes in Alizarin Red-stained cells after osteogenic induction. **d**, **e** qRT-PCR analysis showed increased expression of osteogenesis-specific genes (*Alp* and *Ocn*) during osteogenic differentiation. Expression was normalized to GAPDH and presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with day 0)

Effect of miR-26a-5p on Osteoblast Differentiation of mADSCs

We transfected mADSCs with four different lentiviruses (miR-NC, miR-26a-5p, antimiR-NC, and antimiR-26a-5p) to over-express or inhibit miR-26a-5p, and then measured the expression of GFP by fluorescence microscopy to evaluate the efficiency of transfection. The results indicate successful transfection of 80–90 % of cells (Fig. 3a). We also measured the expression of miR-26a-5p in lentivirus-modified mADSCs by qRT-PCR during differentiation. Mature miR-26a-5p levels were elevated ~4.5-fold in the miR-26a-5p group compared with the miR-NC group ($p < 0.05$), and remained over-expressed up to day 12 of differentiation. By comparison, the level of miR-26a-5p in the antimiR-26a-5p group was reduced by ~fivefold relative to the negative control ($p < 0.05$), and this inhibition continued over time (Fig. 3b). Thus, miR-26a-5p had a sustained and stable over-expression in the miR-26a-5p group during differentiation, but the expression of miR-26a-5p was repressed at all times in the antimiR-26a-5p group.

We also used qRT-PCR to monitor the expression of osteogenesis-specific genes (*Ocn*, *Opn*, *Col1*, *Runx2*, *Alp*, and *Osx*) in mADSCs transfected with miR-NC, miR-26a-5p, antimiR-NC, or antimiR-26a-5p. Relative to the negative controls, transfection with miR-26a-5p reduced the expression of *Ocn*, *Opn*, *Col1*, *Runx2*, *Alp*, and *Osx*, whereas transfection with antimiR-26a-5p increased the expression of these genes (Fig. 3c). Western blot analysis showed similar changes in ALP and OPN expression (Fig. 3d). ALP and ARS staining showed significantly less cell differentiation in the miR-26a-5p group than the miR-NC group, but more differentiation in the antimiR-26a-5p group (Fig. 3e). These results show that over-expression of miR-26a-5p inhibited osteoblast differentiation, and therefore indicate that miR-26a-5p plays a negative role in the osteogenesis of mADSCs.

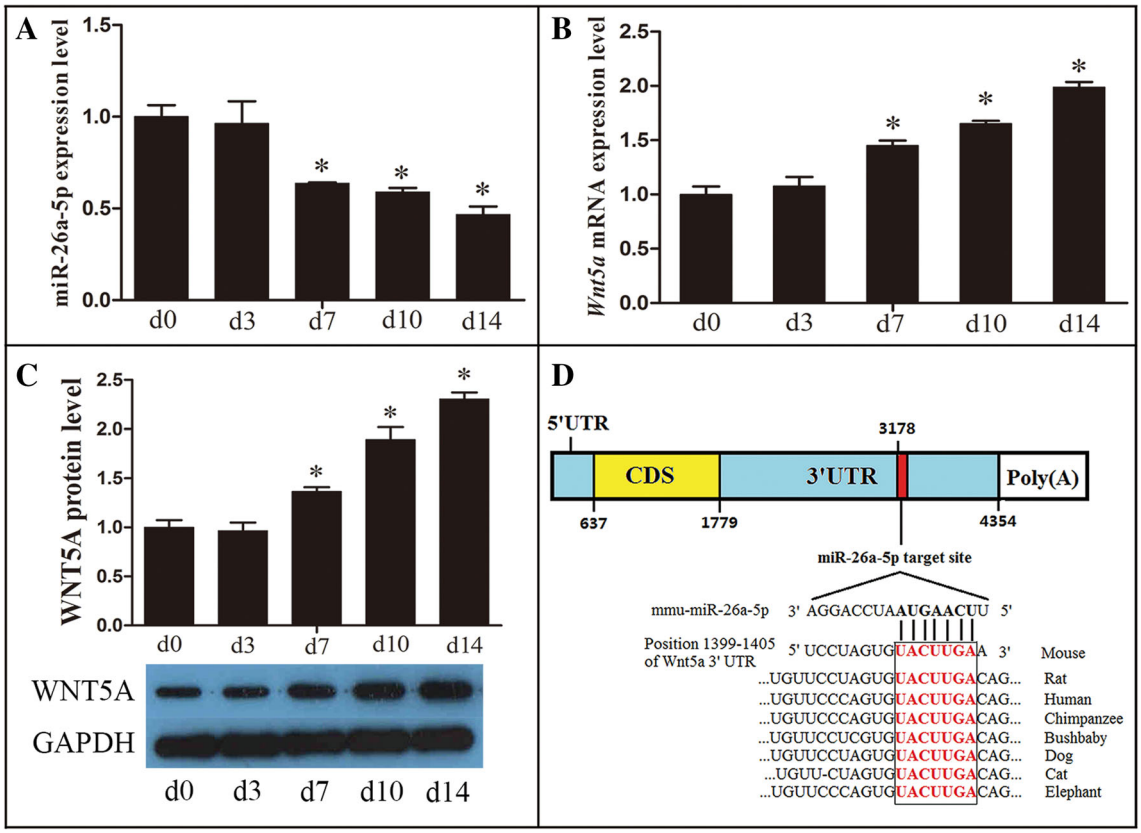

Fig. 2 Changes in expression of miR-26a-5p and WNT5A during osteogenic differentiation of mADSCs. **a** The endogenous expression of miR-26a-5p was measured by qRT-PCR at 0, 3, 7, 10, and 14 days after induction of osteogenesis. Expression was normalized to U6 and presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with day 0). **b** Expression of *Wnt5a* mRNA was measured by qRT-PCR at various times after induction of osteogenesis. Expression was normalized to GAPDH and presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with day 0). **c** Western blotting of WNT5A during osteogenesis and quantifications of these results. Expression was normalized to GAPDH and presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with day 0). **d** Bioinformatics analyses of the complementarity of the miR-26a-5p seed sequence to the 3′UTR of Wnt5a and conservation of the putative-binding site in vertebrates

Direct Association of miRNA-26a-5p with *Wnt5a* 3′UTR

We examined the specific selectivity of miRNA-26a-5p for *Wnt5a* mRNA by use of a dual-luciferase reporter assay in 293T cells. The results show that the Renilla luciferase activity of pmirGLO-*Wnt5a*-WT transfected cells decreased by more than 60 % in miR-26a-5p co-transfected cells compared with miR-NC co-transfected cells or blank controls; however, expression increased more than 2.2-fold in antimiR-26a-5p co-transfected cells compared with negative or blank controls. In addition, co-transfection of lentiviruses and the mutant 3′UTR-binding site (pmirGLO-*Wnt5a*-Mu) had no effect on luciferase levels (Fig. 4a). These results indicated that the 3′UTR of *Wnt5a* was a target of miR-26a-5p.

These results are in agreement with experiments used to detect whether miR-26a-5p binds to *Wnt5a*. In these experiments, we co-transfected GFP/RFP/*Wnt5a*-UTR with miR-26a-5p, antimiR-26a-5p, or their negative controls; GFP was the internal control and RFP was the reporter. Fluorescence microscopy indicated that red fluorescence was notably attenuated compared with green fluorescence in the group co-transfected with miR-26a-5p and GFP/RFP/*Wnt5a*-UTR, but red fluorescence was stronger in antimiR-26a-5p group. There were no obvious differences between the red and green fluorescence in the negative control groups after co-transfection. Further analysis showed that co-transfection with miR-26a-5p and GFP/RFP/*Wnt5a*-UTR decreased luciferase expression compared with the miR-NC group. Moreover, there was an increase of red fluorescence in the antimiR-26a-5p group compared with its negative control (Fig. 4b, d). These data further verify that miR-26a-5p binds to *Wnt5a*.

We also harvested protein and mRNA samples of mADSCs after transfection with miR-26a-5p mimics or inhibitors. The qRT-PCR results indicate a significant decrease in *Wnt5a* mRNA in cells transfected with miR-26a-5p compared with the negative control, but an increase in *Wnt5a* mRNA in the antimiR-26a-5p group. On the other

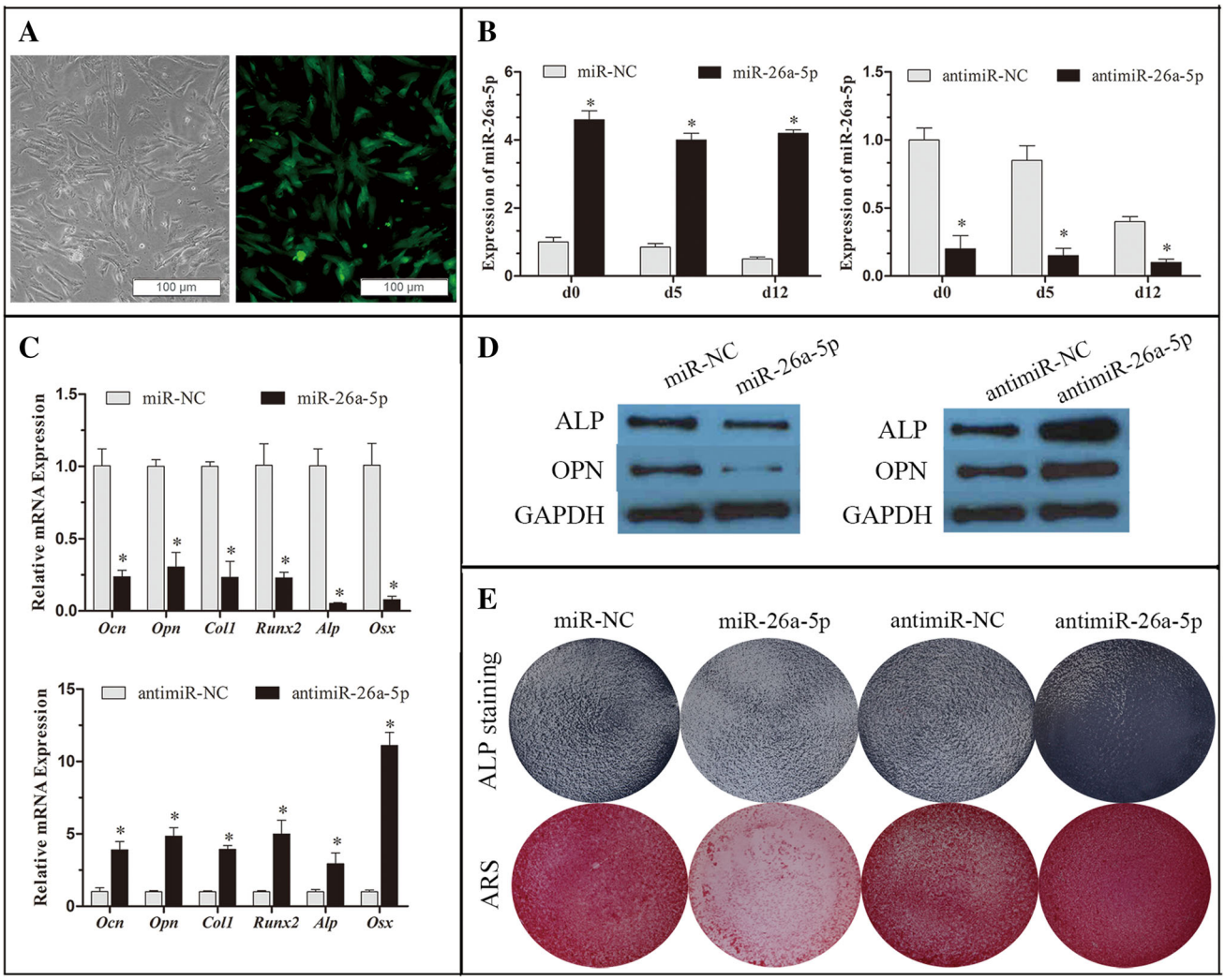

Fig. 3 Effect of up-regulation and down-regulation of miR-26a-5p on osteogenic differentiation of mADSCs. **a** Transfection efficiency of miRNA was estimated as 80–90 % based on GFP expression in mADSCs observed by fluorescence microscopy. **b** Expression of miR-26a-5p in mADSCs infected with miR-26a-5p (*left*) or antimiR-26a-5p (*right*) was assayed by qRT-PCR at 0, 5, and 12 days after induction. Expression was normalized to U6 and presented as means ± SDs ($n = 3$; $*p < 0.05$ compared with the negative control). **c** Expression of osteoblast marker genes (*Ocn*, *Opn*, *Col1*, *Runx2*, *Alp*, and *Osx*) determined by qRT-PCR at 10 days after transfection of mADSCs with miR-26a-5p or antimiR-26a-5p. Expression was normalized to GAPDH and presented as means ± SDs ($n = 3$; $*p < 0.05$ compared with the negative control). **d** Western blotting of ALP and OPN after transfection with miR-26a-5p mimics or an inhibitor. Expression was normalized to GAPDH, and one of three independent experiments is shown. **e** Histological staining for ALP (*top*) at day 7 and Alizarin Red (*bottom*) at day 14 after transfection with miR-26a-5p mimics or inhibitor

hand, the Western blotting results show that WNT5A protein level was lower in miR-26a-5p group than in the miR-NC group, and higher in the antimiR-26a-5p group than in the negative control (Fig. 4c).

Taken together, these results suggest that miR-26a-5p underwent direct binding to the 3′UTR of *Wnt5a* and thereby affecting the expression of *Wnt5a* mRNA and WNT5A protein during the course of osteogenic differentiation.

miR-26a-5p Regulates Osteogenesis via Wnt5a/Ca^{2+} the Signaling Pathway

The results described above suggest that miR-26a-5p depresses osteogenic differentiation by directly inhibiting the expression of *Wnt5a*. WNT5A is a ligand that can activate the Wnt signaling pathway by binding to receptors on the cell surface. We examined the signaling pathway of osteogenesis that is regulated by miR-26a-5p by measuring

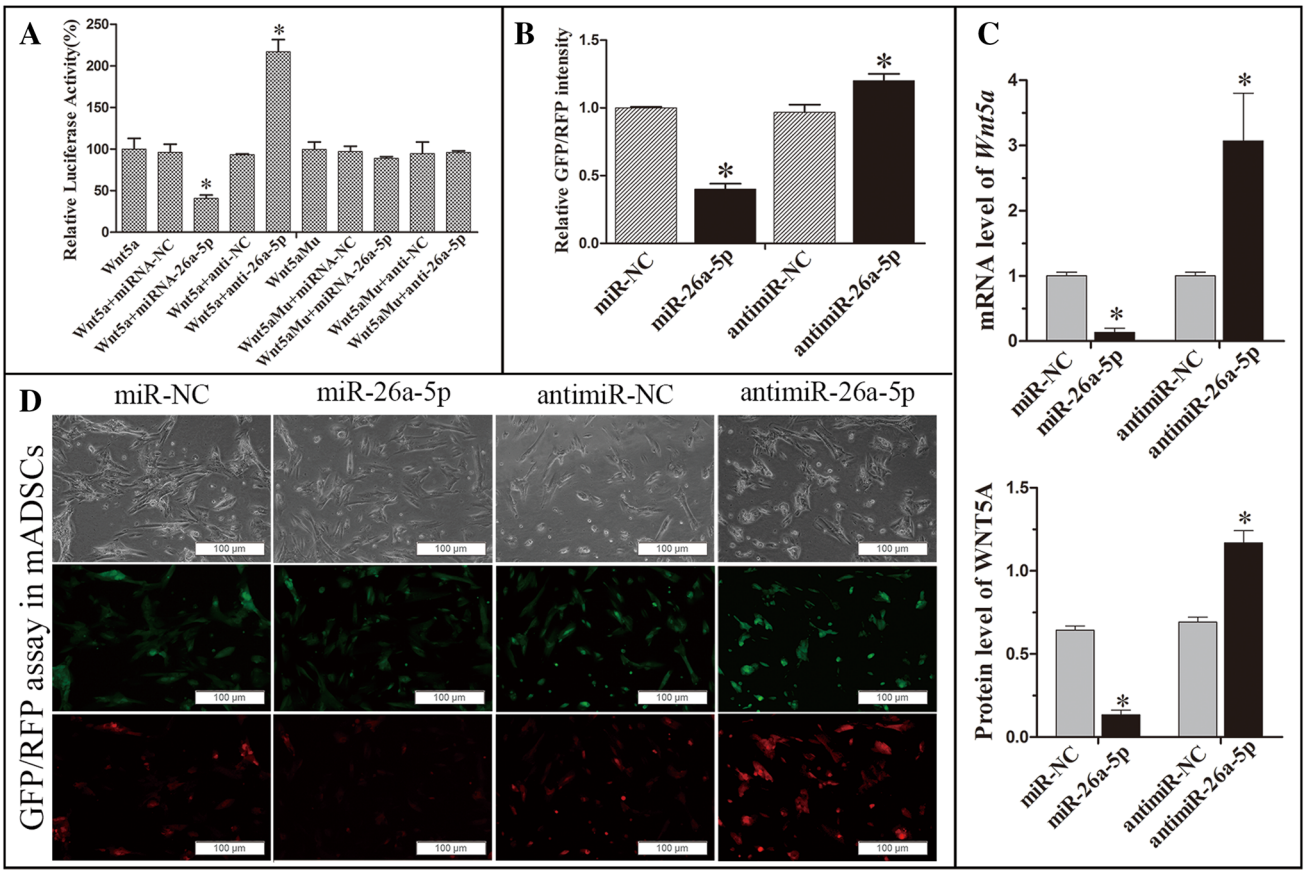

Fig. 4 Interaction of miR-26a-5p with *Wnt5a* 3′UTR and effect on expression of Wnt5a mRNA and protein. **a** pmirGLO-*Wnt5a*-WT or pmirGLO-*Wnt5a*-Mu reporter constructs were co-transfected with miR-26a-5p, miR-NC, antimiR-26a-5p, or antimiR-NC into 293T cells. Renilla luciferase activity was normalized to firefly luciferase. Data are presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with negative control and blank control). **b**, **d** The GFP/RFP/*Wnt5a*-UTR reporter vector was co-transfected with miR-26a-5p or antimiR-26a-5p into mADSCs. GFP and RFP levels were measured by a fluorometer and RFP values were normalized to GFP. Data are presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with separate negative controls). **c** Expression of *Wnt5a* mRNA (top, qRT-PCR) and WNT5A protein (bottom, Western blotting) after transfection with miR-26a-5p or antimiR-26a-5p. The expression was normalized to GAPDH and presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with separate negative controls)

changes of pivotal proteins in this signaling cascade. The results show that over-expression of miR-26a-5p down-regulated WNT5A protein compared with the negative control, and that this decrease accompanied a decrease in CaMKII protein (a crucial factor in the Wnt5a/Ca^{2+} signaling pathway) and COL1 protein (an osteogenesis-related marker protein). Interestingly, relative to controls, cells transfected with miR-26a-5p also had an increased level of β-catenin, which is associated with the canonical Wnt pathway (Fig. 5a).

An assay for PKC, another key factor in Wnt5a/Ca^{2+} signaling, indicated a 58 % reduction in the miR-26a-5p group and an increase in the antimiR-26a-5p group (Fig. 5b, c). A quantitative ALP assay showed that ALP activity declined by 33 % after transfection with miR-26a-5p, but increased by 1.3-fold after transfection with anti-miR-26a-5p (Fig. 5d). Even more remarkable was that the levels of intracellular calcium, an important second messenger, decreased in the miR-26a-5p group but increased in the antimiR-26a-5p group (Fig. 5e). Thus, miR-26a-5p suppressed osteogenic differentiation of mADSCs via the Wnt5a/Ca^{2+} signaling pathway by reducing the expression of *Wnt5a*. In addition, suppression of Wnt5a/Ca^{2+} pathway was associated with an increase of β-catenin expression.

Discussion

ADSCs are a type of mesenchymal stem cells that are considered candidates for the generation of new bone tissue. Various physical, chemical, and biological factors can regulate the osteogenic differentiation potency of ADSCs [20, 27–29]. The results of the present study confirmed that our osteoblast-inducing medium can induce the osteogenic differentiation of mADSCs. Moreover, we assessed the

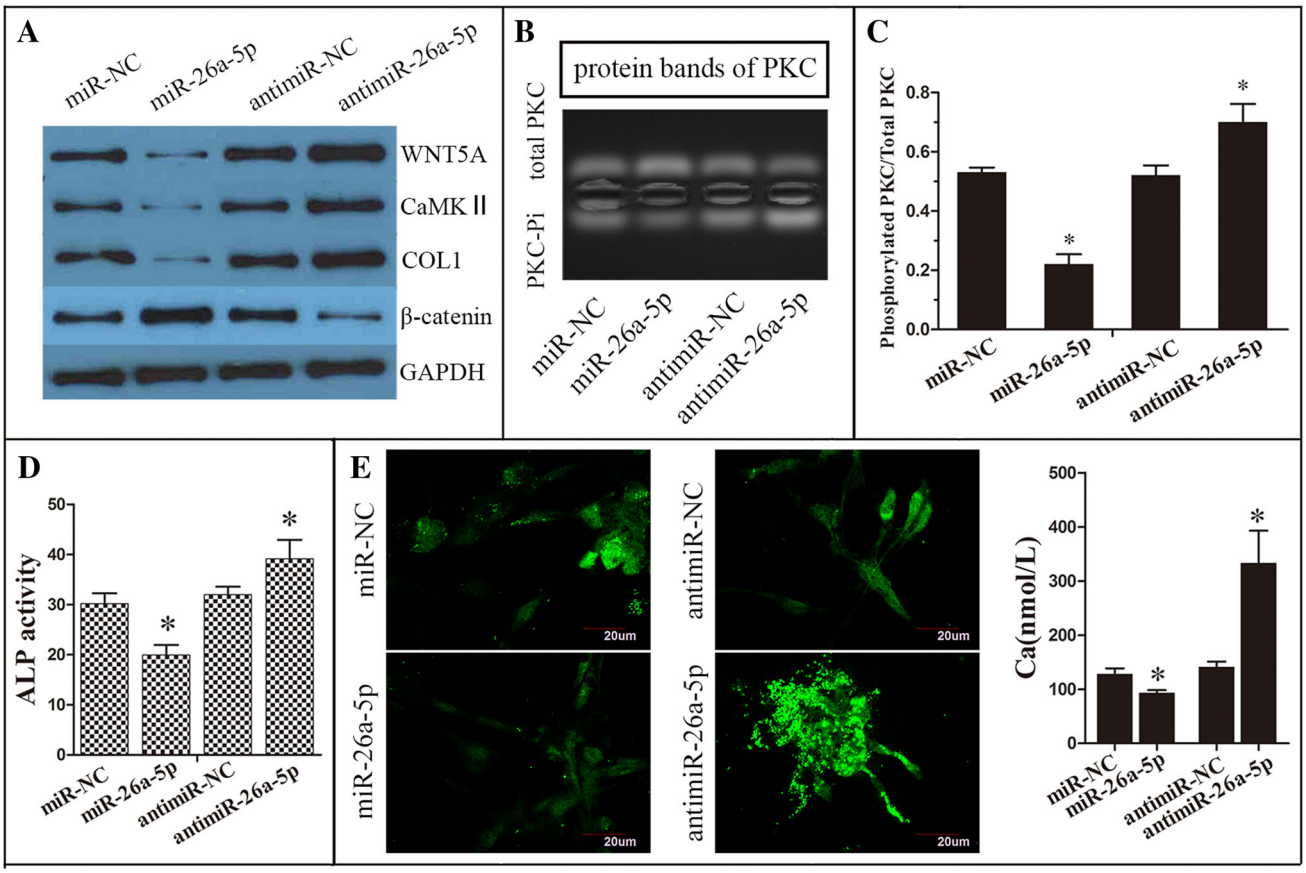

Fig. 5 Effect of miR-26a-5p on WNT5A and the non-canonical Wnt/Ca^{2+} pathway during osteogenic differentiation of mADSCs. **a** Western blotting of key proteins in the Wnt signaling pathway (WNT5A, CaMKII, and β-catenin) and a marker of osteogenesis (COL1) at 10 days after transfection with miR-26a-5p or antimiR-26a-5p. **b, c** Level of phosphorylated PKC and total PKC at 10 days after transfection with miR-26a-5p or antimiR-26a-5p. The level of phosphorylated PKC was normalized to total PKC and data are presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with separate negative controls). **d** Alkaline phosphatase activity at 10 days after transfection with miR-26a-5p or antimiR-26a-5p. Activities are presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with each negative control). **e** Calcium levels (measured by the Fura-2AM assay) at 10 days after transfection with miR-26a-5p or antimiR-26a-5p. The levels are presented as means ± SDs ($n = 3$; $^*p < 0.05$ compared with each negative control)

exact function of miR-26a-5p during osteogenic differentiation of mADSCs by use of transfection experiments. These results showed that inhibition of miR-26a-5p function enhanced the differentiation of mADSCs, whereas miR-26a-5p over-expression inhibited the differentiation of mADSCs. Thus, miR-26a-5p is a negative regulator of osteogenic differentiation. This result is consistent with the results of Luzi et al. [12] but in conflict with those of Trompeter et al. [13]. The discrepancy may be due to the use of cells from different tissues and species, different conditions for induction of differentiation, different methods of gene transfection, and measurement of responses at different time points [17].

Our bioinformatics analysis indicated that the 3′UTR of *Wnt5a* had a perfect 7-nt match to the miR-26a-5p seed region. We also used dual-luciferase reporter assay and GFP/RFP assay to verify the predictions of the bioinformatics analysis and to study the molecular mechanism by which miR-26-5p regulates osteogenic differentiation of mADSCs. The results of these experiments strongly confirmed that the 3′UTR of *Wnt5a* was a direct target of miR-26-5p. There are two major non-canonical Wnt signaling pathways downstream of WNT5A: the PCP pathway and the Wnt/calcium pathway [18, 19]. Previous research indicated that WNT5A has a role in activation of the PCP pathway via the phosphorylation of Dsh and activation of RhoA, ROCK, and JNK [18, 30, 31]. Intracellular calcium is a pivotal second messenger for Wnt/calcium signaling, and WNT5A can influence calcium flux and whereby regulate PKC and CaMKII, key downstream effectors of calcium signaling [19, 32–34]. Recent studies have also implicated an important role for Wnt/calcium signaling in osteogenesis [22, 23, 35].

Although WNT5A plays a crucial role in activation of the non-canonical Wnt pathways [19, 23], the specific molecular mechanisms by which miR-26-5p regulates

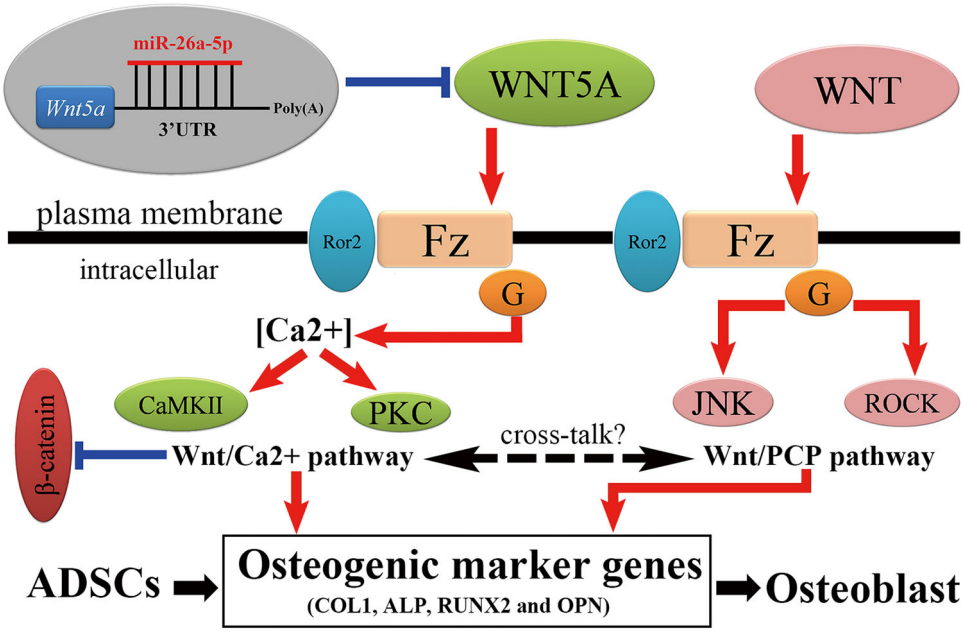

Fig. 6 Proposed regulation of gene expression in the non-canonical Wnt pathway during osteogenic differentiation of mADSCs. miR-26a-5p inhibits the expression of WNT5A by targeting *Wnt5a* 3′UTR. WNT5A activates a G-protein after binding with its receptors on the cell membrane (Fz and Ror2), leading to calcium influx. The increase of intracellular calcium stimulates activation of the calcium-sensitive kinases PKC and CAMKII, implicating the Wnt/calcium pathway. Enhancement of the Wnt/calcium pathway leads to suppression of β-catenin. The Wnt/calcium pathway and the Wnt/PCP pathways (Wnt/JNK and Wnt/ROCK signaling) promote the differentiation of mADSCs into osteoblasts by alteration of osteogenic marker genes expression. Further studies are needed to determine whether there are interactions between the Wnt/calcium pathway and Wnt/PCP pathways

osteogenic differentiation through WNT5A was heretofore unclear. Thus, we examined the effects of transfection of mADSCs with miR-26-5p mimics or inhibitors to investigate the miR-26-5p signaling pathway. After transfection, we incubated mADSCs in osteoblast-inducing medium, and measured the concentrations of calcium and key signaling proteins. The data showed that over-expression of miR-26a-5p reduced the protein content of WNT5A, leading to a decrease of intracellular calcium, an important second messenger. In response, the phosphorylation of PKC and CaMKII was suppressed, leading to an attenuation of cell differentiation. The presence of reduced levels of osteoblast markers (COL1, OPN, and ALP) supports this interpretation.

The opposite outcome occurred when the cells were transfected with antimiR-26a-5p. Furthermore, a comparison of the effects of miR-26a-5p and antimiR-26a-5p on osteogenic differentiation indicated that the osteo-inhibitory effects of miR-26a-5p were much stronger than the osteo-promoting effects of antimiR-26a-5p. This may be attributed to the decreased expression of endogenous miR-26a-5p during the osteogenic differentiation of mADSCs.

Previous studies have shown that WNT5A-mediated activation of different pathways is associated with binding to distinct receptors on the cell surface. The binding of WNT5A to frizzled receptor 3 (Fz3) and the tyrosine kinase-like orphan receptor 2 (Ror2) leads to the activation of the non-canonical Wnt pathways [36]. Moreover, Ror2, by itself or in combination with Frizzled protein through its Frizzled-like cysteine-rich domain [37], mediates diverse Wnt5a signaling by activating the non-canonical Wnt pathway [38] and inhibiting the β-catenin-T cell factor/lymphoid enhancer factor pathway [39]. β-catenin is an important component of the canonical Wnt pathway, and recent research showed that Wnt5a suppressed canonical Wnt signaling during cell differentiation [36]. Moreover, Liu et al. [22] demonstrated that down-regulation of β-catenin leads to activation of the non-canonical Wnt/Ca^{2+} pathway, resulting in the promotion of osteogenic differentiation in periodontal stem cells from chronic periodontitis patients (P-PDLSCs). Consistent with these findings, our study also indicated down-regulation of β-catenin during enhanced Wnt/Ca^{2+} pathway signaling during the inhibition of miR-26a-5p and osteogenic differentiation of mADSCs. The opposite effect occurred in the miR-26a-5p group. This may indicate an interaction between the non-canonical and canonical Wnt pathways.

Based on previous studies, we can hypothesize that the activation of the non-canonical Wnt pathway could restrain the canonical Wnt pathway by several possible mechanisms [19, 22]. Further study is required to determine whether the Wnt canonical and non-canonical pathways interact by one of these mechanisms or by some other mechanism. Moreover, the nature of the interactions

between the two Wnt non-canonical pathways should also be investigated. Based on the foundation provided by the present study and our previous findings, we will examine the cross-talk between the Wnt/Ca^{2+} pathway, which is regulated by miR-26a-5p, and the Wnt/PCP pathway, which is regulated by miR-154-5p, during the osteogenic differentiation of ADSCs.

Conclusion

Previous research indicated that miR-26a, WNT5A, and the Wnt/Ca^{2+} pathway have roles in the osteogenic differentiation of ADSCs. The present study is the first to determine the nature of the inter-relationships of these molecules during osteogenesis (Fig. 6). Reducing the endogenous level of miR-26a-5p led to up-regulation of WNT5A, and thereby increasing the level of intracellular calcium, PKC, and CaMKII, leading to the enhancement of osteogenic differentiation. Suppression of endogenous miR-26a-5p reduced the level of β-catenin. It indicates an interaction between the canonical and non-canonical Wnt signaling pathways. Our analysis of the miR-26a-5p/Wnt5a/Ca^{2+} signaling pathway elucidates the mechanism of osteogenesis of mADSCs and suggests that certain experimental methods can be used to promote new bone formation. Such methods may have potential use for the development of therapies for bone-related diseases.

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Compliance with Ethical Standards

Conflict of interest Shasha Li, Chen Hu, Jianwei Li, Lei Liu, Wei Jing, Wei Tang, Weidong Tian, and Jie Long have declared no conflict of interest.

Human and Animal Rights and Informed Consent All experiments were approved by the Animal Research Committee of Sichuan University and were conducted in accordance with the guidelines for the management and handling of experimental animals.

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