

# Tumor necrosis factor- $\alpha$ and interleukin-6 suppress microRNA-1275 transcription in human adipocytes through nuclear factor- $\kappa$ B

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**Abstract.** Obesity is a confirmed risk factor for hyperlipidemia, type-II diabetes, hypertension, and cardiovascular disease. MicroRNAs (miRs) have emerged as an important field of study within energy metabolism and obesity. A previous study demonstrated miR-1275 to be markedly down-regulated during maturation of human preadipocytes. It has been reported that miR-1275 dysregulates expression in several types of cancer and infections. Little is currently known about the regulation of miR-1275 transcription. The aim of the current study was to explore the mechanism underlying the expression of miR-1275 in mature human adipocytes. After differentiation, human adipocytes were incubated with tumor necrosis factor (TNF)- $\alpha$  and interleukin-6. The results of reverse transcription-quantitative polymerase chain reaction demonstrated that miR-1275 can be down-regulated by TNF- $\alpha$  and IL-6, in human mature adipocytes. Bioinformatic analysis was used to predict nuclear factor (NF)- $\kappa$ B binding sites of miR-1275's promoter region. Luciferase assay and rescue experiments were performed in HEK293T cells. NF- $\kappa$ B was involved in regulating miR-1275 transcription by binding to its promoter. In response to TNF- $\alpha$ , NF- $\kappa$ B was bound to the promoter of miR-1275 and inhibited its transcription. These results indicated that inflammatory

factors could regulate miR-1275 transcription through NF- $\kappa$ B and influencing miR-1275 effects on obesity.

## Introduction

As of 2014, 39% of adults worldwide were overweight and 13% were obese (1). Obesity is a confirmed risk factor for hyperlipidemia, type-II diabetes, hypertension and cardiovascular disease (2). Globally, 44% cases of diabetes, 23% cases of ischemic heart disease and 7-41% cases of certain types of cancer are attributable to excess weight and obesity (1). Therefore, reducing the rates of being overweight and obese will have benefits for all. Recently, new insights have indicated that microRNAs (miRNAs/miRs) serve a role in obesity and may be suitable therapeutic targets (3). miRNAs are a class of small endogenous non-coding RNAs that are involved in post-transcriptional gene repression (4). Epigenetic regulation of adipose functions mediated by miRNAs has emerged as an important mechanism in the study of energy metabolism and obesity in the last decade (5). Numerous miRNAs have been demonstrated to be associated with physiopathological disorders associated with obesity (6).

miR-1275, a 17 base pair (bp) intergenic microRNA, lies on chromosome 6 (7). A previous study demonstrated that miR-1275 was markedly down-regulated during maturation of human preadipocytes (8); less expression of miR-1275 was also observed in obese individuals compared with the non-obese ones (8). miR-1275 inhibits human visceral pre-adipocyte differentiation by silencing of ELK1, an E-twenty-six-domain transcription factor associated with adipocyte differentiation (2,8). These findings indicated that down-regulation of miR-1275 is involved in adipogenesis and obesity. Notably, lower levels of miR-1275 expression have been reported in some other diseases, such as hepatocellular carcinoma (9), breast cancer (10), coronary heart disease and some infections (11). These studies demonstrated that the down-regulation of miR-1275 is involved not only in obesity, but also in cancers and infections. However, little is known about the regulation of miR-1275 transcription. Further study is needed to determine how miR-1275 expression is down-regulated in obesity.

In obese individuals, adipose tissue is in a state of chronic, low-grade inflammation (12). Increased tumor necrosis factor

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(TNF)- $\alpha$  and interleukin (IL)-6 levels have been demonstrated to be notable markers of chronic inflammation in white adipose tissue (13). High levels of TNF- $\alpha$  and IL-6 are associated with the regulation of microRNA transcription in adipocytes (14). For instance, the expression of miR-146b in adipose tissue is up-regulated by TNF- $\alpha$  and IL-6 (15). It was therefore hypothesized that miR-1275 expression in adipose tissue may be regulated by TNF- $\alpha$  and IL-6. In the present study, a series of experiments were performed and the results confirmed the above hypothesis.

## Materials and methods

**Cell culture and treatment.** Human visceral pre-adipocytes (ScienCell Research Laboratories, San Diego, CA, USA) were maintained in preadipocyte medium containing 5% fetal bovine serum, 1% preadipocyte growth supplement, and 1% penicillin/streptomycin solution (PAM, cat. no. 7211; all ScienCell Research Laboratories) at 37°C in an incubator under 5% CO<sub>2</sub> and 95-100% humidity (16). To induce differentiation, serum-free PAM (containing 50 nM insulin, 100 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 100 mM rosiglitazone) was added to confluent human preadipocytes (day 0), and the medium was replaced every 2 days for the next 4 days. Thereafter, the medium was replaced with serum-free PAM containing 50 nM insulin and replaced every 2 days until lipid droplets had accumulated in cells (day 15) (17). Differentiated adipocytes were used for experiments 15 days later, when >80% of cells demonstrated morphological and biochemical properties of adipocytes. After an overnight incubation in serum-free PAM, human adipocytes were treated with a final concentration of 10 ng/ml TNF- $\alpha$  (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or 30 ng/ml IL-6 (Sigma-Aldrich; Merck KGaA, Germany) for 0, 4, 8 or 24 h at 37°C in an incubator under 5% CO<sub>2</sub> and 95-100% humidity (18). Human embryonic kidney 293T (HEK293T) cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Bovogen Biologicals, Keilor East, Victoria, Australia). HEK293T cells were also incubated at 37°C in an incubator under 5% CO<sub>2</sub> and 95-100% humidity (17).

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total miRNA from human adipocytes was extracted using an miRNeasyMiNi kit (Qiagen GmbH, Hilden, Germany). The quality of microRNA was assessed using 1% agarose gel electrophoresis and the concentration of microRNA was measured using spectrophotometry at 260 nm. Equal quantities of microRNA (200 ng) were used to synthesize cDNA using TaqMan microRNA Reverse Transcriptase kit (Applied Biosystems; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol (Table I). qPCR with SYBR (Power SYBR™ Green PCR Master Mix; cat. no. 4368577; Thermo Fisher Scientific, Inc.) was carried out using an Applied Biosystems 7500 Sequence Detection system (ABI 7500 SDS; Thermo Fisher Scientific, Inc.) following manufacturer's protocol (Table II). Relative gene expression was quantified using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (18). Total microRNA expression was

normalized to small nuclear (sn)RNA U6. Primer cat. numbers are: 001973 for snRU6 and 002840 for miR-1275 (Applied Biosystems; Thermo Fisher Scientific Inc.).

**Bioinformatic analysis.** Hsa-miR-1275 precursor (pri-miR-1275) sequence was obtained from Ensembl ([www.asia.ensembl.org/index.html](http://www.asia.ensembl.org/index.html)). The precursor sequence was numbered from +1 to +80, and therefore 1,500 bp upstream of pri-miR-1275 was considered to be the predicted promoter region. To identify the predicted binding sites of NF- $\kappa$ B, the sequence of the predicted promoter was uploaded to Genomatrix ([www.genomatrix.de](http://www.genomatrix.de)), Jaspar ([jaspar.genereg.net](http://jaspar.genereg.net)), and Promo\_v3 ([algggen.lsi.upc.es/cgi-bin/promo\\_v3/promo/promoinit.cgi?dirDB=TF\\_8.3](http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)), and the intersection of putative binding sites was recorded (19-21).

**Plasmids and promoter reporter constructs.** The predicted promoter region of miR-1275 was amplified by PCR with TaqDNA polymerase (cat. no. DR100A; Takara Biotechnology Co., Ltd., Dalian, China). DNA was amplified at 95°C for 40 sec for denaturation, 55°C for 35 sec for renaturation and 130 sec for elongation with 30 cycles. The site-specific mutation vectors aimed at the binding site of NF- $\kappa$ B were constructed by introducing point mutations with overlap-extension PCR. The predicted binding sites of NF- $\kappa$ B are presented in Table III. PCR products were then cloned into the dual-luciferase reporter plasmid PEZX-FR01 using *MluI*/*Bam*HI restriction sites (GeneCopoeia Inc., Rockville, MD, USA). The primer sequences used are listed in Table IV.

**Dual-luciferase assays.** HEK293T cells were cultured in 24-well plates at density of 2x10<sup>4</sup> cells/well. When grown to a density of 60-70%, they were transfected with 2  $\mu$ g/well reporter plasmids and 6- $\mu$ l/well Lipofectamine (Invitrogen; Thermo Fisher Scientific Inc.) for 6 h. Empty PEZX-FR01 plasmid served as a control. Following this, cells were washed with serum-free medium and treated with the NF- $\kappa$ B activator TNF- $\alpha$  (10 ng/ml) for 24 h. For the rescue assay, HEK293T cells were treated with the NF- $\kappa$ B inhibitor 4-methyl-N1-(3-phenyl-propyl)-benzene-1,2-diamine (10  $\mu$ M; Calbiochem; Merck KGaA) for 1 h. Subsequently, cells were washed with serum-free medium and treated with TNF- $\alpha$  (10 ng/ml) for 24 h. After this, luciferase activity was analyzed using a dual luciferase reporter assay (Promega Corporation, Madison, WI, USA) with a GloMax 96 Microplate Luminometer E6501 (Promega Corporation) (22). Firefly luciferase activity was normalized to the *Renilla* luciferase activity.

**Statistical analysis.** The data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). All data are presented as the mean  $\pm$  standard deviation. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression of miR-1275 in human adipocytes treated with TNF- $\alpha$  or IL-6.** Human mature adipocytes were treated with 10 ng/ml TNF- $\alpha$  or 30 ng/ml IL-6 for 24 h, and the expression

Table I. Temperature protocol for the microRNA reverse transcription.

Step	Temp, °C	Time, sec
Hold	16	30
Hold	42	30
Hold	85	5
Hold	4	∞

Table II. qPCR protocol implemented in the present study.

Cycling condition	Temp, °C	Time, sec
Initial denaturation/hot start	95	30
Repeat steps 1-3 through 40 cycles		
Step 1	95	5
Step 2	58	10
Step 3	72	15

of miR-1275 was examined at different times (0, 4, 8 and 24 h). The expression of miR-1275 was normalized to the expression of snRU6. After treatment with TNF- $\alpha$ , miR-1275 relative expression was significantly lower than in untreated control cells (Fig. 1A). A similar reduction in miR-1275 expression was also observed in human adipocytes treated with IL-6 (Fig. 1B). These results indicated that TNF- $\alpha$  and IL-6 could regulate the expression of miR-1275 in human adipocytes.

**Prediction of promoter regions of miR-1275.** Bioinformatic analysis was performed on the 1,500 bp upstream sequence of pri-miR-1275 to further determine how TNF- $\alpha$  and IL-6 regulate miR-1275 expression. Several binding sites of NF- $\kappa$ B, the downstream transcription factor of TNF- $\alpha$  and IL-6, were identified on the 1,500 bp sequence of the predicted promoter. This suggested that TNF- $\alpha$  and IL-6 may regulate miR-1275 expression through NF- $\kappa$ B. To locate the strongest binding site of NF- $\kappa$ B, the sequence of putative promoters was submitted to the following websites: Genomatrix, Jaspas and Promo\_v3. Two sites (from -806 to -792 and from -288 to -274) were predicted by all three websites (Table II). Therefore, these were chosen to be the strongest binding sites of NF- $\kappa$ B (Fig. 2).

**Confirmation of NF- $\kappa$ B binding sites on the promoter region of miR-1275.** To determine whether the two predicted binding sites were functional, different promoters were made and their activity was examined in HEK293T cells. For the deletion assay (Fig. 2A), dual-luciferase reporter plasmids PEZX-FR01 of Pro-1 (including both sites), Pro-2 (only including the -288 site) and Pro-3 (including neither site) were transfected into HEK293T cells. Pro-1 demonstrated >10 times higher luciferase activity compared with the empty reporter plasmids, whereas Pro-2 and Pro-3 showed similar luciferase activity compared with the control (Fig. 3A). These results suggested that the sequence from -840 to +1 was the promoter region of miR-1275, and the -806 site was necessary for the promoter's activity (Fig. 3B and C).

To confirm the role of -288 site, the core binding sequences of the -806 and -288 sites were mutated (Fig. 4A and B). The luciferase activities of Mut-1 (mutation on -806 site), Mut-2 (mutation on -288 site) and Mut-3 (mutation on both -806 and -288 sites) demonstrated no difference compared with the empty reporter plasmids (Fig. 4C). These results suggested that the -288 site was also necessary for the promoter activity.

**Effects of TNF- $\alpha$  and JSH-23 on miR-1275 promoter activity.** To confirm the above-mentioned results, a rescue assay was performed. HEK293T cells were transfected by Pro-1-PEZX-FR01 and then treated with JSH-23 before treatment with TNF- $\alpha$ . As presented in Fig. 5, cells treated with JSH-23 and TNF- $\alpha$  demonstrated significantly more luciferase activity than cells treated with TNF- $\alpha$  alone. These results confirmed that TNF- $\alpha$  decreased miR-1275 promoter activity through NF- $\kappa$ B.

## Discussion

A previous study has demonstrated that miR-1275 is markedly down-regulated during differentiation and maturation of human preadipocytes by miRNA expression microarrays (8). Reduced miR-1275 expression in obese individuals compared with non-obese individuals has been observed. Additionally, miR-1275 has been reported to be dysregulated in certain types of cancer and infection (23). Fawzy *et al* (7) reported that miR-1275 expression is downregulated cirrhotic and cancerous liver tissue. However, the exact mechanism is currently unclear.

In the present study, miR-1275 expression was downregulated by >60% in response to TNF- $\alpha$  and by 50% in response to IL-6 in mature adipocytes, which suggested that inflammatory cytokines could serve an important role in regulating miR-1275 expression in adipocytes. Obesity is characterized by increased production of cytokines such as IL-6, IL-1, and TNF- $\alpha$  (24). The role of miRNAs in inflamed adipose tissues has been highlighted in several studies (14). Shi *et al* (17) reported that IL-6 and TNF- $\alpha$  induce obesity-associated inflammatory responses through transcriptional regulation of miR-146b. Another study also demonstrated Mir-335 to be involved in adipose tissue inflammation (25). The present study was consistent with previous ones. It has confirmed that miR-1275 may be downregulated by TNF- $\alpha$  and IL-6. The role of TNF- $\alpha$  and IL-6 in regulating miR-1275, luciferase assays and rescue experiments were implemented based on bioinformatics analysis.

NF- $\kappa$ B is a downstream transcription factor of TNF- $\alpha$  and IL-6 (26). TNF- $\alpha$  activates NF- $\kappa$ B by enhancing nuclear translocation of P65, which is the active subunit of NF- $\kappa$ B (27,28). Promoter analysis of the miR-146a gene revealed that NF- $\kappa$ B serves a critical role in inducing its transcription by LPS, TNF- $\alpha$ , and IL-1 (29). miR-335 is also proved to be up-regulated by TNF- $\alpha$  and IL-6 through the NF- $\kappa$ B signaling pathway in adipocytes (25). Previous studies have also demonstrated that let-7 and miR-365 are under the control of NF- $\kappa$ B (30,31). To determine whether NF- $\kappa$ B is involved in regulating miR-1275, a 1,500 bp upstream sequence of miR-1275 was uploaded to Jaspas, a database that uses a transcription factor flexible models

Table III. NF- $\kappa$ B binding sites predicted by Genomatrix, Jaspar and Promo\_v3.

Sequence	Position from	To	Core similarity on Genomatrix	Score on Jaspar	Dissimilarity on Promo_v3 (%)
agaggTTCCcc	-1,490	-1,380		6.27	3.01
atagggccTTCCtcc	-947	-933	0.926	5.80	
ctaggggtTTCCttc <sup>a</sup>	-806	-792	0.971	11.19	3.23
ctagggccTTCCtcc	-782	-768	0.926	4.35	
ttgGGGAggcctga <sup>a</sup>	-288	-274	0.932	4.71	9.97

<sup>a</sup>Predicted by all three websites. Uppercase letters in a sequence indicate putative core binding sites of NF- $\kappa$ B. NF- $\kappa$ B, nuclear factor- $\kappa$ B.

Table IV. Primers used in the present study.

Primer	Sequences
Pro-1(-840 to +1)	F: 5'-CAGAACATTTCTCTACTAGTACGCGTTCTTTGGCCAAAAGGACAAGGCAGGCAAAAGTC-3' R: 5'-TTAGCATCGGCCATGGTGGCGGATCCAGTCCTTAGATGGTGATCTTGGTGAAGACC CCAAAG-3'
Pro-3(-240 to +1)	F: 5'-CAGAACATTTCTCTACTAGTACGCGTGGAGGGTCAGTTCCACTTCTTCTGTCTCCAC-3' R: 5'-TTAGCATCGGCCATGGTGGCGGATCCAGTCCTTAGATGGTGATCTTGGTGAAGACC-3'
Mut1	F: 5'-CAGAACATTTCTCTACTAGTACGCGTTCTT-3' R: 5'-TTAGCATCGGCCATGGTGGCGGATCCAGTCCTTAGATGGTGATCTTGGTGAAGACC AAAGAC-3'
Mut2	F: 5'-CAGAACATTTCTCTACTAGTACGCGTTCTTTGGCCAAAAGGACAAGGCAGGCAA AAGTCT-3' R: 5'-TTAGCATCGGCCATGGTGGCGGATCCAGTCCTTAGATGGTGATCTTGGTGAAGACC AAAGAC-3'
Mut3	F: 5'-CAGAACATTTCTCTACTAGTACGCGTTCTT-3' R: 5'-TTAGCATCGGCCATGGTGGCGGATCCAGTCCTTAGATGGTGATCTTGGTGAAGACC AAAGAC-3'

F, forward; R, reverse.

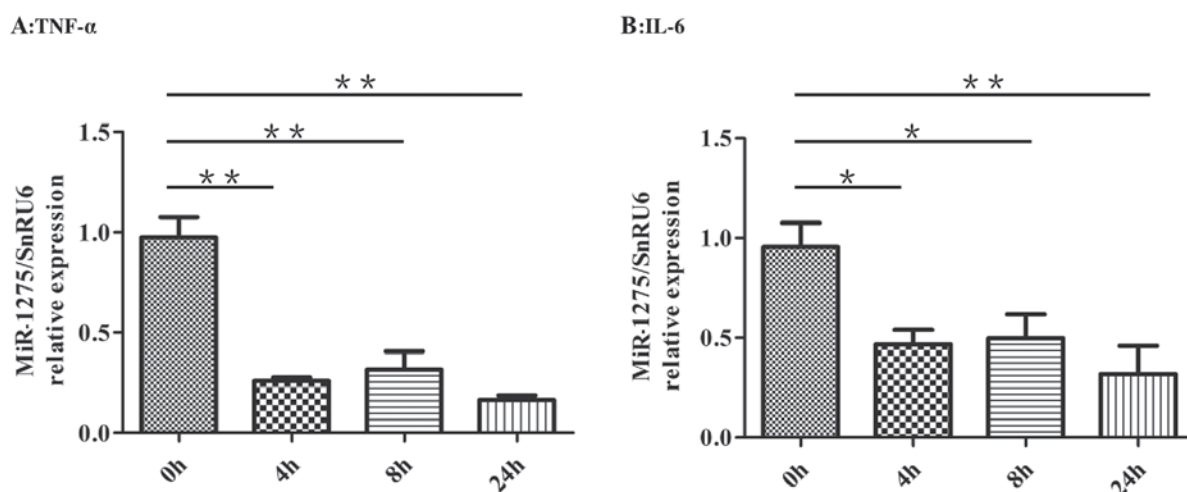


Figure 1. Relative expression levels of miR-1275 in human adipocytes treated with (A) TNF- $\alpha$  and (B) IL-6. Data are presented as the mean  $\pm$  standard deviation of 3 independent experiments. \*\* $P < 0.01$ , \* $P < 0.05$  vs. 0 h. miR, micro-RNA; NF- $\kappa$ B, nuclear factor- $\kappa$ B; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6; snRU6, small nuclear RU6.

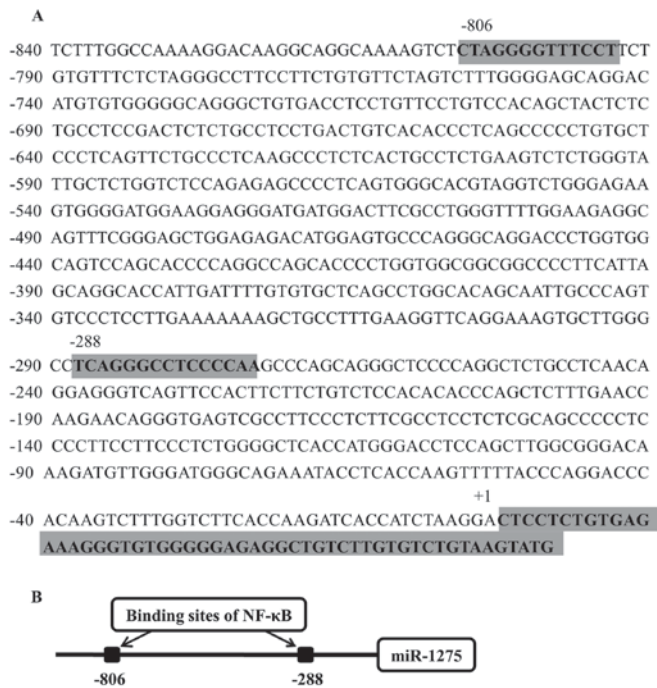


Figure 2. Prediction of miR-1275 promoter region. (A) An upstream sequence of 840 bp, including the two strongest binding sites of NF-κB, was predicted to be the promoter regions of miR-1275. (B) Schematic diagram of the predicted miR-1275 promoter region. miR, micro-RNA; NF-κB, nuclear factor-κB.

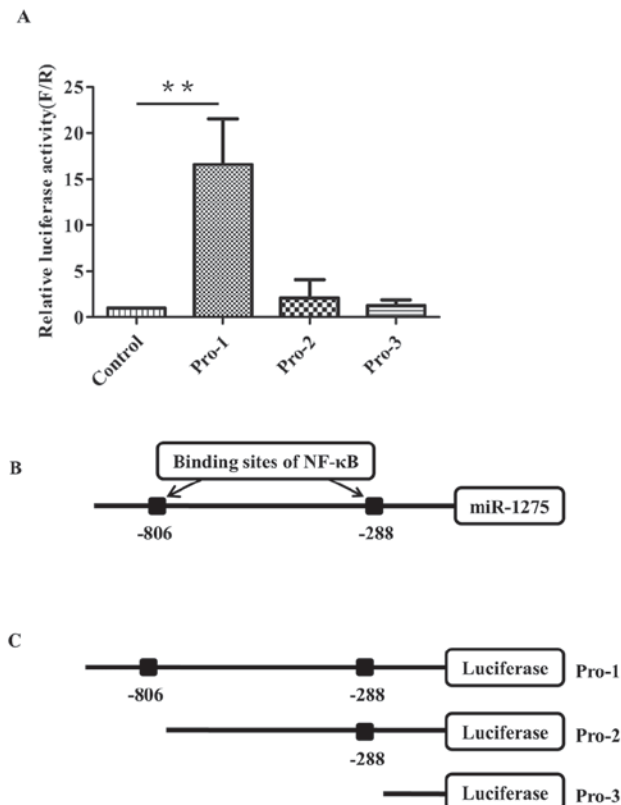


Figure 3. Luciferase activity of miR-1275 promoter region with and without the NF-κB binding sites. (A) Schematic diagram of the predicted promoter region of miR-1275. (B) Schematic diagrams of the plasmids used in the deletion assay. (C) Dual-luciferase activity of reporter plasmids PEZX-FR01 of Pro-1, Pro-2 and Pro-3. Data are presented as the mean ± standard deviation. \*\*P<0.01 vs. control. miR, micro-RNA; NF-κB, nuclear factor-κB.

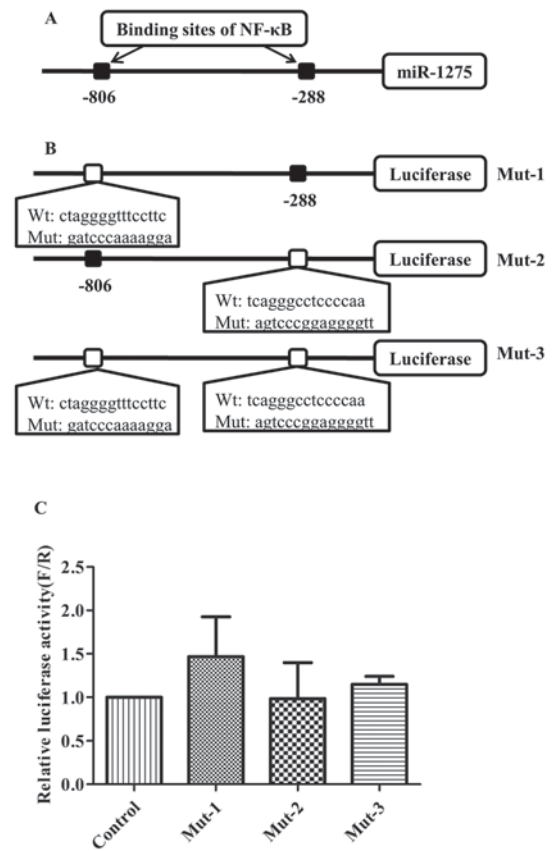


Figure 4. Luciferase activity of the promoter region of miR-1275 with mutation on binding sites of NF-κB. (A) Schematic diagram of the predicted promoter region of miR-1275. (B) Schematic diagrams of mutations of the promoter region of miR-1275's promoter region used in the delete assay. (C) Dual-luciferase activity (F/R) of Mut-1, Mut-2 and Mut-3 in HEK293T cells. Data are presented as the mean ± standard deviation. TNF-α, tumor necrosis factor-α; miR, micro-RNA; mut, mutation; NF-κB, nuclear factor-κB; F, Firefly; R, Renilla.

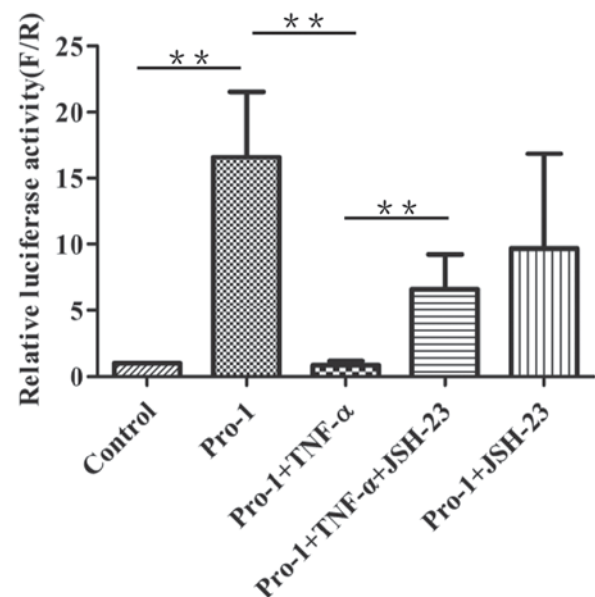


Figure 5. Luciferase activity of the promoter region of miR-1275 treated with TNF-α, JSH-23, or both. The relative luciferase activity (F/R) of the control group was normalized to 1.00. Data are presented as the mean ± standard deviation. \*\*P<0.01 vs. control group. TNF-α, tumor necrosis factor-α; miR, micro-RNA; F, Firefly; R, Renilla.

to predict transcription factors of a given sequence (19). Notably, 23 sites were predicted as binding sites of NF- $\kappa$ B. To confirm the results, the 1,500 bp upstream sequence of miR-1275 was submitted to Genomatix and Promo\_v3 (20,21), and the intersection of binding sites predicted by the three databases was taken (Table II). The region from -806 to -792 (here called site-806) and the region from -288 to -274 (here called site-288) were predicted by all three databases.

A total of six different sequences, termed Pro-1, Pro-2, Pro-3, Mut-1, Mut-2 and Mut-3, were designed to assess the promoter activity of sites-806 and -288. Pro-1 (which has both sites) demonstrated >10 times higher luciferase activity compared with empty reporter plasmids, whereas Pro-2 and Pro-3 demonstrated equal luciferase activity compared with the control. Luciferase activities of Mut-1 (mutation at site -806), Mut-2 (mutation at site -288) and Mut-3 (mutations at both sites) demonstrated no difference compared with the empty reporter plasmids. These results illustrated that both sites are necessary for promoter activity. These data also indicated that NF- $\kappa$ B was involved in the transcription of miR-1275 by binding to sites-806 and -288. The next step was to prove whether NF- $\kappa$ B participates in the regulation of miR-1275 by TNF- $\alpha$ .

HEK293T cells were transfected with Pro-1-PEZX-FR01 and were then treated with JSH-23 prior to TNF- $\alpha$ . TNF- $\alpha$  dramatically decreased promoter activity of Pro-1, while cells treated with JSH-23 and TNF- $\alpha$  demonstrated significantly higher luciferase activity compared with cells treated with TNF- $\alpha$  alone. This meant that JSH-23 could partially reverse the down-regulation of the promoter activity of Pro-1 caused by TNF- $\alpha$ . JSH-23 is an NF- $\kappa$ B inhibitor and acts by inhibiting NF- $\kappa$ B/P65 nuclear translocation (32,33). Notably, a potential mechanism could be the following: In response to TNF- $\alpha$ , with the nuclear translocation of NF- $\kappa$ B/P65, NF- $\kappa$ B is activated via NF- $\kappa$ B/P65 nuclear translocation which then binds to sites-806 and -288 of the miR-1275 promoter region. This leads to inhibiting miR-1275 transcription. Other studies have illustrated a similar association between NF- $\kappa$ B activation and miRNA downregulation. Sasaki and Vageli (34) have reported lower levels of miR-34a, -451a and -375 in response to NF- $\kappa$ B activation; miR-1908 was also down-regulated when NF- $\kappa$ B was bound to its promoter region (22).

In conclusion, the present results demonstrated that miR-1275 expression in mature human adipocytes can be down-regulated by TNF- $\alpha$  and IL-6. NF- $\kappa$ B was proven to be involved in the regulation of miR-1275 transcription by binding to its promoter region. In response to TNF- $\alpha$ , NF- $\kappa$ B was bound to promoter region of miR-1275 and inhibited its transcription. This may partially explain the down-regulation of miR-1275 in mature human adipocytes. More studies are required to explore whether miR-1275 could be a therapeutic target for obesity.

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