MiR-204-5p/FOXC1/GDF7 Axis Regulates Osteogenic Differentiation of Human Adipose-Derived Stem Cells Via the AKT and p38 Signaling Pathways

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Research

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Abstract

Background: Human adipose-derived stem cells (hADSCs) are stem cells with the potential to differentiate in multiple directions. MiR-204-5p is poorly expressed during osteogenic differentiation of hADSCs, and its specific regulatory mechanism remains unclear. Here, we aimed to explore the function and possible molecular mechanism of miR-204-5p involved in the osteogenic differentiation of hADSCs.

Methods: The expression pattern of miR-204-5p, Runx2, Alkaline phosphatase (ALP), Osteocalcin (OCN), and Forkhead box C1 (FOXC1) and growth differentiation factor 7 (GDF7) in hADSCs during osteogenesis were detected by qRT-PCR. Then, ALP and alizarin red staining (ARS) were used to detect the activity of osteoblasts and mineral deposition. Western blot was conducted to confirm the protein levels. The regulation relationship among miR-204-5p, FOXC1 and GDF7 was verified by double luciferase activity and CHIP experiment.

Results: First, miR-204-5p expression was down-regulated and overexpressed miR-204-5p suppressed the osteogenic differentiation. Furthermore, the levels of FOXC1 and GDF7 were decreased in the miR-204-5p mimics group, which indicate that overexpressed miR-204-5p would suppress the expression of FOXC1 and GDF7 through binding the 3’UTR region each. Overexpression of FOXC1 or GDF7 could improve the inhibition of osteogenic differentiation of hADSCs induced by the miR-204-5p mimics. Moreover, FOXC1 could bind to the promoter of miR-204-5p and GDF7, promote the deacetylation of miR-204-5p and reduced the expression of miR-204-5p, thus promoting the expression of GDF7 during osteogenic differentiation. GDF7 could induce hADSCs osteogenesis differentiation by activating the AKT and P38 signaling pathways.

Conclusions: Our results demonstrated that miR-204-5p/FOXC1/GDF7 axis regulates osteogenic differentiation of hADSCs via the AKT and p38 signaling pathways. This study further understood the regulatory mechanism of hADSCs differentiation balance from the perspective of miRNAs regulation.

Introduction

Bone tissue engineering is to combine seed cells with biomaterials under the action of osteogenic inducing factors to differentiate seed cells into osteoblasts and achieve bone regeneration ¹. Therefore, seed cells are the primary link and basic elements of bone tissue engineering. HADSCs are one of the most widely used stem cells as the seed cells in bone tissue engineering². Zuk et al (2001) firstly isolated the hADSCs from human adipose tissue. HADSCs is convenient to access because of adipose tissue in the body widely drawn³. Gronthos et al. have shown that hADSCs resting stage cells 70% of the total number of cells with stem cell properties, existing research has shown that hADSCs equally multi-directional differentiation potential. It can differentiate into adipocytes, cardiomyocytes, nerve cells, osteoblasts, chondrocytes and so on ⁴. HADSCs have many biological characteristics such as self-renewal, multi-directional differentiation, specific migration to the injury site, and functions for tissue
repair and regeneration\textsuperscript{5}. Those advantages make them favoured for clinical application, while the mechanisms in the osteogenic differentiation of hADSCs are far less understood.

MicroRNAs is a class of small molecule RNAs, approximately 22 nt in length, that are endogenously expressed in eukaryotes\textsuperscript{6}. They can inhibit target gene transcription or mRNA degradation by binding to the target gene\textsuperscript{7}. MicroRNAs are involved in the regulation of a variety of biological processes, including cell proliferation and differentiation, metabolic apoptosis, DNA damage repair, cancer development, embryonic development, tissues differentiation and formation, and other physiological processes\textsuperscript{8,9}. Studies have reported that microRNAs can regulate the osteogenic differentiation of mesenchymal stem cells (MSCs)\textsuperscript{10}. Currently, it has reported 27 microRNAs participated in osteogenic differentiation\textsuperscript{11}. MiR-20a has been reported to play a positive role in regulating osteogenic differentiation of hADSCs by co-regulating the BMP signaling pathway, while miR-23a inhibits osteogenic differentiation by acting on the key transcription factor Runx2\textsuperscript{12,13}. MiR-204-5p is a member of the miR-204 family, which is derived from the 5' end arm of the has-miR-204 precursor (pre-miRNA)\textsuperscript{14}. Studies have shown that miR-204 is significantly downregulated during osteogenic differentiation and inhibits osteogenic differentiation by downregulating the important transcription factor Runx2 during osteogenic differentiation\textsuperscript{15}. Nevertheless, the specific mechanism by which miR-204 regulates osteogenic differentiation of hADSCs remains largely unknown.

Forkhead box C1 (FOXCl), a member of the fox superfamily, is widely present in various tissues and organs and regulates biological processes including cell differentiation, embryonic development, tumorigenesis and development\textsuperscript{16}. During embryonic development, FOXC1 activates target gene transcription by binding to the promoter of the target gene or interacting with other transcription factors\textsuperscript{17}. FOXC1 is an important regulator of the normal development and formation of bones originating from both endochondral and intramembranous origins\textsuperscript{18}. The Foxc1\textsuperscript{−/−} mutant mice showed a large number of abnormalities in the skulls, vertebrae, ribs and appendage bones\textsuperscript{19,20}. A recent analysis showed that the ossification centers in the long bones of FOXC1 mutant mice were reduced, and Foxc1 regulated PTHrP expression through interactions with Indian Hedgehog (Ihh)-Gli2 signaling, suggesting that FOXC1 played a role in regulating chondrocyte proliferation and differentiation\textsuperscript{21}. Growth differentiation factor 7 (GDF7), also known as bone morphogenetic protein 12 (BMP12), cartilage-derived morphogenetic protein 3 (CDMP3), is a member of the transforming growth factor beta (TGF\beta) superfamily\textsuperscript{22,23}. Hua Shen et al. found that after GDF7 stimulated ADSCs, it could promote the osteogenic differentiation of ADSCs through the Smad1/5/8 pathway\textsuperscript{24}. However, the roles of FOXC1 and GDF7 in the osteogenic differentiation of hADSCs are still unclear, and the mechanisms also need to further investigate.

Studies reported that growth differentiation factor 7 (GDF7) and FOXC1 are highly expressed during hADSCs osteogenic differentiation, and miR-204-5p is lowly expressed during osteogenic differentiation\textsuperscript{25,26}. Moreover, TargetScan/miRDB predicts that both GDF7 and FOXC1 might be the target genes of miR-204-5p, and JASPAR software shows that the transcription factor FOXC1 is able to bind to the promoter of miR-204-5p and GDF7. Therefore, we speculated that miR-204-5p might act on mRNA of GDF7 and
FOXC1 to inhibit osteogenic differentiation of hADSCs, and FOXC1 might bind to the promoter of miR-204-5p and GDF7, and reduce the expression of miR-204-5p, thus promote the expression of GDF7. In this study, we aim to investigate the role of miR-204-5p/FOXC1/GDF7 regulatory axis in hADSCs osteogenic differentiation. Our data indicated that FOXC1/GDF7/miR-204-5p axis formed cyclic regulation, which plays an important role in the balance of osteogenic differentiation. To our knowledge, the relationship among miR-204-5p, FOXC1 and GDF7 has not been studied during the osteogenic differentiation of hADSCs. Our data are expected to provide new insight into the differentiation of hADSCs in bone regeneration and promote hADSCs clinical application.

Materials And Methods

Cell Culture and Osteogenic Induction

HADSCs used in this study were all derived from discarded adipose tissue after liposuction from patients in the Department of Plastic Surgery, the first hospital of China Medical University. The samples were from 8 female patients aged 29.56 ± 5.85 years, and without metabolic disease, syphilis, HIV, hepatitis and other systemic diseases. The surgical site was abdomen and the surgical method was suction. The above experimental samples were collected with the informed consent of the clinical patients and approved by the Ethics Committee of the first hospital of China Medical University.

The adipose tissue obtained by liposuction of subcutaneous adipose was added into 50 mL centrifuge tubes and washed with sterile phosphate-buffered saline (PBS (Gibco, Grand Island, NY, USA). Then centrifuged at 1000 × g for 5 min to remove residual blood cells and tissue debris. The above procedure was repeated 3–4 times before enzymatic digestion with 0.2% collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 45 min. Dulbecco’s Modified Eagle’s Medium/Nutrient F-12 Ham (DME F12 (HyClone, USA)), containing 10% fetal bovine serum (FBS (Gibco, USA)), was added to the digested lipoaspirates for 5 min to neutralize enzyme activity, this was followed by centrifugation at 1000 × g for 5 min. Finally, the cells were plated in 75 cm2 culture flasks and incubated in culture medium (DME F12, 10% FBS, 1% Penicillin-Streptomycin Solution(Gibco, USA)) at 37°C in 5% CO2 with saturated humidity. The medium was changed for the first time after 24 h, and the medium was changed every 2 days after the first time. HADSCs were passaged until they were 90% confluent; 0.25% trypsin: 0.2% EDTA at a ratio of 1:3 was used to dissociate the cells. HADSCs at passage three were used for subsequent experiments. When the cells were adherent to the wall and grew to about 90%, they were cultured in groups with proliferation medium (PM) or complete OriCell™ osteogenic differentiation medium (Cyagen, USA)(OM).

Flow cytometric analysis

When hADSCs grew up to 80%, removed the old culture medium and washed once or twice with PBS. The trypsin solution was added, and the cells were placed in an incubator at 37°C for 1 minute and observed under an inverted microscope. The cells were about to be separated and presented granular circles, with adding the complete medium of fresh stem cells and were blown evenly with a pipette to prepare a single-cell suspension. The single-cell suspension was transferred to the flow tube, washed with PBS twice, and
centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cells were resuspended with 500 µL 1 × binding buffer and incubated with CD44, CD45, CD90, CD105 and HLA-DR antibody working solution (Abcam, Cambridge, MA, USA) in each group, and the homotype control group was set at the same time. After incubation, the cells were centrifuged at 1000 × g for 5 min. The supernatant was discarded, and the cells were resuspended with 500 µL 1 × buffer and analyzed by flow cytometry with a FACScalibur (Becton Dickinson, Mountain View, CA, USA). The data were analyzed using CELL Quest software.

**RNA oligoribonucleotides and plasmid and transfection**

The RNA oligoribonucleotides used in this study, including miR-204-5p mimics, miR-204-5p inhibitor, the small interfering RNAs (siRNAs) targeting FOXC1 (si-FOXC1) or GDF7 (si-GDF7), and the miR-NC and siRNA control (si-NC), were purchased from GenePharma Co. (Shanghai, China). And the FOXC1 and GDF7 plasmids were provided by Vipotion (Guangzhou, China).

At the time of passage, the surface of the culture vessel was covered with gelatin, and an appropriate amount of 0.1% gelatin was added to the culture vessel to cover the whole bottom of the culture plate. The culture vessel was placed in a super clean table, and the gelatin was discarded after 30 min. After the culture vessel was dried, it could be used for cell inoculation. HADSCs were inoculated into gelatin-coated plates and cultured in the culture medium containing 10%FBS. For transfection, when hADSCs were reached to 70–90% confluence, they were transfected with mimics (100 nM) or inhibitor (100 nM) or with the FOXC1 or GDF7 plasmids (20 ng) or siRNA (20 µM) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. Cells were cultured in PM or OM for 14 days. Discarded the old medium every 3 days, repeated the transfection processes according to the above steps, and replaced the fresh complete medium. The cells harvested at 14 days for RNA and protein analysis.

**RNA extraction and quantitative polymerase chain reaction (qPCR)**

Total cellular RNA was isolated on 14 days or each time point after osteoinduction or normal culturation using TRIZOL® reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the extracted RNA was reverse-transcribed in the presence of a poly-A polymerase with an oligo-dT adaptor. Gene expression levels were measured by the real-time PCR detection system (Bio-Rad, Hercules, CA, USA) by SYBR® Green (Bio-Rad, Hercules, CA, USA) detection. The expression of miR-204-5p, Runx2, ALP, OCN, FOXC1, and GDF7 were quantified by qPCR using SYBR® Green assays (Takara), GAPDH and U6 were used as an internal control for mRNAs and miR204-5p. The data were calculated using the 2ΔΔCt, where RQ is the relative quantity, and expressed as the fold change, relative to the gene expression levels in the control samples.

**Western blot analysis**
After 14 days of osteoinduction or normal culturation, the proteins of hADSCs were extracted by using RIPA buffer containing 1% PMSF (Sigma-Aldrich). BCATM Protein Assay Kit (Pierce, Appleton, WI, USA) was used for quantification of protein samples. An equal amount of protein samples (30 µg) were separated on 12% SDS-PAGE gel and transferred to PVDF membrane (Millipore Corporation, Billerica, MA, USA). After blocking in bovine serum albumin (BSA; Roche) for 1 h at 37°C, the blocked membranes were incubated with corresponding primary antibodies overnight at 4°C, including FOXC1(ab223850, 1:1000), ALP (ab16695, 1:1000), RUNX2 (ab23981, 1 µg/ml), and OCN (ab93876, 1 µg/ml), P38 (ab170099, 1:1000), p-P38 (ab4822, 1:500), AKT (ab179463, 1:1000), p-AKT (ab38449, 1:500) and GAPDH (ab181602, 1:1000) were purchased from Abcam (Cambridge, MA, USA). The membranes were then washed and incubated with appropriate HRP-secondary antibodies (ab6721, 1:2000) (Cambridge, MA, USA) for 1 h at 37°C. The signals were detected and analyzed by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA) and Image Lab™ software (Bio-Rad, Cal, USA).

**Alkaline phosphatase (ALP) staining**

The transfected hADSCs in PM or OM for 14 days were assayed for ALP staining and activity. The medium was removed after 14 days, washed twice with PBS buffer, and fixed with 4% paraformaldehyde (Jianglaibio, Shanghai, China) for 30 min. Remove paraformaldehyde, wash with ddH2O water for three times, and add alkaline phosphatase staining solution (Beyotime, China) for 30 min. Remove the ALP staining solution, wash it with ddH2O for three times, observe under the microscope (Leica DMIRB, Germany) and take photos.

**Alizarin red staining (ARS)**

The transfected hADSCs cultured in PM or OM for 14 days were assayed for ARS. The culture medium was discarded, fixed with 4% paraformaldehyde for 15–20 min, and washed with PBS for 3 times. Alizarin red staining solution (ScienCell, San Diego, CA, USA) was prepared in advance, added to the culture plate, placed in the incubator for 15 min, abandoned the staining solution, washed with PBS solution for three times, drained, and placed under the differential microscope to take photos (Leica DMIRB, Germany).

**Immunofluorescence Staining**

Transient transfected hADSCs with miR204-5p mimics or inhibitors were seeded in 6-well plates. After 14 days in PM or OM, cells grown on sterile glass coverslips were fixed in 4% paraformaldehyde (Jianglaibio, Shanghai, China) for 30 min, permeabilized with 0.1% Triton X-100 for 15 min, and blocked with 5% normal goat serum for 30 min. Then, cells were incubated with a primary antibody-OCN (ab13418, 1:500; Abcam), and incubated with an anti-mouse secondary antibody (ab150117, 1:500; Abcam) for 1 h at room temperature. 40,6-diamidino-2-phenylindole (DAPI) was used to stain the Nuclei, and the coverslips were mounted on a glass slide and observed under a Confocal Zeiss Axiovert 650 microscope with 488 nm (green, OCN) and 405 nm (blue, DAPI). Images were captured using an LSM 5 Exciter confocal imaging system (Carl Zeiss).

**Dual-luciferase reporter assay**
Luciferase reporter assays were carried out as follows. Firstly, the 3′-UTR sequence of wild-type (WT) and mutant-type (MUT) of FOXC1 and GDF7 were conducted into the psiCHEck2 vector, while the WT and MUT region of promoters of miR-204-5p and GDF7 were cloned into the pGL3 vector. For analyzing the interaction between miR-204-5p and FOXC1 or GDF7, hADSCs (1 × 10^5) were grown in a 96-well plate and co-transfected with either miR-204-5p mimics (100 nM) or NC mimics (100 nM), FOXC1-WT/MUT (20 ng), or GDF7-WT/MUT (20 ng) by Lipofectamine 2000 (Invitrogen). For the FOXC1 binding to the promoter of GDF7 and miR-204-5p, the hADSCs were grown in a 96-well plate and co-transfected with either Vector/Vector-FOXC1 or miR-204-5p-WT/MUT, GDF7-WT2/MUT2. Renilla and firefly luciferase activities were measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, Beijing, China)). All luciferase values were normalized to those of firefly luciferase and expressed as fold induction relative to the basal activity.

**Chromatin Immunoprecipitation (ChIP) Assay**

ChIP assays were conducted by using an EZ-Magna ChIP assay kit (Merck Millipore). HADSCs were seeded in 10-cm dishes and transfected with FOXC1 or the vector. The cells were crosslinked with 1% formaldehyde and sonicated to shear DNA. Then the DNA-protein complexes were isolated with antibodies against isotype immunoglobulin G (IgG) and HDAC2, H3K9AC, FOXC1 (Cell Signaling). The protein-DNA complexes were then purified and reverse-crosslinked. The DNA was isolated and quantified by qRT-PCR. Relative enrichment was calculated as the amount of amplified DNA relative to values obtained from Input.

**Statistical Analysis**

All the corresponding experiments were independently repeated for more than three times, and the results of Western Blot were representative. All data in this experiment are expressed as mean ± standard deviation. SPSS 17.0 for Windows statistical software was used for t-test analysis between groups, and P < 0.05 indicated a significant difference.

**Results**

1. **MiR-204-5p is down-regulated during the osteogenic differentiation of hADSCs.**

HADSCs were collected and conducted phenotypic identification, and the results showed that the cells were positive of CD44, CD90, CD105 and negative of CD45 and HLA-DR, which suggested that the primary cultured cells were hADSCs (Fig. 1A). These identified hADSCs were cultured under osteogenic induction conditions until 14 days, and the expression level of hsa-miR-204-5p was detected by qRT-PCR at 3, 5, 7, 10, 14 days. The results showed that the expression level of miR-204-5p was decreased gradually with the induction time (Fig. 1B). At the same time, we detected the expression levels of osteogenic markers RUNX2, ALP, and OCN in cells at various time points. QRT-PCR results showed that the expression levels of RUNX2, ALP and OCN were markedly up-regulated during osteogenesis (Fig. 1B).

2. **miR-204-5p inhibits the osteogenic differentiation of hADSCs in vitro.**
To explore the effect of miR-204-5p on osteogenic differentiation of hADSCs, we transfected miR-204-5p mimics or inhibitor into hADSCs, and cultured in PM or OM, the expression level of miR-204-5p was detected by qRT-PCR at 14d in PM and OM, and it was increased when cells transfected with miR-204-5p mimics but decreased when cells transfected with miR-204-5p inhibitor (Fig. 2A). QRT-PCR and western blot were used to detect the mRNA and protein expression level of osteogenic markers RUNX2, ALP, and OCN. Results showed that miR-204-5p mimics could decrease RUNX2, ALP, and OCN expression, whereas miR-204-5p inhibitor could increase RUNX2, ALP, and OCN expression (Fig. 2A and 2B). Besides, immunofluorescence staining indicated that the protein level of OCN was reduced in the miR-204-5p overexpression group and increased in the miR-204-5p inhibition group (Fig. 2D).

Moreover, western blot showed that the protein level of FOXC1 and GDF7 were also decreased in the miR-204-5p mimics group and increased in the miR-204-5p inhibition group (Fig. 2C).

ALP staining showed that miR-204-5p mimics suppressed the osteogenic differentiation of hADSCs, and miR-204-5p inhibitor enhanced the osteogenic differentiation of hADSCs in PM or OM (Fig. 2E). The extracellular mineralization of hADSCs was measured by ARS assay in PM or OM on days 14, and results showed that the osteogenic differentiation of hADSCs was suppressed with miR-204-5p mimics, but increased with miR-204-5p inhibitor (Fig. 2F). Taken together, these results suggested that miR-204-5p inhibits the osteogenic differentiation of hADSCs in vitro.

3. FOXC1 reduces the suppression effect of miR-204-5p on osteogenic differentiation of hADSCs

To investigate the role of FOXC1 in hADSCs osteogenesis, the cells were co-transfected with miR-204-5p mimics and FOXC1 plasmid or co-transfected with miR-204-5p inhibitor and FOXC1 siRNA. Results showed that overexpression FOXC1 could inhibit miR-204-5p expression, but promote the expression of FOXC1 and GDF7, and also increased the RUNX2, ALP and OCN expression under transfection of miR-204-5p mimics, whereas down-regulation of FOXC1 obtained opposite results in hADSCs with transfection of miR-204-5p inhibitor (Fig. 3A). Then we also carried out western blot to evaluate the protein expression level of FOXC1, GDF7, RUNX2, ALP and OCN and got the same results with qRT-PCR (Fig. 3B). Subsequently, ALP staining and ARS showed that overexpression FOXC1 attenuated the suppression effect of miR-204-5p on osteogenic differentiation and extracellular mineralization of hADSCs, while downregulation of FOXC1 inhibited osteogenic differentiation and extracellular mineralization of hADSCs induced by miR-204-5p inhibitor (Fig. 3C and 3D). These results suggested that FOXC1 reduced the suppression effect of miR-204-5p on osteogenic differentiation of hADSCs.

To explore the relationship between miR-204-5p and FOXC1, we used TargetScan/miRDB to search for potential targets of miR-204-5p. We found that the 3’ untranslated region (UTR) of FOXC1 has two miR-204-5p binding sites (Fig. 3E). Next, we constructed wild-type (WT) reporter and mutant-type (MUT) reporter which contained a mutant 3’UTR of FOXC1 designed with mutated sequences of the miR-204-5p binding site. Results indicated that overexpression of miR-204-5p markedly decreased the luciferase activity in the WT group of FOXC1, whereas this phenomenon was not observed in the MUT group (Fig. 3F), which suggested that FOXC1 is a target of miR-204-5p.
4. GDF7 impairs the suppression effect of miR-204-5p on osteogenic differentiation of hADSCs.

To explore the role of GDF7 in hADSCs osteogenesis, the hADSCs were co-transfected with miR-204-5p mimics and GDF7 plasmid or co-transfected with miR-204-5p inhibitor and GDF7 siRNA. Results showed that overexpression of GDF7 could inhibit miR-204-5p expression, but promote the mRNA and protein expression levels of GDF7, RUNX2, ALP and OCN expression, whereas downregulation of GDF7 obtained opposite results (Fig. 4A and 4B). Recent studies proved that AKT and P38 signaling pathways were involved in osteogenic differentiation, so we next carried out western blot to evaluate the protein expression level of p-AKT, AKT, p-P38 and P38. Results suggested that overexpression of GDF7 could enhance the phosphorylation of AKT and P38 under treatment with miR-204-5p mimics, while the knockdown of GDF7 reduce the phosphorylation of AKT and P38 under inhibition of miR-204-5p (Fig. 4C).

Moreover, ALP staining showed that overexpression GDF7 promoted the osteogenic differentiation of hADSCs transfected with miR-204-5p mimics, while down-regulation of GDF7 reduced this progress in hADSCs transfected with miR-204-5p inhibitor (Fig. 4D). ARS showed that overexpression GDF7 increased extracellular mineralization of hADSCs transfected with miR-204-5p mimics, but downregulation of GDF7 inhibited extracellular mineralization of hADSCs transfected with miR-204-5p inhibitor (Fig. 4E). Taken together, these results indicated that GDF7 impairs the suppression effect of miR-204-5p on osteogenic differentiation of hADSCs via the AKT and p38 signaling pathway.

Finally, we used Dual-luciferase reporter assay to verify whether GDF7 is a potential target of miR-204-5p. We found that there is a miR-204-5p binding site in the 3’ untranslated region (UTR) of GDF7. Next, we constructed wild-type (WT) reporter and mutant-type (MUT) reporter which contained a mutant 3’-UTR of GDF7 designed with mutated sequences of the miR-204-5p binding site. Results indicated that overexpression of miR-204-5p markedly decreased the luciferase activity in the WT group of GDF7, but not in the MUT group (Fig. 4F). This result suggested that GDF7 is also a target of miR-204-5p.

5. FOXC1 as a transcription factor could bind to the promoter of miR-204-5p and GDF7

To further explore the specific regulation mechanism among the miR-204-5p, FOXC1 and GDF7 in osteoblast differentiation of hADSCs, we found that the transcription factor FOXC1 might bind to the promoter of miR-204-5p and GDF7 (Fig. 5A and 5C). We next performed Dual-luciferase reporter assay and the results showed that overexpression of FOXC1 can decrease the luciferase activity of miR-204-5p, but increase the luciferase activity of GDF7 (Fig. 5B and 5D). Moreover, gel electrophoresis and ChIP assay indicted that FOXC1 binds to the promoter of GDF7 (Fig. 5E).

To further investigate the effect of FOXC1 on the transcriptional activity of miR-204-5p promoter, we carried out ChIP assay. Results suggested that compared with IgG control, the histone deacetylase HDAC2 and histone acetylase H3K9AC were all able to bind with the promoter of miR-204-5p. However, compared with empty vector control, overexpression of FOXC1 could promote the interaction between histone deacetylase HDAC2 and miR-204-5p, but inhibit the interaction between histone acetylase
H3K9AC and miR-204-5p (Fig. 5F and 5G). These results proved that FOXC1 epigenetically silenced miR-204-5p transcription through interaction with HDAC2 and H3K9AC.

**Discussion**

In the present study, we investigated the osteogenic differentiation of hADSCs, showing that miR-204-5p was down-regulated during the osteogenic differentiation of hADSCs. Moreover, miR-204-5p can suppress the expression of FOXC1 and GDF7, and FOXC1 could reduce the expression of miR-204-5p, thus promote the expression of GDF7, and activate the AKT and P38 signaling pathways.

Compared with other mesenchymal stem cells, hADSCs stand out because of the abundance in the body, less-invasive extraction procedures, lower incidence rate, and multi-directional differentiation potential. They have become a hotspot in the field of MSCs and one of the important sources of autologous stem cell therapy. In this study, we found a brand new chain which could accelerate the hADSCs osteogenic process.

In recent years, many studies have investigated the role and mechanism of microRNAs in stem cells adipogenesis or osteogenic differentiation. The previous study analyzed the expression profiles of microRNAs in MSCs during osteogenic differentiation, showed that miR-22 expression was up-regulated during osteogenesis, while reduced during MSCs adipogenic differentiation. Studies reported that miR-103-3p regulated osteogenic differentiation of bone marrow mesenchymal cells by inhibiting the target gene Satb2. Moreover, study has shown that miR-204-5p promoted the adipogenic differentiation of hADSCs by modulating DVL3 expression and suppressing Wnt/β-catenin signaling. In this study, we proved that overexpression of miR-204-5p could decrease RUNX2, ALP and OCN expression, and suppress the osteogenic differentiation of hADSCs, whereas inhibition of miR-204-5p could increase RUNX2, ALP, and OCN expression, and promote the osteogenic differentiation of hADSCs, suggesting that miR-204-5p might be an important target miRNA for hADSCs-based therapies related to bone regeneration.

FOXC1 is an important regulator of the initial stage of the osteogenesis process in the membrane and cartilage. Yoshiki et al proved Foxc1 could inhibit stem cells’ adipogenic processes, subsequent research disclosed that FOXC1 could promote RUNX2 express by binding its’ promoter. Moreover, FOXC1 is essential for the maintenance of the mesenchymal niches. This effect may mediated by BMP signaling, because FOXC1 could activate BMP pathway to govern hair follicle stem cells’ quiescence. Besides, inactivation of FOXC1 would result in a dramatic reduction in mice skull vault growth. However, there are some opinions against the positive effect of FOXC1 in osteogenesis. Jordan C. Caddy et al. described that FOXC1 would down-regulate suppresses BMP4-induced osteoblast differentiation. And it has been proved that FOXC1 expression is regulated by microRNA, such as miR-138-5p, miR-374c and miR-133. Our data indicated that FOXC1 is the target gene of miR-204-5p and miR-204-5p could suppress
the expression of FOXC1. Similar to mainstream views, our data also showed that FOXC1 promoted hADSCs osteogenic differentiation.

Moreover, we found that GDF7, also named BMP12, is the downstream key protein in FOXC1 mediated osteogenic differentiation of hADSCs. We found overexpression FOXC1 could promote GDF7 expression and induce osteogenic key protein overexpression such as RUNX2, ALP and OCN, and inactivated GDF7 would obtain opposite results. Although many studies have indicated that GDF7 stimulates proliferation of tendon fibroblasts and induces tendon and ligament formation\textsuperscript{23,37}, Shinji et al. found that GDF7 gene transfer into a rat femoral bone defect induced tendon/ligament-like tissue formation until 4 weeks after the operation and these tissues were eventually replaced with bone-like tissue by 8 weeks after the operation, suggesting that GDF7 initiated the replacement of tendon/ligament-like tissue with bony tissue\textsuperscript{38}. In our study, different from the main opinions, we found that GDF7 also plays an important role in regulating osteoblast differentiation and subsequent bone formation. The reason of diverse conclusion may due to the different species, and the lack of animal experiment might limit our insight.

Interestingly, we also found that GDF7 is also the target gene of miR-204-5p, and miR-204-5p could suppress the expression of GDF7. In other words, miR-204-5p could down-regulate both FOXC1 and GDF7 to inhibit osteogenesis of hADSCs. In order to verify the specific regulation mechanism among the miR-204-5p, FOXC1 and GDF7 in osteoblast differentiation of hADSCs, we performed Dual luciferase reporter assay and CHIP assay, and we found the transcription factor FOXC1 could bind to the promoter of miR-204-5p and GDF7 and enhance the translation expression of GDF7. To our surprise, our results suggested that overexpression of FOXC1 could reverse the inhibition of hADSCs osteogenic differentiation through down-regulating the miR-204-5p, while down-regulation of FOXC1 can weaken the inhibition of miR-204-5p, thus weakening the promotion of miR-204-5p inhibitor on osteogenic differentiation. To further investigate the regulation mechanism between FOXC1 and miR-204-5p, we explored the production of miR-204-5p and found that FOXC1 can deacetylate the promoter region of the miR-204-5p.

The transcription of miRNA is mainly done by RNA polymerase, and the structure of the primary transcript pri-miRNA contains the same 7-methylguanosine cap and poly(A) tail as the primary transcript of the gene which encoding the protein. Besides, miRNAs are also regulated by epigenetic modifications such as DNA methylation and histone acetylation. Research suggested that approximately 5%-10% of mammalian miRNAs are regulated by epigenetics\textsuperscript{39}. For example, the correct expression of miR-148a, miR-34b/c, miR-9 and let-7a-3 depends on the methylation status and DNMT1, DNMT3b or other DNA methylase activities\textsuperscript{39}. Saito et al. found that when DNA demethylation reagents and histone deacetylation inhibitors were used to intervene in human T24 bladder cancer cells, about 5% of miRNAs would be up-regulated by more than 3 times, especially miR-127 in CpG island\textsuperscript{40}. This further illustrated that the regulation of miRNA genes by epigenetic modification is in its promoter region and can be activated by inhibiting DNA methylation and histone acetylation. However, low histone acetylation in special promoter region often leads to the silencing of gene\textsuperscript{41}. In our study, we investigate the effect of FOXC1 on the transcriptional activity of miR-204-5p promoter by ChIP assay. The results showed that
FOXC1 could weaken the H3K9AC acetylation and promote the HDAC2 deacetylation of the miR-204-5p promotor and reduce the transcriptional activity of miR-204-5p during osteogenic differentiation.

To date, studies have confirmed that the activation of p38 MAPK and AKT signaling pathways are the crucial trigger factor of osteogenic differentiation of hADSCs\textsuperscript{42-44}. Our data confirmed that GDF7 overexpression can promote the phosphorylation of P38 and AKT, thus promoting the P38 and AKT signaling pathways. At the same time, this study showed that overexpression GDF7 also reversed the inhibition of osteogenic differentiation of hADSCs by miR-204-5p mimics. Also, FOXC1 could promote the transcription activity of GDF7 through weakening the H3K9AC acetylation and promoting the HDAC2 deacetylation of the miR-204-5p promotor during osteogenic differentiation.

We summarized the mechanism of miR-204 /FOXC1/GDF7 regulatory axis during osteogenic differentiation as shown in Fig. 6. MiR-204-5p is down-regulated during the osteogenic differentiation of hADSCs. Moreover, miR-204-5p can bind to the 3'UTR region of FOXC1 and GDF7, and suppress the expression of FOXC1 and GDF7. FOXC1 could bind to the promoter of miR-204-5p and GDF7, and promote the deacetylation of miR-204-5p and reduced the expression of miR-204-5p, thus promoting the expression of GDF7, which induced hADSCs osteogenesis by activating the AKT and P38 signaling pathways. Finally, FOXC1/GDF7/ miR-204-5p axis formed cyclic regulation, which plays an important role in the balance of osteogenic differentiation. The specific mechanisms of downstream signaling pathways of GDF7 and whether FOXC1 can affect the methylation of the miR-204-5p promoter are our research focuses in the future.

**Conclusion**

Our results demonstrated that miR-204-5p/FOXC1/GDF7 axis regulates osteogenic differentiation of hADSCs via the AKT and p38 signaling pathways. This study further understood the regulatory mechanism of hADSCs differentiation balance from the perspective of miRNAs regulation.

**Abbreviations**

hADSCs: human adipose-derived stem cells; FOXC1: Forkhead box C1; GDF7: growth differentiation factor 7; BMP12: bone morphogenetic protein 12; CDMP3: cartilage-derived morphogenetic protein 3; miRNA: microRNA; MSCs: mesenchymal stem cells; TGF-β: transforming growth factor beta; PBS: phosphate-buffered saline; DME F12: Dulbecco’s Modified Eagle’s Medium/Nutrient F-12; FBS: fetal bovine serum; PM: proliferation medium; OM: osteogenic differentiation medium; ALP: Alkaline phosphatase; ARS: Alizarin red staining; DAPI: 4,6-diamidino-2-phenylindole; WT: wild-type; MUT: mutant-type; ChIP: Chromatin Immunoprecipitation; IgG: immunoglobulin G; 3'-UTR: 3' untranslated region; CXCL: CXC chemokine ligand; CAR cells: CXCL 12-abundant reticular cells

**Declarations**
Authors’ contributions

You Zhou and Shu Guo contributed to research conception and design. You Zhou, Siyu Liu and Wei Wang performed experiments, analyzed and interpreted data. Qiang Sun, Mengzhu Lv obtained the adipose tissue and contributed to cell culture. Shude Yang, Shuang Tong interpreted data and contributed to statistic analysis. You Zhou drafted the manuscript. Shu Guo revised the manuscript critically. All authors approved the final manuscript.

Conflict of Interest

The authors confirm that there are no conflicts of interest.

Ethics approval and consent to participate

The above experimental samples were collected with the informed consent of the clinical patients and approved by the Ethics Committee of the first hospital of China Medical University [AF-SOP-07-1.1-01].

Availability of data and materials

Please contact author for data requests.

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Consent for Publication

Not applicable.

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and Adipocyte Differentiation. Biological pharmaceutical bulletin. 2019;42(6):968–76.

Figures

Figure 1

miR-204-5p is down-regulated during the osteogenic differentiation of hADSCs. (A) Flow cytometry was used to identify hADSCs cells by detecting specific marker proteins CD44, CD45, CD90, CD105, and HLA-DR. (B) Relative expression of miR-204-5p at each time points during the osteogenic differentiation of hADSCs detected by qRT-PCR. U6 was used for reference gene. Relative mRNA levels of the osteogenic markers RUNX2, ALP, and OCN at each time points during the osteogenic differentiation of hADSCs detected by qRT-PCR. GAPDH was used for reference gene. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.01 (n = 3 independent experiments).
Figure 2

miR-204-5p overexpression inhibits the osteogenic differentiation of hADSCs. HADSCs were transfected with miR-204-5p mimics, miR-204-5p inhibitors or NC, and cultured in proliferation medium (PM) or osteogenic medium (OM). (A) Relative mRNA levels of miR-204-5p, RUNX2, ALP and OCN measured by qRT-PCR on day 14 of osteogenic induction. U6 and GAPDH was used for reference genes respectively. (B) Western blot of RUNX2, ALP and OCN proteins level on day 14 after osteogenic induction. GAPDH was used as the internal control. (C) Western blot of FOXC1 and GDF7 proteins level on day 14 after osteogenic induction. GAPDH was used as the internal control. (D) Confocal microscopy of OCN with DAPI counterstaining on day 14 after osteogenic induction. Scale bars, 20 µm. (E-F) Alkaline phosphatase (ALP) staining (E) and alizarin red staining (ARS) (F) on day 14 after osteogenic induction. Data are presented as mean ± SD. * indicated hADSCs transfected with miR-204-5p mimics or inhibitors vs NC in PM; # indicated hADSCs transfected with miR-204-5p mimics or inhibitors vs NC in OM. * or # p < 0.05, ** or ## p < 0.01, *** or ### p < 0.001 (n = 3 independent experiments).
Figure 3

FOXC1 reduces the suppression effect of miR-204-5p on osteogenic differentiation of hADSCs. HADSCs were co-transfected with miR-204-5p mimics and FOXC1 overexpressed vector, or co-transfected with miR-204-5p inhibitors and si-FOXC1, and cultured in osteogenic medium (OM). (A) Relative expression of miR-204-5p, FOXC1, GDF7, RUNX2, ALP and OCN measured by qRT-PCR on day 14 of osteogenic induction. U6 and GAPDH was used for reference genes respectively. (B) Western blot of FOXC1, GDF7, RUNX2, ALP and OCN.
RUNX2, ALP and OCN proteins level on day 14 after osteogenic induction. GAPDH was used as the internal control. (C-D) ALP staining (C) and ARS (D) on day 14 after osteogenic induction. (E) Predicted binding site of miR-204-5p in the 3′-UTR of FOXC1 mRNA. (F) Relative luciferase activity of cells in different groups. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, *** p < 0.001 (n = 3 independent experiments).

**Figure 4**

GDF7 impairs the suppression effect of miR-204-5p on osteogenic differentiation of hADSCs. HADSCs were co-transfected with miR-204-5p mimics and GDF overexpressed vector, or co-transfected with miR-204-5p inhibitors and si-GDF, and cultured in osteogenic medium (OM). (A) Relative expression of miR-204-5p, GDF7, RUNX2, ALP and OCN measured by qRT-PCR on day 14 of osteogenic induction. U6 and GAPDH was used for reference genes respectively. (B) Western blot of GDF7, RUNX2, ALP and OCN proteins level on day 14 after osteogenic induction. (C) Western blot of p-AKT, AKT, p-P38, and P38 proteins level on day 14 after osteogenic induction. GAPDH was used as the internal control. (D-E) ALP staining (D) and ARS staining (E) on day 14 after osteogenic induction. (F) Left panel: Predicted binding site of miR-204-5p in the 3′-UTR of GDF7 mRNA. Right panel: Relative luciferase activity of cells in different groups. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 (n = 3 independent experiments).
Figure 5

FOXC1 as a transcription factor could bind to the promoter of miR-204-5p and GDF7. (A) Predicted binding site between FOXC1 and the promoter of miR-204-5p. (B) Relative luciferase activity of cells transfected with FOXC1 and miR-204-5p-WT, or FOXC1 and miR-204-5p-MUT. Data are presented as mean ± SD. *p < 0.05 (n = 3 independent experiments). (C) Predicted binding site between FOXC1 and the promoter of GDF7. (D) Relative luciferase activity of cells transfected with FOXC1 and GDF7-WT, or
FOXC1 and GDF7-MUT. Data are presented as mean ± SD. ***p < 0.001 (n = 3 independent experiments). (D) Gel electrophoresis and ChIP assay indicated that FOXC1 binds to the promoter of GDF7. (F) ChIP assay proved that FOXC1 promote the interaction between histone deacetylase HDAC2 and miR-204-5p. (G) ChIP assay proved that FOXC1 inhibit the interaction between histone acetylase H3K9AC and miR-204-5p. Data are presented as mean ± SD. *** indicated Anti-HDAC2 or Anti-H3K9AC vs IgG, ### indicated overexpression of FOXC1 vs Vectors in HDAC2 or H3K9AC samples. ***p < 0.001, ### p < 0.001 (n = 3 independent experiments).

Figure 6

The mechanism of miR-204-5p/FOXC1/GDF7 regulatory axis in the osteogenic differentiation of hADSCs.