Original Paper

miR-137 Controls Proliferation and Differentiation of Human Adipose Tissue Stromal Cells

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Key Words

MicroRNA • hADSC • Adipogenic differentiation • Proliferation • miR-137

Abstract

Background/Aims: Demonstrating the molecular mechanisms of human adipose tissue-derived mesenchymal stem cells (hADSCs) differentiation and proliferation could develop hADSCs-based cell therapy. **Methods:** The microRNA-137 (miR-137) and cell division control protein 42 homolog (*CDC42*) levels were regulated by oligonucleotides transfection. The adipogenic differentiation was induced for 10 days in an adipogenic medium and assessed by using an Oil Red O stain. The regulation of miR-137 on *CDC42* expression was determined by western blot, real-time PCR and luciferase reporter assay. **Results:** We confirmed the roles of miR-137 on hADSCs proliferation and adipogenic differentiation. We showed that overexpression of miR-137 inhibited both hADSCs proliferation and adipogenic differentiation. Overexpression of miR-137 also downregulated protein and mRNA levels of *CDC42*, a predicted target of miR-137. In contrast, inhibition of miR-137 with 2'-O-methyl antisense RNA increased proliferation and adipogenic differentiation in hADSCs. Luciferase reporter activity in the miR-137 target site within the *CDC42* 3'UTR was lower in miR-137-transfected hADSCs than in control miRNA-transfected hADSCs. RNA interference-mediated downregulation of *CDC42* in hADSCs inhibited their proliferation and adipogenic differentiation. **Conclusion:** Our results indicate that miR-137 regulates hADSCs adipogenic differentiation and proliferation by directly targeting *CDC42*. These findings improve our knowledge of the molecular mechanisms governing hADSCs differentiation and proliferation.

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Introduction

MicroRNAs (miRNAs) are endogenous 19- to 25-nucleotide non-coding RNAs that bind to partially complementary recognition sequences of mRNA, resulting in translational repression or cleavage and degradation of mRNA, thus regulating the expression of miRNA targets [1]. Furthermore, each miRNA can regulate multiple mRNAs and each mRNA can be targeted by a number of miRNAs. Thus, it is evaluated that as high as 30% of protein-coding genes could be targets of miRNAs. miRNAs have been involved in diverse biological processes including differentiation [2], proliferation, apoptosis [3, 4], metabolism [3] and neuronal patterning [5]. miRNAs play important roles in the self-renewal and pluripotency of stem cells [6, 7]. The functions of miR-137 have been mostly investigated in several cancer cells. In glioblastoma [8-11], oral squamous cell carcinoma [12], lung cancer [13], colorectal cancer [14, 15] and gastric cancer [16], miR-137 either induced cell cycle arrest in G1 or inhibited invasion through interaction with various target molecules. MiR-137 plays a significant role in the neuronal maturation and dendritic morphogenesis of young neurons [8, 17]. miR-137 is one of the two miRNAs in embryonic stem cells (ESCs) that are co-occupied by the key transcription factors: Oct4, Sox2, and Nanog [18], and is mainly expressed in embryonic stem cells at mitotic stages of the cell cycle. The expression of miR-137 is significantly upregulated when ESCs [19] and neural stem cells [20, 21] are differentiated into neuronal lineage.

Adipose tissue, like bone marrow, is a mesodermally-derived organ with a stromal cell population [22]. This population, termed adipose tissue-derived mesenchymal stem cells or adipose tissue stromal cells (ADSCs), shares many of its characteristics with bone marrow. These characteristics include extensive proliferative potential and the ability to differentiate toward adipogenic, osteogenic, chondrogenic and myogenic lineages [23-25]. Understanding the molecular events involved in adipocyte differentiation is interesting for the development of therapeutics for metabolic diseases such as obesity and diabetes. Our laboratory reported that several microRNAs control proliferation and differentiation of human adipose tissue-derived mesenchymal stem cells (hADSCs) [26, 27].

In this study, we examined the role of miR-137 on proliferation and adipogenic differentiation of hADSCs. Our data showed that miR-137 inhibits hADSCs proliferation and adipogenic differentiation by binding to specific target sequences harbored in the 3' untranslated region (UTR) of cell division control protein 42 homolog (*CDC42*) mRNA.

Materials and Methods

Cell culture

All protocols involving human subjects were approved by the Institutional Review Board of Pusan National University. Superfluous materials were collected from four individuals undergoing elective abdominoplasty after informed consent was given by each individual. The hADSCs were isolated according to the methods described in previous studies [28]. hADSCs were maintained in Low-glucose DMEM with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 mg/ml penicillin in 5% CO₂ environment at 37°C.

Induction of differentiation

Adipogenic differentiation was induced by culturing ADSCs for 10 days in an adipogenic medium (10% FBS, 1 µM dexamethasone, 0.5 mM/mL 3-isobutyl-1-methylxanthine, and 200 µM indomethacin in α -MEM) and assessed by the use of an Oil Red O stain as an indicator of intracellular lipid accumulation. In order to obtain quantitative data, 1 mL of isopropyl alcohol was added to the stained culture dish. The dye was extracted into a 96-well plate and the absorbance read in a plate reader at 562 nm.

Evaluation of cell proliferation

hADSCs were transfected with miRNA or siRNA. After a 48 hr incubation, the cells were detached using Hank's balanced salt solution containing 0.05% trypsin and 0.02% EDTA, then plated in 6 well plates at a density of 1×10^4 cells/well. The number of cells was counted using a Countess® Automated Cell Counter (Invitrogen, Carlsbad, CA, USA).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted using Trizol (Invitrogen), according to the manufacturer's instructions and reverse-transcribed into cDNA with the Reverse Transcriptase M-MLV (Promega, Madison, WI, USA). Internal control for expression analysis was *GUSB* (Beta-D-glucuronidase). Primer sequences to be used in the experiment were as follows: *CDC42* FW, 5'-ATC GGA ATA TGT ACC GAC TG-3' and RV, 5'-GTC TTT GGA CAG TGG TGA GT-3'; *GUSB* FW, 5'-GCG TCC CAC CTA GAA TCT GC-3' and RV, 5'-CAT ACG GAG CCC CCT TGT C-3'; *AP2* (fatty acid binding protein) FW, 5'-GGT GGT GGA ATG CGT CAT G-3' and RV, 5'-CAA CGT CCC TTG GCT TAT GC-3'; *PPAR γ* (peroxisome proliferator-activated receptor gamma) FW, 5'-GAG CCC AAG TTT GAG TTT GC-3' and RV, 5'-CTG TGA GGA CTC AGG GTG GT-3'; *PPAR γ 2* FW, 5'-CCA TGC TGT TAT GGG TGA A-3' and RV, 5'-TGG GAG TGG TCT TCC ATT A-3'; *C/EBP- α* (CCAAT/enhancer binding protein alpha) FW, 5'-GCA AGG CCA AGA AGT CGG TGG AC-3' and RV, 5'-TGC CCA TGG CCT TGA CCA AGG AG-3'. For each primer pair, the linearity of detection was confirmed by measuring a dilution curve with cDNA isolated from hADSCs. *GUSB* mRNA, of which expression was maintained at a constant levels during differentiation, was amplified as an internal control. The data were presented as the ratio of a target gene level to *GUSB* level of each sample.

For miRNA quantitative RT-PCR (qPCR), small RNA species enriched RNA isolation was performed as per the manufacturer's instructions (mirVana miRNA isolation kit; Ambion, Austin, TX, USA). miRNA was reverse-transcribed according to the manufacturer's specified guidelines, using Ncode miRNA first-strand cDNA synthesis kits (Invitrogen). Forward primer sequence was designed as the corresponding mature miRNA sequences and 5S rRNA was used as normalizing control. The miR-137-specific forward primer sequence was designed on the basis of miRNA sequences obtained from the miRBase database. Real time PCR was performed using a Power SYBR Green PCR Master Mix on the ABI 7500 Instrument (Applied Biosystems, Warrington, UK).

Western blot analysis

Samples were homogenized in RIPA buffer (Sigma, St. Louis, MO, USA). The isolated proteins were separated by SDS-PAGE and electro-transferred to PVDF membranes (Millipore, Bedford, MA, USA). Blots were probed with primary antibodies, followed by HRP-conjugated secondary antibodies. Antibodies used in this study were purchased as follows: *CDC42* from Abcam (Cambridge, MA); *GAPDH* from Cell Signaling Technologies (Boston, MA). Bound antibodies were detected using an ECL detection kit (Pierce Biotechnology, Rockford, IL, USA) and visualized using LAS 3000 Luminoimage Analyzer (Fujifilm, Tokyo, Japan). Protein level was quantified using the National Institutes of Health ImageJ software.

miRNA / siRNA transfection

hADSCs were seeded with complete medium without antibiotics. On the following day, 20nM of miRNAs (miR-137 mimics or inhibitors, Dharmacon, Thermo Scientific, Epsom, UK) and 100nM of siRNA (on-TARGET plus SMART pool, Dharmacon) for *CDC42* were transfected using DharmaFECT Transfection Reagent I (Dharmacon) according to manufacturer's protocol with miRNA negative control or non-targeting siRNA as a negative control.

Reporter Vectors and DNA Constructs

A putative miR-137-recognition element (as single copy) from the *CDC42* gene was cloned in the 3'-untranslated region (UTR) of the firefly luciferase reporter vector according to the manufacturer's specified guidelines. The oligonucleotide sequences were designed to carry the HindIII and SpeI sites at their extremities facilitating ligation into the HindIII and SpeI sites of pMIR-Report (Ambion). The oligonucleotides used in these studies were: pMIR-*CDC42* FW, 5'-CTAGT TTAATAATTCGTTTTCGCAATAAT A-3' and RV, 5'-AGCTT ATTATTGCAAAAACGAATTTTAA A-3'; pMIR-*CDC42*-mut FW, 5'-CTAGT TTAATAATTCGTTTTCGTTATTT A-3' and RV, 5'-AGCTT AAATAACGAAAAACGAATTTTAA A-3'.

Reporter gene assay

All transient transfections were conducted using Lipofectamine Plus Reagent (Invitrogen). The pMIR-*CDC42* and pMIR- β -gal plasmids were used as reporter constructs. The cells were harvested 48 hr after transfection, lysed in RLB buffer, and subsequently assayed for their luciferase activity (Luciferase Assay System, Promega). The transfections were performed in duplicate, and all experiments were repeated several times. The luciferase assays were normalized according to their β -galactosidase activity.

Fig. 1. Overexpression of miR-137 inhibits adipogenic differentiation and proliferation of hADSCs. (A) miR-137 levels were determined in control- (miR-con) or miR-137-transfected hADSC using real-time PCR. The data were presented as the relative ratio of miR-137 to 5S RNA level of each sample. (B) hADSC proliferation was determined by direct cell counting after oligonucleotide transfection. (C) Oligonucleotide-transfected hADSC were grown to 80-90% confluence. Adipogenic differentiation was induced for 10 days and determined by Oil Red O staining to visualize intracellular lipid accumulation, which was quantified by absorbance at 562nm. (D and E) Real-time PCR analysis of PPAR γ and PPAR γ 2 in miR-137 mimic-transfected undifferentiated cells (D). Changes in expression of α P2 and C/EBP- α in differentiated cell to adipogenic lineage (E). Total RNA was isolated at days after induction of differentiation. Data represent mean $\pm$ SEM of the ratio of a target gene level to GUSB level (n = 4). *p < 0.05 compared with miR-con-transfected hADSCs.

Statistical analysis

All of the results are presented as the means $\pm$ SEM. Comparisons between groups were analyzed via t-tests (2-sided) or ANOVA for experiments with more than 2 subgroups. Post hoc range tests and pairwise multiple comparisons were conducted using the t-test (2-sided) with Bonferroni adjustments. Probability values of P<0.05 were considered to be statistically significant.

Result

Effects of miR-137 on the adipogenic differentiation and proliferation of hADSC

We chose the microRNAs that showed high levels of expression in preliminary microRNA microarray study and then targeted differentiation-related molecules using miRWalk database [29]. To examine the role of miR-137 in hADSCs functions, we transfected 20 nM of miR-137 mimics into hADSCs for the overexpression of miR-137. miRNA mimics augments

Fig. 2. Inhibition of miR-137 increases adipogenic differentiation and proliferation of hADSCs. (A) miR-137 levels were determined in control- (anti-miR-con) or anti-miR-137-transfected hADSC using real-time PCR. The data were presented as the relative ratio of miR-137 to 5S RNA level of each sample. (B) hADSC proliferation was determined by direct cell counting after oligonucleotide transfection. (C) Oligonucleotide-transfected hADSC were grown to 80-90% confluence. Adipogenic differentiation was induced for 10 days and determined by Oil Red O staining to visualize intracellular lipid accumulation, which was quantified by absorbance at 562nm. (D and E) Real-time PCR analysis of *PPARγ* and *PPARγ2* in anti-miR-137-transfected undifferentiated cells (D). Changes in expression of *aP2* and *C/EBP-α* in differentiated cell to adipogenic lineage (E). Total RNA was isolated at days after induction of differentiation. Data represent mean $\pm$ SEM of the ratio of a target gene level to GUSB level ($n = 4$). * $p < 0.05$ compared with anti-miR-con transfected hADSCs.

the function of endogenous miRNAs for easier detection of a phenotypic change. Real-time PCR analysis showed that miR-137-transfected hADSCs increased miR-137 expression (Fig. 1A).

To test the effect of miR-137 on hADSCs proliferation, we plated miR-137-transfected hADSCs (1×10^4 cells/well) on culture plates and counted the number. Cell counting on the days indicated in Fig. 1B showed that miR137-transfected cells had slower growth than control cells. We induced adipogenic differentiation of miR-137-overexpressing hADSCs with the appropriate media. After incubating hADSCs with adipogenic media for 10 days, miR-137-overexpressing hADSCs significantly inhibited adipogenic differentiation. The inhibition was quantified by absorbance of Oil Red O staining (Fig. 1C). We then used real-time PCR

Fig. 3. miR-137 targets the 3'UTR of *CDC42* mRNA. (A, B) *CDC42* expression in hADSCs transfected with oligonucleotide was analyzed by western blot (A) and real-time PCR (B). (C) pMIR-*CDC42* or pMIR-*CDC42*-mut luciferase constructs were transfected into miR-con or miR-137-mimic-transfected hADSCs and anti-miR-con or anti-miR-137-transfected hADSCs. Data represent mean $\pm$ SEM of the ratio of a target gene level to GUSB level ($n = 4$), * $p < 0.05$, compared with miR-con-transfected hADSCs, # $p < 0.05$ compared with anti-miR-Con-transfected hADSCs.

to determine whether miR-137 overexpression decreased the expression of adipogenesis-related genes. The results showed that miR-137-overexpressing hADSCs revealed lower levels of *PPAR γ* and *PPAR γ* expression than control microRNA-transfected cells (Fig. 1D). Real time PCR analysis of differentiated cells showed that the transfection of miR-137 mimic inhibited *aP2* and *C/EBP- α* expression at 4 days and 7 days after differentiation (Fig. 1E).

Effect of miR-137 inhibition on the differentiation and proliferation of hADSCs

To investigate the effect of miR-137 inhibition on hADSCs differentiation, we transfected hADSCs with a specific miRNA inhibitor (anti-miR-137). Real-time PCR analysis showed that transfection of anti-miR-137 effectively downregulated miR-137 expression in hADSCs (Fig. 2A). Cell number counts showed that anti-miR-137 enhanced hADSCs proliferation compared with control oligonucleotide-transfected cells (Fig. 2B).

To examine the effect of miR-137 inhibition on adipogenic differentiation, anti-miR-137-transfected hADSCs were induced to differentiate into adipogenic lineages. Oil red O staining revealed that the inhibition of miR-137 enhanced adipogenic differentiation of hADSCs (Fig. 2C). To further confirm the effect, we determined adipogenic marker genes expression by

Fig. 4. *CDC42* RNAi inhibits adipogenic differentiation and proliferation of hADSC. (A) *CDC42* mRNA levels were determined in control- (si-con) or *CDC42* oligonucleotide- (si-*CDC42*) transfected hADSC using real-time PCR. (B) hADSC proliferation was determined by direct cell counting after siRNA oligo transfection. (C) siRNA oligo-transfected hADSC were grown to 80-90% confluence. Adipogenic differentiation was induced for 10 days and determined by Oil Red O staining to visualize intracellular lipid accumulation, which was quantified by absorbance at 562nm. Silencing of the *CDC42* gene inhibited the adipogenic differentiation of hADSCs. (D) Real-time PCR analysis of *PPAR γ 2* and *C/EBP- α* in si-*CDC42*-transfected undifferentiated cells. Data represent mean $\pm$ SEM of the ratio of a target gene level to GUSB level (n = 4). *p < 0.05 compared with si-con-transfected hADSCs.

real-time PCR. Anti-miR-137 transfection increased the expression of marker genes (*PPAR γ 2*, *aP2* and *C/EBP- α*) during adipogenic differentiation of hADSCs (Figs. 2D and 2E).

miR-137 targets the 3'UTR of CDC42 mRNA

To determine the potential targets of miR-137 in hADSCs, we analyzed the miR-137-induced changes in gene expression profile by microarray. Using miRWalk database for target gene prediction, *CDC42* in genes where expression was downregulated in microarray analysis was selected as the potential target of miR-137.

To determine the relationship between miR-137 and *CDC42*, we analyzed *CDC42* expression with real time PCR and western blot analysis in miR-137 mimic transfected cells. The transfection of miR-137 mimic led to a significant decrease in *CDC42* expression at both protein (Fig. 3A) and mRNA levels (Fig. 3B) in the hADSCs.

To test whether miR-137 directly targeted *CDC42* in hADSCs, we constructed luciferase reporter genes with *CDC42* 3'UTR with or without mutation at the miR-137 binding regions and confirmed their expression with miR-137 transfection. The results showed that a decrease in relative luciferase activity was demonstrated when the *CDC42* 3'UTR (pMIR-*CDC42*) was transfected into miR-137-transfected hADSCs, while no decrease in relative luciferase activity was observed in hADSCs transfected with mutant *CDC42* 3'UTR (pMIR-*CDC42*-mut). On the contrary, transfection of anti-miR-137 increased the luciferase activity of pMIR-*CDC42* in hADSCs compared with transfection of the miRNA control (Fig. 3C).

Effect of CDC42 on adipogenic differentiation and proliferation of hADSCs

To determine the role of *CDC42* in adipogenic differentiation and proliferation of hADSCs, we suppressed *CDC42* expression in hADSCs with an RNA interference technique using *CDC42* siRNA (si-*CDC42*) transfection. Real-time PCR analysis confirmed that si-*CDC42* effectively inhibited *CDC42* expression in hADSCs (Fig. 4A). We also determined the effect of *CDC42* downregulation on hADSCs proliferation. Direct cell counting showed that si-*CDC42*-transfected hADSCs proliferated less than the control cells (Fig. 4B).

To further study the effect of *CDC42* downregulation on the efficiency of hADSCs differentiation, we induced adipogenic differentiation of si-*CDC42*-transfected hADSCs. Oil red O staining and real-time PCR analysis of adipogenic marker genes indicated that downregulation of *CDC42* inhibited adipogenic differentiation of hADSCs (Figs. 4C and 4D).

Discussion

While several reports have demonstrated the function of miR-137 in neurogenesis within ESCs [19, 20], no studies to date have examined the effects on other lineage differentiation. In the present study, we investigated whether miR-137 was involved in proliferation and adipogenic differentiation of hADSCs. We demonstrated that miR-137 overexpression with miR-137 mimic in hADSCs inhibited proliferation and adipogenic differentiation. We also demonstrated that miR-137 inhibition by transfection of inhibitor oligonucleotids increased adipogenic differentiation and proliferation. These results indicated that miR-137 negatively regulated adipogenic differentiation and proliferation in hADSCs.

Adipogenesis is a very complex process significantly involved in diseases including obesity, insulin resistance, and type 2 diabetes [30, 31]. It is regulated by multiple transcription factors, especially the CCAAT/enhancer-binding protein (*C/EBP*) family and peroxisome proliferator-activated receptor γ (*PPAR* γ) [32, 33]. *PPAR* γ is certainly necessary for the adipogenic differentiation in *in vivo* models [34]. In this study we showed that the modulation of miR-137 level altered the expression of *PPAR gamma* in undifferentiated hADSCs.

We identified from microarray experiment that one of the downregulated genes in miR-137 mimic-transfected cells is *CDC42*. The data in this experiment showed that overexpression of miR-137 downregulated the expression of *CDC42* at the protein and mRNA levels. An assay with a firefly luciferase reporter plasmid containing the predicted *CDC42* target gene sequences showed that overexpression of miR-137 decreased luciferase activity and inhibition of miR-137 increased luciferase activity, confirming the direct binding of miR-137 to 3'UTR of *CDC42* in hADSCs. The role of miR-137 in the proliferation and adipogenic differentiation of hADSCs was further supported by determining the effect of *CDC42* downregulation. In this study, the downregulation of *CDC42* using RNA interference inhibited the adipogenic differentiation and proliferation of hADSCs (Fig. 4)

CDC42, as a key member of the Rho GTPase family, is well known to be involved in proliferation, migration, invasion, apoptosis and angiogenesis, in several types of cancers. *CDC42* contributes to the regulation of cell cycle progression in G1/S-phase transition by activation of downstream molecules such as ERK1/2 and cyclin D1 [35]. Consistent with our findings, miR-137 has been reported to regulate cell proliferation and invasion by targeting *CDC42* gene in several cancers [13, 14, 16].

Although the role of *CDC42* in cell fate determination has been reported in neural progenitor cells [36], epidermal stem cells [37] and hematopoietic stem cells [38], the relationship between *CDC42* and adipogenic differentiation has not been reported. In spite of high sequence analogies among Rho GTPase family members, many Rho GTPases play functionally unique and essential roles, and these roles are often cell type specific. In this study we showed that miR-137 inhibits adipogenic differentiation through inhibition of *CDC42* function. The Rho GTPase family members involved in adipogenesis have shown various actions according to subtypes. Sordella et al [39] reported that activation of Rho inhibits adipogenesis in Mouse embryonic fibroblasts (MEFs) or 3T3-L1 cells, while Mcbeath

et al [40] showed that cell shape changes induced by expression of dominant-negative RhoA committed human mesenchymal stem cells (hMSCs) to become adipocytes. Rho guanine nucleotide exchange factors (RhoGEFs), which stimulate the exchange of GDP for GTP in order to generate the activated form of GTPases and activate RhoA, CDC42 and Rac1, inhibits adipogenesis in 3T3-L1 cells [41]. In contrast, Nishizuka et al [42] reported that the overexpression of TC10-like/TC10_Long, a member of Rho GTPase, induced adipogenic differentiation in the presence of the ligand for *PPAR gamma* in NIH3T3 cells. Differential effects of Rho family members on cellular functions also have been reported in mouse fibroblast [43], intestinal epithelial cells [44] and hematopoietic cells [45].

miR-137 that regulates adipogenic differentiation and proliferation of hADSCs through the modulation of *CDC42* levels can provide mechanistic insights into the molecular processes of stem cell differentiation and proliferation. These findings present miR-137 as a potential target for adipose tissue engineering through miRNAs and in the management of obesity and other metabolic diseases.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

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