# Obesity related microRNA-424 is regulated by TNF-α in adipocytes

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**Abstract.** In recent years, obesity has become a major public health concern. Obesity has been previously associated with low-grade inflammation and TNF-α induction in adipose tissue, which subsequently disrupts adipocyte metabolism. MicroRNAs (miRNAs/miRs) are important metabolic factors and their dysregulation has been associated with obesity-related metabolic syndromes. In fact, it has been directly suggested that miR-424 may be functionally associated with adipogenesis, although its exact role in this process remains unclear. The present study aimed to identify the function of miR-424 in adipogenesis. In the present study, miR-424 expression levels were analyzed during adipogenesis and the differential expression of this miRNA in the adipose tissue of obese and non-obese children was also assessed. Furthermore, the interaction between miR-424 and the adipocytokine TNF-α was determined. Finally, miR-424 target genes and downstream signaling pathways were predicted via bioinformatics and analyzed by performing a luciferase reporter assay to elucidate the functional mechanisms of miR-424 in adipogenesis of visceral adipocytes. The results revealed that the expression levels of miR-424 upregulated in the adipose tissue biopsies from obese children compared with the biopsies of non-obese children. However, in cultured adipocytes, the expression levels of miR-424 were discovered to be gradually downregulated during the adipogenesis process. TNF-α treatment significantly downregulated the expression levels of miR-424 via binding to its promoter region and reducing its transcriptional activity. Through bioinformatic prediction analysis, miR-424 target genes were analyzed, of which several were identified to be involved in signaling pathways that are known to regulate adipogenesis, such as the Wnt signaling pathway. In conclusion, the present study indicated that miR-424 was regulated by TNF-α and served an important role in adipogenesis.

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## Introduction

Obesity has been a serious health problem worldwide for decades and it is now considered as a potential trigger of other metabolic disorders, including cardiovascular diseases, diabetes and cancer (1,2). The accumulation of adipose tissue is the most common cause of obesity (3). It has been reported that 30% of adipose tissue is derived from preadipocytes that undergo adipogenesis and develop into mature adipocytes (4). Adipogenesis is a multifactorial process that is regulated by various elements, including microRNAs (miRNAs/miRs), transcription factors, epigenetic regulators and diverse signaling pathways, such as PPARγ/MAPK, PI3K/Akt and Wnt/β-catenin (5). In addition, the development of obesity is known to be accompanied by low-grade inflammation (6). In this context, adipose tissue functions as an endocrine organ, secreting a variety of inflammation-related adipocytokines, including IL-6, TNF-α and IL-8 (2). TNF-α is highly induced in adipose tissue compared with in other tissues and its expression has been discovered to affect adipocyte metabolism, including glucose consumption, lipolysis and adipocyte differentiation (7).

miRNAs are a class of small non-coding RNAs of 20-24 nucleotides in length, which negatively regulate the expression of target proteins. Therefore, miRNAs are involved in a variety of biological events, including stem cell differentiation, cell proliferation and death, neurogenesis, hematopoiesis and immune responses (8,9). In adipocytes, miRNAs function as regulators of differentiation by targeting adipocyte-related factors, such as peroxisome proliferator-activated receptor (PPAR)-γ, which was reported to be downregulated by miR-27b, miR-31 and miR-138, resulting in the inhibition of the adipogenic process (9-12). The dysfunction or abnormal

expression of miRNAs has been associated with the development of cancer (13,14), cardiovascular diseases (15), diabetes and obesity (16,17). Therefore, the potential association of miRNA dysregulation and the onset of obesity has rapidly become a topic of interest. For example, Ortega *et al* (18) compared the miRNA expression profiles in pre- and mature adipocytes of obese and non-obese people, and observed significant differences in the expression of 71 distinct miRNAs between the two groups.

miR-424 in an intragenic miRNA, and a member of the miR-16 family, which clusters with miR-503 on chromosome Xq26.3 (19). Previous studies have reported the functional relationship between miR-424 and several types of disease and biological processes, including cancer (20-23), cell differentiation (24), diabetes (25), angiogenesis (26), vascular diseases (27) and inflammatory diseases (28,29). The role of miR-424 in adipogenesis has been studied in recent years, and its expression has been associated with waist-to-hip ratio and to body fat mass related parameters (30,31). Functionally, the expression of miR-424 was identified to be regulated by PPAR-γ, an important transcription factor during adipogenesis (32). Interestingly, in non-obese women, those with higher fat droplet measurements had upregulated expression levels of miR-424 (30). These reports provided novel information about the role of miR-424 in obesity. However, to the best of our knowledge, the molecular mechanisms of this miRNA during adipogenesis are yet to be determined.

A previous study revealed that miR-424 expression levels were closely associated with fat deposition in women (30); hence, in the present study, it was questioned whether a similar expression pattern would be observed in children. In the current study, the expression levels of miR-424 in the adipose tissue of obese and non-obese children were compared. To further elucidate the mechanisms of miR-424, the changes in miR-424 expression levels during adipogenesis were also analyzed. Furthermore, the association between miR-424 and the adipocytokine TNF-α was investigated, and the results demonstrated a negative regulatory effect of TNF-α on miR-424. Finally, TargetScan, PicTar and microRNA.org softwares were used to predict the target genes of miR-424. The putative downstream miR-424 signaling pathways were analyzed and an association between miR-424 and signaling pathways closely associated with adipogenesis, including the Wnt signaling pathway, were identified.

## Materials and methods

*Study participants*. A total of 40 male pediatric patients (age, 6-12 years) undergoing surgery for abdominal disorders were prospectively chosen to obtain abdominal fat biopsies in The Affiliated Hospital of Nantong University between August 2019 and September 2019. The following exclusion criteria were used: Presence of malignancy, an endocrine disorder or severe systemic illness. Subjects considered obese were chosen according to the Working Group on Obesity in China (WGOC) in 2003 [body mass index (BMI) above the age- and sex-appropriate 95th percentile] (33). Written informed consent was obtained from the parents or legal guardians of all participants. The methods and experiments were approved by the Ethics Committee of The Affiliated Hospital of Nantong University (approval no. 2019-K050; Nantong, China).

*Cell culture*. Human visceral preadipocytes (HPA-V cells) were obtained from ScienCell Research Laboratories, Inc. Preadipocyte medium (PAM) containing 1% preadipocyte growth supplement, 1% penicillin/streptomycin solution and 5% FBS was also obtained from ScienCell Research Laboratories, Inc. (cat. no. 7211). Preadipocytes were cultured in a humidified atmosphere at 37˚C with 5% $CO_2$. Serum-free PAM (containing 100 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 50 nM insulin and 100 mM rosiglitazone) was used to induce the differentiation of confluent human preadipocytes at day 0, followed by replacement of the medium every 2 days for the next 4 days in the 37˚C incubator. Subsequently, the medium was replaced with serum-free PAM (containing 50 nM insulin), which was then replaced every 2 days until lipid droplets started accumulating in the cells (day 15). Cells were collected at different time periods (days 0, 1, 4, 7, 10 and 15) during the cell culture period during the adipogenesis.

Human preadipocytes were cultured at 37˚C overnight in serum-free PAM media. Following the incubation, the cells were treated with 10 ng/ml TNF-α (Sigma-Aldrich; Merck KGaA) or the same volume of PBS for 0, 12, 24 and 48 h at 37˚C, as previously described (34). 293T cells (American Type Culture Collection) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37˚C with 5% $CO_2$.

*RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)*. Total RNA was extracted from the human adipocytes and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. DNA was removed by DNaseI digestion (Takara, Bio, Inc.). Total RNA was then reverse transcribed into cDNA using the Reverse Transcriptase kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) with 200 ng total RNA as the template. qPCR was subsequently performed using SYBRGreen kits (Vazyme Biotech Co., Ltd.) on an Applied Biosystems 7500 Sequence Detection system (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The following thermocycling conditions were used for qPCR: Initial denaturation of 95˚C for 10 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1. miRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (35) and normalized to the internal reference gene U6. miR-424 and U6 snRNA were designed and synthesized by Guangzhou RiboBio Co., Ltd. (36,37).

*Fluorescence reporter constructs and dual luciferase reporter assay*. The potential TNF-α binding sites were predicted using Genomatix software (www.genomatix.de). Luciferase wild-type (WT) and mutant (mut) miR-424 reporter plasmids, pro-miR-424-WT and pro-miR-424-mut, respectively, were synthesized and inserted into the luciferase reporter cloning vector pEZX-FR01 (GeneCopoeia, Inc.) by Shanghai Generay Biotech Co., Ltd. Both plasmids contained the 1,500 bp proximal promoter sequences of miR-424 (chrX:

134546712-134548211). The pro-miR-424-mut reporter plasmid had a mutated fragment of 5'-TTATTTTAGGAA GGA-3' at chrX: 134546762-134546776 to replace the original sequence 5'-GGCGGGGCTTCCTTC-3'.

Briefly, 293T cells were seeded in six-well plates ($5x10^5$/well) and incubated at 37˚C for 24 h before transfection. Following the incubation, 250 ng/well pro-miR-424-WT and pro-miR-424-mut plasmids were co-transfected with 25 ng/well of Renilla luciferase vector (pRLTK; Invitrogen; Thermo Fisher Scientific, Inc.), using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). Following 24 h of incubation at 37˚C, 10 ng/ml TNF-α was added to the cells, together with culture medium replacement for an additional 24 h of incubation. Finally, the relative luciferase activity was measured using a Dual Luciferase Reporter Assay system (cat. no. E1901; Promega Corporation), according to the manufacturer's protocol. Luciferase activity was normalized to *Renilla* luciferase activity.

*miRNA target prediction and functional annotation*. TargetScan (www.targetscan.org/vert_72; version 7.0), PicTar (pictar.mdc-berlin.de) and microRNA.org programs (www.microrna.org) were used to predict the target genes of miR-424, using the default parameters. miRNA targets were obtained by manually selecting the intersecting elements identified in the miR-424 target prediction program. All target genes were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/pathway.html) and Gene Ontology (GO) databases (geneontology.org). GO functional term enrichment analysis, which organizes genes into hierarchical categories and uncovers the gene regulatory network on the basis of biological processes was used to analyze the main functions of the miR-424 target genes (38,39). Specifically, a two-sided Fisher's exact test and a $\chi^2$ test were used to classify the GO category. Multiple-test correction was performed by calculating the false discovery rate (FDR) (40). The FDR was defined as FDR=1-($N_k$)/T, where $N_k$ was the number of Fisher's test P-values $<\chi^2$ test P-values and T was the theoretical frequency. P-values were computed for the GOs of all the miR-424 target genes. Enrichment provides a measure of the significance of the function: As the enrichment increases, the corresponding function is more specific, which helps to identify GO terms with more concrete function description in the experiment. Within the significant category, the enrichment (Re) was given by the equation: Re=(nf/n)/(Nf/N) where nf was the number of flagged genes within the particular category, n is the total number of genes within the same category, Nf is the number of flagged genes in the entire microarray and N is the total number of genes in the microarray (41,42).

Signaling pathway enrichment analysis was performed for the differentially expressed genes, using KEGG (43), Biocarta (44) and Reactome databases (45). Significant signaling pathways were selected on the basis of the Fisher's exact test and $\chi^2$ test, and the threshold of significance was defined by the P-value (P<0.01) and FDR (q<0.05). The enrichment Re was calculated as above (46-48).

*Statistical analysis*. All experiments were performed independently ≥3 times and the data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc test was used to compare data of ≥3 groups, whereas a paired Student's t-test was used to compare the statistical differences between two groups. P≤0.05 was considered to indicate a statistically significant difference.

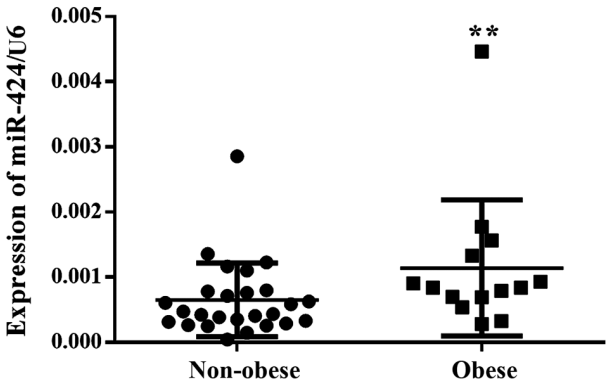


Figure 1. miR-424 expression levels in obese and non-obese children. miR-424 expression levels in abdominal fat biopsies from obese (n=14) and non-obese (n=26) participants were determined using reverse transcription-quantitative PCR. **P<0.01 vs. non-obese. miR, microRNA.

## Results

*Differential miR-424 expression levels between obese and non-obese individuals*. Concerning the clinicopathological data of the study participants (Table SI), there was no differences identified between age, whereas subjects in the obese group exhibited significantly increased BMIs compared with subjects in the non-obese group. To determine the association between miR-424 and obesity in children, miR-424 expression levels were analyzed in the abdominal fat biopsies from patients undergoing surgery for abdominal disorders. The results revealed that the miR-424 expression levels were significantly upregulated in the biopsies from obese patients compared with non-obese patients, suggesting a positive association between miR-424 expression levels and obesity in children (Fig. 1).

*miR-424 expression levels are downregulated during the maturation of human preadipocytes*. The variation in miR-424 expression levels during different stages of human preadipocyte maturation was investigated. As shown in Fig. 2, miR-424 expression levels were the highest in the preadipocytes, and then continuously decreased with the maturation progression and reached the lowest point between days 7-15. According to the literature review (49,50), it was predicted that at day 10 after induction, >80% of cells exhibited a typical adipocyte phenotype, suggesting that miR-424 expression levels may be downregulated during the differentiation of human preadipocytes into adipocytes.

*miR-424 expression levels are downregulated by TNF-α in human adipocytes*. To determine whether proinflammatory cytokines were involved in regulating miR-424 expression levels, the effects of IL-6 and TNF-α on the expression

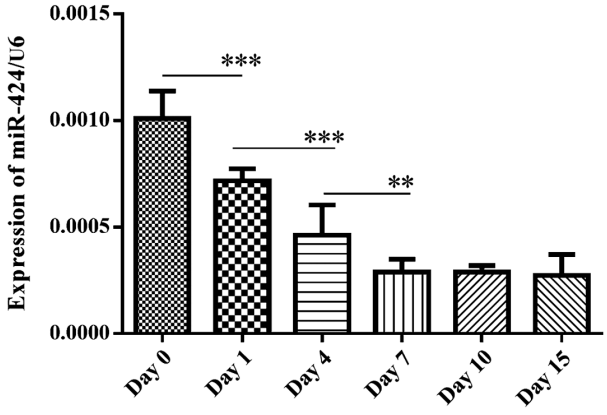


Figure 2. miR-424 expression levels throughout preadipocyte differentiation. Cells were collected at different time points (day 0, 1, 4, 7, 10 and 15) throughout preadipocyte differentiation and the expression levels of miR-424 were determined using reverse transcription-quantitative PCR. U6 was used as the internal control. $^{**}$P<0.01, $^{***}$P<0.001. n=3 independent biological replicates. miR, microRNA.

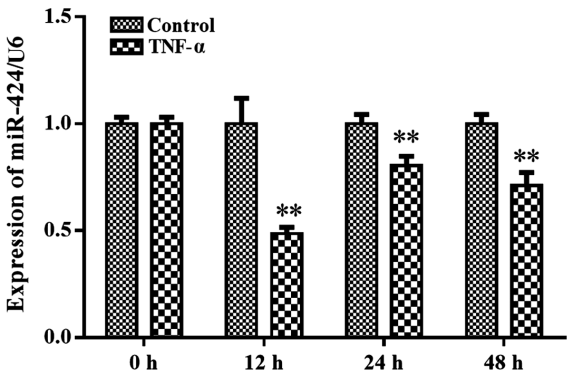


Figure 3. miR-424 expression levels in mature adipocytes treated with TNF-α. miR-424 expression levels in mature adipocytes treated with 10 ng/ml TNF-α for different time points (0, 12, 24 and 48 h). Control cells were treated with an equivalent volume of PBS. Data were normalized to U6 expression levels. $^{**}$P<0.01 vs. control at each time point. n=3 independent biological replicates. miR, microRNA.

levels of miR-424 in human adipocytes were investigated. Mature adipocytes were treated with 10 ng/ml TNF-α and the miR-424 expression levels were analyzed at different time points (12, 24 and 48 h) following normalization to U6 expression levels. While IL-6 had no significant effects on miR-424 expression levels (data not shown), TNF-α treatment significantly downregulated the expression levels of miR-424 at 12, 24 and 48 h compared with the control cells (Fig. 3). The lowest expression levels of miR-424 were observed at 12 h post TNF-α treatment, with ~2.17-fold lower expression levels compared with the control group. These results suggested that TNF-α may downregulate miR-424 expression levels in adipocytes.

*TNF-α downregulates miR-424 expression levels by reducing its promoter activity*. To support the aforementioned findings, dual luciferase reporter assays were performed using 293T cells. The 1.5 kB upstream sequence of the miR-424 precursor (chrX: 134546712-134548211) was selected as the candidate promoter fragment, synthesized and inserted into the

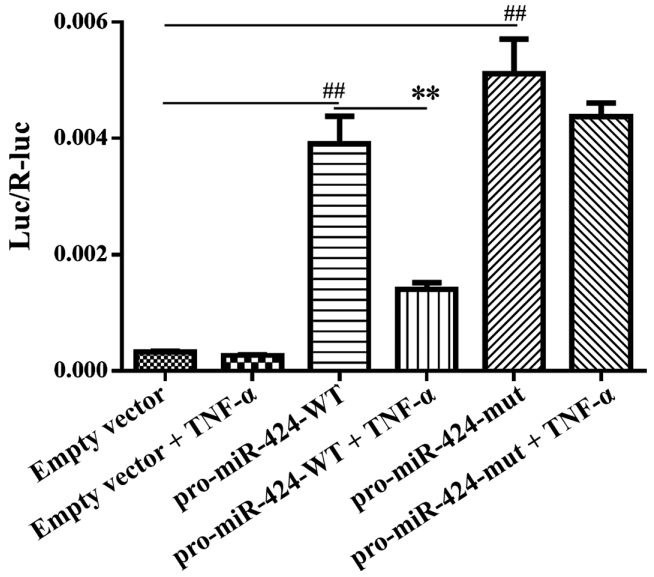


Figure 4. Dual luciferase reporter assays were performed following cell transfection with pro-miR-424-WT or pro-miR-424-mut and treatment with or without of TNF-α in 293T cells. $^{**}$P<0.01 vs. pro-miR-424-WT and $^{\#\#}$P<0.01 vs. Empty vector. n=3 independent biological replicates. miR, microRNA; WT, wild-type; Mut, mutated.

pEZX-FR01 vector to generate a pro-miR-424-WT plasmid. The results revealed that pro-miR-424-WT had promoter activity, which drove the expression of the downstream reporter gene when compared with the vector alone (Fig. 4).

It was hypothesized that TNF-α may bind to the promoter region of miR-424, therefore, the potential TNF-α binding sites were predicted using Genomatix software. One of the predicted binding sites, located at chrX: 134546762-134546776, was selected for experimental validation and then mutated to generate the pro-miR-424-mut plasmid. To analyze and compare the effects of TNF-α on pro-miR-424-WT and pro-miR-424-mut, 293T cells were used. TNF-α treatment lead to a decrease in the relative luciferase activities of both miR-424 promoters; however, only the pro-miR-424-WT showed a significant reduction in relative luciferase activity following TNF-α treatment compared with the pro-miR-424-WT alone (Fig. 4), indicating that the effect of TNF-α may be attenuated by a mutation at the promoter binding site. Overall, these results suggested that TNF-α may inhibit miR-424 transcription by binding to specific sites in its promoter region.

*GO functional term and signaling pathway enrichment analyses of miR-424 target genes*. To study the biological implications of miR-424, target genes of miR-424 were predicted through GO functional term enrichment analysis. Three areas, including biological process, molecular function and cellular component are primarily covered in GO annotation, which provides controlled annotations to describe genes and their products in a given organism (51). As the present study aimed to identify the function of miR-424, biological process was assessed. The GO analysis results identified that the target genes of miR-424 were classified into diverse categories, among which the most frequent GO term was 'Catabolism' followed by 'protein ubiquitination during

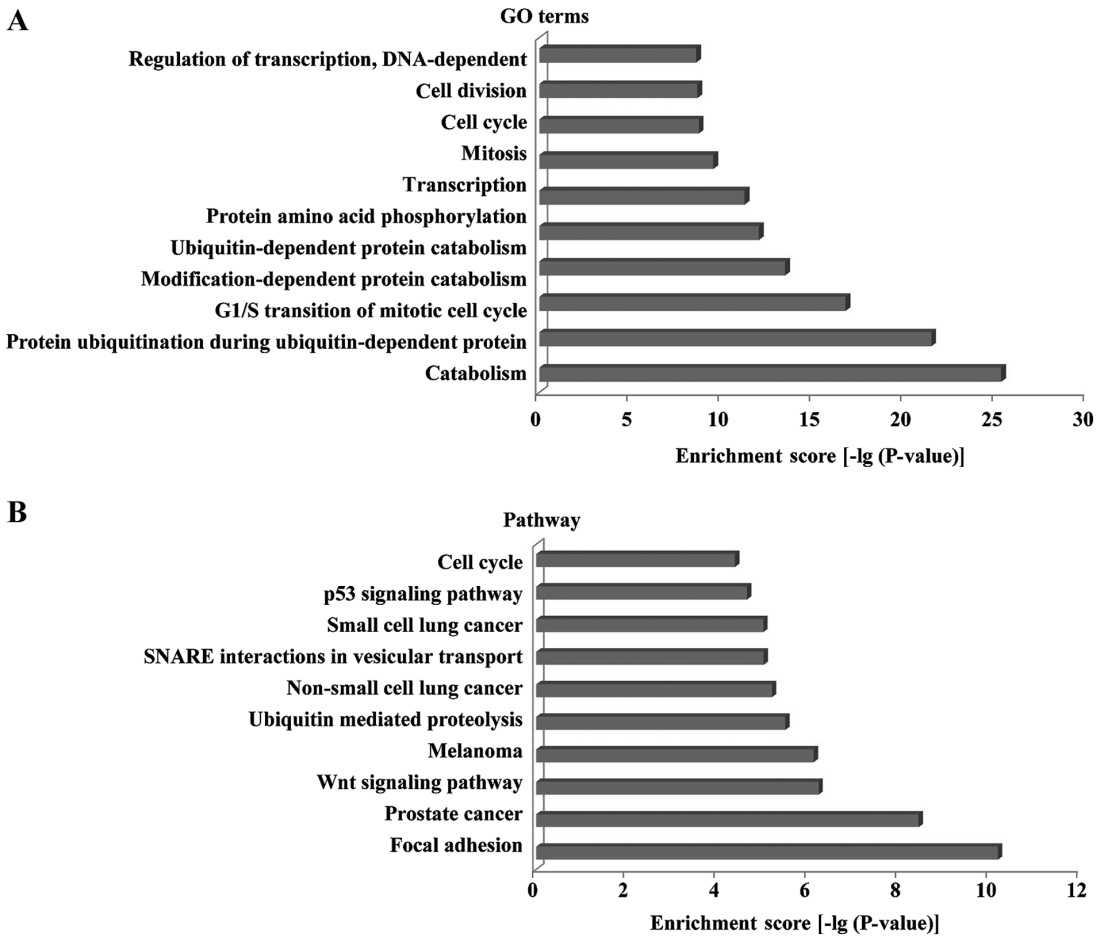


Figure 5. Analysis of miR-424 target genes. (A) GO functional term enrichment analysis was used to determine the main functions of the miR-424 target genes. (B) Signaling pathway enrichment analysis was used to determine the significantly represented pathways according to KEGG, Biocarta and Reactome databases. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

ubiquitin-dependent protein' and 'G1/S transition of mitotic cell cycle' (Fig. 5A). Moreover, signaling pathway enrichment analysis predicted that the miR-424 target genes were involved in diverse processes, including 'Wnt signaling pathway', 'p53 signaling pathway', 'Ubiquitin mediated proteolysis', 'SNARE interactions in vesicular transport', 'Focal adhesion' and 'Cell cycle' (Fig. 5B). These results further implied that miR-424 may target genes in prostate cancer, melanoma and lung cancer (small-cell and non-small cell).

## Discussion

Obesity has become a serious public health problem, with the number of affected individuals increasing worldwide. Since 1980, the number of cases of obesity has doubled in >70 countries. In 2015, there were 100 million and 600 million cases of obesity reported in children and adults, respectively (52). The dysregulation of preadipocyte differentiation is a main cause of obesity (53). Therefore, it is important to study the regulatory mechanisms of preadipocyte differentiation. miRNAs have been discovered to have an important role in the regulation of inflammatory responses by adipocytes, and are thus associated with the development of obesity (34,54). miR-424, an inflammation-related miRNA implicated in diverse cellular events and diseases, has been studied for its potential as a clinical and diagnostic biomarker of cancer (55), heart failure (56) and diabetes (57,58). In the context of obesity, miR-424 was identified to negatively regulate adipocyte differentiation (59). However, the mechanistic details on the miR-424-mediated inhibition of adipocyte differentiation remain unclear.

In the present study, the differential expression levels of miR-424 in abdominal fat biopsies of obese and non-obese children were analyzed. BMI is the gold standard measurement of obesity; however, children cannot be identified as obese by only calculating BMI. According to the WGOC in 2003, the criteria for determining obesity in children is a BMI > age- and sex-appropriate 95th percentile (33). For example, 6-year-old children with a BMI of >18.1 are categorized as obese, while 11-year-old children with the same BMI are recognized as non-obese, and are diagnosed as obese when the BMI is >22.7 (60). Thus, BMI is not suitable for the diagnosis of obesity in children and is not useful for detecting the relevance between BMI and miRNA expression, the present study revealed that the expression levels of miR-424 in the adipose tissue were positively associated with obesity. Our previous data using a miRNA microarray demonstrated that the expression levels of miR-424 were upregulated in mature adipocytes compared with in preadipocytes, both in human adipose-derived mesenchymal stem cells and human stromal vascular cells (61). However, in subsequent PCR analysis, miR-424 expression levels were observed to continuously decline

during preadipocyte differentiation, maintaining an low, but steady, level in mature adipocytes (59). Aiming to find an appropriate explanation for these opposing results, Gene Expression Omnibus (GEO) datasets related to miR-424 and obesity were searched. Interestingly, one of the profiles (ID: 30947926; www.ncbi.nlm.nih.gov/geoprofiles/?term=30947926) revealed that the expression levels of miR-424 were downregulated during preadipocyte differentiation in subcutaneous and mesenteric adipose cells (62,63). Conversely, in omental cells, miR-424 expression levels were upregulated, suggesting that miR-424 expression may be tissue or cell-dependent. Therefore, it was hypothesized that the results of the present study may be caused by environmental factors in the patient abdominal tissues, which are absent from cultured adipocyte cells (30). Another possibility is that miR-424 may be downregulated during adipocyte differentiation due to its negative roles in adipogenesis. However, when an elevated number of adipocytes accumulate, there may be a feedback effect, which in turn would upregulate miR-424 to exert its inhibitory functions, thereby restricting the further development of adipose tissues (64).

Proinflammatory cytokines are essential factors in regulating adipocyte maturation (65). TNF-α, a secreted inflammatory factor, was discovered to be positively associated with obesity, both in adults and children (66,67) and has been shown to affect adipogenesis by regulating gene expression (7). Our previous studies revealed that several miRNAs are positively regulated by TNF-α during preadipocyte differentiation, including miR-1908 and miR-146b (34,54,58,68). In the present study, it was observed that miR-424 expression levels were inhibited by TNF-α treatment in mature adipocytes, suggesting that miR-424 may have opposing functions to TNF-α, miR-1908 and miR-146b. This was further confirmed through dual luciferase reporter assays, in which TNF-α significantly inhibited the transcription of a reporter gene via binding to the miR-424 promoter.

In biological systems, miRNAs normally exert their cellular functions by regulating the expression of target proteins (69). Aiming to further understand the function of miR-424 in adipogenesis, its target genes were predicted and their functions were bioinformatically analyzed. The target genes were found to be enriched in several processes, among which focal adhesion, the Wnt signaling pathway and ubiquitin mediated proteolysis have all been previously associated with adipogenesis (70-72). These results improved the current understanding of the mechanisms through which miR-424 may regulate adipogenesis.

In conclusion, the results of the present study revealed that miR-424 expression levels were upregulated in abdominal fat biopsies from obese children. In cell culture experiments, miR-424 expression levels were discovered to be significantly downregulated during preadipocyte differentiation. Furthermore, TNF-α treatment was discovered to downregulate miR-424 expression through binding to its promoter region. The prediction of miR-424 target genes involved in adipogenesis revealed signaling pathways and biological processes that may regulate preadipocyte differentiation. However, additional studies on miR-424 regulatory mechanisms are required to provide a deeper understanding of the function of miR-424 in adipocyte differentiation and its potential association with obesity onset. The present study provided novel insights into the association between adipogenesis and miR-424 expression levels, opening the possibility of miR-424 being characterized in the future as a candidate biomarker for the diagnosis of obesity and related diseases.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

XRG, XC and LJZ conceived and designed the study. QZX performed the experiments. ZYF analyzed the data and drafted the manuscript. XRG and XC reviewed and edited the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Written informed consent was obtained from the parents or legal guardians of all participants, and the study protocol was approved by the ethics committees and the institutional review board of The Affiliated Hospital of Nantong University (approval no. 2019-K050; Nantong, China).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests

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