Original Article
miR-141 promotes differentiation of adipocyte and contributes to the obesity by targeting to FOXA2

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Abstract: MicroRNAs (miRNAs) were considered as powerful, post-transcriptional regulators in adipogenesis progress. However, the function of miR-141 was still unclear. Here, upregulation of miR-141 was found during adipocyte differentiation. In miR-141 transfected cells, triglyceride accumulation was increased, the adipogenesis related transcription factors and adipocyte markers levels of C/EBPα, PPARγ, and AP2 were up-regulated. Bioinformatic analyses and luciferase reporter assays were used to validate FOXA2 as a direct target gene of miR-141. By over-expressing miR-141, the expression of FOXA2 was remarkably repressed from both RNA and protein level. Using wildtype and mutant 3'-UTR of the FOXA2, we identified the targeting sites for miR-141. The levels of c-myc and CCND1 were upregulated in miR-141 inhibitor group as well as the FOXA2 overexpression group, which indicated the Wnt/β-catenin signaling was involved in the miR-141/FOXA2 cascade. Consistently, the expression of miR-141 was significantly increased in ob/ob, db/db, and diet-induced obese mice. Using miR-141 antagomir, the body weight, the white adipose tissue weight and the expression of C/EBPα, PPARγ, AP2 were reduced effectively. In conclusion, we demonstrated that miR-141 was a positive regulator of adipocyte differentiation through regulation of FOXA2.

Keywords: miR-141, FOXA2, adipocyte differentiation, obesity, Wnt/β-catenin

Introduction

MicroRNAs (miRNAs) are groups of highly conserved, small noncoding RNAs that regulate gene expression by binding to the 3' untranslated region (UTR) of mRNAs [1] to control mRNA stability or degradation at the post-transcriptional level. MicroRNAs are small but important modulators that are involved in physiological and pathological processes [2], such as energy homeostasis [3], lipid metabolism, adipogenesis [4] and tumor progression. MiRNAs have been identified as oncogenes or tumor suppressors in different types of cancer [5]. Among them, miR-141 has previously been shown to function as a tumor suppressor in various cancers including colorectal cancer [6], pancreatic cancer [7], gastric cancer [8], head and neck squamous cell carcinoma [9]. However, whether miR-141 was involved in the adipogenic differentiation was still unknown.

Obesity is one of the largest medical conditions on the health care systems and is a major risk factor in the developed countries [10, 11], which is usually associated with insulin resistance, the metabolic syndrome, hyperlipidemia, hypertension and atherosclerosis [12, 13]. Understanding the molecular mechanism governing adipogenesis is of great importance in biomedical research. The regulation of adipogenesis may have clinical relevance in treating obesity and the metabolic syndrome.

The forkhead transcription families in mammals include several members, FOXA proteins which include three genes are the first identified ones and designate as FOXA1, FOXA2 and FOXA3 [14]. In preadipocytes, FoxA2 is reported to inhibit adipocyte differentiation by activating the expression of Pref-1 [15]. However, the regulation of FOXA2 in adipocyte is still unknown.

In our study, we demonstrate that miR-141 can indirectly inhibit FOXA2 expression during the adipogenic differentiation, and find that miR-141 is an effective positive regulator of adipogenesis.

Materials and methods

Cell culture and reagents

3T3-L1 fibroblasts cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin at 10% CO_2 and 37°C. For differentiation, at the point of day 0, cells were switched to DMEM supplemented with 10% FBS with MDI (0.5 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10 µg/ml insulin). Dexamethasone was removed on day 2, and cells were cultured with insulin containing 10% FBS media for two additional days. On day 4 and every 2 days thereafter, cells were incubated with 10% FBS in DMEM. On day 8, 90% of the control cells were differentiated into mature adipocytes. Human adipose-derived mesenchymal stem cells (hADSCs) was obtained from donors who underwent elective liposuction or other abdominal surgery in Shanghai East Hospital. All the patients were given written consent and approval forms. miR-141 mimics, miR-141 inhibitors and the scramble negative controls were purchased from RiboBio (Shanghai, China). The following primary antibodies used were as follows: FOXA2 (ab108422; Abcam, Cambridge, UK), Anti-beta Actin antibody (ab8226, Abcam). C/EBPα (18311-1-AP; Proteintech) and PPARγ (16643-1-AP; Proteintech), CCND1 (ab134175, Abcam) and c-myc (ab32072, Abcam).

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For miRNA extraction, cells or tumor samples were lysed in RNAiso for Small RNA (TaKaRa, Dalian, China). Then 2 µg total RNAs or miRNAs in each group was used for reverse transcription to get the first strand cDNA by using the PrimeScript Reverse Transcriptase (TaKaRa, Dalian, Liaoning, China).

Luciferase reporter assay

TargetScan Human 7.0 predicted that FOXA2 was a potential target of miR-141. The wild-type or mutant (without miR-141 binding site) human

FOXA2 3'UTR sequences were synthesized using Quik Change Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and were separately cloned into the luciferase reporter respectively. The recombinant plasmids were named as pGL3-FOXA2-WT and pGL3-FOXA2-MUT. These plasmids were co-transfected with 50 nm miR-141 mimic or inhibitor or their negative control using Lipofectamine 2000. The pRL-TK vector containing Renilla luciferase was co transfected as a reference control. 48 h after transfection, cells were collected to analyze the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla.

Western blot

Cells were lysed using lysis buffer for protein extraction (Beyotime, Shanghai, China), and the concentration was measured using a bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific Pierce Chemical, Rockford, IL, USA). Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Roche). After blocked with 5% nonfat milk for 30 min at room temperature, the membranes were incubated with the specific primary antibodies at 4°C overnight. The membranes were washed 5 times with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:5000, anti-mouse IgG, 1:3000, Merck Milipore). At last, the signals were visualized using the ECL Western blotting substrate (Thermo Scientific). GAPDH was used as the internal control.

Lipid metabolism assay

The concentration of triglycerides (TGs) in the lysates of adipocytes was measured using the tissue triglyceride assay kit (Applygen Technologies, Beijing, China) according to the manufacturer's instructions. The concentration of the TGs was normalized to the protein content using a BCA assay kit.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)22,5-diphenyltetrazolium bromide (MTT) assay was used to detect cells proliferation on the 0, 1, 2, 3, and 4 days. The relative 3T3-L1 cells or hADSCs in each group were placed into 96-wellplates and transfected with the relative construction. The
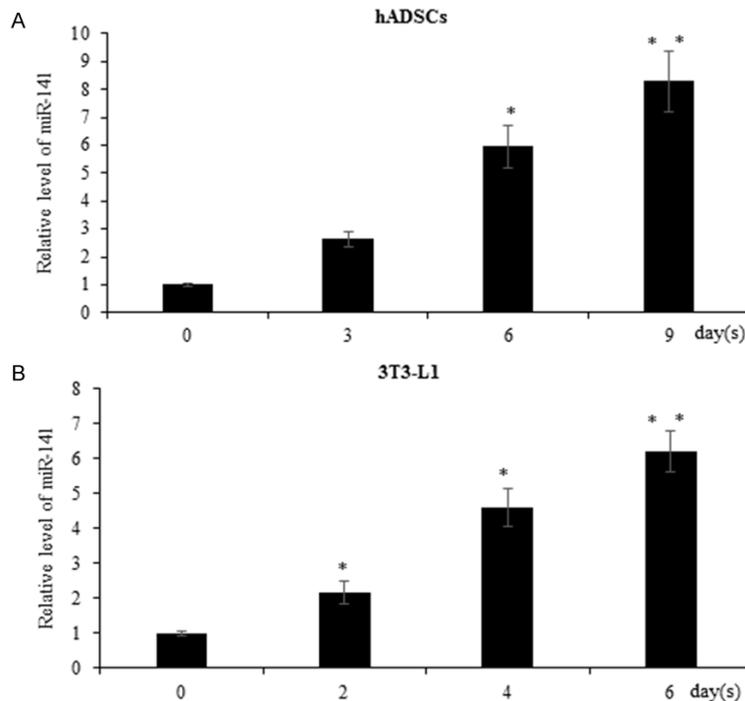
Figure 1. Upregulation of miR-141 was found during adipocyte differentiation. A: The miR-141 expression level was measured at intervals of 3 days using hADSCs following a 9-day induction of adipogenic differentiation, as determined by RT-qPCR. Data was represented as the means ± SD; of 3 independent experiments, in comparison to the relative expression levels on day 0. *P<0.05, **P<0.01. B: Total RNA was prepared at the indicated time points using the 3T3-L1 cells, subjected to RT-qPCR for miR-141 abundance. Data was represented as the mean ± SD of at least three independent experiments. *P<0.05, **P<0.01.

tiation of hADSCs in vitro at different time points such as 0, 3, 6 and 9 days, respectively. We found that the expression of miR-141 was gradually increased during adipogenesis, compared to the undifferentiated cells on the 0 day (**Figure 1A**). And then, mouse 3T3-L1 preadipocyte cell line was chosen for further study. The level of miR-141 was investigated during 3T3-L1 differentiation. MiRNA expression profile revealed that the expression of miR-141 was also upregulated during adipogenesis (**Figure 1B**). Therefore, we hypothesized that miR-141 may play an important role during the differentiation of adipogenesis.

MiR-141 promoted adipocyte differentiation of 3T3-L1 cells

To further elucidate whether miR-141 was involved in regulating adipocyte differentiation, we transiently transfected miR-141 mimics or miR-141 inhibitor or negative control (NC) into 3T3-L1 preadipocytes and examined the differentiation of 3T3-L1 cells. The expression of miR-141 was remarkably increased when miR-141 mimics was transfected into 3T3-L1 preadipocytes. In contrast, transfection of miR-141 inhibitor decreased miR-141 expression (**Figure 2A**). The triglyceride quantification analysis showed increased triglyceride accumulation in lipid droplets transfected with miR-141 mimics, while the miR-141 inhibitor showed the opposite tendency (**Figure 2B**). Consistent with these data, we measured the expression of various adipocyte differentiation markers. The mRNA expression levels of adipogenic markers such as key adipogenic transcription factors, CCAAT/enhancer binding protein alpha (C/EBPα), PPARγ and adipocyte fatty acid binding protein (AP2) were increased in miR-141 transfected cells, and adipogenic differentiation was promoted (**Figure 2C**), while the transfection of miR-141 inhibitor showed the opposite tendency (**Figure 2D**). We also measured

MTT reagent (5.0 mg/ml) was added to each well and incubated for 2 h. The absorbance was measured at 490 nm using a multiwell plate reader and the extraction buffer was used as the blank.

Statistical analysis

All the experiments were repeated at least 3 times unless otherwise mentioned. The data was expressed as the mean ± SD values. Student's t-test and one-way ANOVA were applied to analyze the differences between the groups using SPSS software version 17 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered as statistically significant difference.

Results

Upregulation of miR-141 was found during adipocyte differentiation

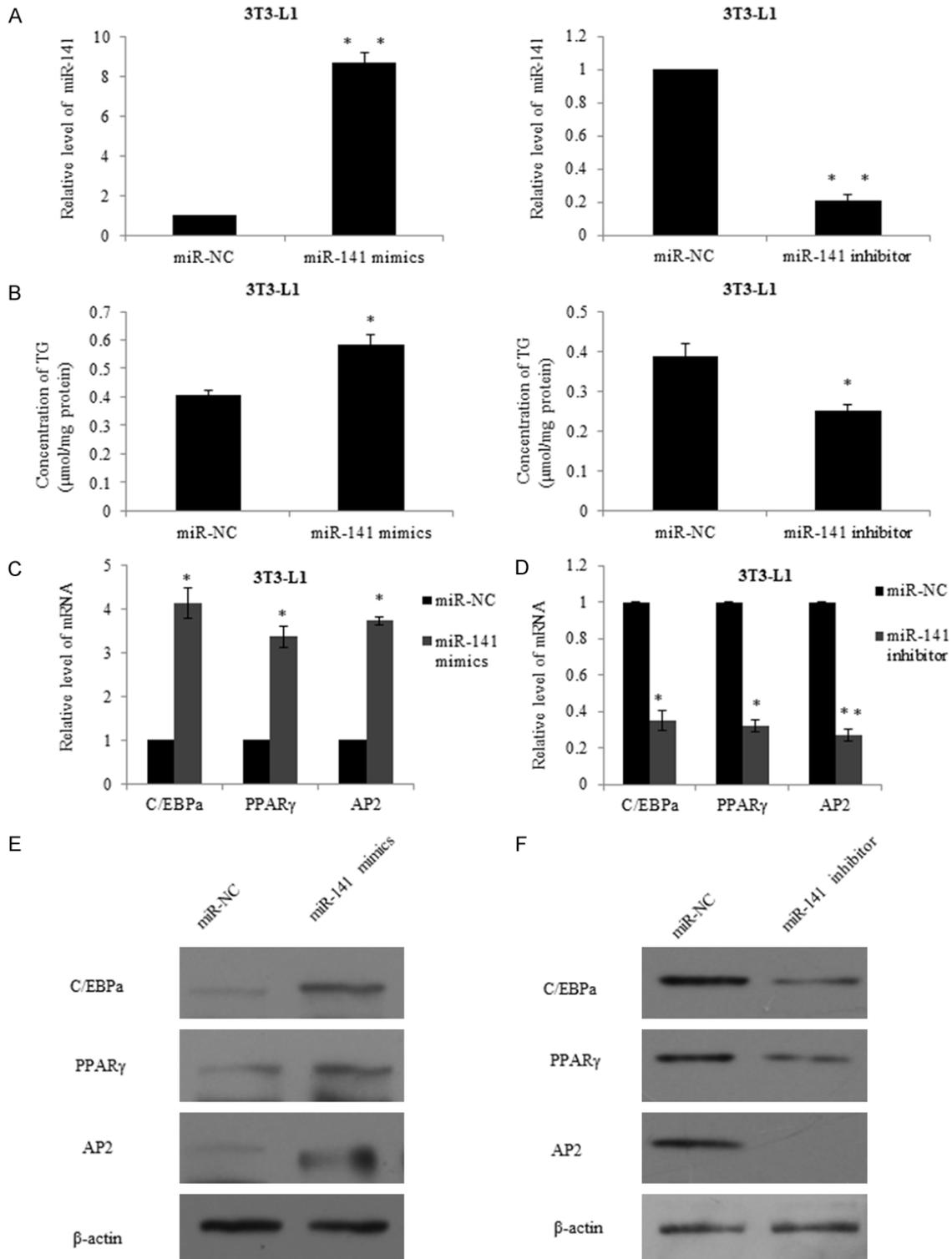We first determined the changes of miR-141 using RT-qPCR during the adipogenic differen-

Figure 2. MiR-141 promoted adipocyte differentiation of 3T3-L1 cells. A: Undifferentiated 3T3-L1 cells were transfected with miR-141 mimics or miR-141 inhibitor or negative control (NC) maintained in DMEM supplemented with 10% FBS for 8 days. Expression of miR-141 was calculated relative to NC. Data was represented as the means ± SD; of 3 independent experiments, *P<0.05, **P<0.01. B: The Triglyceride accumulation was quantified and normalized to that of the protein amount at 8 day of differentiation after transfection. C: qRT-PCR analysis was used to measure the relative level of several adipogenic marker genes including C/EBPα, PPARγ, and AP2 in 3T3-L1 cells transfected with miR-141 mimics or miR-141 control. Data was represented as the mean ± SD, of three independent experiments, *P<0.05, **P<0.01. D: The relative qRT-PCR analysis was performed in 3T3-L1 cells transfected with

miR-141 inhibitor or negative control (NC). β-actin was used as an internal control. E: Western blot analysis was used to measure the relative level of several adipogenic marker genes including C/EBPα, PPARγ, and AP2 in 3T3-L1 cells transfected with miR-141 mimics or control miRNA. F: Western blot analysis was usedto measure the relative level of several adipogenic marker genes including C/EBPα, PPARγ, and AP2 in 3T3-L1 cells transfected with miR-141 inhibitor or negative control (NC).
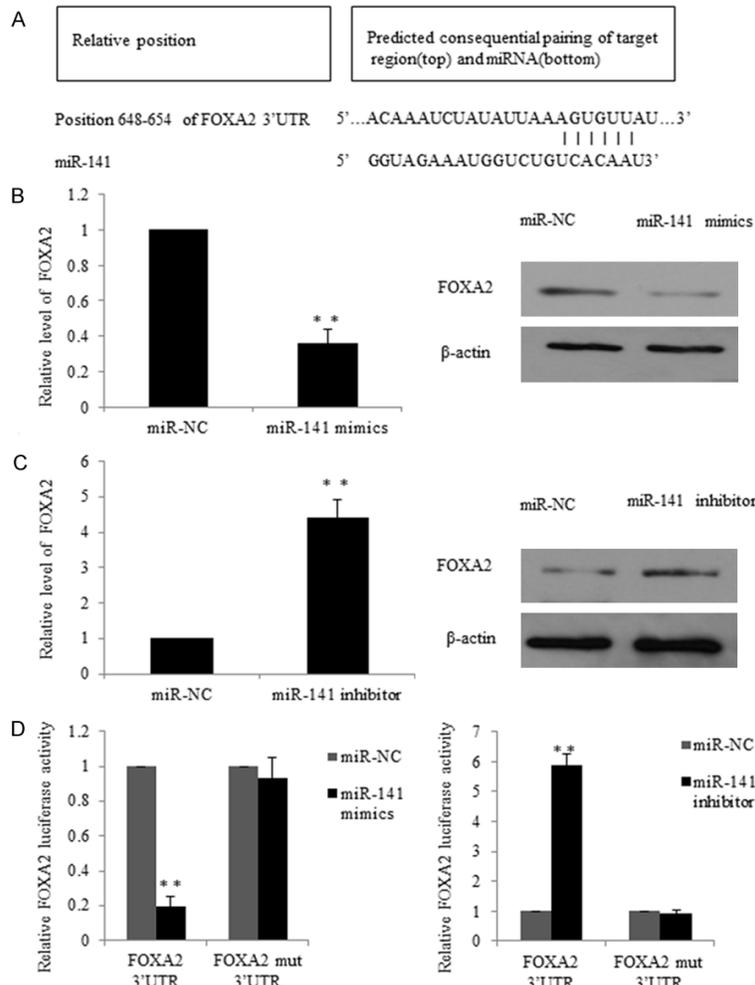

Figure 3. Identification of FOXA2 as miR-141 target genes. A: Prediction of the miR-141 binding site in the FOXA2-3'UTR. B: The effect of miR-141 on the FOXA2 mRNA and protein level in undifferentiated 3T3-L1 cells transfected with the miRNA control (NC) or miRNA mimics. The relative mRNA was calculated to the control cells. Data was represented as the mean ± SD, of three independent experiments, *P<0.05, **P<0.01. C: The effect of miR-141 on FOXA2 mRNA and protein expression was measured in undifferentiated 3T3-L1 cells transfected with the miRNA control (NC) or miRNA inhibitor. D: pGL3-FOXA2-WT or pGL3-FOXA2-MUT(E) was transfected into 3T3-L1 cells combined with the control or miR-141 mimic or miR-141 inhibitor. Renilla luciferase activity was normalized to that of firefly luciferase. Data was represented as means ± SD. *P<0.05.

down-regulated in miR-141 inhibitor transfected cells compared to the negative control (**Figure 2F**).

Identification of FOXA2 as miR-141 target genes

To search for putative direct target of miR-141, several computational prediction programs such as TargetScan, miR-Gen, and miRanda (mircorna. org) were used to select the miR-141 target genes. Among the candidate genes, FOXA2 was reported to inhibit adipocyte differentiation. As shown in **Figure 3A**, FOXA2 had putative binding site of miR-141 in its 3'UTR, and this indicated that FOXA2 may be a potential target of miR-141. As shown **Figure 3B**, Treatment with miR-141 mimics remarkably decreased FOXA2 mRNA and protein expression compared with control-miRNA treated 3T3-L1 cells. While the cells were treated miR-141 inhibitor, the FOXA2 mRNA and protein expression increased (**Figure 3C**). To confirm whether miR-141 was able to recognize the FOXA2-3'UTR, we generated two luciferase reporter DNA constructs containing either with the FOXA2 wild type 3'UTR or FOXA2 mutant 3'UTR. When 3T3-L1 cells were co-transfected with miR-141 mimics and the luciferase reporter vector, the luciferase activity was significantly suppressed compared with the control cells, while the cells transfected with miR-141 inhibitor, the luciferase activity was significantly increased. Unlike the wild-type luciferase reporter, expression of miR-141 expression could not affect the

the protein expression of the above markers and confirmed the increase expression of adipogenesis markers were observed after miR-141 transfection (**Figure 2E**). In contrast, the protein levels of C/EBPα, PPARγ, and AP2 were
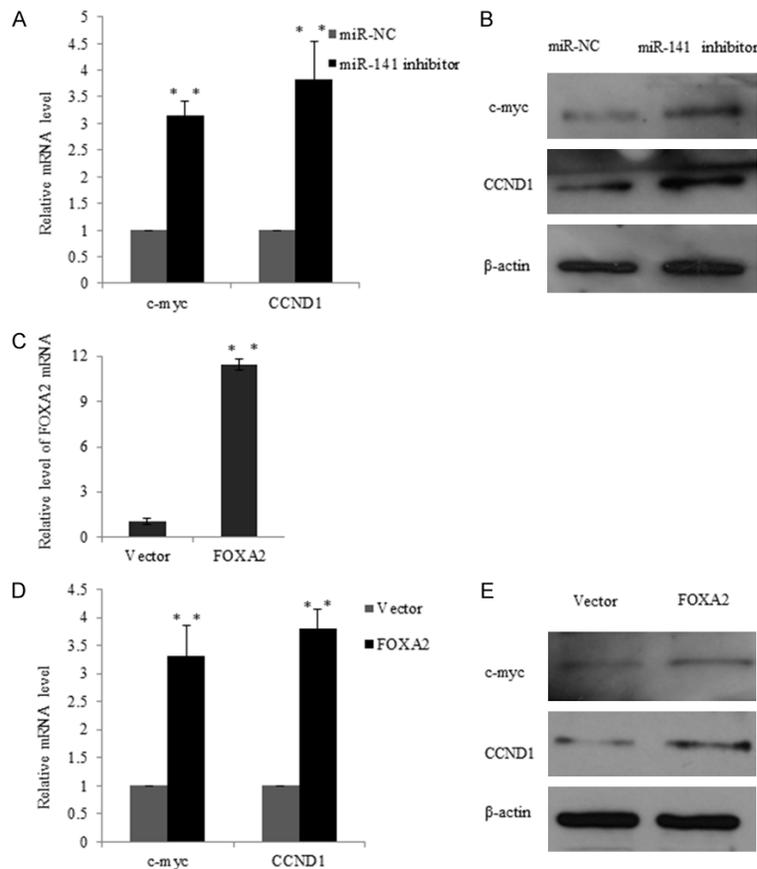
Figure 4. MiR-141 regulated adipogenesis through Wnt/β-catenin signaling. A: mRNA expression of c-myc and cyclin D1 (CCND1) was detected by qRT-PCR in 3T3-L1 transfected with miR-141 inhibitor or the control group. B: Protein expression of c-myc and cyclin D1 was detected by western blot in 3T3-L1 transfected with miR-141 inhibitor or the control group. C: The expression of FOXA2 in 3T3-L1 cells was measured by qRT-PCR in the different treated groups with FOXA2 construct or control plasmid (Vector). D: mRNA expression of c-myc and cyclin D1 (CCND1) was measured by qRT-PCR in 3T3-L1 transfected with FOXA2 construct or control plasmid. E: Protein expression of c-myc and cyclin D1 was measured by qRT-PCR in 3T3-L1 transfected with FOXA2 construct or control plasmid.

mutant reporter activity (**Figure 3D**). These results clearly indicated that miR-141 directly recognized and bound to the 3'UTR of FOXA2.

MiR-141 regulated adipogenesis through Wnt/β-catenin signaling

The above data suggested that miR-141 may exert an impact during adipogenesis through targeting FOXA2. To further delineate the mechanism, we focused our attention on the Wnt signaling pathway. Not only canonical but also non-canonical Wnt signaling were shown to negatively regulate adipogenesis. We first monitored the expression of c-myc and CCND1, both of which were downstream targets of the canonical Wnt signaling pathway. The levels of c-myc and CCND1 were upregulated in miR-141 inhibitor group compared with the control group, as shown from the mRNA and protein level (**Figure 4A and 4B**). Consistently, we induced the expression of FOXA2 in 3T3-L1 cells using FOXA2 construct (**Figure 4C**). It was found that the mRNA level of c-myc and CCND1 was activated by FOXA2 overexpression group compared with the control (**Figure 4D**). Western blot analysis showed the similar result (**Figure 4E**).

Silencing of miR-141 ameliorated obesity through upregulation of FOXA2

To further understand the role of miR-141 in the development of obesity, we compared the expression of miR-141 in adipose tissues from obese mice and the matching control mice. As shown in **Figure 5A**, compared with the control mice, the expression of miR-141 was significantly increased in ob/ob, db/db, and diet-induced obese mice. Conversely, there was a substantial downregulation of FOXA2 in genetically and diet-induced obese mice (**Figure 5B**). These data suggested that in the white adipose tissue of obese mice, miR-141 was upregulated while the expression of FOXA2 was subsequently downregulated. In order to investigate the role of miR-141 on body weight and adiposity of fat mice. We injected a locked nucleic acid (LNA)-miR-141 antagomir (LNA-miR-141) or LNA-scrambled negative control (LNA-NC) into the obese mice. The expression of miR-141 was analyzed in various tissues, compared with the LNA-NC mice, the administration of LNA-miR-141 resulted in effective silencing of miR-141 with subsequent upregulation of FOXA2 (**Figure 5C**). Consistently, LNA-miR-141 reduced the body weight and white adipose tissue area effectively (**Figure 5D**). qRT-PCR analysis showed the
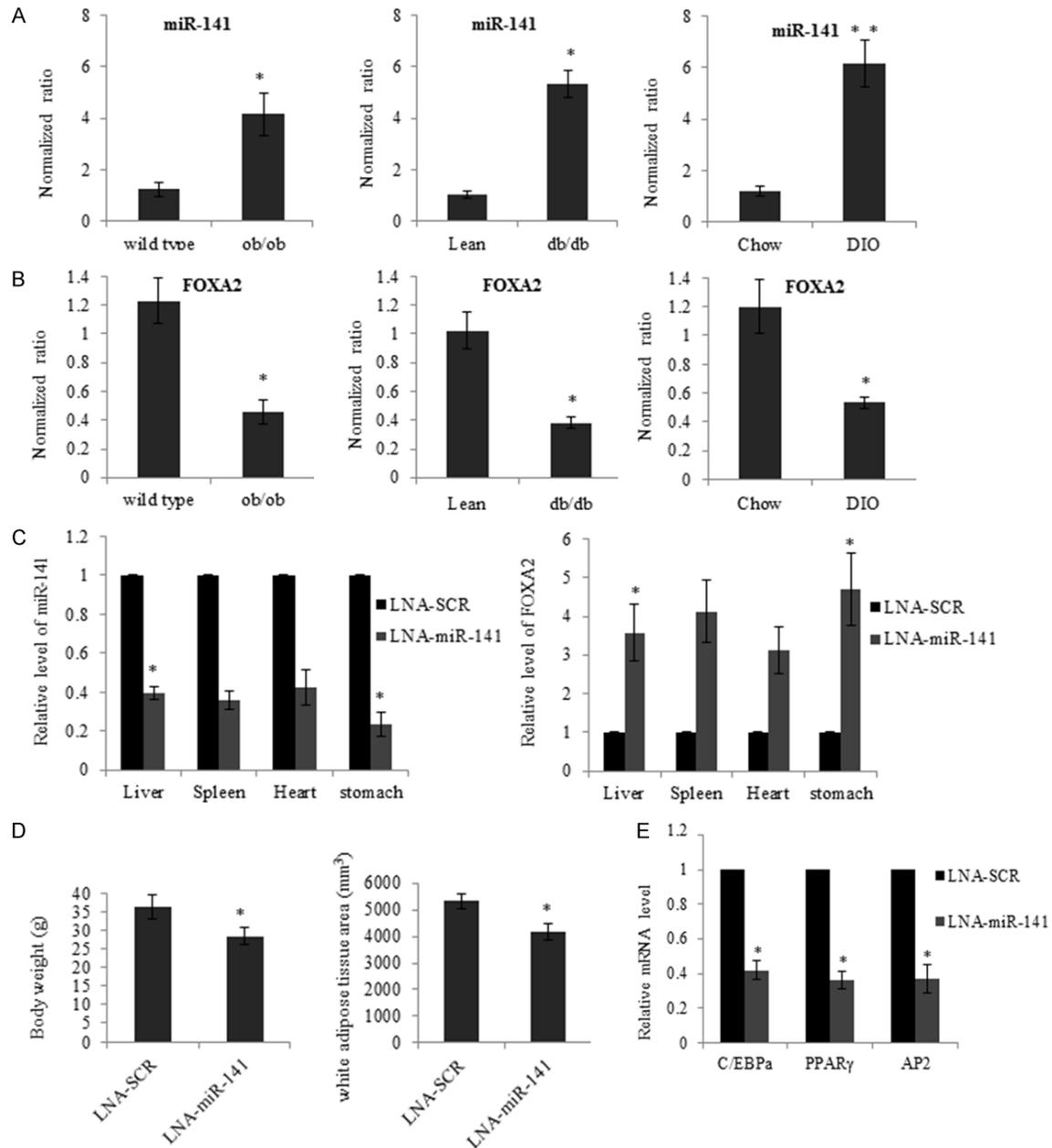
Figure 5. Silencing of miR-141 ameliorated obesity through upregulation of FOXA2. A: The expression of miR-141 was measured in the adipose tissue from ob/ob, db/db, and diet-induced obese mice (DIO), n=5. Values are means ± SEM. *P<0.05, **P<0.01. B: The expression of FOXA2 was measured in the adipose tissue from ob/ob, db/db, and diet-induced obese mice. (n=5). Values were means ± SEM. *P<0.05, **P<0.01. C: Expression levels of miR-141 and FOXA2 were measured by qRT-PCR in various tissues treated with LNA-miR-141 and those treated with LNA-scrambled negative control (LNA-SCR) (n=5). Values were means ± SEM. *P<0.05, **P<0.01. D: Knockdown of miR-141 by LNA-miR-141 induced weight loss and white adipose tissue in mice. Body weight and the white adipose tissue area were measured 72 h after the last injection. Values were means ± SEM. *P<0.05, **P<0.01. E: qRT-PCR analysis was used to measure the expression of adipogenesis-related markers C/EBPα, PPARγ, and AP2 from LNA-141-injected mice. β-actin was used as a negative control.

inhibitor of LNA-miR-141 remarkably decreased adipogenesis related transcription factors and adipocyte markers such as C/EBPα, PPARγ, and AP2 (**Figure 5E**).

Discussion

Obesity has reached epidemic proportions world-wide, which is characterized as increased lipid storage in adipocytes and increased number of adipocytes. By the presence of metabolic disorders, obesity constituted a substantial risk factor for hypertension, type 2 diabetes, cardiovascular diseases and certain cancers [16, 17].

Evidence indicated that the involvement of miRNA in obesity and may serve as sensitive and specific biomarkers. For example, miR-122 and miR-199a showed great increase in children with obesity compared with the controls [18]. miR-132 worked as a key regulator of hepatic lipid homeostasis and steatosis by synergistic multiple targets suppression with cumulative synergistic effects [19]. miR-204-5p could promote the adipogenic differentiation of human adipose-derived mesenchymal stem cells [20]. By suppressing the SIRT1-FOXO1 cascade, microRNA-146b promoted adipogenesis [21]. In human adipose tissue-derived stromal cells, MicroRNA-29b promotes the adipogenic differentiation [22]. Oppositely, MicroRNA-215 impaired adipocyte differentiation and co-represses FNDC3B [23]. miR-27a and miR-130 suppressed adipogenesis by inhibiting PPARγ [24]. The above results suggested that miRNAs played important roles in regulating adipocyte differentiation.

In this study, we examined the dynamical upregulated expression of miR-141 during adipogenesis in hADSCs and 3T3-L1. The overexpression of miR-141 enhanced the adipogenesis. The triglyceride quantification showed increased triglyceride accumulation in lipid droplets and the increase expression of C/EBPα, PPARγ, and AP2 in cells transfected with miR-141 mimics. Conversely, the knockdown of miR-141 suppressed adipogenic differentiation. We demonstrated that miR-141 directly bound to the 3'-UTR of FOXA2 and the downregulation of FOXA2 was from the transcriptional and translational levels. Furthermore, as known, Wnt/β-catenin signaling negatively regulated adipogenic differentiation in preadipocytes [25-27]. Our data strongly suggested that the regulation of c-myc and CCND1 was involved in the progress of miR-141 regulating the adipogenesis. Increased miR-141 level was observed in the white adipose tissue of ob/ob, db/db and diet-induced obesity (DIO) mice. Knockdown of miR-141 by LNA-miR-141 could reduce body weight and adiposity in obese mice and in consequence resulted in the upregulation of FOXA2 and downregulation of adipogenesis related markers in adipose tissues. In our study, we described the involvement of miR-141 in the pathogenesis of obesity and associated metabolic disorders. miR-141 represented as a potential diagnostic biomarker and promising therapeutic targets. Our topic may advance the understanding of the molecular basis about microRNAs in the obesity pathogenesis.

So far, regulation of energy intake by dietary and pharmacological treatments has met limited success. Our study provides the first evidence of the role for miR-141 in adipogenic differentiation through the regulation of FOXA2, and this miRNA could represent a new therapeutic target for anti-obesity drugs. We characterized the miR-141/FOXA2 pathway for preventing obesity.

Disclosure of conflict of interest

None.

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