MicroRNA-139-5p elevates skeletal myogenic differentiation of human adult dental pulp stem cells through Wnt/β-catenin signaling pathway

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Abstract. The aim of the present study was to identify a microRNA (miRNA or miR)-based biomarker and therapeutic target for skeletal myogenic differentiation of human adult dental pulp stem cells (ADSCs). miRNA expression was measured using reverse transcription-quantitative polymerase chain reaction; cell viability assay and lactate dehydrogenase (LDH) activity levels were measured using MTT and LDH activity kits, respectively. Apoptosis assay and caspase-3/9 activity levels were measured using flow cytometry and caspase-3/9 activity kits, respectively. Western blot analysis and immunofluorescence microscopy measured the protein expression of myocyte-specific enhancer factor 2C, myogenic differentiation 1, myosin heavy chain, Wnt and β-catenin. Overexpression of miR-139-5p promoted cell growth and induced skeletal myogenic differentiation of ADSCs. Downregulation of miR-139-5p inhibited cell growth and reduced skeletal myogenic differentiation of ADSCs. Overexpression of miR-139-5p induced Wnt/β-catenin signaling pathway and Wnt/β-catenin signaling pathway was suppressed by anti-miR-139-5p in ADSCs. Wnt inhibitor reduced the effect of miR-139-5p on skeletal myogenic differentiation of ADSCs. In conclusion, miR-139-5p elevates skeletal myogenic differentiation of human ADSCs through the Wnt/β-catenin signaling pathway.

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Introduction

Tooth development derives from the interaction between the outer epithelial cell and the mesenchymal cell (1). The enamel organ is initially formed, followed by dental papilla and the dental sac. Mesenchymal stem cells (MSCs) can form other structures of tooth, such as dentin, dental pulp and the primary periodontal pocket (1). Dental pulp serves a vital role in tooth tissue regeneration following tooth eruption (1). Furthermore, cells in the dental pulp will form new odontoblasts in the presence of dental tissue trauma and irreversible disease damage. In this way, pulp can secrete the dentin matrix, thus forming the so-called reparative dentin (1).

Currently, various applications of false teeth remain the major repair method for missing teeth (2). Tooth regeneration is one of the major challenges in oral medicine at present. Biological tissue engineering technology has been applied in organ reconstruction, tissue regeneration and tooth regeneration for years (2). However, the research status of global tooth regeneration is far beyond the reach of providing tissue-engineered teeth for clinical application. Dental pulp stem cells are important for tooth tissue engineering. Cells that can be used in tooth tissue engineering include dental pulp stem cells (DPSCs) and odontogenic mesenchymal cell (3). DPSCs are located in the dental pulp, which possesses self-renewal ability and can differentiate into odontoblasts (4). Previous research has indicated that there are several factors affecting DPSC proliferation and differentiation (5). They include basic fibroblast growth factor, transforming growth factor, bone morphogenetic protein, β-phosphoglycerol and dentin (5).

In recent years, research on non-coding small RNA has also affected the development of dental pulp regeneration research (6). microRNAs (miRNAs or miRs) are a class of endogenous non-coding small RNAs that can bind with the 3'-untranslated region of target gene mRNA (6) inhibiting target gene transcription and regulating the target gene. miRNA is closely associated with multiple physiological processes in mammals, including cell proliferation, migration and development (7). In other cases, miRNA serves a vital regulatory role in certain functions, physiological activities

(including anti-inflammatory and anti-apoptotic effects) and cell differentiation of stem cell (3,7).

DPSCs serve a crucial role in research on tooth repair and regeneration. Multiple signal transduction pathways, such as Notch and Wnt, can regulate DPSC proliferation and differentiation (8). Typically, the Wnt signaling pathway serves a key role in medical engineering research, including signal regulation in tooth development and tooth regeneration (9). Wnt1, adenomatous polyposis coli (APC) and β-catenin are important factors in the Wnt signaling pathway, which can affect the interaction between dental epithelium and mesenchyme (10). Furthermore, the Wnt/β-catenin signaling pathway impacts the development, proliferation and differentiation of dentin and tooth root (10). The aim of the present study was to identify an miRNA-based biomarker and therapeutic target for skeletal myogenic differentiation of human adult DPSCs (ADSCs).

Materials and methods

Cell culture and transfection. A total of 12 Female C57/BL6 mice (age, 5-6 weeks; weight, 16-18 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., (Shanghai, China). Mice were housed at a temperature of 22-23˚C and a humidity of 55-60%, with a 12 h light/dark cycle and free access to food and water. All mice were used in the present study and anesthetized using 35 mg/kg pentobarbital sodium (intraperitoneal inkection; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The present study was approved by the Ethics Committee of Ninth People's Hospital, Shanghai JiaoTong University School of Medicine (Shanghai, China). Mice were sacrificed via decapitation, mandibular tissues were removed and pulp tissues were separated and incubated with α-minimum essential medium (a-MEM) supplemented with 5% fetal bovine serum (FBS), 0.004% DNase I, 1.5 mg/ml collagenase type I and 2 mg/ml dispase (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 12 h at 37˚C. The dissociated cells were separated, resultant single-cell suspensions of dental pulp were counted and incubated into six-well cell culture plates (BD Biosciences, Franklin Lakes, NJ, USA) using a-MEM supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) in a 5% CO_2 atmosphere at 37˚C for 14 days.

Subsequently, cells were incubated with a-MEM with 2% FBS and 0.5 mM 5-Aza-20-deoxycytidine (Sigma-Aldrich; Merck KGaA) for 1 day at 37˚C and subsequently incubated with a-MEM with 10% FBS for 3 days at 37˚C.

The sequences of each transfected miRNA were as follows: miR-139-5p forward, 5'-UCUACAGUGCACGUGUCUCCA GU-3' and reverse, 5'-UGGAGACACGUGCACUGUAGA UU-3'; anti-miR-139-5p forward, 5'-CAGUACUUUUGU GUAGUACAA-3' and reverse, 5'-CAGUACUUUUGUGUA GUACAA-3'; negative forward mimics, 5'-UUCUCCGAA CGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUU CGGAGAATT-3'. Each miRNA was purchased from Sangon Biotech Co., Ltd (Shanghai, China). miR-139-5p (100 ng), anti-miR-139-5p (100 ng) and negative mimics (100 ng) were co-transfected into the cells with 1 ml Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 6 h transfection, the medium was replaced with a-MEM with 10% FBS and treated with the Wnt inhibitor (1 μmol/l;

Wnt-C59; MedChemExpress USA, Monmouth Junction, NJ, USA) for 42 h.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cell samples using a Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China). cDNA was prepared using a SuperScript III First-Strand Synthesis Supermix kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. miR-139-5p expression was measured using a 7500 Realtime PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a Kapa SYBR Fast qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA). The Primer sequences utilized for RT-qPCR were as follows: miR-139-5p forward, 5'-ACACTCCAGCTGGGTCTACAG TGCACGTG-3' and reverse, 5'-CTCAACTGGTGTCGT GGA-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCCGT-3'. The PCR conditions consisted of an initial 3 min denaturation at 95˚C, 40 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec. miR-139-5p expression was calculated using the $2^{-\Delta\Delta Cq}$ method (11).

Cell viability assay and lactate dehydrogenase (LDH) activity levels. At 24, 48 and 72 h following transfection, cells were stained with MTT (20 μl; 5 mg/ml) for 4 h at 37˚C. Dimethyl sulfoxide was added to samples and incubated for 30 min at 37˚C. The absorbance was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 492 nM.

At 48 h following transfection, cellular LDH activity levels were measured using LDH activity kits (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's protocol. The absorbance was measured using a microplate reader at 450 nM.

Apoptosis assay. At 48 h following transfection, cells were washed with PBS for 15 min and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were then stained with 5 μl Annexin V-APC and 5 μl propidium iodide (1 mg/ml; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 15 min in darkness at room temperature. Apoptosis rate was measured using a FACScan flow cytometer (BD Biosciences) and analyzed using Flowjo 7.6.1 (FlowJo, LLC, Ashland, OR, USA).

Western blot analysis. At 48 h following transfection, total protein was extracted from the transfection cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) for 15 min at 4˚C and protein was quantified using a BCA assay (Beyotime Institute of Biotechnology). Protein samples (30 μg) were loaded on 10-12% SDS-PAGE for electrophoresis and were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk in TBS-Tween 20 (TBST) for 1 h at 37˚C and incubated with myocyte-specific enhancer factor 2C (MEF2C; cat. no. sc-13268; 1:1,000), myogenic differentiation 1 (MyoD; cat. no. sc-304, 1:1,000) and myosin heavy chain (MyHC; cat. no. sc-20641; 1:1,000), anti-Wnt (cat. no. sc-50360; 1:1,000), anti-β-catenin (cat. no. sc-376959; 1:1,000), and anti-GAPDH antibodies (cat. no. sc-25778; 1:5,000; each Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Membranes were
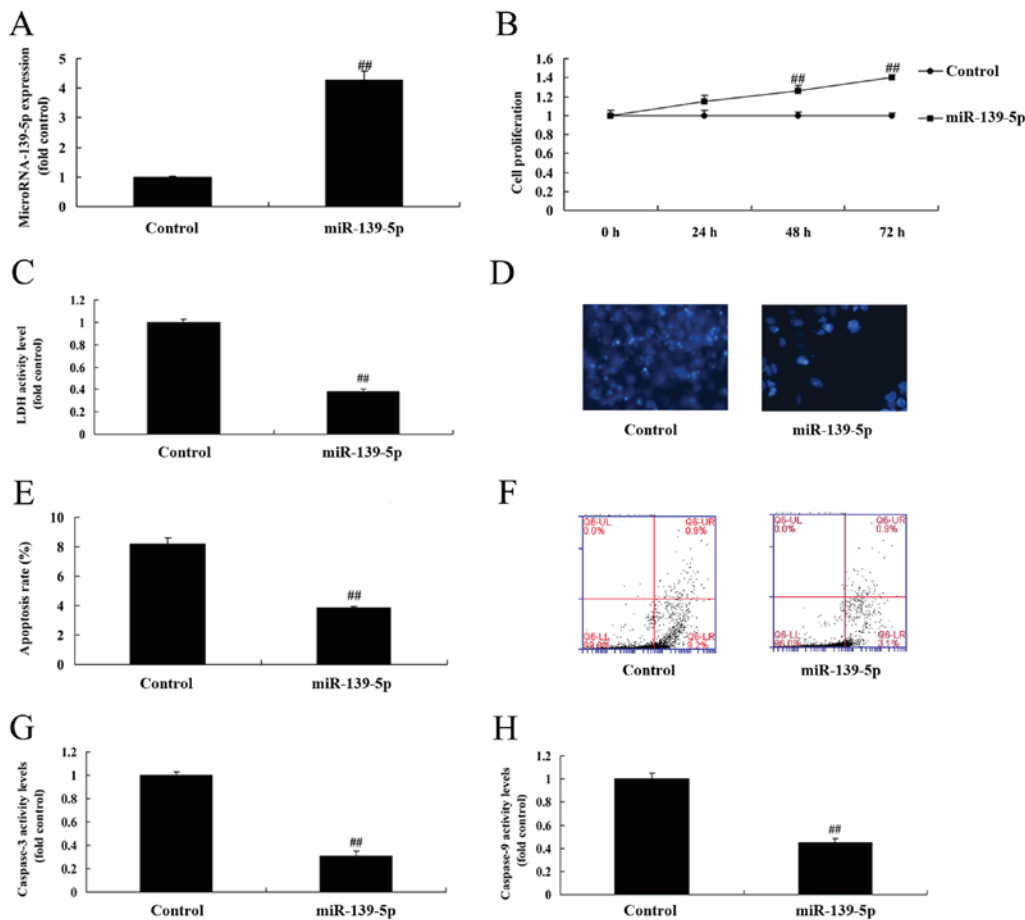
Figure 1. Overexpression of miR-139-5p promoted cell growth of adult dental pulp stem cells. (A) miR-139-5p expression, (B) cell growth, (C) LDH activity level, (D) DAPI staining, (E and F) apoptosis rate, (G and H) caspase-3/9 activity levels. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control. miR, microRNA; LDH, lactate dehydrogenase.

washed with TBST for 15 min and subsequently incubated with goat-anti-rabbit or anti-mouse horseradish peroxidase conjugated immunoglobulin G antibodies (cat. nos. sc-2004 or sc-2005, respectively; 1:5,000; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. Membranes were observed using BeyoECL Plus (Beyotime Institute of Biotechnology) and analyzed using Image Lab 3.0 software (Bio-Rad Laboratories, Inc.).

Luciferase reporter assays. 100 ng of Wnt1 3'UTRs (forward, 5'-CCATGGGGGCTCTGGGCGCTG-3' and reverse, 5'-TTATAGATAAAAG-3') containing miRNA-139-5p (forward, 5'-ACACTCCAGCTGGGTCTACAGTGCACG TG-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3') were amplified in psiCHECK™-2 Vectors (Promega Corporation, Madison, WI, USA) and co-transfected into cells using 1 ml Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection for 48 h, cells were lysed in Passive Lysis Buffer (Invitrogen; Thermo Fisher Scientific, Inc.) and luciferase activity was measured using a DualGlo Luciferase Assay System (Promega Corporation).

Immunofluorescence microscopy. At 48 h following transfection, Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were incubated with 0.01% Tris-X100 in PBS for 15 min at room temperature and blocked with 5% bovine

serum albumin (Beyotime Institute of Biotechnology) in PBS for 1 h at room temperature. Cells were then incubated with anti-Wnt (cat. no. sc-5210; 1:1,000) and anti-β-catenin antibodies (cat. no. sc-65480; Santa Cruz Biotechnology, Inc.) at 4°C overnight and washed with PBS for 15 min. Cells were then incubated with 488 and 555 fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies for 1 h at 37°C and washed with PBS for 15 min. Cells were stained with DAPI for 30 min at room temperature in darkness and washed with PBS for 15 min. Images were captured using an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan; magnification, x200).

Statistical analysis. Data are presented as the mean ± standard deviation (n=3). Differences between groups were determined using Student's t-test, or one-way analysis of variance with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of miR-139-5p promotes cell growth of ADSC. The function of miR-139-5p on skeletal myogenic differentiation of ADSCs was analyzed, and miR-139-5p mimics were observed to increase the expression of miR-139-5p in ADSCs
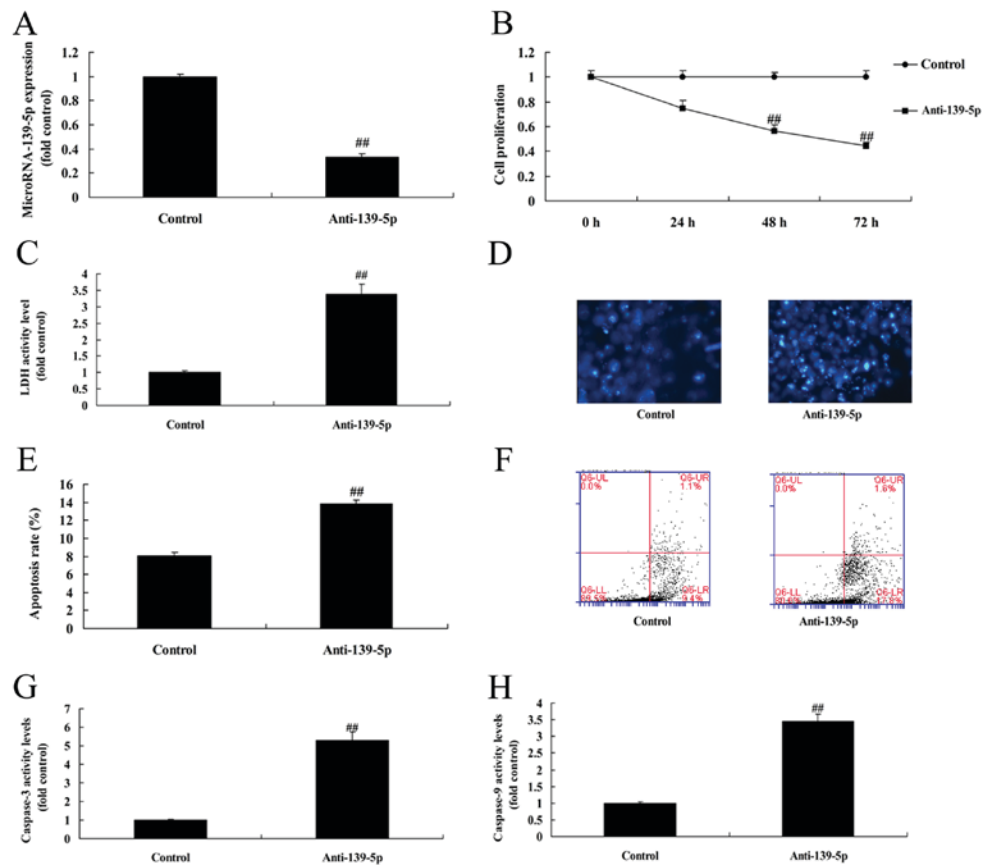
Figure 2. Downregulation of miR-139-5p inhibited cell growth of adult dental pulp stem cells. (A) miR-139-5p expression, (B) cell growth, (C) LDH activity level, (D) DAPI staining, (E and F) apoptosis rate, (G and H) caspase-3/9 activity levels. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control. miR, microRNA; LDH, lactate dehydrogenase.

compared with control negative group via RT-qPCR (Fig. 1A). Overexpression of miR-139-5p promoted cell growth, inhibited LDH and caspase-3/9 activity levels, and reduced apoptosis rate of ADSC, compared with the control negative group (Fig. 1B-H).

Downregulation of miR-139-5p inhibits cell growth of ADSCs. Anti-miR-139-5p was used to decrease the protein expression of miR-139-5p in ADSCs, compared with control negative group (Fig. 2A). Downregulation of miR-139-5p inhibited cell growth, increased LDH and caspase-3/9 activity levels, and promoted apoptosis rate of ADSCs, compared with control negative group (Fig. 2B-H).

miR-139-5p regulates skeletal myogenic differentiation of ADSCs. It was hypothesized that miR-139-5p would upregulate skeletal myogenic differentiation of ADSC. As presented in Fig. 3A-D, overexpression of miR-139-5p inducedMEF2C, MyoD and MyHC protein expression in ADSCs, compared with control negative group. However, downregulation of miR-139-5p suppressed MEF2C, MyoD and MyHC protein expression in ADSCs, compared with control negative group (Fig. 3E-H). These results indicated that overexpression of miR-139-5p induced skeletal myogenic differentiation of ADSCs.

miR-139-5p regulates skeletal myogenic differentiation of ADSC through Wnt/β-catenin signaling pathway. The

mechanism of miR-139-5p on skeletal myogenic differentiation of ADSC was investigated, and miR-139-5p was identified as a direct target of Wnt (Fig. 4A). Immunofluorescence microscopy analysis demonstrated that overexpression of miR-139-5p induced Wnt and β-catenin protein expression in ADSCs, compared with control negative group (Fig. 4B). Overexpression of miR-139-5p induced Wnt and β-catenin protein expression in ADSCs, compared with control negative group (Fig. 4C-E). Downregulation of miR-139-5p suppressed Wnt and β-catenin protein expression in ADSCs, compared with control negative group (Fig. 4F-H). These data indicated that miR-139-5p regulates skeletal myogenic differentiation of ADSCs through the Wnt/β-catenin signaling pathway.

Wnt inhibitor reduces the effect of miR-139-5p on skeletal myogenic differentiation of ADSCs. The role of Wnt/β-catenin signaling pathway on the effect of miR-139-5p on skeletal myogenic differentiation of ADSCs was investigated. As presented in Fig. 5A-C, Wnt inhibitor suppressed Wnt and β-catenin protein expression in ADSCs induced by miR-139-5p, compared with the miR-139-5p group. Wnt inhibitor also suppressed MEF2C, MyoD, and MyHC protein expression in ADSCs induced by miR-139-5p, compared with the miR-139-5p group (Fig. 5D-G). Wnt inhibitor also reduced the effect of miR-139-5p on cell growth, LDH and caspase-3/9 activity levels, and apoptosis rate of ADSCs induced by miR-139-5p, compared with the miR-139-5p group (Fig. 6).
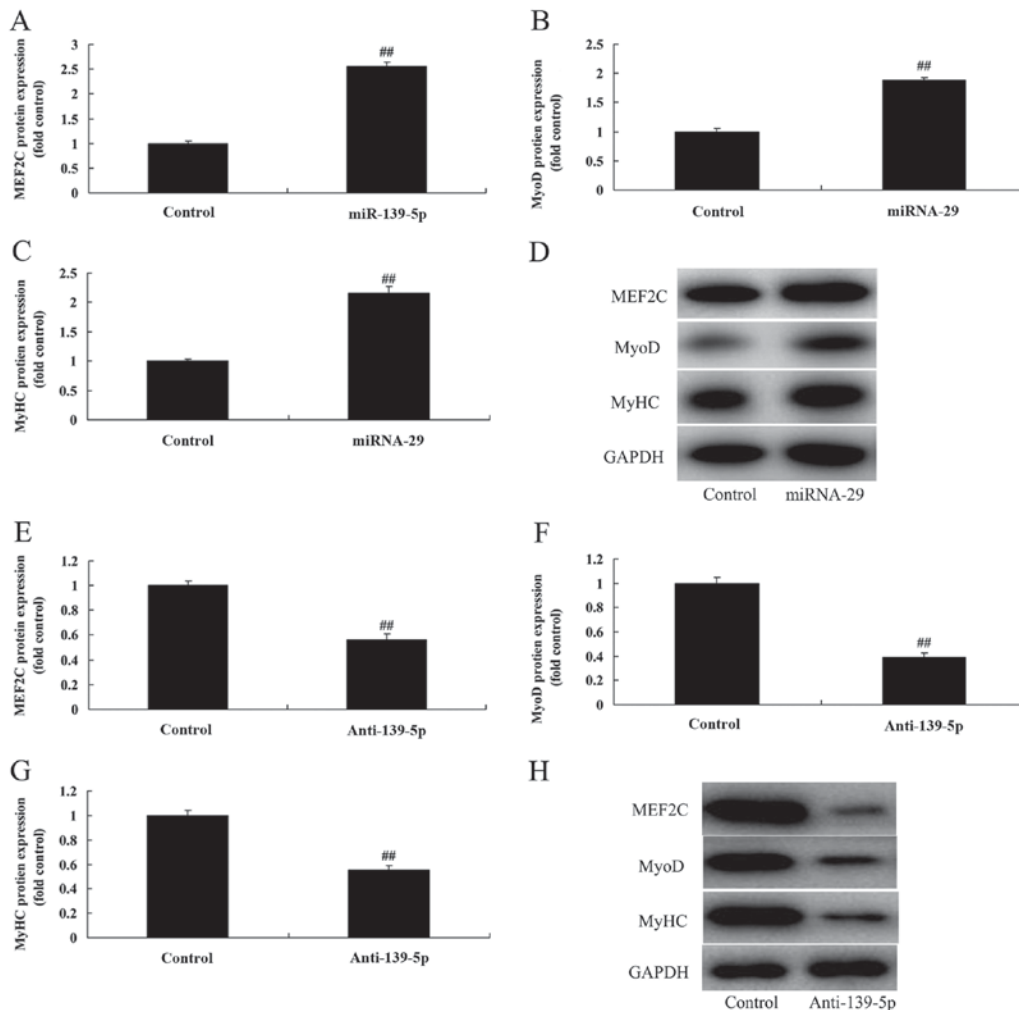
Figure 3. miR-139-5p regulates skeletal myogenic differentiation of adult dental pulp stem cells. Quantified data of (A) MEF2C, (B) MyoD, and (C) MyHC protein expression and (D) western blotting images following overexpression of miR-139-5p. Quantified data of (E) MEF2C, (F) MyoD, and (G) MyHC protein expression and (H) western blotting images following downregulation of miR-139-5p. Data are presented as the mean ± standard deviation. [##]P<0.01 vs. control. miR, microRNA; MEF2C, myocyte-specific enhancer factor 2C; MyoD, myogenic differentiation 1; MyHC, myosin heavy chain.

Discussion

Endodontic disease is a common disease in the oral cavity, with an incidence rate of 72% (4). It mainly results from irreversible inflammatory response, liquidation or necrosis in dental pulp. Eventually, the diseased dental pulp should be removed (4). Consequently, conventional root canal therapy should be conducted to relieve the disease and repair tooth function (12). Tissue engineering technology has been rapidly developed in recent years (12). As a result, regenerative medicine has gradually penetrated into all areas of medicine (12). Meanwhile, regenerative medical research on oral endodontic disease has attracted attention from scholars worldwide. The focus of research has typically been dental pulp regeneration (6). miRNAs can also regulate the expression of genes associated with dental germ formation quantity (6). Researchers have previously knocked out Dicer enzyme, which is essential for the formation of mature miRNAs, and observed an increase in the number of dental germs in mice, as well as partial edentia (13). miRNAs also serve important regulatory roles in dental pulp regeneration research (13). Results of the present study demonstrated that overexpression

of miR-139-5p promoted cell growth and induced skeletal myogenic differentiation of ADSCs. Mi *et al* (14) recently demonstrated that miR-139-5p suppresses 3T3-L1 preadipocyte differentiation.

The Wnt signaling pathway is an extremely conserved signal transduction pathway (9). Furthermore, it serves a key role in the dynamic balance of maintaining adult tissue (9). Recent findings have suggested that Wnt signaling can also regulate stem cell differentiation and self-renewal (9). In addition, recent research has also indicated that inhibiting canonical Wnt signal transduction can promote adipogenic differentiation of preadipocytes and differentiation of adipocytes (9). Upregulating peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 can activate the canonical Wnt pathway, and inhibit expression of CCAAT/enhancer-binding protein-α and peroxisome proliferator-activated receptor γ (9). Thus, it can suppress the adipogenic differentiation of DPSCs (15). These findings indicate that the Wnt pathway serves a key regulatory role in the adipogenic differentiation of MSC (15). The Wnt pathway is an important target of the adipogenic differentiation of pluripotent stem cells (16). In the present study, it as demonstrated that overexpression of
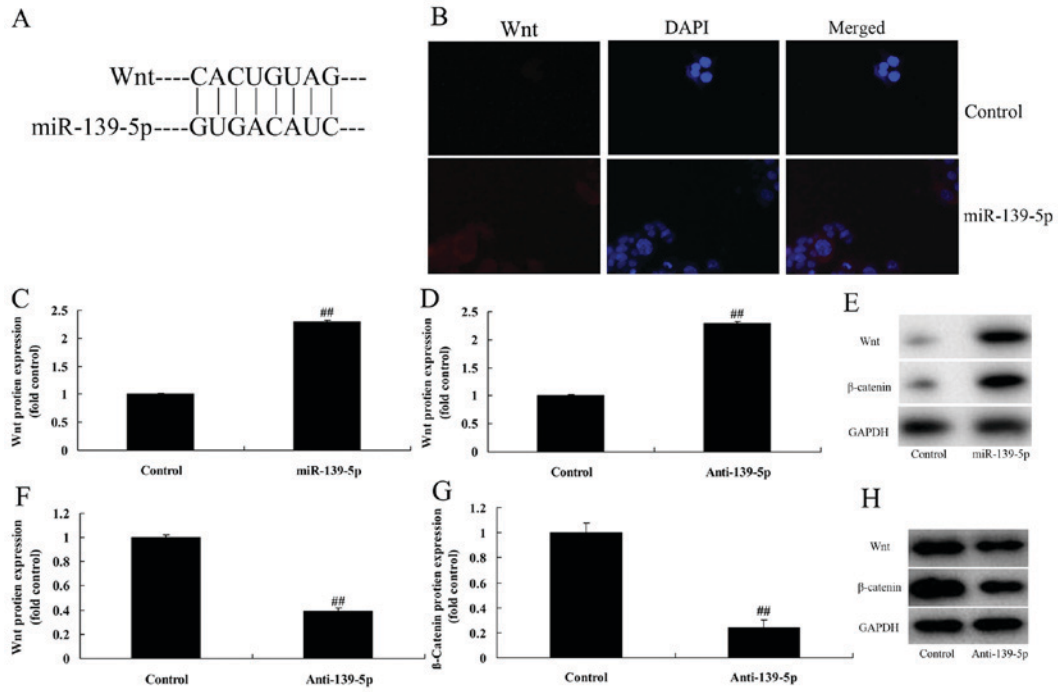
Figure 4. miR-139-5p regulates skeletal myogenic differentiation of adult dental pulp stem cells through Wnt/β-catenin signaling pathway. (A) miR-139-5p was identified as a direct target of Wnt. (B) Immunofluorescence analysis for Wnt protein expression. (C) Wnt and (D) β-catenin protein expression and (E) western blotting images following overexpression of miR-139-5p. (F) Wnt and (G) β-catenin protein expression and (H) western blotting images following downregulation of miR-139-5p. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control. miR, microRNA.
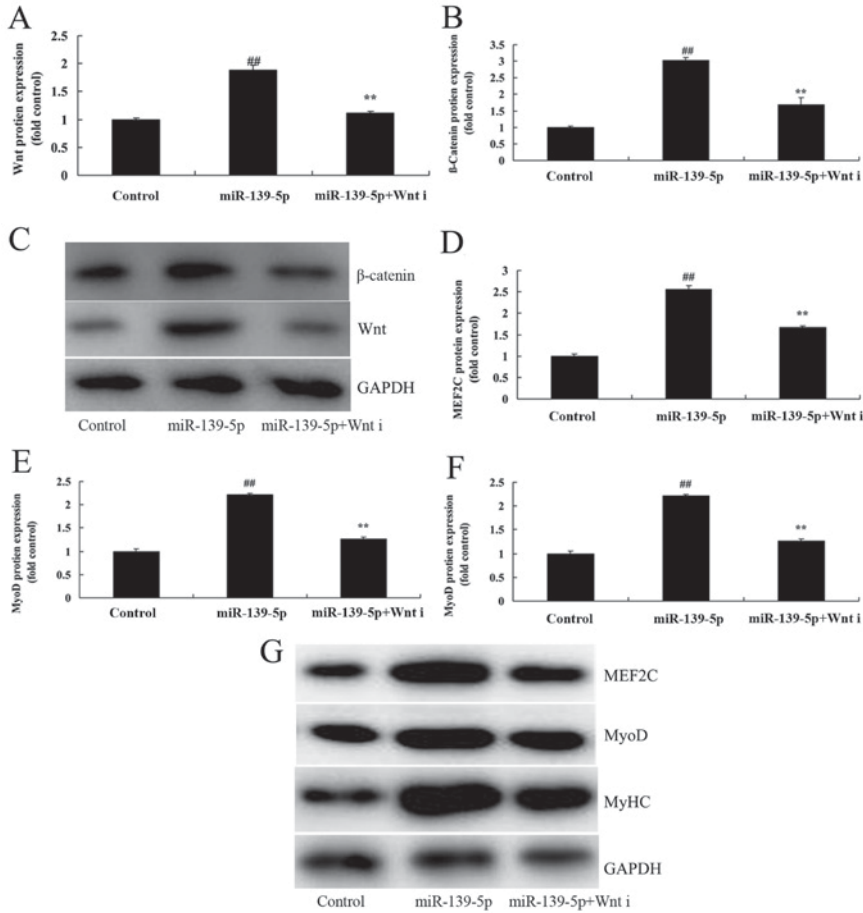

Figure 5. Wnt i reduced the effect of miR-139-5p on Wnt/β-catenin signaling pathway. (A) Wnt and (B) β-catenin protein expression and (C) western blotting images following Wnt inhibition. (D) MEF2C, (E) MyoD, and (F) MyHC protein expression and (G) western blotting images following Wnt inhibition. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control; **P<0.01 vs. miR-139-5p. Wnt i, Wnt inhibitor; miR, microRNA; MEF2C, myocyte-specific enhancer factor 2C; MyoD, myogenic differentiation 1; MyHC, myosin heavy chain.
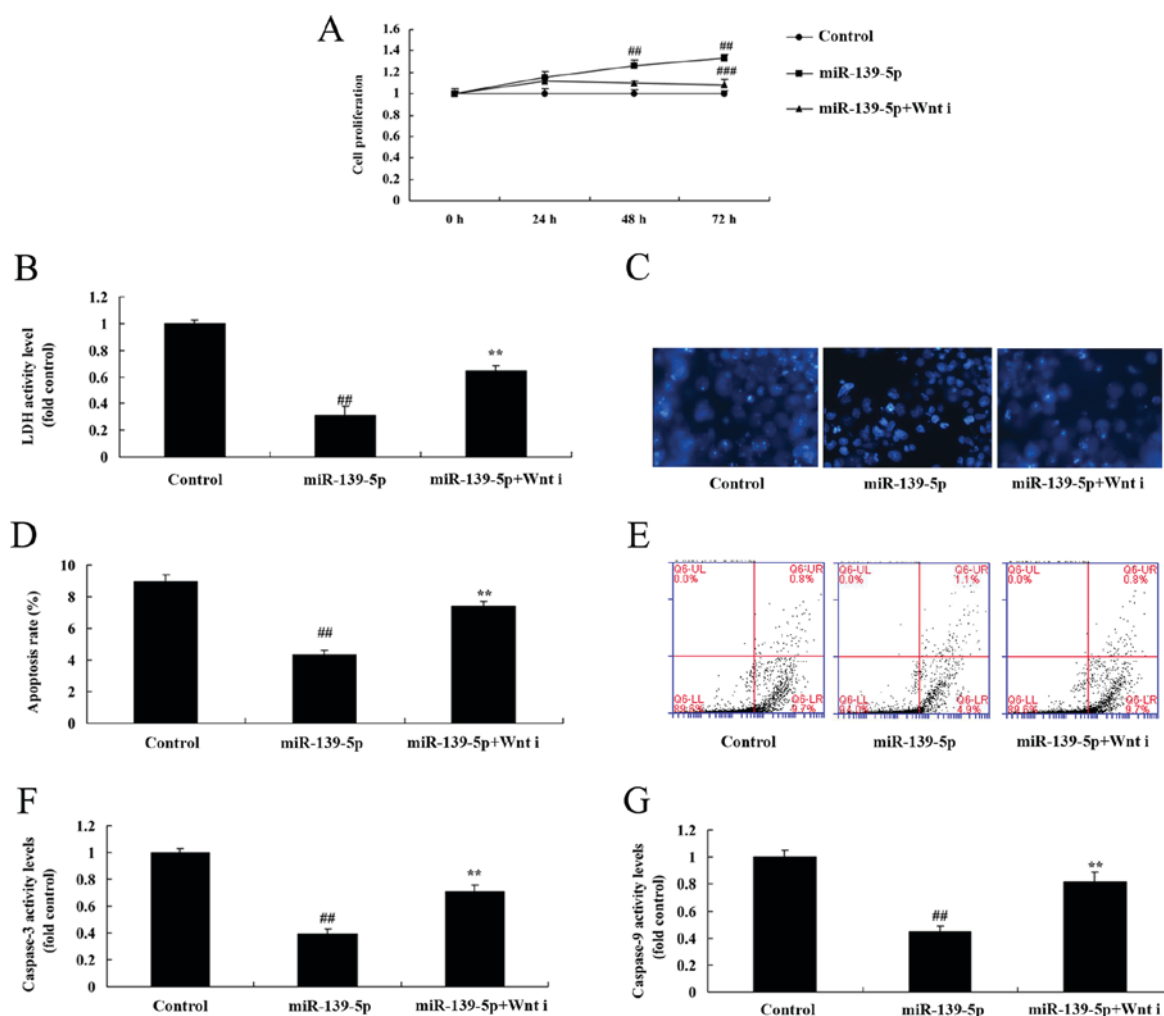
Figure 6. Wnt i reduced the effect of miR-139-5p on skeletal myogenic differentiation of adult dental pulp stem cells. (A) Cell growth, (B) LDH activity level, (C) DAPI staining, (D and E) apoptosis rate, (F and G) caspase-3/9 activity levels. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control; **P<0.01 vs. miR-139-5p. Wnt i, Wnt inhibitor; miR, microRNA; LDH, lactate dehydrogenase.

miR-139-5p induced the Wnt/β-catenin signaling pathway of ADSCs. Mi et al (17) recently reported that miR-139-5p regulates myogenesis through blocking Wnt/β-catenin signaling pathway.

Wnt protein ligand, Wnt receptor, β-catenin, glycogen synthase kinase, axin and APC are the major members of the Wnt signaling pathway (18). Specifically, β-catenin is the important mediator of Wnt signal transduction. APC also serves an important role in Wnt signal transduction pathway (19). Wnt/β-catenin is a canonical intracellular Wnt signaling pathway. Previous studies have demonstrated that Wnt signal serves an irreplaceable role in dental epithelium-mesenchyme interaction, dentin and dental root development (18,20). The present study demonstrated that Wnt inhibitor reduced the effect of miR-139-5p on skeletal myogenic differentiation of ADSCs. Luo et al (21) recently demonstrated that miR-139-5p inhibits bladder cancer proliferation by targeting the Wnt signaling pathway. However, this study is limited as only in vitro experiments were performed. Future studies should therefore perform in vivo experiments in order to confirm these results.

In conclusion, the results of the present study demonstrated that miR-139-5p elevates skeletal myogenic differentiation of human ADSCs through Wnt/β-catenin signaling pathway. miR-139-5p may therefore be a novel marker of skeletal myogenic differentiation of human ADSCs.

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

GS designed the experiment; YX and GS performed the experiments, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Ninth People's Hospital, Shanghai JiaoTong University School of Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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