miR-103/107 promote ER stress mediated apoptosis via targeting the Wnt3a/β-catenin/ATF6 pathway in preadipocytes

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Running Title: miR-103/107 promote preadipocytes apoptosis
Abstract

Both miR-103 and 107 have been demonstrated to restrain cell proliferation, regulate lipid metabolism and inflammation. However, the effects of miR-103/107 on preadipocytes apoptosis remain unknown. In the present research, we have investigated how miR-103/107 regulated preadipocytes apoptosis. We found that miR-103/107 aggravated Endoplasmic Reticulum (ER) stress mediated apoptosis in preadipocytes. We confirmed that miR-103/107 targeted Wnt3a (WNT family member 3a) in preadipocytes. It was found that overexpressing Wnt3a resulted in suppression of ER stress mediated apoptosis, while restoration of miR-103/107 counteracted the effects of Wnt3a in preadipocytes. Moreover, bioinformatics and luciferase assays indicated that Activating Transcription Factor 6 (ATF6) is a key player linking miR-103/107 induced ER stress to apoptosis. ATF6 is regulated by Lymphoid Enhancer-Binding Factor 1 (LEF1), a transcription factor downstream of Wnt3a/β-catenin signaling pathway, and ATF6 binds to the B-cell lymphoma 2 (Bcl2) promoter to regulate apoptosis further. In conclusion, miR-103/107 promoted ER stress mediated apoptosis by targeting the Wnt3a/β-catenin/ATF6 signaling pathway in preadipocytes. This study revealed that miR-103/107-Wnt3a/β-catenin-ATF6 pathway is critical to the progression of apoptosis in preadipocytes, which suggested that approaches to activate miR-103/107 could potentially be useful as new therapies for treating obesity and metabolic syndrome related disorder.

Key words: Preadipocytes; miR-103/107; Endoplasmic Reticulum stress; Apoptosis; Wnt3a/β-catenin-ATF6 pathway
Introduction

Now a day, the obesity is a global epidemic with the morbidity increasing at a particularly high rate (1). Obesity, characterized by excessive adipose tissue, is related to some metabolic disease such as hypertension, cardiovascular disease, type 2 diabetes mellitus (T2DM) (2,3). As an important factor of obesity, adipose tissue has become a new targeting for obesity treatment. Except for adipogenesis and lipolysis, adipocyte death due to apoptosis has been demonstrated to play a very important role in the loss of adipose tissue. The adipose tissue contains a considerable amount of fibroblast-like preadipocytes. Preadipocytes have the ability to proliferate to produce new adipocytes and differentiate into mature adipocytes as well, which account for healthy adipose tissue turnover. Thus, induction of preadipocyte apoptosis may be an effective approach for obesity treatment.

MicroRNAs (miRNAs) are a class of small (18~25 nt) single-stranded noncoding RNAs, which are function as endogenous regulators by targeting mRNAs (4). miRNAs dysfunction is related to a variety of human diseases, such as obesity and metabolic disorders, suggesting that miRNAs play a promising role in obesity (5,6). Aberrant expression of miRNAs, including miR-221, miR-519d, miR-141 and miR-520e is associated with human obesity and related metabolic syndrome (7-9). miR-103 and 107 belong to the same family, differing only at one nucleotide residue close to their 3’ ends. It has been documented that miR-103/107 can retard tumor angiogenesis, promote adipogenesis, suppress cell proliferation, regulate lipid metabolism and control insulin sensitivity (10-12). However, to date, the roles of
miR-103 and miR-107 in preadipocyte apoptosis remained unknown.

The Wnt signaling pathway plays a pivotal role in embryonic development and apoptosis regulation. Studies have shown that the canonical Wnt/β-catenin pathway is participated in multiple biological functions, including apoptosis and proliferation by targeting downstream genes (13). Wnt3a is a canonical Wnt ligand that is an important regulator of various developmental processes, especially osteogenesis, apoptosis, adipogenesis and mitochondrial biogenesis (14). Wnt ligands, including Wnt3a, bind to cell surface Frizzled receptor family, and regulate β-catenin stabilization and translocation to nucleus where it can interact with the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family to regulate the transcription of target genes (15,16). In the absence of Wnts, β-catenin in cytoplasm is constantly degraded by the degradation compounds, which prevents β-catenin reaching the nucleus, resulting target genes being restricted by the TCF/LEF family (17).

Endoplasmic reticulum (ER) is an important organelle in cells. It is not only the place for protein folding and transportation, but also important for intracellular Ca$^{2+}$ storage and cholesterol, steroids and many lipid synthesis (18,19). In a variety of physiological or pathological conditions, such as inhibition protein glycosylation and imbalance of calcium homeostasis will cause unfolded or misfolded protein in the ER accumulation, and thus damage normal ER physiological function, termed ER stress (20,21). Under these conditions, the ER actives the unfolded protein response (UPR) to pause early protein synthesis and reduce the unfolded or misfolded protein
in ER, thus restoring normal physiological function and protecting cells finally. However, if the ER stress continues or cannot be alleviated, the signal will ultimately cause apoptosis (22,23).

In the present study, we treated preadipocytes with palmitate or serum free medium and found that miR-103 and 107 levels increased sharply. Bioinformatics analysis and dual-luciferase reporter assays revealed that Wnt3a, which is involved in processes related to adipocytes, is a novel target of miR-103/107. miR-103/107 promoted ER stress mediated apoptosis, and Wnt3a decreased ER stress mediated apoptosis. Furthermore, miR-103/107 were found to inhibit the canonical Wnt/β-catenin signaling pathway, with LEF1/ATF6/Bcl2 transcription playing a critical role in miR-103/107 aggravated ER stress mediated apoptosis. Considering these results, it could be concluded that miR-103/107 played an important role to facilitate ER stress mediated apoptosis in preadipocytes by targeting Wnt3a.

MATERIALS AND METHODS

Primary preadipocyte culture

The proposal of Primary preadipocyte culture was as described before (24). Briefly, the eWAT tissues were washed three times with PBS buffer containing 200 U/mL penicillin (Sigma, St. Louis, MO, USA) and 200 U/mL streptomycin (Sigma, St. Louis, MO, USA). And the connective fiber and blood vessels were removed, and then preadipocytes were seeded onto culture dishes at 30% confluency and incubated at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air until
Differentiation of preadipocytes was performed as follows (25). Cells grown to 100% confluence (Day 0) were induced to differentiation using DMEM/F12 medium containing dexamethasone (1 μM, Sigma, St. Louis, MO, USA), insulin (10 μg/mL, Sigma, St. Louis, MO, USA), IBMX (0.5 mM, Sigma, St. Louis, MO, USA) and 10% FBS. Four days after the induction (from Day 2), cells were maintained in the induction medium containing insulin (10 μg/mL, Sigma, St. Louis, MO, USA) and 10% FBS.

**Cell stimulation**

The miR-103 and 107 mimics, inhibitor, miRNA negative control, siATF6 and siBcl2 were purchased from Gemma (Shanghai, China). Wnt3a and ATF6 CDS were cloned into pc-DNA 3.1(+) to generate plasmid vector. Preadipocytes were transfected at 70% confluence using X-tremeGENE HP DNA Transfection Reagent (Roche, Carlsbad, CA, USA). Transfection procedure was followed protocol provided by the manufacturer.

For stimulation, preadipocytes were treated with 250 nM palmitate (Sigma, St. Louis, MO, USA) for 24 h. 50 μM of 4-phenylbutyric acid (4-PBA, Selleck, Shanghai, China) was incubated for 2 h to relieve ER stress. 150 ng/mL of Dickkopf1 (DKK1, Selleck, Shanghai, China) was incubated for 3 h to block canonical Wnt pathway.

**RNA extraction and cDNA synthesis**
Total RNA, including miRNA, was extracted from cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA was synthesized using a PrimeScript™ Ⅱ 1st Strand cDNA RT Synthesis Kit (Takara Biotechnology Co., Ltd., Dalian, China). Especially, the mature miRNA was reverse transcribed extra with the miRNA-specific stem-loop primer (5’ GAAAGAAGGGAGGAGCAGATCGAGGAAGAAGACGGAAGAATGTGCGTCTCGCTICTTCTCNNNNN 3’).

**Quantitative PCR analysis**

QPCR was used to detect the relative mRNA expression levels with AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China) as described in Gan et al (26). GAPDH and U6 were used to normalize mRNA and miRNA levels, respectively. The relative genes expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are as follows:

- miR-103-F: AGCAGCATTGTACAGGGCTATGA,
- miR-107-F: AGCAGCATTGTACAGGGCTATCA,
- miRNA-R: AAGGCGAGACGCACATTCTT,
- U6-F: CTCGCTTCGGCAGCACA,
- U6-R: AACGCTTCACGAATTTGCGT,
- Wnt3a-F: ATGCCTCAGAGATGTTGCCTCACT,
- Wnt3a-R: TCAGATGGGTCTGAAACAACCCT.
Bax-F:CAGGATGCGTCCACCAA,
Bax-R:AAAGTAGAAGAGGGCAACCAC,
Bad-F:TGAGCCGAGTGAGCAGGAA,
Bad-R:GCCTCCATGATGACTGTTGGT,
Bim-F:GACAGAACCAGCAAGGTAATCC,
Bim-R:ACTTGTCAACACTCATGGGTG,
Bcl2-F:GGGAGAACAGGAGGTACGATAA,
Bcl2-R:TACCCAGCCTCCGTTATCC,
Grp78-F:GCATCAGCGCCGTCGTATGT,
Grp78-R:ATTCCAAGTGCTCCGATGAG,
Chop-F:CTCGCTCTCCAGATTCCAGTC,
Chop-R:CTTCATGCGTTGCTTCCCA,
Atf4-F:CCTGAACAGCGAAGTGTTGG,
Atf4-R:TGGAGAACCCATGAGGTTCAA,
Atf6-F:TCGCTTTTTAGTCCGGTTCTT,
Atf6-R:GGCTCCATAGGTCTGACTCC,
GAPDH-F:AATGGATTTGGACGCATTGGT,
GAPDH-R:TTTGCACTGGTACGTGTTGAT.
**miRNA target gene prediction**

To predict the miRNA target gene, we used three different bioinformatics softwares: TargetScan ([http://www.targetscan.org/vert_71/](http://www.targetscan.org/vert_71/)), miRBase ([http://www.mirbase.org/](http://www.mirbase.org/)) and PicTar ([http://www.pictar.org/](http://www.pictar.org/)). In order to improve the accuracy of the forecast, we choose their intersection to carry out our research.

**Reporter constructs and Dual Luciferase Reporter Assays**

Dual luciferase reporter assay proposal was performed as described in Liu et al (27). The fragment of the 3′-untranslated region (UTR) of Wnt3a for miR-103 and miR-107 was synthesized and cloned into the region of the PGL3 vector. The mutant of Wnt3a vector (PGL3-Wnt3a–mut) was cloned as the same.

For luciferase assay, renal luciferase expression plasmid (pRL-TK) vector and pGL3 vector containing wild type fragment of the 3′-UTR of Wnt3a or mutation fragment were transfected into 293T cells with miR-103 mimics or control. 48 h later, cells were harvested to analyze the luciferase activity using the Dual Luciferase Reporter Assay kit (Promega Corporation, Madison, WI, USA).

**Caspase3 activity Measurement**

Caspase3 activities were determined using a Caspase-Glo® 3 Assay Systems (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

**Apoptosis analysis by flow cytometry**
Preadipocytes were transfected with miR-103/107 mimics, inhibitor or control prior to apoptosis assessment. Annexin V-FITC Apoptosis Detection Kit (Vazyme Biotech Co., Ltd, Nanjing, China) was used to analyze cell apoptosis. The procedure was followed protocol provided by the manufacturer. The data were analyzed with FCS express software.

**TUNEL staining**

TUNEL BrightGreen Apoptosis Detection Kit (Vazyme, Nanjing, China) was further used to detect apoptosis. The procedure was followed protocol provided by the manufacturer.

**Western blot**

Western blot analysis was performed using standard methods as described in Liu et al (28). Primary antibodies used were anti-Wnt3a (Abcam, ab28472), anti-Bax (Abcam, ab32503), anti-Bcl2 (Bioworld, bs1511), anti-GRP78 (Abcam, ab108615), anti-CHOP (Abcam, ab179823), anti-Caspase-3 (Bioworld, bs6428), anti-active Caspase-3 (Bioworld, bs7004), anti-β-actin (Bioworld, ap0060), anti-GAPDH (Bioworld, ap0063). The rabbit secondary antibody was purchased from Abcam.

**Nuclear protein extraction**

The nuclear fractions were prepared as described in Liu et al (25). Briefly, cells were lysed with cytoplasmic lysis buffer. The lysates were centrifuged and supernatants were collected as cytoplasmic fractions. The pellets were resuspended in nuclear extraction buffer. Then the nuclear fractions were centrifuged and the
supernatant was collected to obtain nuclear proteins. The proteins were denatured by boiling and kept for further studies.

**Immunofluorescent Staining**

Immunofluorescent staining were performed as described (25). After fixed with 4% paraformaldehyde and blocked with 5% bovine serum albumin (BSA), the cells were incubated with rabbit polyclonal primary antibody overnight, followed by incubation with fluorescein isothiocyanate- conjugated goat anti-rabbit IgG antibody (Boster, Wuhan, China). DAPI was used for nuclear staining. Finally, cells were observed and photographed using a fluorescence microscope.

**Data analysis**

All experiments were repeated at least three times. The data were expressed as mean ± standard deviation (SD). The statistical analysis of differences was performed in GraphPad Prism 5.0 using t-test. $P<0.05$ (*) was considered to show statistical significance, and $P<0.01$ (**) means very significant.

**RESULTS**

**Wnt3a is a target gene of miR-103/107**

In order to understand the biological functions of miR-103/107, we used three different programs to identify the potential molecular targets of miR-103/107. For miRNAs are known to regulate target mRNAs by binding to their 3’-UTR regions, Wnt3a was identified as a potential target of miR-103/107 based on sequences in its
3'-UTR region that were complementary to the seed sequences of miR-103/107. Moreover, we found that these sequences of Wnt3a were evolutionarily conserved (Fig.1A). Dual luciferase reporter assays were performed to determine if miR-103/107 directly targets Wnt3a. Both co-transfection of miR-103 or 107 mimics with a wt-Wnt3a-3'UTR luciferase reporter vector resulted in about 28% decline of luciferase activity than that in the transfection with mutation groups (Fig.1B). Furthermore, western blot analysis indicated that overexpression of miR-103/107 by transfection miR-103 or miR-107 mimics decreased Wnt3a expression by 25% and 12% compared to the control respectively, whilst inhibition of miR-103 or 107 by transfection miR-103/107 inhibitor resulted in an approximately 35% increase of Wnt3a levels (Fig.1C). In addition, we also detected the mRNA levels of miR-103/107 and Wnt3a during the process of adipocyte differentiation, founding that miR-103/107 and Wnt3a had opposite trends (Fig.1D). In summary, these results clearly suggested that miR-103/107 directly target and suppress Wnt3a.

**miR-103/107 were upregulated in preadipocytes on treatment with palmitate and serum free medium**

Two different types of apoptosis model were used to investigate the roles of miR-103/107 in preadipocyte apoptosis. We found that treatment with serum free medium and 250 nM palmitate resulted in induction of apoptosis in a time-dependent manner, with 24 h incubation period sufficient to induce apoptosis, for qPCR showed that mRNA levels of Bcl-2-associated X protein (Bax) were increased by about 78% and 191% respectively, while Bcl2 mRNA experienced a degradation of
43% and 56%, respectively (Fig.2A and 2F). Furthermore, western blot results also indicated that serum free medium caused a 50% increase of Bax and an 18% decreased of Bcl2 (Fig.2B). And 250 nM palmitate resulted in a 53% increase of Bax and a 55% decreased of Bcl2 (Fig.2G). We also examined levels of miR-103/107 and Wnt3a in these models over several time points and found that palmitate and serum starvation treatment induced an about 150% and 110% increase expression of miR-103/107 respectively, while expression of Wnt3a was downregulated significantly (Fig.2C and 2H). Reports have indicated that continuous ER stress leads to apoptosis ultimately, so we decided to probe the role of ER stress in this process. The levels of ER stress factors, Glucose-Regulated Protein 78 (GRP78) and CCAAT/enhancer-binding protein-homologous protein (CHOP) were detected to evaluate ER stress level. After 24 h serum free medium treatment, GRP78 was found to increase by 97% and 75% at mRNA and protein level respectively, and CHOP was found to elevate to 312% and 141% at mRNA and protein level respectively (Fig.2D and 2E). 250 nM palmitate incubation resulted in GRP78 and CHOP mRNA increased to 297% and 206% respectively (Fig.2I). The protein levels of GRP78 and CHOP were found to elevate by 31% and 37% respectively (Fig.2J). These results indicated that both serum starvation and palmitate treatment active ER stress, and miR-103/107 may play important roles in serum starvation and palmitate treatment actived ER stress and apoptosis.

miR-103/107 mimics promoted preadipocytes ER stress and apoptosis
Based on this data and previous literature reports, 250 nM palmitate was chosen as an apoptotic model to elucidate the roles of miR-103/107 in preadipocyte ER stress and apoptosis. In this study, preadipocytes were transfected with miR-103/107 mimics or miRNA mimics negative control. ER stress and apoptotic makers were determined by qPCR and western blot. As displayed in Fig.3A, miR-103/107 mimics effectively enhanced miR-103 or 107 levels respectively. We found miR-103/107 mimics dramatically increased the mRNA levels of GRP78, CHOP, Transcription Factor 4 (ATF4) and ATF6 (Fig.3B), and the protein levels of GRP78 and CHOP were also upregulated significantly (Fig.3C), indicating miR-103/107 activated ER stress. It was also found that the mRNA levels of Bcl2-associated death protein (Bad), Bcl-2 interacting mediator of cell death (Bim) and Bax were increased, while Bcl2 mRNA level was decreased to about 55% compared to control (Fig.3D). Interestingly, except for the upregulated Bax and downregulated Bcl2 protein levels, we also observed the expression of active caspase-3 was elevated by 10%-30% (Fig.3E). So we examined Caspase-3 activity and found Caspase-3 activity was raised to $1.82 \pm 0.18$, $1.60 \pm 0.30$ and $1.62 \pm 0.16$ after miR-103, 107 or both respectively (Fig.3G). Moreover, with TUNEL staining, we found miR-103/107 mimics increased positive cell number (Fig.3F). After Annexin V-FITC/PI staining, flow cytometry analysis also showed miR-103/107 mimics treatment increase the proportion of apoptotic cells from $52.94\% \pm 2.13\%$ to $66.61\% \pm 3.25\%$, $64.90\% \pm 4.06\%$ and $62.85\% \pm 2.78\%$, respectively (Fig.3H).

miR-103/107 knockdown suppressed ER stress and apoptosis in preadipocytes
To confirm the roles of miR-103/107 on preadipocytes ER stress and apoptosis, we carried out studies using miR-103/107 inhibitor to knockdown miR-103/107 (Fig.4A). As expected, miR-103/107 inhibitor downregulated the mRNA levels of GRP78, CHOP, ATF4 and ATF6 (Fig.4B). Western blot analysis revealed that GRP78 experienced a degradation of approximately 30%-40% after miR-103/107 inhibitor transfection, and CHOP had the same change (Fig.4C). In addition, the mRNA levels of Bad, Bim and Bax were also reduced significantly, whilst the level of Bcl2 mRNA was raised to 150%-170% in miR-103/107 inhibitor groups compared to miRNA inhibitor negative control group (Fig.4D). Western blot analysis revealed that miR-103/107 inhibitor decreased the protein levels of Bax and active caspase 3, as well as upregulated the expression of Bcl2 (Fig.4E). Caspase-3 activity was found to be reduced by 40%-60% after miR-103/107 inhibitor treatment (Fig.4G). Furthermore, TUNEL staining and flow cytometry analysis revealed that suppressing miR-103/107 decreased the number of apoptotic cells compared to control (Fig.4F and 4H). Therefore, these findings suggested that miR-103/107 promoted ER apoptosis in preadipocytes as well.

Wnt3a relieved the effects of miR-103/107 on preadipocytes ER stress and apoptosis

Since we had shown that miR-103 and 107 directly targeted Wnt3a, we carried out a set of experiments to study the effect that interactions between miR-103/107 and Wnt3a on ER stress and apoptosis. Preadipocytes were treated with Wnt3a in the presence/absence of miR-103/107 mimics. Wnt3a had an inhibitory effect on
miR-103/107 levels (Fig. 5A). It was found that Wnt3a reduced ER stress, with the mRNA levels of GRP78, CHOP, ATF4 and ATF6 being decreased by approximately 40%-65%, and the protein levels of GRP78 and CHOP being reduced to 50%-70% (Fig. 5B and 5C). Besides, the mRNA levels of Bad, Bim and Bax and the protein levels of Bax and Caspase-3 were also downregulated, whilst Bcl2 mRNA and protein level was increased (Fig. 5D and 5E), indicating that Wnt3a inhibited apoptosis and ER stress. Besides, Wnt3a was also found to decreased Caspase-3 activity to 63% compared to control (Fig. 5G). TUNEL staining showed Wnt3a treatment reduced the number of apoptotic cells (Fig. 5F). Flow cytometric analysis also indicated that Wnt3a reduced the number of apoptotic cells from 54.47% ± 5.22% to 39.68% ± 4.94% (Fig. 5H), which was similar to the results previously observed with miR-103/107 inhibitor. In addition, miR-103/107 mimics extra addition reversed the repressive effects of Wnt3a on ER stress and apoptosis, as the levels of GRP78, CHOP, ATF4, ATF6, Bad, Bim, Bax and Caspase-3 were elevated, while Bcl2 level was decreased compared to the Wnt3a treatment group (Fig. 5A-E). On the other hand, the proportion of apoptotic cells was shown to increase in the Wnt3a and miR-103/107 mimics group than that in the Wnt3a individual treatment group (Fig. 5F and 5H), which was consistent with our previous results.

**miR-103/107 promoted preadipocytes ER stress and apoptosis by inhibiting the canonical Wnt/β-catenin pathway**

As a canonical Wnt ligand, Wnt3a is qualified for activating the canonical Wnt/β-catenin signaling pathway. So we next to determine whether this pathway was
involved in mediating miR-103/107 induced ER stress and apoptosis. The expression of nucleus β-catenin was monitored. Compared with the control, Wnt3a increased nucleus β-catenin level by 70%, and miR-103/107 mimics addition reduced β-catenin level in nucleus (Fig.6A). Similarly, p-β-catenin immunofluorescent staining showed that Wnt3a treatment decreased β-catenin phosphorylation level, and miR-103/107 mimics addition reversed this effect (Fig.6B). Furthermore, Dickkopf1 (DKK-1), a specific inhibitor of the canonical Wnt/β-catenin signaling pathway, was used for verification. We found that DKK-1 raised the expression levels of GRP78, CHOP, Caspase-3 and Bax by 39%, 62%, 73% and 38% respectively, and the Bcl2 level was decreased by 36% (Fig.6C), indicating canonical Wnt/β-catenin signaling inhibition blocked miR-103/107 induced ER stress and apoptosis. Wnt3a addition relieved the effects of DKK-1, and combination with miR-103/107 mimics reversed these effects again (Fig.6C). These data suggested that miR-103/107 promoted preadipocytes ER stress and apoptosis by inhibiting the canonical Wnt/β-catenin pathway at least partly.

**ATF6 is essential in miR-103/107 promoted ER stress and apoptosis**

To clarify the mechanism of how miR-103/107 promoted ER stress, a series of bioinformatics analyses were conducted. We found two putative binding sites of LEF1, an important transcription factor downstream of canonical Wnt/β-catenin signaling pathway, in the ATF6 promoter region (from -1 to -2000). It had been found that activating canonical Wnt/β-catenin signaling by Wnt3a treatment resulted in decreased ATF6 mRNA level (Fig.5B). To determine whether LEF1 is critical for
the regulation of \( ATF6 \) promoter, two sites were mutated, and \( ATF6 \) promoter activity was determined. Overexpressing LEF1 resulted in a 36% decrease of \( ATF6 \) promoter activity, and mutation of the -1538 binding site experienced a 73% increase in \( ATF6 \) promoter activity compared to the wild type group, while there was no difference between the mutation of the -1639 binding site group and wild type group (Fig.7A), suggesting that the -1538 binding site is critical for LEF1 negative regulation of \( ATF6 \).

siATF6 was then used to silence ATF6 to determine whether ATF6 plays a key role in miR-103/107 promoted ER stress and apoptosis. It was found that siATF6 downregulated \( ATF6, GRP78, CHOP, ATF4 \) and \( Bax \) mRNA levels, and upregulated \( Bcl2 \) mRNA level (Fig.7B). Furthermore, siATF6 was shown to raised \( Bcl2 \) mRNA level by 86%, and decrease \( GRP78, CHOP \) and \( Bax \) mRNA levels to 43%, 75% and 51% respectively, which were elevated by miR-103/107 mimics (Fig.7C). Western blot analysis was consistent to the results of mRNA (Fig.7D). These results suggested ATF6 is essential in miR-103/107 promoted ER stress and apoptosis.

**Preadipocytes apoptosis induced by miR-103/107 was mediated by ER stress**

We next addressed whether apoptosis induced by miR-103/107 was related to ER stress, we used 4-phenylbutyric acid (4-PBA) as an ER stress inhibitor to relieve ER stress. These results demonstrated that the expression levels of \( GRP78, CHOP, Caspase 3 \) and \( Bax \) were significantly repressed and \( Bcl2 \) expression level was enhanced in 4-PBA pretreated group (Fig.8A). Compared to miR-103/107 mimics
treatment, 4-PBA pretreatment rescued the levels of GRP78, CHOP, Caspase 3, Bax and Bcl2 (Fig.8A).

In order to understand how ER stress mediated miR-103/107 induced apoptosis, bioinformatics analyses revealed that the Bcl2 promoter (from -1 to -2000) contained a putative ATF6 binding site (-385). We found overexpressing ATF6 inhibited the mRNA level of Bcl2 (Fig.8B). The role of ATF6 on Bcl2 transcription was confirmed by dual luciferase reporter assays. Wild type of the binding site resulted in a 54% decrease in Bcl2 promoter activity, and the mutation of Bcl2 promoter led to a 95% increase compared to wild type group (Fig.8C), suggesting that ATF6 negative regulates Bcl2 transcription. It was also found that knockdown Bcl2 by siBcl2 had no effect on ER stress, as the mRNA levels of ATF6, GRP78 and CHOP remained unchanged (Fig.8D). In contrast, Bax mRNA level was increased to 153%, while Bcl2 mRNA level was decreased to 68% (Fig.8D), suggesting Bcl2 plays a role in apoptosis. We then determined the levels of ER stress and apoptosis after miR-103/107 mimics transfection in the presence/absence siBcl2. These studies revealed that siBcl2 had no effect on miR-103/107 activated ER stress (Fig.8E). On the contrary, siBcl2 treatment upregulated Bax and Caspase-3, and Bcl2 was downregulated (Fig.8E). These results indicated ER stress mediated miR-103/107 induced apoptosis and ATF6 transcriptional regulates Bcl2 was essential during this process.

DISCUSSION
With social development, the number of adolescent obesity is on the rise. More seriously, obesity-related lifestyles and their health hazards will continue to adulthood, increasing the risk of obesity, high blood pressure, heart disease and other chronic non-communicable diseases (29,30). Obesity has become one of the serious public health problems that endanger the health of children and adolescents. Except for exercise, there are two methods for obesity treatment at this stage: drug treatment and bariatric surgery. However, drug treatment has too much unacceptable side effects and bariatric surgery not suit for any obese patient. Considering these problems, inducing apoptosis to reduce adipocyte number by may be an effective approach for obesity treatment. Besides, our previous studies have shown that regulation of apoptosis in adipose tissue having the potential to form the basis of a future treatment of obesity (31-35).

A great number of miRNAs have been implicated in the apoptosis process. For example, miR-125b resisted breast cancer cells via suppressing Bak expression (36). miR-187, miR-181c, and miR-34a also have been proved to regulate apoptosis and inflammation by directly targeting TNF-α (37-39). miR-103 and 107, are paralogs, which differ only at one nucleotide near their 3’ends and reside on different human chromosomes. Their host genes are conserved in all known vertebrates (40).

It has been reported previously that miR-103/107 regulated adipose insulin sensitivity (12). In order to study the effect of miR-103/107 on preadipocytes, bioinformatic analyses were carried out to predict their target genes. We found 3’-UTR region of Wnt3a includes a potential binding sit for miR-103/107 with a 7-nt
match. Interestingly, a previous study in epithelial stem cells had shown that miR-103/107 increased the proliferative capacity of keratinocytes by targeting Wnt3a (41). We employed dual-luciferase reporter system to confirm that miR-103/107 targets Wnt3a in preadipocytes.

As Wnt3a is a canonical Wnt ligand, we examined β-catenin phosphorylation level, and found that miR-103/107 blocked the canonical Wnt/β-catenin signaling pathway. Canonical Wnt/β-catenin signaling pathways are involved in a variety of cell activities, including apoptosis (42,43). As a main ligand of Wnt/β-catenin signaling pathways, Wnt3a binds to the specific receptor, delivering the signal to β-catenin, which accumulates as an important substance in nucleus and regulates the expression of the relevant target genes (15,16). In the deficiency of Wnt ligands, the intracellular β-catenin is degraded by a polyprotein complex consist of Axin/APC/GSK3/DK1, resulting in a low level of β-catenin. In this study, we found LEF1 regulated ATF6 promoter activity, indicating ATF6 is a target gene of the Wnt/β-catenin signaling pathway. In preadipocytes, miR-103/107 were shown to suppress wnt3a expression, block Wnt/β-catenin signaling and degraded β-catenin, resulting in releasing the inhibitory effect of LEF1 on ATF6, and the onset of activated ATF6 related ER stress (Fig.9).

Apoptosis exists through the whole life process of multicellular organisms, which can remove the excess and damaged cells in the body in time and maintain the stability of tissues and organs. Apoptosis as a mechanism of programmed cell death is critical for cell homeostasis which allows deleting redundant cells in a controlled
manner (44). There are three apoptotic pathways in eukaryotic cells: the death receptor-mediated extrinsic pathway, the intrinsic mitochondrial pathway and ER stress mediated pathway which we studied in our research (45). When the intracellular environment changes, such as hypoxia, hypoglycemia and oxidative stress, unfolded proteins accumulate on endoplasmic reticulum, thus resulting in UPR through the ER stress sensors located in the ER membrane: IRE1α, PERK and ATF6. However, if ER stress continues at a high level, the adaptation mechanisms are useless, ER stress is irreparable, and the sensors produce signal transduction to promote apoptosis by modulating Bcl2 family proteins or regulating calcium channels on ER (46). In this study, we have shown ATF6 transcriptionally inhibited Bcl2 promoter activity, thus firmly demonstrating a link between ER stress and apoptosis in preadipocytes.

This study is concentrated on the effect of miR-103/107 on preadipocytes apoptosis. Studies have shown that miR-103/107 is highly expressed in adipose tissue and palmitate and serum free medium treatment induced ER stress and apoptosis accompanied by upregulated miR-103/107 levels, suggesting miR-103/107 may play important roles in the control of ER stress and apoptosis. We had demonstrated that overexpression of miR-103/107 by miR-103/107 mimics promoted ER stress and apoptosis, whilst knockdown miR-103/107 by miR-103/107 inhibitor suppressed ER stress and apoptosis. Using the bioinformatics, cellular and molecular biology methods we confirmed that miR-103/107 targeted Wnt3a to promote ER stress and apoptosis in preadipocytes. Moreover, bioinformatic and
luciferase assays indicated that ATF6 and Bcl2 are key players linking miR-103/107 induced ER stress to apoptosis in preadipocytes. ATF6 is regulated by LEF1, and ATF6 binds to the Bcl2 promoter to further regulate apoptosis.

In conclusion, we have demonstrated that miR-103/107 suppressed Wnt3a expression, blocked Wnt/β-catenin signaling pathway and degraded β-catenin, resulting in releasing the inhibitory effect of LEF1 on ATF6, and the onset of activated ATF6 related ER stress. Furthermore, ATF6 transcriptionally inhibited Bcl2 promoter activity, activating apoptosis finally (Fig.9). We propose that this miR-103/107 induced apoptosis pathway may provide an important target for developing new therapies for the treatment of obesity and metabolic syndrome.

Conflicts of interest

None.

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Figure 1. Wnt3a is a target gene of miR-103/107.

(A) Scheme of the potential binding sites of miR-103/107 in the Wnt3a 3′UTR. (B) Double luciferase assay was performed in 293T cells 48 h after miR-103/107 mimics transfected with Wnt3a-wt 3′UTR or Wnt3a-mut 3′UTR. (C) Changes of Wnt3a protein level after 48 h transfected with miR-103/107 mimics, inhibitors or control were detected by western blot. (D) Changes of miR-103/107 and Wnt3a mRNA levels in the process of adipocytes differentiation. Data represented the mean ± SD (*P < 0.05; **P < 0.01. n≥3).

Figure 2. miR-103/107 were upregulated in apoptosis models.

Preadipocytes were treated with serum free medium (A-E). (A) The mRNA levels of Bax and Bcl2. (B) The protein levels of Bax and Bcl2. (C) Changes of miR-103/107 and Wnt3a mRNA levels. (D) The mRNA levels of GRP78 and CHOP. (E) The protein levels of GRP78 and CHOP. Preadipocytes were treated with palmitate (F-J). (F) The mRNA levels of Bax and Bcl2. (G) The protein levels of Bax and Bcl2. (H) The changes of miR-103/107 and Wnt3a mRNA levels. (I) mRNA levels of GRP78 and CHOP. (J) The protein levels of GRP78 and CHOP. Data represented the mean ± SD (*P < 0.05; **P < 0.01. n≥3).

Figure 3. Overexpressing of miR-103/107 promoted preadipocytes ER stress and apoptosis.

Preadipocytes were transfected with miR-103/107 mimics or control. (A) The changes of miR-103 and 107. (B) The changes of GRP78, CHOP, ATF4 and ATF6 mRNA levels. (C) The changes of GRP78 and CHOP protein levels. (D) The changes of Bad, Bim, Bax and Bcl2 mRNA levels. (E) The changes of Bax, Bcl2 and
Caspase-3 protein levels. (F) TUNEL staining assessed the effects of miR-103/107 mimics on preadipocytes apoptosis. (G) Caspase-3 activity was measured. (H) Flow cytometry was used to analyze the effects of miR-103/107 mimics on preadipocytes apoptosis. Data represented the mean ± SD. scale bars: 200μm (*P < 0.05; ** P < 0.01. n ≥ 3).

**Figure 4. Inhibition of miR-103/107 suppressed ER stress and apoptosis in preadipocytes.**

Preadipocytes were transfected with miR-103/107 inhibitor or control. (A) The changes of miR-103 and 107. (B) The changes of *GRP78, CHOP, ATF4* and *ATF6* mRNA levels. (C) The changes of GRP78 and CHOP protein levels. (D) The changes of Bad, Bim, Bax and Bcl2 mRNA levels. (E) The changes of Bax, Bcl2 and Caspase-3 protein levels. (F) TUNEL staining assessed the effects of miR-103/107 inhibitor on preadipocytes apoptosis. (G) Caspase-3 activity was measured. (H) Flow cytometry was used to analyze the effects of miR-103/107 inhibitor on preadipocytes apoptosis. Data represented the mean ± SD. scale bars: 200μm (*P < 0.05; ** P < 0.01. n ≥ 3).

**Figure 5. Wnt3a relieved the effects of miR-103/107 on preadipocytes ER stress and apoptosis.**

Preadipocytes were transfected with Wnt3a vector combine with or without miR-103/107 mimics or control. (A) The changes of Wnt3a, miR-103 and 107. (B) The changes of *GRP78, CHOP, ATF4* and *ATF6* mRNA levels. (C) The changes of
GRP78 and CHOP protein levels. (D) The changes of Bad, Bim, Bax and Bcl2 mRNA levels. (E) The changes of Bax, Bcl2 and Caspase-3 protein levels. (F) TUNEL staining assessed the effects of Wnt3a and miR-103/107 on preadipocytes apoptosis. (G) Caspase-3 activity was measured. (H) Flow cytometry was used to analyse the effects of Wnt3a and miR-103/107 on preadipocytes apoptosis. Data represented the mean ± SD. scale bars: 200μm (*P < 0.05; ** P < 0.01. n≥3).

Figure 6. miR-103/107 promoted preadipocytes ER stress and apoptosis by inhibiting the canonical Wnt/β-catenin pathway.

Preadipocytes were treated with Wnt3a vector combined with or without miR-103/107 mimics or control, and DKK-1 was added into medium 3 h before collected. (A) The changes of nucleus β-catenin level. (B) p-β-catenin immunofluorescent staining. (C) The changes of protein levels of GRP78, CHOP, Bax, Bcl2 and Caspase-3. Data represented the mean ± SD. scale bars: 200μm (*P < 0.05; ** P < 0.01. n≥3).

Figure 7. ATF6 is essential in miR-103/107 promoted ER stress and apoptosis.

(A) Dual-Luciferase Reporter Assays was performed to test ATF6 promoter activity. (B) qPCR was used to analyze the effects of siATF6 on GRP78, CHOP, ATF4, Bax and Bcl2. Preadipocytes were treated with miR-103/107 mimics combine with or without siATF6 (C and D). (C) qPCR was performed to detect the changes of GRP78, CHOP, Bax and Bcl2 mRNA level. (D) The protein levels of GRP78,
CHOP, Bax, Bcl2 and Caspase-3. Data represented the mean ± SD (*P < 0.05; ** P < 0.01. n≥3).

**Figure 8. miR-103/107 promoted preadipocytes apoptosis was mediated by ER stress.**

(A) Preadipocytes were treated with miR-103/107 mimics combined with or without 4-PBA. Western blot was performed to detect the expression changes of GRP78, CHOP, Bax, Bcl2 and Caspas 3. (B) qPCR was used to detect the effect of ATF6 on Bcl2. (C) Dual-Luciferase Reporter Assays was performed to detect Bcl2 promoter activity. (D) The effect of siBcl2 on ATF6, GRP78, CHOP, Bax and Bcl2 mRNA levels. (E) Preadipocytes were treated with miR-103/107 mimics combined with or without siBcl2. Western blot analyses were performed to detect the changes of GRP78, CHOP, Bax, Bcl2 and Caspase-3. Data represented the mean ± SD (*P < 0.05; ** P < 0.01. n≥3).

**Figure 9. miR-103/107 promoted ER stress mediated apoptosis via the Wnt3a/β-catenin /ATF6 pathway in preadipocytes.**

miR-103/107 blocked wnt/β-catenin signaling by targeting Wnt3a, released the inhibitory effect of LEF1 on ATF6, and activated ATF6 related ER stress. On the other hand, ATF6 transcriptional inhibited Bcl2, inducing apoptosis finally.
Figure 1:

A. Diagram showing the interaction of RL with wnt3a 3'UTR.

B. Graph showing relative luciferase activity with different treatments.

C. Western blot analysis of Wnt3a and GAPDH with miR-103 and miR-107 mimics and inhibitors.

D. Graph showing the relative level of Wnt3a over time with different treatments.
Figure 3:
Figure 4:
Figure 5:
Figure 6:

A

B

C

[Graphical representation of experimental results related to nuclear β-catenin level and relative protein level across different conditions.]
Figure 7:
Figure 8:

A

miR-103 mimics  -  +  -  -  +  -
miR-107 mimics  -  -  +  -  -  -
4-PBA  -  -  +  +  +  +
GRP78  +  +  +  +  +  +
CHOP  +  +  +  +  +  +
Active Caspase-3  +  +  +  +  +  +
T-Caspase-3  +  +  +  +  +  +
BAX  +  +  +  +  +  +
BCL2  +  +  +  +  +  +
β-actin  +  +  +  +  +  +

B

Relative mRNA Level

+4-PBA  |

C

Relative Luciferase Activity

ATF6  -  -  +  +
Bc2-wt  +  +  +  -
Bc2-mut  -  -  -  +

D

Relative mRNA Level

GRP78  +  +  +  +  +  +
CHOP  +  +  +  +  +  +
Active Caspase-3  +  +  +  +  +  +
T-Caspase-3  +  +  +  +  +  +
BAX  +  +  +  +  +  +
BCL2  +  +  +  +  +  +
β-actin  +  +  +  +  +  +

E

Relative Protein Level

GRP78  +  +  +  +  +  +
CHOP  +  +  +  +  +  +
Active Caspase-3  +  +  +  +  +  +
T-Caspase-3  +  +  +  +  +  +
BAX  +  +  +  +  +  +
BCL2  +  +  +  +  +  +
β-actin  +  +  +  +  +  +
Figure 9: