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Original Article

miR-27 inhibits adipocyte differentiation via suppressing CREB expression

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miR-27 plays a negative role in the regulation of adipogenesis. However, the molecular mechanism still remains to be clarified. In the present study, we found that miR-27 inhibits adipogenesis partially by repressing the early adipogenic transcription factor cAMP response element-binding protein by directly targeting its 3′ untranslated region. In addition, we demonstrated that tumor necrosis factor-α (TNF-α) treatment up-regulates miR-27 through the NF-κB pathway. Furthermore, anti-miR-27 reduces the TNF-α-induced inhibition of adipogenesis. Simultaneously, the levels of miR-27 expression were decreased in mature adipocytes of obese mice when compared with lean mice. Our data revealed a novel mechanism of miR-27 in the regulation of adipogenesis.

Keywords miR-27; 3T3-L1 cells; TNF-α; adipogenesis

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Introduction

Obesity is increasing every year worldwide and it increases the likelihood of various diseases, particularly Type 2 diabetes, cardiovascular disease and certain types of cancer [1]. The program of adipogenesis is regulated by the sequential activation of transcription factors and several signalling pathways. Adipogenic differentiation is accomplished by a cascade of three major transcriptional events: (i) the early genes C/EBPβ and C/EBPδ, (ii) the determination genes PPARγ and C/EBPα, and (iii) adipocyte-specific genes such as those encoding fatty acid synthase and fatty acid-binding proteins [2–4].

miRNAs are small non-coding RNA molecules of 21–23 nucleotides in length, which function in the post-transcriptional regulation of gene expression [5]. Recent studies indicated that numerous inhibitory and stimulatory miRNAs were involved in the regulation of adipogenesis, such as the commitment stage and the terminal differentiation [6]. For example, it was shown that inhibition of miR-143 in differentiating human adipocytes reduced the level of triglyceride accumulation and ultimately abolished adipocyte differentiation [7]. The miR-17-92 cluster was found to accelerate adipocyte differentiation by inhibiting the tumor suppressor Rb2/p130 during early clonal expansion of preadipocytes [8]. It was also shown that over-expression of miR-138 reduced triglyceride accumulation and inhibited the expression of adipogenic markers such as C/EBPα and PPARγ [9]. miR-27a/b and miR-130 are known adipogenic inhibitors that target PPARγ directly [10–12]. Decreased expression of miR-155, miR-221, and miR-222 has been found during the adipogenic programing, suggesting that they act as negative regulators of differentiation [13]. miR-448 selectively targets KLF5 to down-regulate its induction of PPARγ expression, thereby reducing adipocyte differentiation [14]. Tumor necrosis factor (TNF-α)-mediated over-expression of miR-155 via the NF-κB pathway results in the down-regulation of C/EBPβ and cAMP response element-binding protein (CREB), thereby inhibiting the transcription of later adipogenic markers PPARγ and C/EBPα [15].

In the present study, we investigated the role of miR-27 in adipogenesis. We found that miR-27 inhibits 3T3-L1 adipocyte differentiation by repression of CREB. Simultaneously, TNF-α treatment up-regulates miR-27 expression through the NF-κB pathway. In addition, the level of miR-27 expression was down-regulated in adipocyte tissues of obese mice compared with that of lean mice. These results demonstrated that miR-27 can be a potential target for the treatment of obesity.

Materials and Methods

Mice

C57BL/6J and *ob/ob* mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences

(Shanghai, China). All animals were housed and maintained under pathogen-free conditions. All animal experiments were performed in compliance with the guide for the care and use of laboratory animals and were approved by the institutional biomedical research ethics committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Cell culture and differentiation

Mouse embryonic fibroblast-derived 3T3-L1 preadipocytes were maintained and differentiated in a 37°C incubator with 10% CO_2 as previously described [16,17]. The differentiation of the 3T3-L1 adipocyte monolayers was analyzed with an Oil-Red-O staining assay.

Western blot analysis

The protocol was used as previously described [16]. All primary antibodies were incubated with the membrane at 4°C overnight: CREB (#9197; Cell Signaling, Boston, USA), FOXO1 (#9454; Cell Signaling), and tubulin (T6199; Sigma, California, USA). Membranes were washed with 1 × Tris buffered saline-with Tween-20 and incubated with either anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, California, USA).

RNA isolation and real-time PCR analysis

Total RNA from 3T3-L1 cells was isolated with the miRVana Isolation Kit (Ambion, California, USA) according to the manufacturer's instructions. About 0.5 μg of total RNA from each sample was reverse-transcribed into complementary DNA using the PrimeScript™ RT Reagent Kit (Takara, Dalian, China). The miRNA levels were quantitatively assessed by an SYBR green-based quantitative real-time polymerase chain reaction (PCR) with gene-specific primers (**Supplementary Table S1**) in a PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, California, USA) according to the manufacturer's instructions. U6 was used as an internal normalization control.

ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed with a commercial kit (Upstate Biotechnologies, Inc., New York, USA) according to the manufacturer's instructions. Chromatin samples were prepared from 3T3-L1 cells at the indicated time points after TNF-α treatment and then immunoprecipitated with the corresponding antibodies according to the protocol described previously [17].

Luciferase reporter transfection and dual-luciferase assay

In the 3′ untranslated region (3′ UTR)-reporter assay, 293T cells cultured in 24-well plates were grown to 80%–90% confluence and then transfected with 50 ng of the 3′ UTR reporter (pGL3-CREB, mutant-pGL3-CREB, pGL3-PPARγ, pGL3-RXRA, or pGL3-RBL2), 20 ng of the transfection control Renilla vector (pRL-TK; Promega, Wisconsin, USA), and 100 nM miRNA precursor molecules (Ambion) along with 1 μl of Lipofectamine 2000 (Life Technologies, California, USA). Lysates were harvested 24 h after transfection, and reporter activity was measured with the dual-luciferase assay (Promega). Mutation of the sites of miR-27 binding to CREB 3′ UTR was performed using the KOD-Plus-Mutagenesis Kit (SMK-101; Toyobo, Shanghai, China) according to the manufacturer's instructions. Relative luciferase levels were calculated with the formula $(S_{Luc}/S_{renilla})/(C_{Luc}/C_{renilla})$, where Luc is raw firefly luciferase activity, renilla is the internal transfection control of renilla activity, S is the sample, and C is the control pre-scramble.

Results

miR-27 inhibits 3T3-L1 adipocyte differentiation

Several studies have demonstrated that miR-27 inhibits adipogenic differentiation. In the present study, we demonstrated that the expressions of miR-27a and miR-27b are down-regulated after hormone induction (**Fig. 1A,B**). This down-regulation implies that miR-27 may play a negative role for 3T3-L1 adipocyte differentiation. Over-expressed miR-27a, miR-27b or miR-27a/b inhibits 3T3-L1 cell differentiation compared with pre-miR-NC (**Fig. 1C**). To further determine whether miR-27a/b inhibits adipogenesis specifically, we investigated the effect of miR-27a/b on the myogenic differentiation of the C2C12 myoblast cells. Formation of myofibers was not adversely affected by miR-27 over-expression, indicating that miR-27 does not play an important role in myogenic differentiation (**Fig. 1D**).

miR-27 inhibits 3T3-L1 cell differentiation by directly targeting CREB 3′ UTR

CREB plays a critical role in the early stage of 3T3-L1 cell differentiation after hormone stimulation [18,19]. By searching miRNA target prediction websites from TargetScan, miRanda and PicTar, we found that CREB is a putative target of miR-27, having a 3′ UTR element that is partially complementary to this miRNA (**Fig. 2A,B**). We transfected 3T3-L1 cells with exogenous mouse-miR-27 precursor molecules and analyzed the expression of CREB by western blotting. The results showed that the expression of mature miR-27a or miR-27b increased by 10–15 folds compared with the control (**Fig. 2C**), and that CREB was partially down-regulated in miR-27 transfected cells (**Fig. 2D**). Simultaneously, miR-27 inhibits the FOXO1 expression which is consistent with the previous study [20].

We also performed a luciferase reporter assay to test whether miR-27 directly binds to the 3′ UTR of CREB. The results showed that over-expression of miR-27a or miR-27b

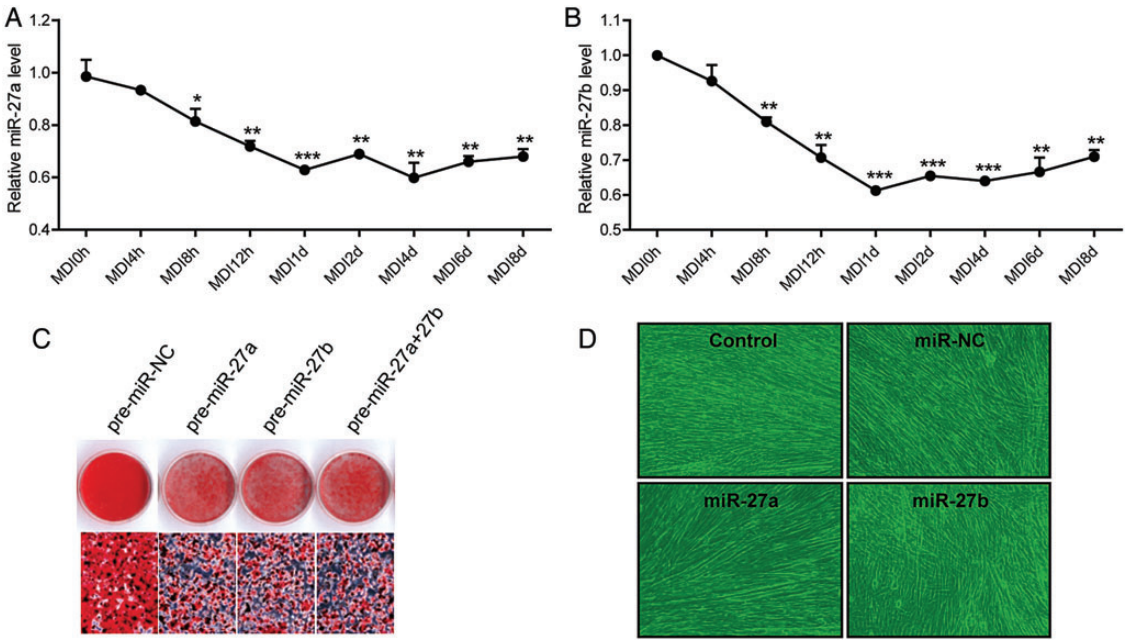

Figure 1. miR-27 is a negative regulator of 3T3-L1 adipogenesis (A,B) The endogenous expression pattern of miR-27a (A) and miR-27b (B) at the stage of 3T3-L1 adipocyte differentiation. The expressions of miR-27a or miR-27b at indicated times were assessed by an SYBR green-based quantitative real-time PCR. The data shown are averages of three independent experiments (mean ± SD). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (C) Over-expression of miR-27 results in the inhibition of adipocyte differentiation. 3T3-L1 preadipocytes were transfected with pre-miR-27a, pre-miR-27b or pre-miR-27a + 27b (100 nM) and then subject to adipocyte differentiation. Adipocytes were assayed with Oil-Red-O on day 8 of differentiation. (D) C2C12 myoblast cells were transfected with indicated miRNAs or untransfected. Myogenic differentiation was initiated at 48 h post-transfection by maintaining the cells in culture medium containing 2% horse serum.

effectively inhibits the luciferase activity of pGL3-CREB (**Fig. 2E**). In addition, when the miR-27 binding sites in the 3′ UTR are mutated, the luciferase activities of mutant-pGL3-CREB under the conditions of miR-27 over-expression are similar to that of the control, which indicates that these binding sites directly mediate the miR-27 repression (**Fig. 2E**). Furthermore, miR-27 also inhibits the luciferase activities of pGL3-PPARγ and pGL3-RXRA. In contrast, the luciferase activity of pGL3-RBL2 was not changed by miR-27 over-expression (**Fig. 2E**). Taken together, these *in vitro* results suggested that miR-27 may inhibit the expression level of CREB directly.

TNF-α induces miR-27 expression through the NF-κB pathway and repression of miR-27 reduces the TNF-α-induced inhibition of 3T3-L1 adipogenesis

The role of TNF-α in the inhibition of 3T3-L1 adipocyte differentiation has been investigated in several studies [15,19,21,22]. In order to investigate whether miR-27 can be regulated by TNF-α, we analyzed the expression level of miR-27a/b after TNF-α treatment. The results demonstrated that TNF-α up-regulates miR-27a/b expression in 3T3-L1 preadipocytes in a dose- and time-dependent manner (**Fig. 3A,B**).

Previous reports showed that TNF-α activates the transcription factor NF-κB in response to many types of stresses [23–26]. Therefore, we proposed that TNF-α might up-regulate miR-27a/b expression through the NF-κB pathway. Furthermore, we determined that NF-κB can localize to miR-27 gene regulatory elements based on online databases (http://microrna.sanger.ac.uk; http://jaspar.genereg.net). Our immunofluorescence results demonstrated that NF-κB translocates into the nucleus within 30 min after TNF-α treatment in 3T3-L1 preadipocytes (**Fig. 3C**). These results suggest that the NF-κB nuclear translocation induced by the TNF-α treatment is involved in miR-27 up-regulation. Four putative NF-κB binding sites were identified in 5-kb genomic sequence upstream of the mouse-miR-27 gene (**Fig. 3D**). We designed four PCR amplicons to check the presence of these putative binding sites in a ChIP assay (**Supplementary Table S1**). The results showed that miR-27 gene might be directly regulated by the specific binding of NF-κB to its putative promoter (**Fig. 3D**). This result was confirmed by ChIP-qPCR (**Fig. 3D**). These results indicated that TNF-α treatment up-regulates miR-27 expression through specific binding of NF-κB to the putative promoter of miR-27 in 3T3-L1 preadipocytes.

Given that the expression of CREB can be repressed by both TNF-α [15] and miR-27, and that TNF-α treatment can up-regulate the expression of miR-27 in 3T3-L1 adipocytes, we proposed that the down-regulation of miR-27 expression can reduce the TNF-α-induced inhibition of 3T3-L1 adipocyte differentiation. In order to verify this hypothesis, we

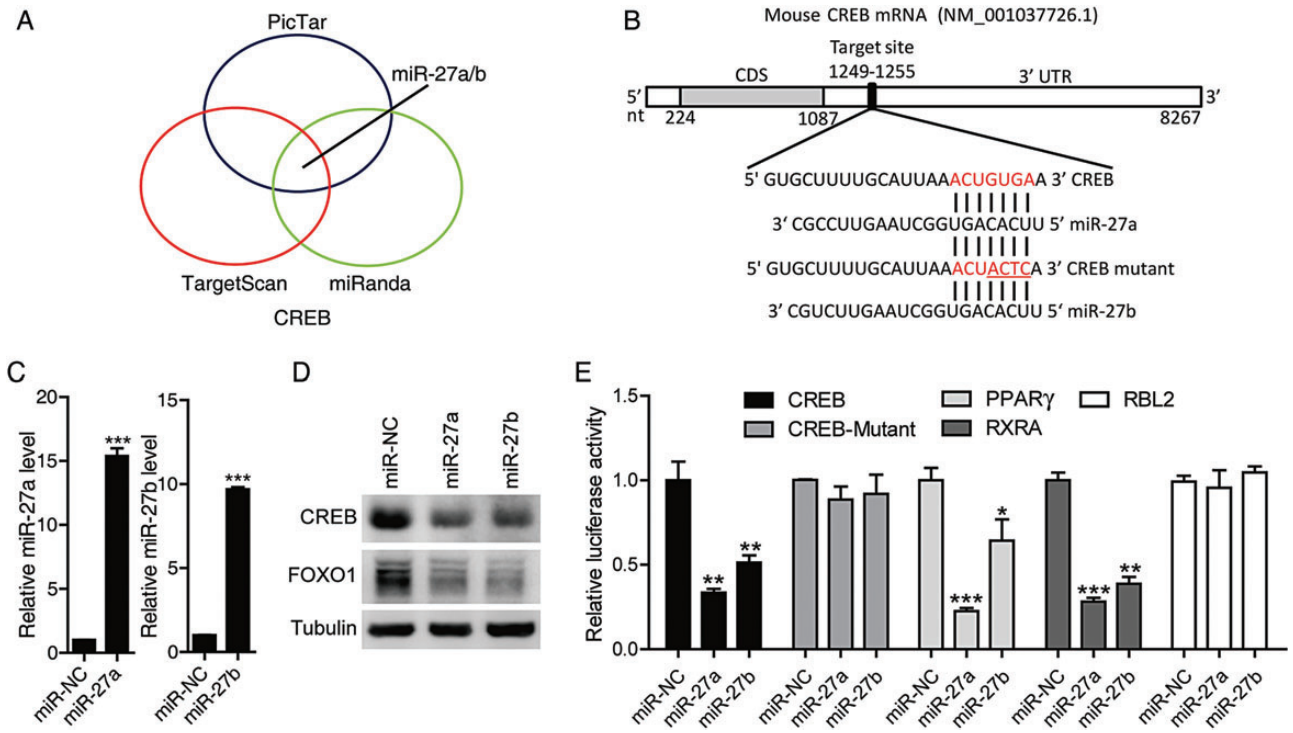

Figure 2. Over-expression of miR-27 suppresses CREB expression directly (A) CREB is a potential target gene of miR-27 predicted by TargetScan, miRanda, and PicTar. (B) Schematic diagram of the predicted miR-27 target site in the 3′ UTRs of CREB. The underlined nucleotides (target sites) were mutated. (C) Detection of exogenous miR-27a or miR-27b expression in 3T3-L1 preadipocytes. Expression of transfected miR-27 was assessed by an SYBR green-based quantitative real-time PCR. (D) Confluent 3T3-L1 preadipocytes were transfected with the miR-27a or miR-27b precursor molecules (100 nM). The expressions of CREB and FOXO1 were analyzed by western blotting. Tubulin was used as a loading control. (E) miR-27 regulates CREB by directly binding to its 3′ UTR. 293T cells in 24-well plates were transfected with indicated 3′ UTR reporters (50 ng), renilla vector (pRL-TK, 20 ng), and miRNA precursor molecules (100 nM). Lysates were harvested 24 h after transfection, and reporter activity was measured by the dual-luciferase assay. Data sets marked by brackets in the panel were compared using the Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The error bar represents the standard deviation (SD) from three independent experiments.

transiently transfected 3T3-L1 preadipocytes with miR-27 antisense (anti-miR-27) and then subjected the cells to adipogenesis analysis. The results showed that TNF-α-induced inhibition of adipogenesis is partially reversed by the transfection of anti-miR-27 in cells treated with 2 ng/ml TNF-α (**Fig. 3E**). This result indicated that up-regulation of miR-27 expression was involved in TNF-α-induced inhibition of adipogenesis, and down-regulation of miR-27 could counteract the inhibition of adipogenesis in TNF-α treatment.

Down-regulation of miR-27 in mature adipocytes of obese mice

In order to gain insights into the potential biologically relevant role of miR-27 in the regulation of adipose tissue function *in vivo*, we examined the expression of miR-27 in the genetically obese *ob/ob* mice. Compared with that of C57BL/6J mice, the weight of *ob/ob* mice was increased significantly (**Fig. 4A**). Simultaneously, the expression level of CREB was increased in epididymal adipocytes (epi. ad.) of the *ob/ob* mice (**Fig. 4B**). In contrast to CREB expression, the expression levels of miR-27 were decreased in the epididymal adipocytes (epi. ad.), subcutaneous adipocytes (sub. ad.) and omental adipocytes (ome. ad.) from the *ob/ob* mice, when compared with that of the genetically matched lean C57BL/6J mice of the same gender and age (**Fig. 4C,D**). In addition, the levels of miR-27 expression were not changed in other tissues of both *ob/ob* and C57BL/6J mice (**Fig. 4C,D**). These data imply that miR-27 plays a negative role in adipocyte differentiation, and down-regulation of miR-27 may contribute to the dysregulated adipogenesis in fat tissues of obese mice.

Discussion

Adipose tissue is not only an energy depot [27], but also a source of endocrine factors [28,29], which is essential for energy metabolism. Adipogenic differentiation is accomplished by several key stages, and C/EBPβ and CREB play crucial roles in the early stage of differentiation. In our previous study, we demonstrated that miR-155 inhibits adipocyte differentiation by repressing both C/EBPβ and CREB expressions through targeting their 3′ UTR. However, whether there are other miRNAs that inhibit adipocyte

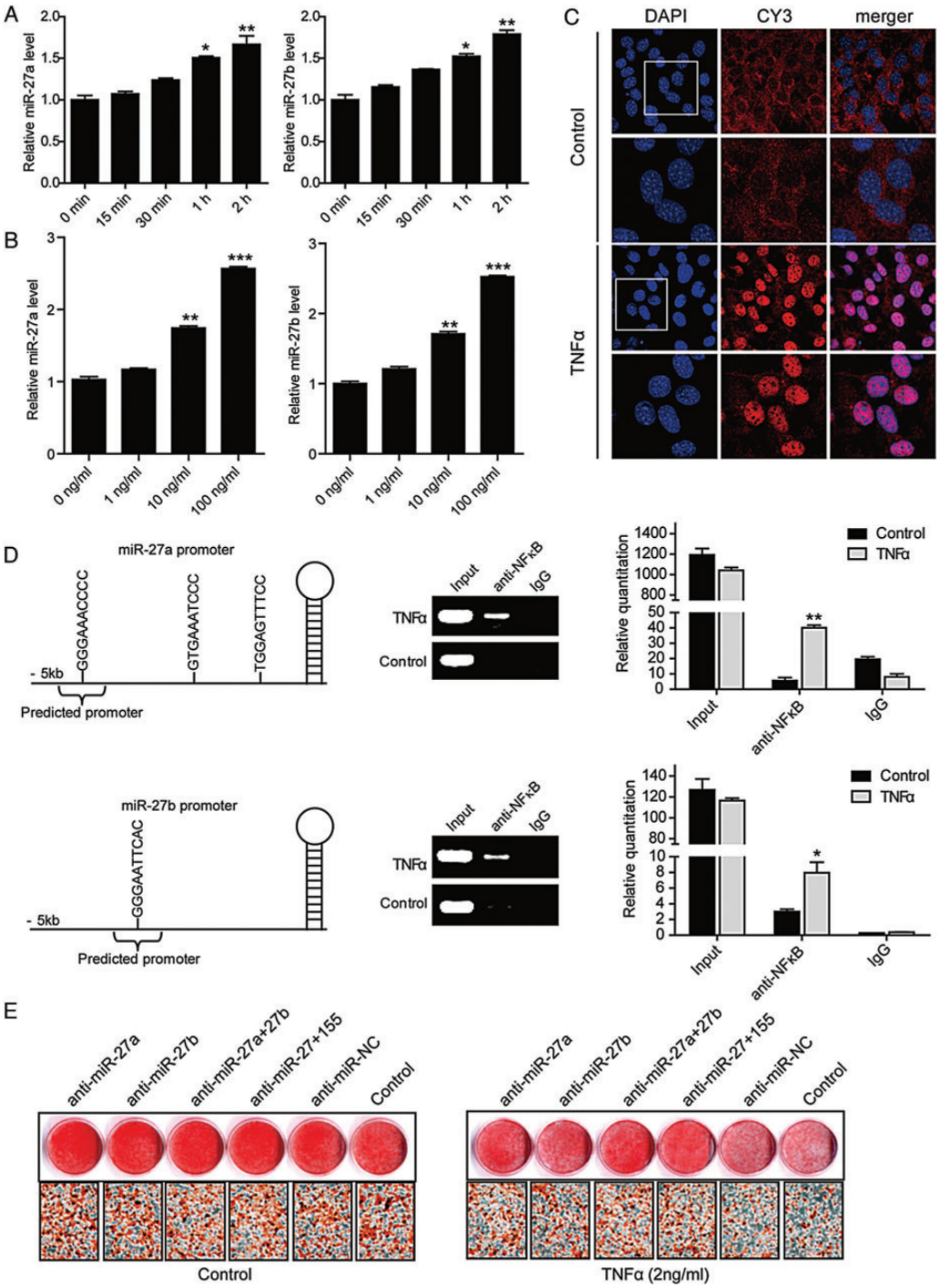

Figure 3. TNF-α induces miR-27 expression through the NF-κB pathway and anti-miR-27 reduces the TNF-α-induced inhibition of 3T3-L1 adipocyte differentiation (A) TNF-α induces miR-27 expression in a time-dependent manner. Cells were treated with 10 ng/ml TNF-α, and RNA samples were collected at indicated times for qPCR analysis. (B) TNF-α induces miR-27 expression in a dose-dependent manner. Confluent 3T3-L1 preadipocytes were treated with TNF-α at the indicated concentrations for 2 h, after which miR-27 expression was detected with a quantitative real-time PCR. (C) 3T3-L1 preadipocytes grown on coverslips were treated with 10 ng/ml TNF-α for 30 min and analyzed by immunofluorescence. (D) NF-κB regulates miR-27a or miR-27b expression by directly binding to its predicted promoter. The indicated PCR amplicons are putative binding sites for NF-κB. Confluent 3T3-L1 preadipocytes were treated with or without TNF-α (10 ng/ml) for 30 min, then chromatin samples were prepared as indicated and subject to ChIP assays using antibodies against p65 or IgG. PCR or real-time PCR was performed with primers specific for indicated sites of miR-27a or miR-27b promoter. The results are presented as mean ± SD from three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (E) Confluent 3T3-L1 preadipocytes were transfected with indicated anti-miRNAs (100 nM) or anti-miR-NC (100 nM, negative control). At 48 h post-transfection, adipogenesis was induced with or without TNF-α (2 ng/ml) treatment. Adipocytes were assayed with Oil-Red-O on day 8 of differentiation.

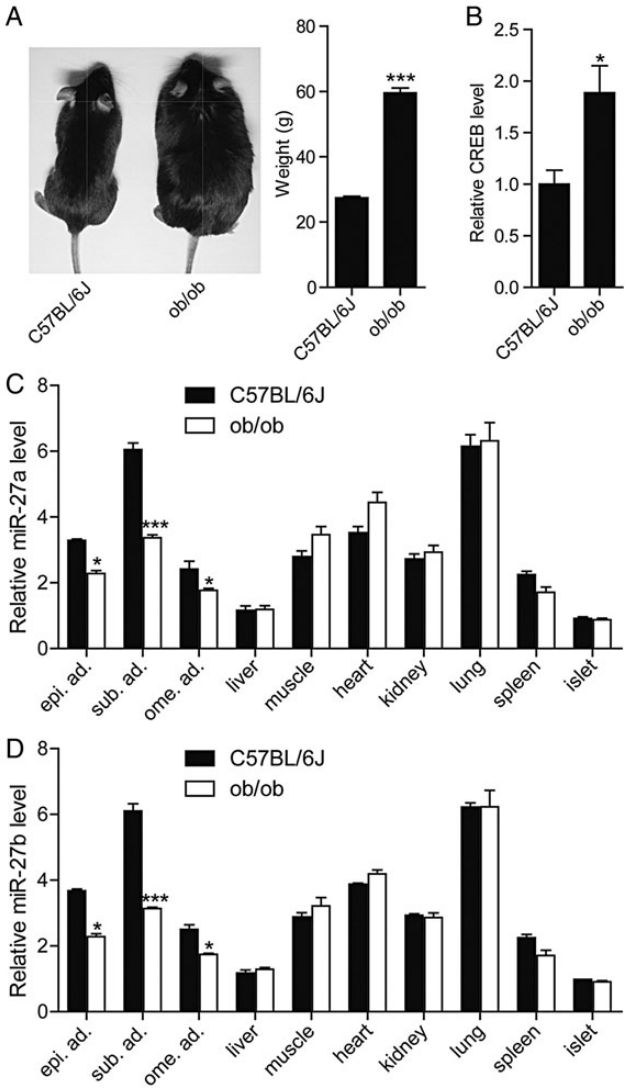

Figure 4. Expression levels of miR-27 in adipocyte tissue of C57BL/6J or *ob/ob* mice (A) Weight of C57BL/6J and *ob/ob* mice. Nineteen weeks old mice were sacrificed for analysis, $n = 5$ for each group. (B) q-RT-PCR analyses were conducted to detect the expression levels of CREB at epididymal fat pads harvested from *ob/ob* mice and genetically matched lean mice ($n = 5$). (C and D) Total RNA was prepared from indicated tissues harvested from *ob/ob* mice and genetically matched lean mice. Levels of miRNA expression were analyzed by q-RT-PCR. Data are presented as mean ± SD from five individual mice of each group. $^*P < 0.05$, $^{***}P < 0.001$ (*ob/ob* versus lean).

differentiation through early transcription factors still remains to be clarified.

Although miR-27 has been found to be a negative regulator of adipocyte differentiation via suppressing PPARγ expression [10,30], our results demonstrated that miR-27 plays a role in the early stage of adipocyte differentiation but not in the late stage, which is consistent with the previous study [11]. Therefore, the inhibition of adipocyte differentiation by miR-27 is not fully through targeting PPARγ. In the present study, miR-27 was predicted to target CREB 3′ UTR by searching several miRNA target gene prediction websites. We investigated the role of miR-27 in the regulation of early transcription factor CREB during the differentiation process of 3T3-L1 preadipocytes. We found that miR-27 represses CREB expression by targeting its 3′ UTR in a direct manner, which confirms that miR-27 inhibits 3T3-L1 adipogenesis in the early stage.

Simultaneously, TNF-α treatment up-regulates miR-27 expression, which results in the inhibition of 3T3-L1 adipocyte differentiation by repression of CREB. In addition, the TNF-α-induced inhibition of adipocyte differentiation is reduced by anti-miR-27. In addition, our results are consistent with a previous research which revealed that miR-27 expression was lower in mature adipocytes from obese mice than those from lean mice, indicating that miR-27 down-regulation may be necessary for adipocyte hypertrophy [30].

Taken together, our results provide a new perspective into the role of miR-27 in adipogenesis, but the precise mechanism of miR-27 in the inhibition of adipocyte differentiation still needs further investigation *in vivo*.

Supplementary Data

Supplementary data are available at *ABBS* online.

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