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Original Paper

Obesity-Associated MiR-342-3p Promotes Adipogenesis of Mesenchymal Stem Cells by Suppressing CtBP2 and Releasing C/EBPα from CtBP2 Binding

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Key Words
Mesenchymal stem cells • miR-342-3p • Obesity • Adipogenic differentiation • CtBP2

Abstract
Background/Aims: The elucidation of the molecular mechanism of adipocyte differentiation in mesenchymal stem cells is of essential importance for the development of treatments for metabolic diseases, such as obesity and diabetes. ***Methods:*** The expression levels of miR-342-3p and carboxy-terminal binding protein 2 (CtBP2) were regulated by oligonucleotide transfection. Adipogenic differentiation was induced by adipogenic medium containing indomethacin, dexamethasone and 3-isobutyl-1-methylxanthine on day 12, as determined by Oil Red O staining and triglyceride concentration assay to assess intracellular lipid accumulation. The induction of adipocyte-specific transcription factors and markers was detected by qRT-PCR and western blot. The regulation of CtBP2 expression by miR-342-3p was determined by western blot, qRT-PCR, luciferase reporter assay, ChIP assay and functional experiments. ***Results:*** We revealed that miR-342-3p was enriched in the adipose tissue of obese mice, and its expression was significantly elevated during adipogenic differentiation in both human mesenchymal stem cells (hMSCs) and 3T3L1 cells. Using gain- and loss-of-function assays, we demonstrated that the overexpression of miR-342-3p markedly promoted the differentiation of hMSCs into an adipogenic lineage. Adipogenesis was significantly blocked by miR-342-3p downregulation. We identified and validated that CtBP2 was a direct target of miR-342-3p in this process. The effects of the inhibition of CtBP2 were similar to those of miR-342-5p overexpression on adipogenic differentiation, promoting the release of C/EBPα from CtBP2 binding. ***Conclusion:*** miR-342-3p is a powerful enhancer of the adipogenesis of human adipose-derived MSCs that acts by inhibiting CtBP2 and releasing the key adipogenic regulator C/EBPα from CtBP2 binding, subsequently activating the expression of adipogenic transcription factors and markers.

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Introduction

Obesity is a major health concern worldwide and is associated with increased risks of chronic diseases, such as metabolic syndrome (hyperlipidemia, insulin resistance, type 2 diabetes, etc.), atherosclerosis, chronic inflammation, cardiovascular disease and cancer [1-3]. Adipocytes are derived from mesenchymal stem cells or progenitor cells, which undergo a lineage-specific differentiation process termed adipogenesis or adipogenic differentiation that occurs in two phases, namely commitment to the preadipocyte lineage and terminal differentiation; ultimately, these cells undergo differentiation into mature adipocytes [4]. The stromal cell population derived from adipose tissue has been termed adipose-derived mesenchymal stromal cells or adipose tissue-derived mesenchymal stem cells (AMSCs). These cells have extensive proliferative potentials and are able to differentiate into adipogenic, osteogenic, chondrogenic and myogenic lineages [5-8]. The elucidation of the molecular mechanisms involved in the adipocyte differentiation of AMSCs is of essential importance for the development of therapeutics for metabolic diseases, such as obesity and diabetes.

MicroRNAs (miRNAs) are ~22 nucleotide-long, small, non-coding, single-stranded RNAs that mediate gene suppression by binding to the 3' untranslated regions (3'UTRs) of target mRNAs and either promoting degradation or inhibiting translation [9]. They have been shown to play regulatory roles in several biological processes, including cell proliferation, differentiation, apoptosis and tumor oncogenesis [10-13]. Recently, increasing evidence has demonstrated that miRNAs play critical roles in osteoblast differentiation. For example, miR-196a, miR-335-5p, miR-322, miR-26a, miR-26b, miR-29b, miR-1192, miR-218, miR-378, miR-548d-5p and miR-302a accelerate osteogenic differentiation [14-22], while miR-26a, miR-133, miR-135b, miR-138, the miR-30 family, miR-155, miR-100, miR-433, miR-145, miR-143, miR-542-3p, miR-338-3p, miR-140-5p and miR-146a impair osteoblast differentiation [23-35]. Many miRNAs have been reported to be involved in the regulation of adipogenic differentiation, such as miR-143, miR-17-92, miR-130, miR-138, miR-17-5p, miR-106a, miR-210, miR-709, miR-137, and miR-302 [36-44]. However, few miRNAs have been reported in association with obesity and the positive regulation of adipogenesis [45]. Therefore, future studies are warranted to understand the exact role of obesity-associated miRNAs in the adipogenic differentiation of AMSCs.

In this study, we characterized miR-342-3p, which is upregulated during the development of obesity [46], and investigated its effects on the adipogenic differentiation of human adipose-derived MSCs (hAMSCs). Our results indicate that miR-342-3p is an important positive regulator of adipogenesis. Using target prediction and luciferase reporter assays, we demonstrated that carboxy-terminal binding protein 2 (CtBP2), a corepressor of C/EBPα [47], is a direct target of miR-342-3p. Our findings provide further insights into the mechanisms of the regulation of adipocyte differentiation by miRNAs.

Materials and Methods

Cell culture

This study was approved by the Ethical Committee of the Shengli Oilfield Central Hospital. hAMSCs were isolated from five donors who provided informed consent. These cells were harvested and cultured according to previously described methods [8, 48] and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 mg/ml penicillin in a 5% CO_2 environment at 37°C according to standard protocols. All hAMSC experiments were performed at passage 3 to 4.

Induction and identification of adipocyte *differentiation*

Adipocyte differentiation was induced by culturing hAMSCs for 12 days in an adipocyte induction medium (10% FBS, 200 μM indomethacin, 1 μM dexamethasone, and 0.5 M 3-isobutyl-1-methylxanthine in

α-MEM). The extent of differentiation was assayed by measuring adipogenesis-related genes and was also evaluated by Oil Red O staining and measuring the triglyceride concentration as indicators of intracellular lipid accumulation.

Oil Red O staining

Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature. They were then washed twice with deionized water, stained with saturated Oil Red O solution (stock solution: 3 mg/ml in isopropanol; working solution: 60% Oil Red O stock solution and 40% distilled water) for 30 min at room temperature. After staining, the cells were fully washed with distilled water to remove unbound dye, visualized by light microscopy, and photographed. To obtain quantitative data, intracellular absorbed Oil Red O was extracted into a 96-well plate with 100% isopropanol, and absorbance was measured at 510 nm.

Triglyceride concentration measurement

Cells were washed with PBS after the culture medium was removed. The triglyceride concentration in the washed cells was then measured using a glycerol assay kit (Applygen, Beijing, China).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Beijing, China) for mRNA and a miRVana miRNA Isolation Kit (Life Technologies, Beijing, China) for miRNA, respectively, according to the manufacturer's instructions. For mRNA amplification, reverse transcription was performed using 1 μg of total RNA with M-mlv reverse transcriptase (TaKaRa).

For miRNA amplification, reverse transcription was performed using 1 μg total RNA with a Ncode miRNA First-strand cDNA Synthesis Kit (Invitrogen, USA), according to the manufacturer's instructions. Beta-actin was used as a normalizing control for the mRNA, and U6 was used as a normalizing control for the miRNA. PCR was performed using Power SYBR Green PCR Master Mix with an ABI 7500 Instrument (Applied Biosystems, Foster City, CA). To perform qRT-PCR, cDNA prepared from mRNA was mixed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and miRNA-specific cDNA was mixed with TaqMan Universal Master Mix (Life Technologies, USA). All samples were analyzed with an Applied Biosystems 7500 Fast Real-Time PCR system. Each reaction was performed at least in triplicate and repeated with at least three different samples. Quantifications of the fold changes in gene and miRNA expression were performed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Samples were harvested and lysed in ice-cold radio immunoprecipitation assay (RIPA) lysis buffer plus protease inhibitors (Beyotime, Nanjing, China). Equal protein amounts were loaded and separated by 10% SDS–PAGE. They were then transferred to a PVDF membrane (Millipore, USA), blocked by incubation with 5% fat-free milk, and probed with the primary antibodies anti-CtBP2, anti-PPARγ, anti-C/EBPα, anti-FABP4 and anti-LPL at 4°C overnight. After washing, the blots were incubated with horseradish peroxide-conjugated secondary antibodies at room temperature for 1 h. The blots were developed with chemiluminescent ECL reagent (Millipore, USA). GAPDH was used as a loading control.

Cell transfection assay

Transfections were performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells were seeded with complete medium without antibiotics. On the following day, 100 nM of miRNAs (miR-342-3p mimics or inhibitors, GenePharma, Shanghai, China) and 100 nM of siRNAs (GenePharma, Shanghai, China) for CtBP2 were transfected into cells with a miRNA/inhibitor negative control (NC/NCI) or non-targeting siRNA as a negative control (siNC). Six hours after transfection, the medium was replaced with a maintenance medium or induction medium, according to the requirements of further experiments.

Vectors, DNA constructs and dual luciferase reporter assay

A putative miR-342-3p-recognition element in CtBP2 was cloned into the 3' untranslated region (UTR) of a firefly luciferase reporter vector according to the manufacturer's instructions. Oligonucleotide

sequences were designed and ligated into a luciferase reporter vector (p-MIR-Report; Ambion). The following oligonucleotides were used in these experiments: pMIR-CtBP2 (pMIR-CtBP2-wt) FW, 5'-GAGCT CAGAAGTATGTTTTGTTTTA **GTGTGAG** TTACCGTTACTGTATTTGTT A-3' and RV, 5'- CGCGT AACAAATACAGTAACGGTAA **CTCACAC** TAAAACAAAACATACTTCTG C-3'; and pMIR-CtBP2-WT-mut (pMIR-CtBP2-M) FW, 5'- GAGCT CAGAAGTATGTTTTGTTTTA **GAATCCG** TTACCGTTACTGTATTTGTT A-3' and RV, 5'-CGCGT AACAAATACAGTAACGGTAA **CTTAGGC** TAAAATAAAACATACTTCTG C-3'. The sequences of the miR-342-3p binding site and mutant site are shown in bold. Each vector, along with 100 ng pMIR-β-gal plasmids and 100 nM miR-342-5p mimics or NC, was cotransfected into 293T cells using Lipofectamine 2000 reagent (Invitrogen, USA). At 24 h after transfection, cells were harvested. Renilla and firefly luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Luciferase Assay System, Promega, USA). Transfections were performed in triplicate, and all experiments were repeated three times. Luciferase activity was normalized to β-galactosidase activity.

ChIP assay

A total of 2×10^6 cells were fixed with 1% formaldehyde at room temperature for 10 min. Cross-linking was stopped with 0.125 M glycine, and the cultures were incubated for 2 min. The cells were then scraped, and nuclear extracts were prepared and sonicated with an EZ-Magna ChIP G Chromatin Immunoprecipitation (ChIP) Kit (Millipore, USA) according to the manufacturer's instructions. The lysates were pelleted and precleared, and each lysate was incubated with 1 mg of one of the following antibodies and rotated at 4°C overnight with magnetic protein G beads (Roche, Switzerland): anti-CtBP2 (Santa Cruz, China) or immunoglobulin G (IgG). The complexes were eluted with buffer containing 1% SDS and 0.1 M NaHCO3, and crosslinks were reversed at 65°C. DNA was recovered by phenol–chloroform extraction and ethanol precipitation and then subjected to PCR analysis.

Statistical analysis

All results are presented as the mean ± SEM. Comparisons between groups were analyzed using student *t*-tests (two-sided) with SPSS 17.0. Statistical significance was defined as a P value <0.05.

Results

Obesity-associated miR-342-3p is upregulated during adipogenesis

MiR-342-3p had been demonstrated to be upregulated during the development of obesity in C57BLJ6 mice fed a high-fat diet [46]. We found that the expression of miR-342-3p was higher in obese C57BLJ6 mice compared with normal mice (Fig. 1A). TargetScan confirmed that this miR is conserved between humans and mice (Fig. 1B). To investigate the role of miR-342-3p in the adipogenesis of hAMSCs, its expression pattern was analyzed in these cells in response to adipogenic induction medium by qRT-PCR. The results showed that adipogenic induction led to an 8.78-fold upregulation in miR-342-3p expression in the hAMSCs (Fig. 1C). To further verify that miR-342-3p is induced during adipocyte differentiation, we detected its expression in 3T3L1 cells cultured in adipogenic induction medium and found that it was also significantly increased in these cells (Fig. 1D). Therefore, we postulated that miR-342-3p may play an important role in adipocyte differentiation.

MiR-342-3p overexpression promotes adipogenesis of hAMSCs

To study the effects of miR-342-3p on the adipogenic differentiation of hAMSCs, synthetic miR-342-3p mimics (342-3p) along with their respective negative control (NC) were transiently transfected into hAMSCs. qRT-PCR analysis confirmed that the expression of miR-342-3p was increased by 17.58-fold in the hAMSCs transfected with miR-342-3p mimics (Fig. 2A). Next, hAMSCs were transfected with 100 nM of miR-342-3p mimics or 100 nM of NC and induced with adipogenic induction medium containing 10% FBS, 200 μM indomethacin, 1 μM dexamethasone and 0.5 mM/ml 3-isobutyl-1-methylxanthine in α-MEM. We found that the amount of lipid droplets was significantly increased in the miR-342-3p mimic-transfected cells compared with the control-transfected cells on day 12 after

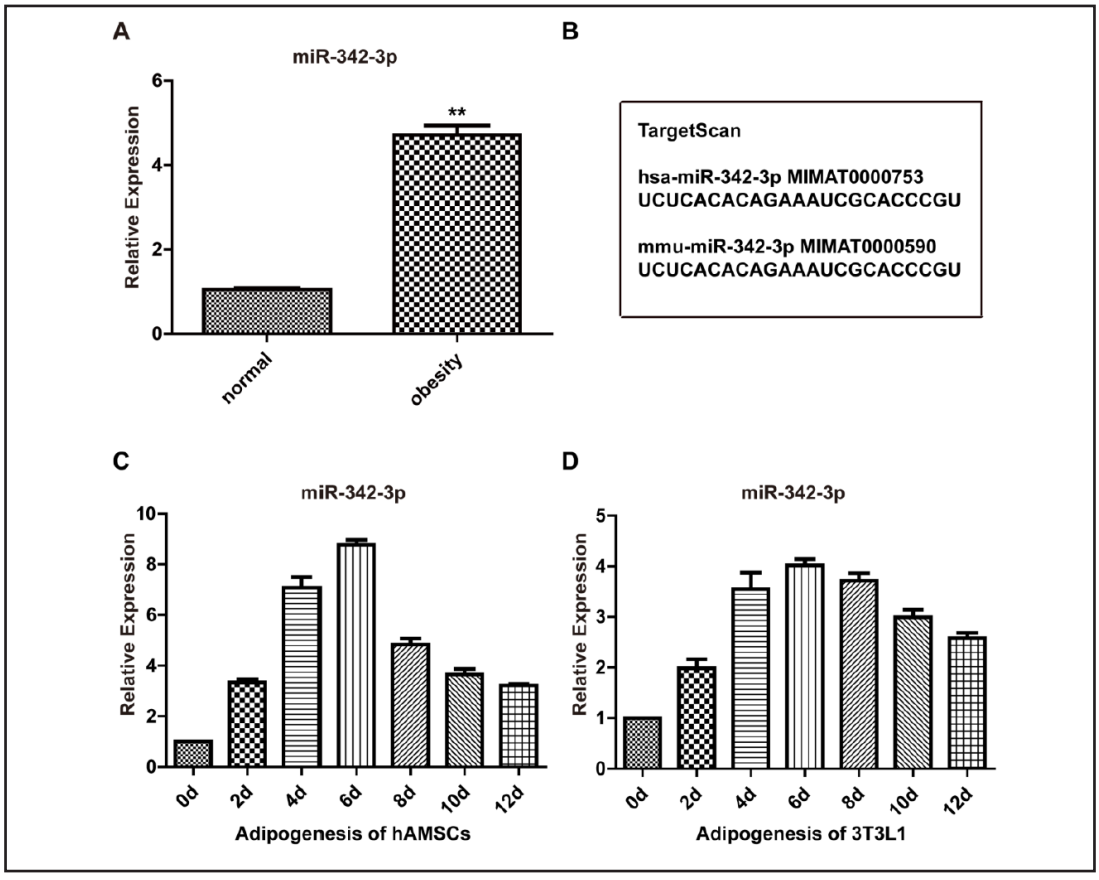

Fig. 1. MiR-342-3p expression profile. qRT-PCRwas performed to determinethe expression of miR-342-3p in obesemice compared with normal mice (n=5). (B) The conservation of miR-342-3p between human-sand mice. (C and D)Thedynamic expression pattern of miR-342-3p during the adipogenic differentiation of hAMSCs and 3T3L1, respectively, as detected by qRT-PCR.

induction, as determined by Oil Red O staining (Fig. 2B). The overexpression of miR-342-3p enhanced absorbance by approximately 1.88-fold, as determined by measuring the OD of Oil Red O extractions at 510 nm (Fig. 2C), and resulted in a significant increase in the triglyceride concentration compared with the negative control-transfected cells, as determined by triglyceride concentration assays (Fig. 2D).

The induction of adipocyte-specific transcription factors and markers was also detected by qRT-PCR. The mRNA levels of PPARγ, C/EBPα, FABP4 and LPL were all significantly increased following the transfection of miR-342-3p mimics into hAMSCs during adipogenic differentiation (Fig. 2E). Consistent with these results, western blot analysis showed that the protein levels of PPARγ, C/EBPα, FABP4 and LPL were elevated following adipogenic treatment in the cells transfected with miR-342-3p mimics (Fig. 2F).

Inhibition of miR-342-3p blocks adipogenic differentiation of hAMSCs

To further investigate the effects of miR-342-3p on hAMSC differentiation, we transfected hAMSCs with a specific miRNA inhibitor (miR-342-3p inhibitor, 342-3pI). qRT-PCR analysis showed that the transfection of miR-342-3p inhibitor effectively downregulated miR-342-3p expression in the hAMSCs (Fig. 3A). To clarify the effect of miR-342-3p inhibition on adipogenic differentiation, miR-342-3pI-transfected AMSCs were induced to differentiate into adipogenic lineages. Oil Red O staining revealed that the inhibition of miR-342-3p suppressed lipid droplet accumulation in the process of adipogenic differentiation of the hAMSCs (Fig. 3B), which was quantified by measuring the OD of Oil Red O extractions at

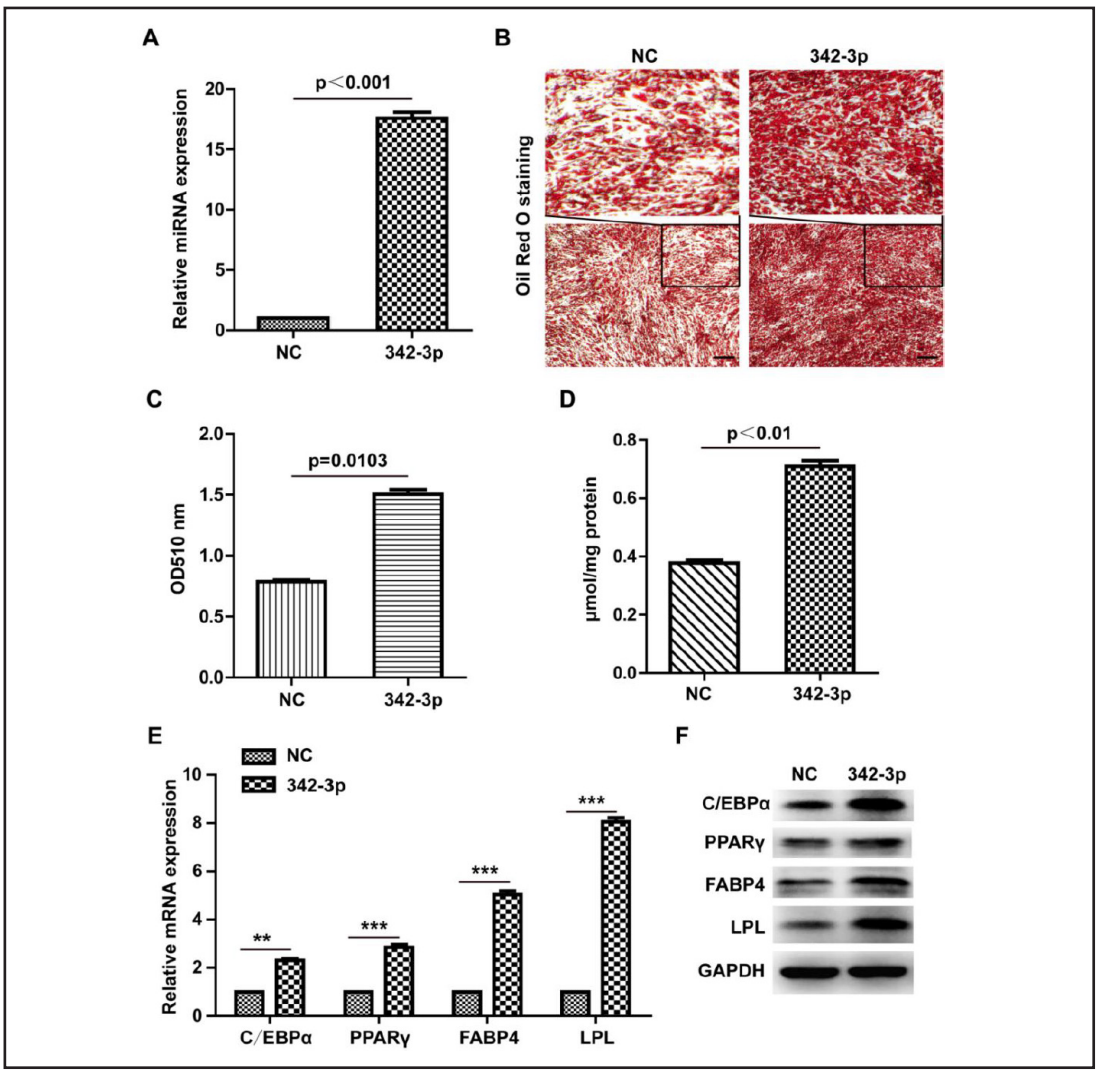

Fig. 2. MiR-342-3p overexpression promotes adipogenic differentiation. miR-342-3p levels were determined in negative control (NC)- or miR-342-3p (342-3p)-transfected hAMSCs using qRT-PCR. The data are presented as the relative ratio of the level of miR-342-3p to that of U6 for each sample. (B and C) Lipid accumulation was determined by Oil Red O staining and quantified by measuring absorbance at 510 nm. (D) The triglyceride concentrations in induced cells on day 12 of differentiation. (E and F) The mRNA and protein levels of adipogenic regulators and marker genes in induced cells were analyzed on day 9 of differentiation by qRT-PCR and western blot, respectively. The qPCR data are presentedas the mean± SEM (n=3). **P<0.01, and *** P<0.001.

510 nm (Fig. 3C). Consistent with these results, triglyceride concentration assays indicated a decrease in the triglyceride concentration compared with the inhibitor negative control (NCI)-transfected cells (Fig. 3D). To further confirm this finding, we analyzed the expression of adipocyte-specific transcription factors and marker genes by qRT-PCR and western blot. The results showed that the inhibition of miR-342-3p decreased the expression of PPARγ, C/EBPα, FABP4 and LPL during the adipogenic differentiation of the hAMSCs (Fig. 3E and F). These results demonstrated that adipocyte formation was significantly blocked by miR-342-3p downregulation.

MiR-342-3p targets the 3'UTR of CtBP2 mRNA

To investigate the molecular mechanism underlying the regulation of the adipogenic differentiation of hAMSCs by miR-342-3p, several miRNA target prediction tools were used

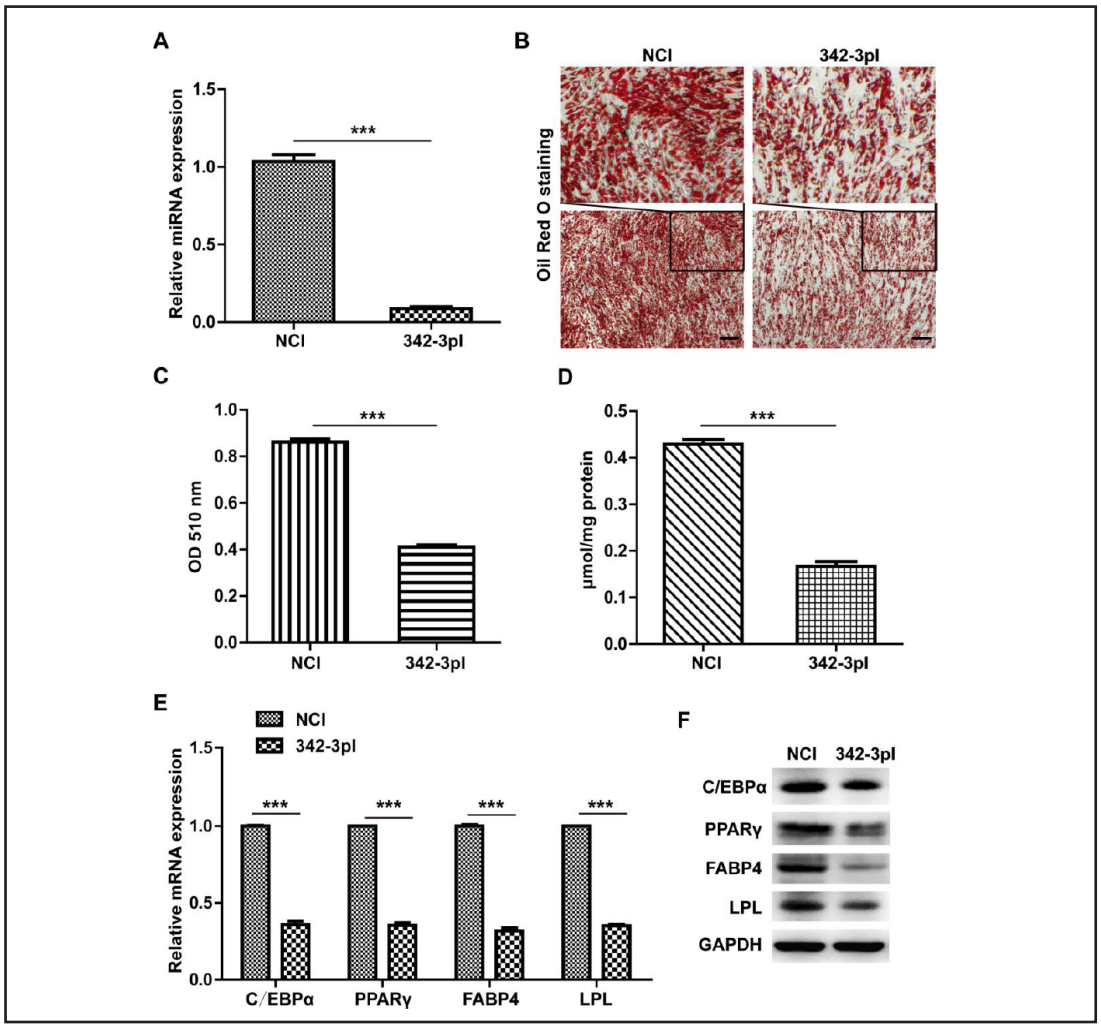

Fig. 3. Inhibition of miR-342-3p blocks adipogenic differentiation. miR-342-3p levels were determined in miR-342-3p inhibitor (342-3pI)- or inhibitor negative control (NCI)-transfected hAMSCs using qRT-PCR. The data are presented as the relative ratio of the level of miR-342-3p to that of U6 for each sample. (B and C) Lipid accumulation was determined by Oil Red O staining and quantified by measuring absorbance at 510 nm in the miR-342-3pI- and miR-NCI–transfected hAMSCs during adipogenic differentiation on day 12. (D) The triglyceride concentrations in induced hAMSCs transfected with miR-342-3pI or miR-NCI on day 12 of differentiation. (E and F) The mRNA and protein levels of adipogenic regulators and markers in induced cells were analyzed on day 9 of adipogenic differentiation by qRT-PCR and western blot, respectively. ***P<0.001.

to predict potential targets of this miR. TargetScan suggested a very high likelihood of the targeting of CtBP2 mRNA 3'UTR sequences by mature miR-342-3p (Fig. 4A). CtBP2 mRNA sequences were found to be highly conserved among mammals (Fig. 4B).

To clarify whether miR-342-3p directly targets CtBP2, we constructed luciferase reporter genes with the CtBP2 3'UTR with or without a mutation in the miR-342-3p binding region. The 3'UTR constructs were then cotransfected into 293T cells along with miR-342-3p mimics or NC. Luciferase assay revealed a decrease in relative luciferase activity when the CtBP2 3'UTR (pMIR-CtBP2-W) was transfected into miR-342-3p-transfected 293T cells, while no decrease in activity was observed in 293T cells transfected with the mutant CtBP2 3'UTR (pMIR-CtBP2-M) (Fig. 4C). In contrast, the miR-342-3p mimics significantly decreased the luciferase activity of the CtBP2 3'UTR construct by 54.9%, whereas transfection with NC had no impact on the mutant 3'UTR construct of CtBP2 (Fig. 4C). To further determine the relationship between miR-342-3p and CtBP2, we detected CtBP2 expression by qRT-PCR and

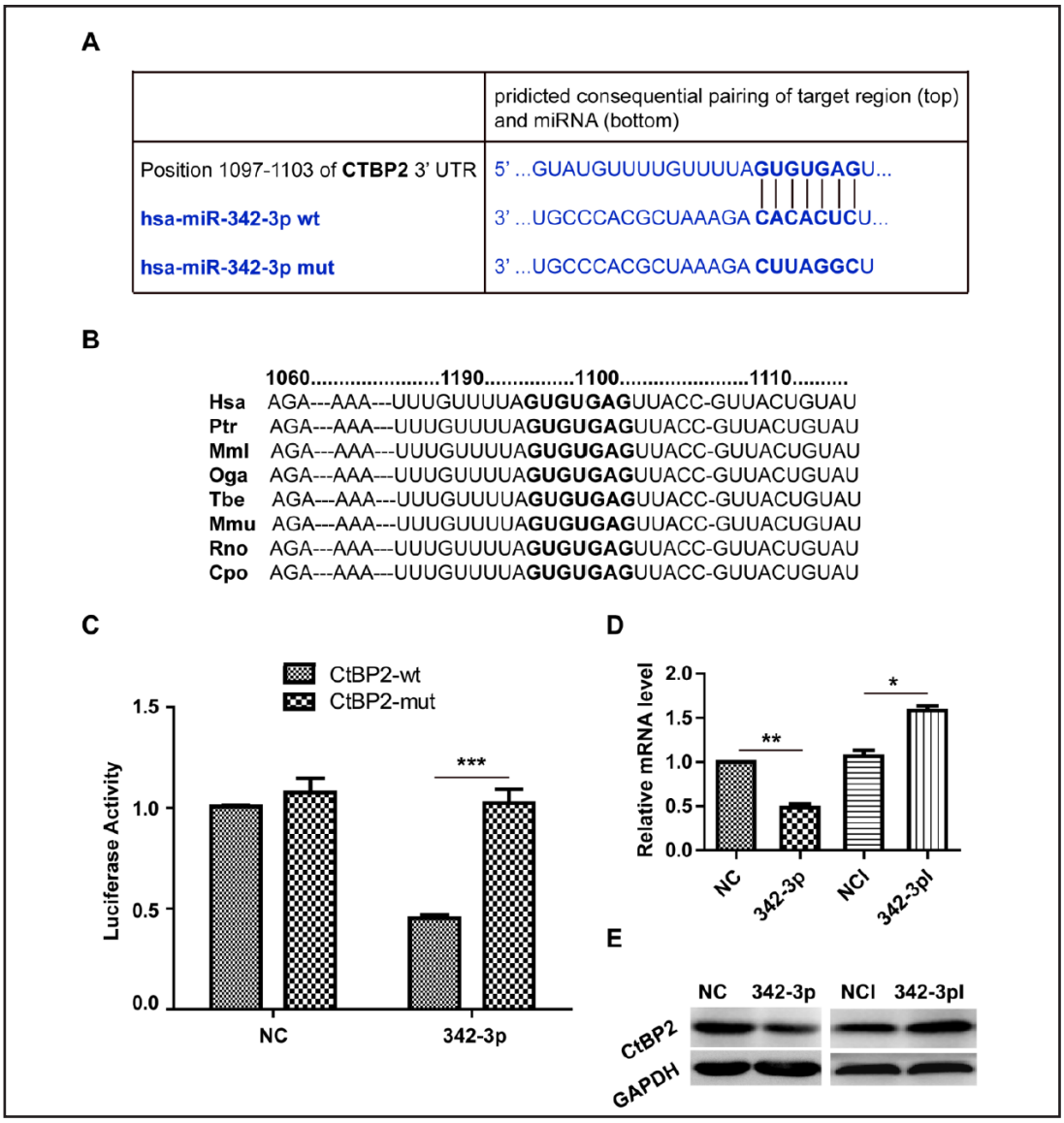

Fig. 4. MiR-342-3p directly targets the 3'UTR of CtBP2 mRNA. (A and B) One potential binding site for miR-342-3p in human CtBP2 3'UTR was predicted by TargetScan, and this binding site of miR-342-3p (in bold characters) is evolutionally conserved. Four nucleotides in the seed region were mutated to abolish the interaction between miR-342-3p and mRNA 3'UTR of CtBP2. (C) The relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System. The transfections were performed in triplicate, and experiments were repeated three times. The luciferase activity was normalized to its β-galactosidase activity. (D and E) The mRNA and protein levels of CtBP2 were analyzed by qRT-PCR and western blot respectively. GAPDH was used as a loading control. The qRT-PCR data are presented as mean ± SEM (n=3). *P<0.05. **P<0.01. *** P<0.001.

western blotting during adipocyte differentiation and found that miR-342-3p overexpression led to a marked decrease in CtBP2 expression at both the mRNA and protein levels (Fig. 4D and E).

Inhibition of CtBP2 mimics effects of miR-342-5p on adipogenesis by releasing C/EBPα from CtBP2 binding

To investigate the influence of CtBP2 on the adipogenic differentiation and proliferation of hAMSCs, we suppressed CtBP2 expression in hAMSCs with two siRNAs against CtBP2

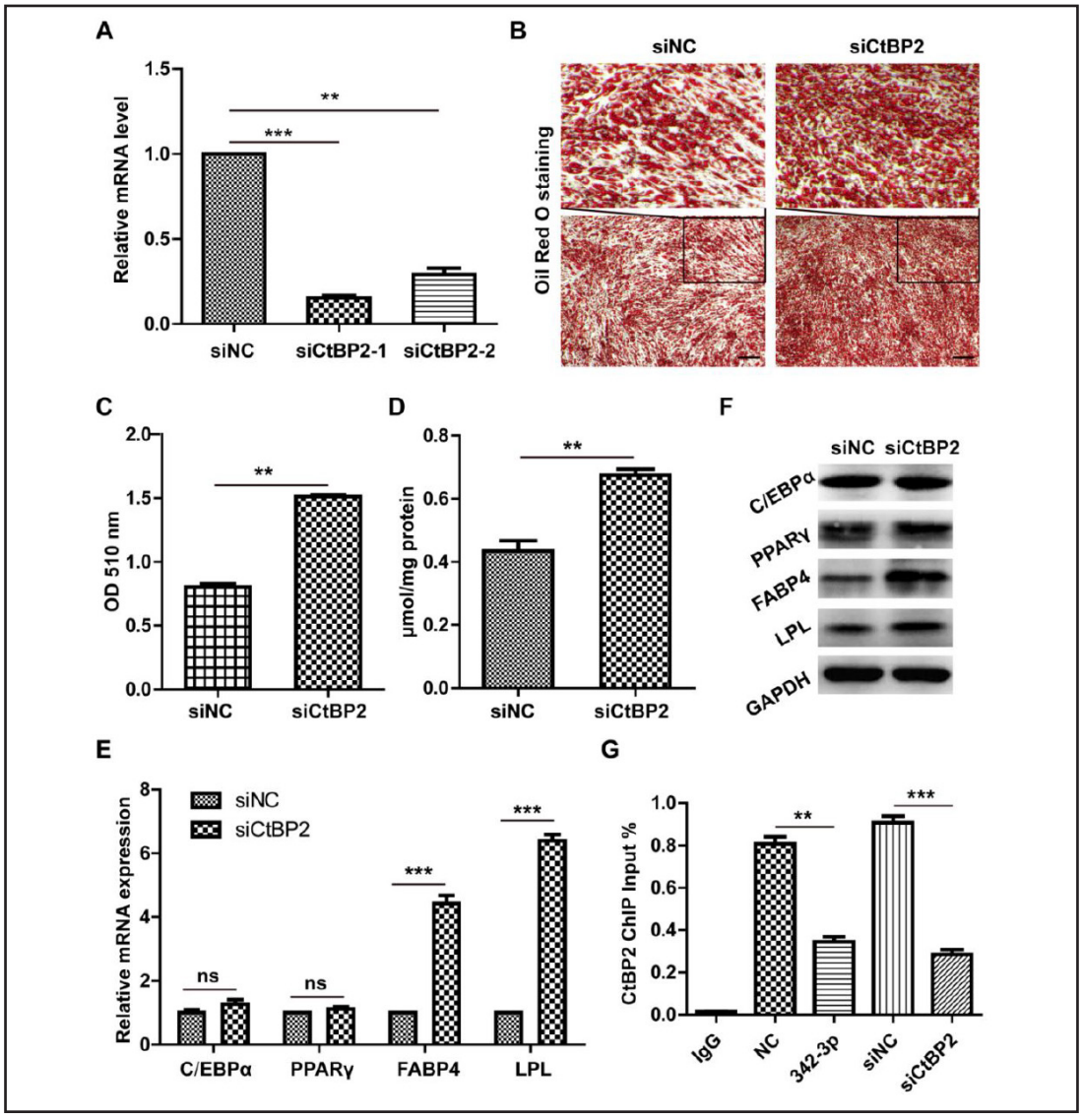

Fig. 5. Inhibition of CtBP2 promotes adipogenic differentiation by releasing C/EBPα and activating adipogenic genes. (A) CtBP2 levels were determined in siCtBP2-1- and siCtBP2-2-transfected or negative control (siNC)-transfected hAMSCs using qRT-PCR. The data are presented as the relative ratio of the level of CtBP2 to that of GAPDH for each sample. (B and C) Lipid accumulation was determined by Oil Red O staining and quantified by measuring the absorbance at 510 nm for the siCtBP2-1- and siNC-transfected hAMSCs during adipogenic differentiation on day 12. (D) The triglyceride concentrations in induced hAMSCs transfected with siCtBP2-1 or siNC on day 12 of adipogenesis. (E and F) The mRNA and protein levels of adipogenic regulators and marker genes in induced cells were analyzed on day 9 of adipogenic differentiation by qRT-PCR and western blotting, respectively. (G) ChIP analysis was performed to confirm the interaction of CtBP2 with C/EBPα. The qPCR data are presentedas the mean± SEM (n=3). **P<0.01, and *** P<0.001.

(siCtBP2-1 and siCtBP2-2) using an RNA interference technique. First, qRT-PCR analysis was performed, confirming that both siCtBP2-1 and siCtBP2-2 effectively inhibited CtBP2 expression in the hAMSCs (Fig. 5A). Then, siCtBP2-transfected hAMSCs were induced to differentiate into an adipogenic lineage. Oil Red O staining and triglyceride concentration assays showed that the downregulation of CtBP2 resulted in an increase in the accumulation of lipid droplets (Fig. 5B and C). Triglyceride concentration assays also indicated a 55.3% increase in the triglyceride concentration in the siCtBP2-transfected cells compared with the

siRNA negative control (siNC)-transfected cells (Fig. 5D). qRT-PCR and western blot analysis also indicated that the downregulation of CtBP2 led to significant increases in the mRNA and protein levels of the adipogenic marker genes FABP4 and LPL but not those of PPARγ or C/EBPα (Fig. 5E and F), implying that CtBP2 acts downstream of C/EBPα as a transcriptional corepressor [47]. To investigate this possibility, we immunoprecipitated C/EBPα complexes in hAMSC adipocytes treated with miR-342-3p mimics or their respective negative control (NC) and analyzed the associated proteins by PCR. Consistent with siCtBP2, the results showed that miR-342-3p can significantly decrease the interaction of CtBP2 with C/EBPα during adipogenic differentiation and subsequently activate the expression of adipogenic genes, such as FABP4 and LPL (Fig. 5G).

Discussion

MiR-342-3p as a relatively new member of the miRNA family, and there are few studies that have evaluated it. miR-342-3p had been found to be upregulated in experimental and idiopathic prion disease, suggesting that it may be used as a novel marker for animal and human bovine spongiform encephalopathy [49]. A high level of miR-342-3p has been associated with significantly worse survival in patients with colon cancer [50]. More recently, it has been reported that miR-342-3p suppresses proliferation, migration and invasion by targeting FOXM1 in human cervical cancer [51]. This miR has also been found to be significantly elevated in irritable bowel syndrome patients [52]. However, there has been no report of the involvement of miR-342-3p in regulating cell differentiation to date. Previous studies have found that it is activated and upregulated in the adipose tissue of mice fed a high-fat diet [53, 54]. However, its functional roles were not been investigated in these earlier reports. In this study, we revealed that the level of miR-342-3p was significantly elevated during the adipogenic differentiation of hAMSCs and 3T3L1 cells. Using gain- and loss-of-function assays, we demonstrated that miR-342-3p significantly promoted the differentiation of hMSCs into an adipogenic lineage. We also identified and validated that CtBP2 was a direct target of miR-342-3p in this process.

Adipogenesis, which plays roles in diseases including obesity, insulin resistance and type 2 diabetes [55, 56], is a dynamic and complex process that involves clonal expansion, cell cycle exit and terminal differentiation into mature adipocytes [57, 58]. It is regulated by multiple adipogenic transcription factors, and CCAAT/enhancer-binding protein (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) are two master regulators of preadipocyte differentiation [4, 59, 60]. During the process of adipocyte differentiation, lipid droplets are gradually produced, and cells are filled with fatty droplets until a final large fat droplet is formed [61]. Here, we found that the overexpression of miR-342-3p promoted the expression of adipogenic marker genes (C/EBPα, PPARγ, FABP4 and LPL) as well as lipid droplet accumulation and increased triglyceride concentrations, indicating its important role in the adipogenesis of hAMSCs. We applied several target prediction tools to predict potential targets for miR-342-3p. Then, luciferase reporter gene assay was performed, and the results indicated that miR-342-3p directly targeted the 3'UTR of C-terminal-binding protein 2 (CtBP2) and that both the mRNA and protein levels of this protein were regulated by miR-342-3p. These results suggest that the suppression of CtBP2 by miR-342-3p may occur via either promoting its degradation or inhibiting its translation.

CtBP family proteins are conserved among vertebrates and invertebrates and function as transcriptional corepressors [62-64]. Previous studies have found that CtBP1 and CtBP2 repress gene transcription and play important roles in development and oncogenesis [65]. Enriched CtBP2 expression has been reported in stem cells, and this protein has been demonstrated to have an important role in stem cell maintenance and the regulation of differentiation in ESCs [66]. CtBP2 has also been identified as a coactivator that is critical for retinoic acid (RA)-induced transcription in mouse F9 cells [67]. CtBP1 and CtBP2 have been reported to directly interact with PRDM16 and selectively mediate the repression

of white fat genes by recruiting a PRDM16/CtBP complex to their promoters [68]. The repression of adipogenic maker expression involves the recruitment of CtBP1/2, as directed by C/EBPα, to the minimal promoters of corresponding genes in response to the PPARγ ligand [47]. However, the role of CtBP2 in the adipocyte differentiation of hMSCs has not been explored to date.

Using two specific siRNAs for CtBP2, we downregulated CtBP2 expression in hMSCs during adipocyte differentiation and found that its inhibition resulted in the enhanced accumulation of lipid droplets, increased triglyceride concentrations, and increases in the expression levels of the adipogenic marker genes FABP4 and LPL. However, the expression levels of PPARγ and C/EBPα did not change in response to the downregulation of CtBP2 during adipogenic differentiation, implying that this protein may act downstream of C/EBPα as a transcriptional corepressor. Co-immunoprecipitation assay confirmed that the overexpression of miR-342-3p in hAMSCs significantly decreases the interaction of CtBP2 with C/EBPα during the process of adipogenesis. CtBP2 downregulation promoted an increase in adipogenesis similar to that caused by the miR-342-3p mimics, and its effects were not influenced by the adipogenic inhibition caused by the miR-342-3p oligonucleotide inhibitor. Furthermore, the inhibition of miR-342-3p was largely counteracted by the inhibition of CtBP2 during adipogenic differentiation. However, the suppression of CtBP2 was not responsible for the moderate activation of PPARγ or C/EBPα by miR-342-3p overexpression, suggesting that there may be other target genes that also play roles in this process.

In conclusion, our study demonstrated that miR-342-3p, which is a powerful enhancer of adipogenesis, directly targeted and inhibited the expression of CtBP2 (a corepressor of the initial factor, C/EBPα) at the posttranscriptional level. Further, we showed that C/EBPα was released from CtBP2 binding and in turn recruited and activated the transcription of adipogenic marker genes. Our findings indicate that miR-342-3p is a potential target for adipose tissue engineering and the management of obesity and other metabolic diseases.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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