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The Correlation between MicroRNA-199a and White Adipose Tissue in C57/BL6J Mice with High-Fat Diet

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Abstract

Understanding is emerging about microRNAs as mediators in the regulation of white adipose tissue (WAT) and obesity. The expression level of miR-199a in mice was investigated to test our hypothesis: miR-199a might be related to fat accumulation. And we try to find its target mRNA, which perhaps could propose strategies with a therapeutic potential affecting the fat storage. C57/BL6J mice were randomly assigned to either a control group or an obesity model group (*n* = 10 in both groups). Control mice were fed a normal diet (fat: 10 kcal %) *ad libitum* for 12 weeks, and model mice were fed a high-fat diet (fat: 30 kcal %) *ad libitum* for 12 weeks to induce obesity. At the end of the experiment, we used DEXA to test body fat mass and used enzyme-linked immunoassays method to measure the free fatty acids (FFAs) and triglycerides (TGs) in WAT. Fat cell size was measured by hematoxylin-eosin (H&E) staining method and counted by MetaMorph-based method. QRT-PCR method was used to test the expression level of microRNA-199a and key adipogenic transcription factors, including peroxisome proliferator activated receptor gamma2 (PPARγ2), CCAAT/enhancer binding proteins alpha (C/EBPα), adipocyte fatty acid-binding protein (aP2). The fat mass of the model group was higher than that of the control group (*P* < 0.05). In addition, the concentrations of the FFAs and TGs were higher (*P* < 0.05) and the adipocyte size was bigger (*P* < 0.05) in the model group. Up-regulated expression of miR-199a was observed in control group. Decreased levels of miR-199a were accompanied by high expression levels of SREBP-1c. We found that the 3'-UTR of SREBP-1c mRNA has a predicted binding site for miR-199a. Based on the current discoveries, we suggest that miR-199a may exert its action by binding to its target mRNA and mediated SREBP-1c inactive or down-regulated to inhibit WAT hypertrophy. Therefore, if the predicted binding site is confirmed by further research, miR-199a may have therapeutic potential for obesity.

Introduction

Obesity is closely linked to metabolic syndrome, and it is a risk factor for various metabolic diseases, including type 2 diabetes, hypertension, hyperlipidemia and atherosclerosis [1-3]. Excessive storage of white adipose tissue (WAT) is the main manifestation of obesity. WAT has been characterized as an endocrine organ that participates in energy metabolism [4]. Although WAT is important in the regulation of metabolism, excessive fat accumulation in WAT is associated with metabolic syndrome [5]. Several key adipocytokines (e.g., adipocyte fatty acid-binding protein [aP2], CCAAT/enhancer binding proteins alpha [C/EBPα], peroxisome proliferator activated receptor gamma2 [PPARγ2], and sterol regulatory element binding protein-1c [SREBP-1c]) play a significant role in the regulation of fat storage [6,7], which in turn will affects the normal function of WAT.

MicroRNAs (miRNAs) are highly conserved, single-stranded non-coding RNAs [8]. At present, numerous studies have confirmed that miRNAs are involved in the regulation of various human tumors, such as ovarian cancer, colorectal cancer, and others [9,10]. Accumulating evidence has recently shown that miRNAs participate in the regulation of adipose tissue function, affecting adipose tissue metabolism and related diseases [11,12]. Many miR-

NAs are dysregulated in the WAT of obese animals and human subjects, potentially contributing to the pathogenesis of obesity-associated complications [4,13,14]. Despite wide studies above [4,11-14], we also want to know the relationship between miRNAs and key adipocytokines, and whether their interaction leads to adipose tissue dysfunction. Further exploration of more miRNAs is required. Identification and exploration of more obesity-related miRNAs will be helpful for better understanding of fat accumulation and WAT dysfunction.

Among the many miRNAs that have been identified in humans, we focus on miR-199a because of its potential effect on obesity. Previous studies showed miR-199a was highly expressed in 3T3-L1 preadipocytes, and was a suppressor of adipogenic differentiation [15,16]. Several studies have reported that miR-199a participates in the regulation of adipogenesis [17,18], which indicates that miR-199a may play an important regulatory role in obesity. However, there is currently no report on the relationship between miR-199a and obesity.

In the present study, an obesity model was established in C57/BL6J mice. Free fatty acids (FFAs), triglycerides (TGs), adipocyte counts, and fat mass were observed among the model and control groups. The expression level of miR-199a in mice was investigated to test our hypothesis that miR-199a may be associated with fat accumulation. Furthermore, the study aimed to establish its target mRNA, which may propose strategies with a therapeutic potential that affect fat storage.

Materials and Methods

Animals

All experimental protocols were approved by the Shandong University Institutional Animal Ethics Committee, and all procedures were performed in accordance with ethical standards. Male C57/BL6J mice (n = 20) were obtained from PLA Nanjing Military Medical College (Nanjing, China). The weight of mice were 18~22 g (4~6 weeks), and housing conditions was as follows: environment temperature 22~25 °C, relative humidity 55%~65%, 12 h light and 12 darkness cycle. The C57/BL6J male mice were randomly assigned to one of two groups (n = 10 per group for this pilot study). In the control group, mice were fed a normal diet (10% kcal from fat) *ad libitum* for 12 weeks. In the model group, mice were fed a high-fat diet (30% kcal from fat) *ad libitum* for 12 weeks to induce obesity. The diet was ordered from Shandong University. Weight measurements were performed weekly. At the end of the experiment, adipose tissue mass was scanned *in vivo* using digital dual-energy X-ray (DEXA) scanners (Norland at Swissray. Fort Atkinson, WI, USA). After all of the mice were euthanized, WAT samples were dissected to detect associated indicators of obesity.

Body fat mass

Fat mass was measured using digital DEXA scanners. DEXA data were processed for study of morphometry. Mice were anesthetized for ~1 h by injection of pentobarbital sodium (30 mg/kg bodyweight). The anesthetized mice were individually placed on a foam board and the scanning arm of the DEXA scanner assessed the fat mass (in grams) from neck to tail. After several minutes, the fat mass was recorded (Figure 1).

FFAs and TGs levels in WAT

Subcutaneous adipose tissue and visceral adipose tissue were dissected, weighed, and stored at -80 °C. Lipids from subcutaneous adipose tissue were extracted by the
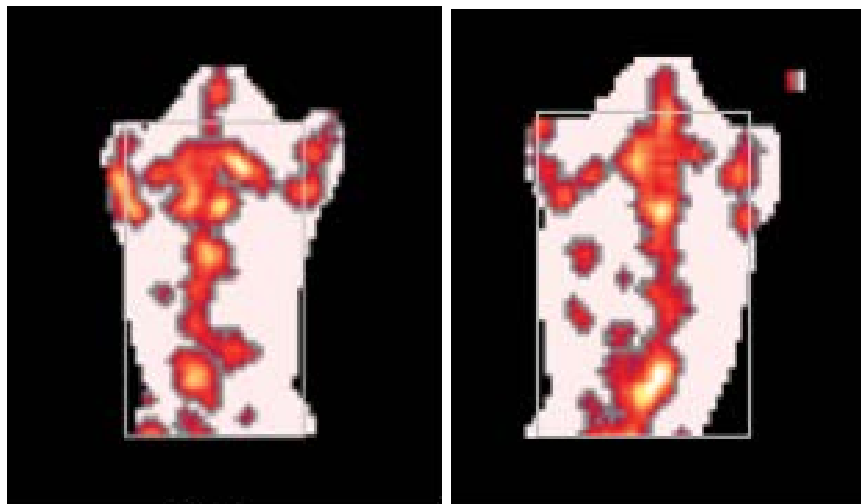
Figure 1: Body fat mass (from neck to tail) were scanned and tested by using digital DEXA scanners to process for morphometric studies. White is the color of fat mass, and red is the color of bone mineral content. The fat mass of model group is higher than that of control group.
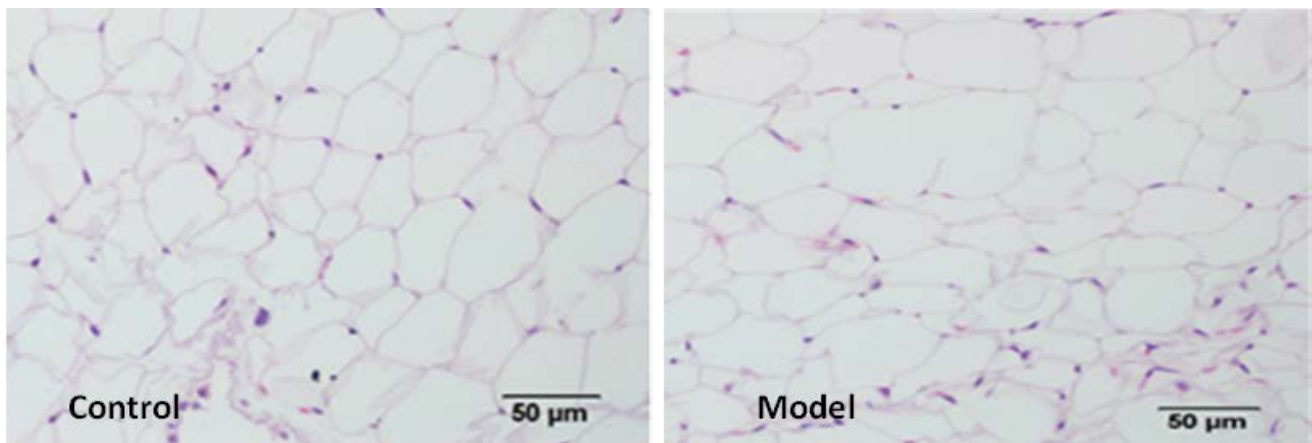
Figure 2: Adipocytes were stained with hematoxylin and eosin, and counted under the light microscope (magnification, x200; scale bar = 50 µm). An average number of 236 and 200 adipocytes were counted in the control and model groups, respectively.

Table 1: List of gene specific primers for real-time PCR.

Genes	Accession number	Sequences	Size (bp)	Tm (°C)	Ct
miR-199a	-	F: GCC ATC CCA GTG TTC AGA CT	118	60	40
		R: ACT GTA CAT GTC CCA GCC TC			
PPARγ2	-	F: TCG CTG ATG CAC TGC CTA TG	103	60	40
		R: GAG AGG TCC ACA GAG CTG ATT			
C/EBPα	NM-007678	F: CAA GAA CAG CAA CGA GTA CCG	124	60	40
		R: GTC ACT GGT CAA CTC CAG CAC			
aP2	NM-024406	F: ATC AGC GTA AAT GGG GAT TTG G	105	60	40
		R: GTC TGC GGT GAT TTC ATC GAA			
SREBP-1c	NM-011480.3	F: GCG CTA CCG GTC TTC TAT CA	113	60	40
		R: GGA TGT AGT CGA TGG CCT TG			
β-actin (house-keeping genes)	NM-007393.3	F: ACC CCA GCC ATG TAC GTA GC	101	60	40
		R: GTG TGG GTG ACC CCG TCT C			

Folch method as previously described [19], and the levels of FFAs and TGs were measured with the use of enzyme-linked immunoassays (Shanghai Tongwei reagent biological technology Co., Ltd., China) and the kit catalogue numbers were TWp002083 and TWp003393. The concentrations of FFAs and TGs were calculated based on a standard curve, which is made standard concentration (µmol/L) as abscissas and optical density (OD) value (l g (1/trans)) as ordinate.

Adipocyte size measurement

Adipose tissue was harvested, fixed, sectioned and stained with hematoxylin-eosin (H&E). And ~5000 adipocytes per mouse were counted using the MetaMorph-based method [20] under the light microscope (× 200 X, scale bar = 50 µm, OLYMPUS C o., Ltd., Japan) to show the distribution of adipocytes sizes (Figure 2).

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

Total RNA was extracted from adipose tissue, which was isolated using an RNeasy Lipid Tissue Mini kit (Qiagen GmbH, Hilden, Germany) and stored at -80 °C. The purity, concentration, and integrity of the RNA were analyzed by micro-ultraviolet spectrophotometry (Thermo Scientific NanoDrop 2000/2000c, USA) and agarose gel electrophoresis. Total RNA was reverse-transcribed with the ReverTra Ace qP CR RT kit (TOYOBO life science Co., Ltd., Japan) to obtain total cDNA. qRT-PCR was performed with SYBR® Green qPCR Master Mix (Shanghai TOYOBO biological technology Co., Ltd., China) in a LightCycler® 480 system (Roche Group, Switzerland). The expression level of each mRNA was normalized to β-actin. SYBR Green fluorescence quantification method was used and thermocycling was 40 Ct. DNAase (Shanghai TOYOBO biological technology Co., Ltd., China) was used to eliminate DNA interference. The primer sequences and cycling conditions used are listed in Table 1. TaqMan™ MicroRNA reverse transcription kit (Applied Biosystems, USA) was optimized to convert total RNA into cDNA when used with specific microRNA primers supplied with the TaqMan™ MicroRNA Assay (has-miR-199a-000498, Thermo Fisher Scientific, USA). The expression level of miRNA was normalized to endogenous snRNA U6 (ThermoFisher Company, USA).

Table 2: Male C57/BL6J mice fat metabolism parameters (control, normal diet; model, high-fat diet), medians (P25, P75) or mean ((mean - SD) - (mean + SD)).

Group	Control	Model	P value
Fat mass (g)	6.1 (4.9 - 7.3)	8.1 (6.8, 9.3)	0.009
Adipocyte count	236 (194 - 276)	200 (187 - 213)	0.012
FFAs (µmol/L)	319.9 (305.5, 334.8)	545.2 (450.8, 635.6)	0.046
TGs (µmol/L)	5.5 (3.9 - 7.1)	11.9 (10.8 - 13.1)	0.014
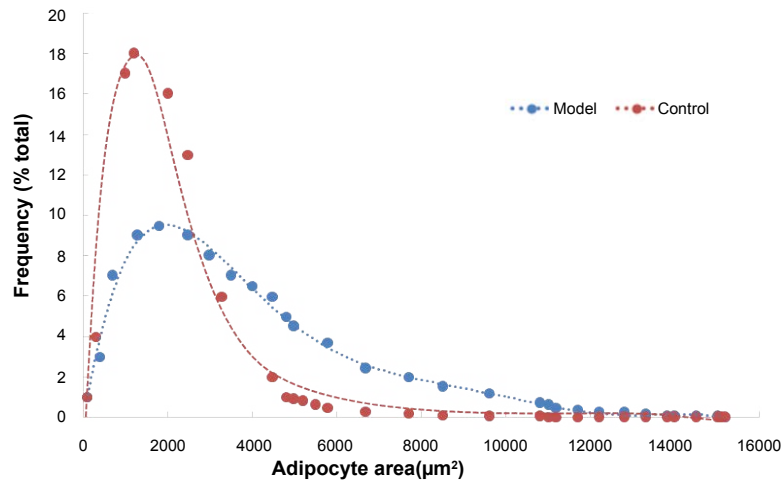
Figure 3: The distribution of adipocyte sizes was used to calculate the mean radius of adipocytes in subcutaneous WAT of mice fed normal diet (27.1 ± 2.1 µm) or high-fat diet (34.3 ± 3.2 µm).

Functional analyses

We check the structure of these molecules by nucleotide database (http://www.microrna.org, https://www.ncbi.nlm.nih.gov/nuccore), and the functional link between these molecules was analyzed using the Miranda program [21] to find theoretical binding site for miR-199a.

Statistical analyses

We used SPSS 22.0 and Excel software to analyze the data. The distribution of adipocyte sizes and the linear relationship between average adipocyte volume and subcutaneous WAT weight was calculated. The distribution of adipocytes sizes was showed using the frequency function in Excel to calculate the mean radius of adipocytes. Results are expressed as medians (P25, P75) or means ± SD according to normality test, which correspondingly used two independent-sample nonparametric tests or t-test to compare the medians or means between the two groups. A correlational analysis was calculated and is shown with correlation coefficient. A P-value < 0.05 was considered statistically significant.

Results

Changes in lipid metabolism induced by high-fat diet

The body fat mass (from neck to tail) was determined using DEXA scanners (Figure 1). The fat mass of the model group was higher than that of the control group (Table 2; P = 0.009). Under a magnified visual field (x 200) of the light microscope, an average number of 236 adipocytes were counted in the control group and 200 in the model group (Figure 2). The adipocyte count was lower in the model group than in the control group (Table 2; P = 0.012). In addition, the distribution of adipocyte size was showed in Figure 3 and the mean radius of adipocyte in subcutaneous WAT of mice was 27.1 µm and 34.3 µm in control group and model group (Figure 3). A linear relationship to weight of subcutaneous WAT observed between model group and control group (Figure 4) and the model group was higher in adipocyte volume. The FFA and TG levels in the WAT are presented in Table 2. The concentrations of FFA (Table 2; P < 0.05) and TG (Table 2; P < 0.05) were higher in the model group than in the control group.

Changes in the expression levels of miR-199a, aP2, C/EBPα, PPARγ2, and SREBP-1c
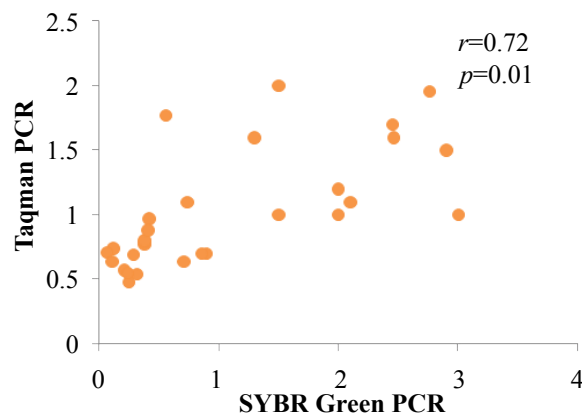
The miR-199a expression level in WAT was measured using RT-qPCR and TaqMan Probe method (Table 3). Correlation of the expression between the SYBR Green PCR method and TaqMan probe PCR method in microRNA-199a was showed in Figure 5 and it showed a very good consistency. The miR-199a expression level was upregulated in the control group and was higher when compared with the model group (Table 3, $P < 0.01$).

To further confirm the role of miR-199a in obesity, the expression levels of four key adipogenic transcription fac-
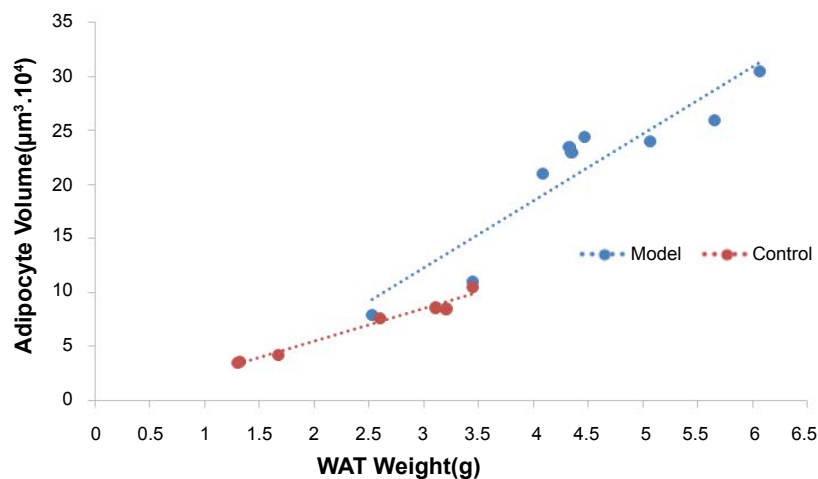
Figure 4: Average adipocyte volume was calculated based on the radius of adipocytes from WAT of mice and a linear relationship to weight of subcutaneous WAT observed between model group and control group.

Figure 5: Correlation of the expression between the SYBR Green PCR method and TaqMan probe PCR method in microRNA-199a.

Table 3: The relative expression level ($2^{-\Delta\Delta Ct}$) of miR-199a, aP2, C/EBPα, PPARγ2 and SREBP-1c in two groups, medians (P25, P75) or mean ((mean - SD) - (mean + SD)).

Group	qPCR	Control	Model	P-value
miR-199a	SYBR Green	0.63 (0.61 - 0.65)	0.27 (0.23 - 0.31)	0.01
	TaqMan Probe	0.74 (0.70 - 0.78)	0.38 (0.35 - 0.42)	0.006
aP2		0.25 (0.23 - 0.27)	0.44 (0.38 - 0.50)	0.021
C/EBPα		0.29 (0.25 - 0.33)	0.34 (0.25 - 0.43)	0.246
PPARγ2		0.05 (0.02, 0.07)	0.10 (0.07, 0.15)	0.013
SREBP-1c		0.43 (0.38, 0.48)	0.64 (0.5 - 0.74)	0.035

tors, including PPARγ2, C/EBPα, aP2, and SREBP-1c were evaluated. The expression levels of aP2, C/EBPα, PPARγ2 and SREBP-1c were higher in the model group than in the control group. Aside from the expression level of C/EBPα (Table 3; P = 0.246), the increased expression levels of the three other indicators (SREBP-1c, PPARγ2, aP2) were statistically significant (Table 3; P = 0.0035, 0.013 and 0.021).

Correlation between miR-199a and aP2, C/EBPα, PPARγ2, and SREBP-1c

The correlation (*r*) between miR-199a and the four

key adipogenic transcription factors is presented in Table 4. SREBP-1c expression levels were negatively correlated with the miR-199a expression level (*r* = -0.7222, *P* = 0.002). No statistically significant correlation was observed between miR-199a and aP2, C/EBPα, or PPARγ2 expression levels (P > 0.05). The functional link between these molecules was analyzed using the Miranda program [21]. It was found that the 3'-UTR of SREBP-1c mRNA has a theoretical binding site for miR-199a. The miR-199a processing and recognition of the mRNA target sites are presented in Figure 6.
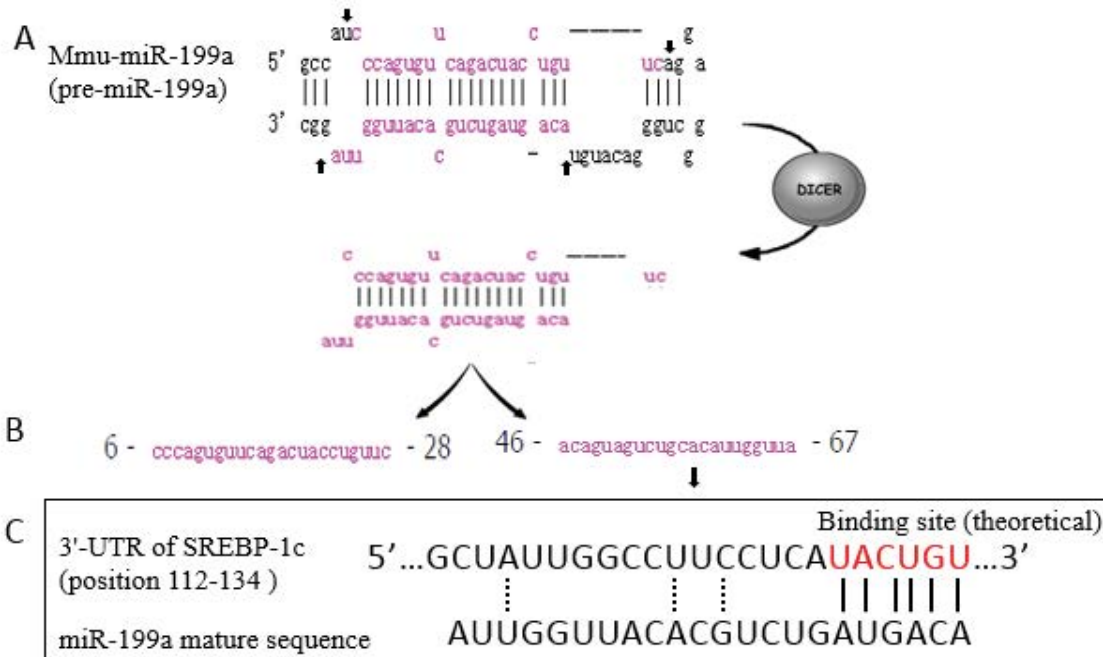
Figure 6: MiR-199a processing and recognition of the mRNA target sites A) The hairpin structure of a pre-miRNA is processed into an miRNA duplex using the RNase Dicer via cleavage at various sites in the hairpin (indicated by the arrows); B) The miRNA is unwound into two strands: miR-199a-3p (right) and miR-199a-5p (left); C) The theoretical binding site (highlighted, vertical lines) is presented. The dotted lines mark interactions that influence and strengthen target recognition.

miR: microRNA; UTR: untranslated region.

Table 4: The correlation (r) between miR-199a and aP2, C/EBPα, PPARγ2 and SREBP-1c.

	miR-199a	
	r	P-value
aP2	-0.17	0.54
C/EBPα	-0.32	0.25
PPARγ2	-0.33	0.23
SREBP-1c	-0.722	0.002

Discussion

The results of our study showed decreased expression level of miR-199a in the WAT of the model group. The expression levels of key transcriptional regulation factors related to adipocyte differentiation and fat accumulation, including aP2, PPARγ2, and SREBP-1c, were up-regulated in the WAT of the model group. Among them, SREBP-1c was negatively correlated with the miR-199a expression level.

Obesity is an energy balance disorder that is characterized by increased lipid storage in adipocytes (hypertrophy) as well as an increased number of adipocytes (hyperplasia) [22]. Adipocyte hypertrophy is the main reason of adult obesity. Generally speaking, it is not only caused by increased fatty acid synthesis from carbohydrates and fat intake by organs and low energy expenditure, but also by a malfunction of fat tissue. The typical diameter of an adipocytes is 0.3-0.9 μm, but it can be about 20-fold larger in hypertrophic adipose tissue [20,23]. In our study, a high-fat diet was used to create an obesity model. The fat mass was higher in the model group, and adipocyte sizes in the model group were bigger than in the control grouped.

The function of WAT is to store excess energy in the form of TGs and to convert them into FFAs and glycerol to provide energy upon demand. When the excess calories overwhelm the storage capacity of adipocytes, excess fat is stored in the liver, leading to fat deposition and insulin resistance [24]. As an endocrine organ, WAT plays a crucial role in controlling whole body metabolism by secreting adipokines and storing FFAs [25], and increased WAT mass via hyperplasia and hypertrophy results in adipocyte dysfunction [26]. In our study, the FFA and TG levels of the model group were higher than those of the control group. This finding implies a disorder in lipid metabolism in the hypertrophic adipose tissue.

MiRNAs, as transcription factors in the adipocyte differentiation process, were previously demonstrated to regulate adipogenesis and fat storage [11]. A better understanding of the regulation of adipogenesis is crucial to the development of novel therapeutic strategies for obesity and its associated metabolic syndromes. Existing data demonstrated that miR-33 reduces the oxidation of fatty acids and inhibits the production of high-density lipoprotein (HDL), and it appears to be up-regulat-

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ed in the individuals with obesity [27]. In addition, the miR-222 expression level was found to be up-regulated in the serum of obese subjects, whereas miR-221 was down-regulated [28]. The expression levels of both types of miRNAs are related to body mass index (BMI), waist circumference measurement, fat distribution, and HDL concentration [28]. Many studies have also shown that miRNAs can be used as targets in the treatment of obesity and obesity-related chronic diseases [4,13,29].

As shown in Figure 6, pre-miR-199a has a hairpin structure. This hairpin structure is processed into a miR-199a duplex by the RNase Dicer through cleavage at several sites in the hairpin. The miRNA is then unwound into two strands: miR-199a-3p (right) and miR-199a-5p (left). A previous study showed that miR-199a was highly expressed in 3T3-L1 preadipocytes [30], and was a suppressor of adipogenic differentiation [15]. Other several researches have shown that miR-199a participates in the regulation of adipogenesis [17,18], suggesting that miR-199a may play an important regulatory role in obesity. However, the potential role of miR-199a in adipogenesis and hypertrophy of WAT has not yet been demonstrated in animal models or in human subjects. In our study, the result showed a decreased expression level of miR-199a in the WAT of the model group. Based on these results, we hypothesized that the decreased miR-199a expression was associated with lipid accumulation. We then conducted the experiments to test this hypothesis.

We also measured the expression levels of key transcriptional regulation factors that are related to adipocyte differentiation and fat storage. PPARγ, which is specifically expressed in WAT, is involved in lipid formation in mature adipocytes [31]. C/EBPα also stimulates adipogenesis and works together with PPARγ in the process of adipocyte differentiation [32]. In addition, other transcription factors, including aP2 and SREBP-1c are related with to fatty acid metabolism and glucose metabolism [33-35]. Our results showed that the expression levels of aP2, PPARγ2, and SREBP-1c were up-regulated in the WAT of the model group. The results were consistent with previous reports [31]. Moreover, SREBP-1c expression was negatively consistent with the miR-199a expression level (Table 4), implying a potential mechanism between obesity and the miR-199a. In the control group, SREBP-1c expression level was higher. When induced by a high-fat diet, the expression decreased significantly in line with miR-199a expression. It showed that miR-199a may be relevant to obesity induced by a high-fat diet and the potential mechanism might be associated with SREBP-1c.

We analyzed the functional link between SREBP-1c and miR-199a and found that the 3'-UTR of SREBP-1c mRNA has a theoretical binding site for miR-199a (Figure 6). Based on our findings, we speculate that miR-199a may exert its action by binding to its target mRNA and mediated SREBP-1c inactive or down-regulated to inhibit WAT hypertrophy. As reported in the literature, miRNAs function is by partially pairing to sequences located in the 3'UTR of target mRNA [36]. If the predicted binding site for miR-199a in the 3'UTR of SREBP-1c is confirmed in further research, therapeutic strategies affecting the function of miR-199a may be possible (e.g., chemically modified complementary inhibitors). However, future research would first need to verify the direct role of miR-199a by using gene silencing or knockout technology specifically in WAT.

In conclusion, the present study showed that miR-199a expression is decreased by a high-fat diet, and a higher level of miR-199a is correlated with increased expression of SREBP-1c. We analyzed the functional link of these molecules and found that the 3'-UTR of SREBP-1c mRNA has a theoretical binding site for miR-199a. Further research is needed to confirm our speculation. If the predicted binding site is confirmed in future research, potential therapeutic strategies affecting the function of miR-199a may be proposed for obesity and related-diseases.

Acknowledgments

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