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miR-1934, downregulated in obesity, protects against low-grade inflammation in adipocytes

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Abstract

It has been previously demonstrated that miR-1934 was specially regulated by adiponectin, an anti-inflammatory adipokine, in adipose tissue (AT) *in vivo*. Herein we investigated the role of miR1934 in the regulation of inflammatory response. Compared with chow-diet fed mice, miR1934 expression was down-regulated in epididymal AT of high-fat diet-induced obese mice. miR1934 expression was down-regulated as well in omental AT of obese subjects in comparison with lean subjects. The circulating miR-1934 was also lower in obese subjects and its levels were correlated negatively with body mass index (BMI), waist circumference (WC), low-density lipoprotein cholesterol (LDL-c), insulin resistance and high-sensitivity C-reactive protein (hs-CRP). The down-regulation of miR1934 in obesity was mimicked by TNF- α treatment of 3T3-L1 adipocytes. Moreover, overexpression of miR1934 suppressed the TNF- α -induced gene expression of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in 3T3-L1 adipocytes. *De novo* formed AT in nude mice transplanted with 3T3-L1 preadipocytes overexpressing miR1934 displayed a reduction in IL-6, TNF- α , IL-1 β and an enhancement in IL-10 gene expression when compared with transplant with 3T3-L1 preadipocytes overexpressing miR1934 scrambled control sequence. These results suggest that miR1934 is an important anti-inflammatory factor and may represent a novel mechanism for controlling AT inflammation.

Keywords

Obesity; Low-grade inflammation; microRNA; Adipose tissue; Adipocyte

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Disclosure statement

The authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2016.03.026>.

1. Introduction

During the past decade, it became clear that a low-grade pro-inflammatory state in adipose tissue (AT) is a key feature of obesity. This low-grade inflammation is considered to build the common soil for the development of obesity-linked disorders like insulin resistance. Resetting the immunological balance in AT may be a crucial approach for the future management of the metabolic syndrome (MS) (Hotamisligil, 2006; Maury and Brichard, 2010). Recently, microRNAs (miRNAs), the endogenous ~22 nt RNAs, have been shown as a new mechanism mediating AT inflammation. A number of miRNAs have been found dysregulated in AT during obesity and closely associated with obesity-related metabolic disorders including type 2 diabetes and atherosclerosis (Chartoumpekis et al., 2012; Ortega et al., 2010; Kloting et al., 2009; Heneghan et al., 2011; Arner et al., 2012; Arner and Kulyte, 2015). Moreover, inflammation *per se* could alter the expression pattern of miRNAs in AT (Ortega et al., 2015; Xie et al., 2009). Furthermore, specific miRNAs have been identified as crucial regulators of AT inflammation (Arner et al., 2012; Parra et al., 2010; Strum et al., 2009; Ge et al., 2012; Zhuang et al., 2012). These small oligonucleotides can also be secreted by adipocytes in the form of micro-vesicles (ADMs). The miRNAs-contained ADMs released by adipocytes may either go into circulation or transfer into the neighboring adipocytes or macrophages, probably acting as inflammatory communicators (Ogawa et al., 2010; Muller et al., 2011; Ge et al., 2014; Fernandez-Messina et al., 2015). Thus, these findings make miRNAs attractive therapeutic targets in AT inflammation.

Adiponectin (ApN), which is secreted almost exclusively by adipocytes and is decreased in obesity, has emerged as a master regulator of inflammation/immunity in various tissues, including AT, its own production site (Ouchi and Walsh, 2007; Ge et al., 2011). The anti-inflammatory property of ApN led to raise the hypothesis that miRNAs targeted by ApN in AT may have functional roles in AT inflammation. By taking advantage of two mouse models: the transgenic mouse overexpressing ApN specifically in white AT (ApN-Overex) (Bauche et al., 2007) and the ApN-knockout mouse characterized by a complete lack of ApN (ApN-KO) (Maeda et al., 2002), Ge et al. have identified one miRNA, miR-1934, which was upregulated in white AT of ApN-Overex mice and showed a reverse undetectable levels in the ApN-KO mice (Ge et al., 2012).

The aim of the present work was to further investigate the functional roles of miR-1934 in the regulation of AT inflammatory response. We examined whether this miRNA was abnormally expressed in obesity, and characterized its function by *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Animals

Sixteen 4-week old male C57BL/6J mice were housed at a constant temperature (22 °C) with a 12 h-light, 12 h-dark cycle. After 2 weeks of adaptive feeding, mice were randomly assigned to receive a chow diet (CD) (15% fat, TD, 2018s; Harlan, Kentucky, USA) or a high-fat diet (HFD) (45% fat, TD 08811; Harlan) for 8 weeks. Body weight was measured weekly during the feeding regimen. At the end of experiment, the measurements of serum

levels of glucose, insulin and triglycerides (TG), the decapitation of mice and the isolation of inguinal and epididymal fat pads were performed as described (Yang et al., 2014). For *de novo* fat pad formation, eight 6-week old male BALB/c Nude mice (Huafukang, Beijing, China) were used. These mice were kept in micro-isolation cages and received an HFD (TD 08811; Harlan) for 4 weeks as described (Ge et al., 2012; Lijnen et al., 2006). At the end of the experiment, the *de novo* formed fat pad was removed, weighed, and stored at -80°C .

The University Animal Care Committee approved all procedures.

2.2. Subjects

Omental adipose tissue was obtained from 21 lean and 14 obese subjects undergoing laparoscopic cholecystectomy. Serum was obtained from another cohort with 25 lean subjects and 24 obese subjects. Obesity was defined as BMI $\geq 25\text{ kg/m}^2$ based on the criteria of World Health Organization (WHO, 1998) adapted for Asian adults (Weisell, 2002). The two groups of participants in each cohort were sex- and age-matched. Body weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively, while subjects were in light clothes and bare feet. WC was measured at the level of the umbilicus in the late exhalation phase while the subject was standing. Blood pressure (BP) was measured on the non-dominant arm in a seated position after a 10-min rest, using an electronic BP monitor (Omron BP-203RVIIIC, Matsuaaka, Japan). Fasting plasma glucose (FPG) and 2-h post-load glucose (2hPG) were measured by the hexokinase-UV/NAD method (Olympus, Tokyo, Japan). Fasting insulin (FINS) levels were determined by chemiluminescence (Beckman, California, USA). Homeostasis model assessment for insulin resistance (HOMA-IR) and homeostasis model assessment for insulin sensitivity (HOMA-IS) were calculated using the formula $\text{FPG (mmol/L)} \times \text{FINS (\mu IU/ml)} / 22.5$ and $1 / \text{FPG (mmol/L)} \times \text{FINS (\mu IU/ml)}$ respectively. Plasma lipids were measured as follows: total cholesterol (TC) using the cholesterol oxidase-HDAOS method (Wako, Osaka, Japan); triglycerides (TGs) using the GPO-HDAOS glycerol blanking method (Wako); high-density-lipoprotein cholesterol (HDL-C) using the immunoinhibition (direct) method (Wako), and low-density-lipoprotein cholesterol (LDL-C) using the selective protection enzymatic (direct) method (Wako). The plasma levels of high-sensitivity C-reactive protein (hsCRP) were measured by immunoturbidimetry on the autoanalyzer (HITACHI 7180, Ichige, Japan). The plasma glycosylated hemoglobin (HbA1c) was measured using boronate affinity HPLC (Trinity Biotech, Dublin, Ireland).

Patients provided written informed consent and the study protocol had the approval of the local Ethical Committee of First Affiliated Hospital of Chongqing Medical University.

2.3. miRNA or mRNA quantification by real-time quantitative polymerase chain reaction (RT-qPCR)

For miRNA quantification, total RNA was extracted from epididymal adipose tissue using a miRNeasy Mini kit (Qiagen, Hilden, Germany) and from serum using a miRNeasy Serum/Plasma kit (Qiagen). A total of 1 μg RNA from AT or a total of 300 ng RNA from serum was subjected to reverse transcription using a miScript Reverse Transcription kit (Qiagen). The resultant cDNA was amplified using a miScript SYBER Green PCR Kit (Qiagen) on a

CFX96 Real-time System (Bio-Rad, California, USA). The miR-1934-specific forward primer was purchased from Qiagen, and a universal primer (provided in the miScript SYBER Green PCR Kit) was used as the reverse primer.

For mRNA quantification, total RNA was extracted and subjected to reverse transcription and RT-qPCR with the designated primers (Supplementary Table 1) as previously described (Ge et al., 2011).

Cyclophilin, GAPDH, β -actin or RNU6B (Qiagen) was used as the internal control. Relative changes in the expression level of one specific gene were presented as 2^{-Ct} .

2.4. Culture of 3T3-L1 adipocytes

Murine 3T3-L1 preadipocytes were grown at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Gibco, California, USA) and 1% antibiotics (Hyclone, Logan, USA). Two days after 90% confluence (day 0), adipocyte differentiation was induced by adding 100 nmol/L insulin (Sigma–Aldrich, Bornem, Belgium), 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma–Aldrich) and 0.25 mmol/L dexamethasone (Sigma–Aldrich) in the original medium. At day 2, the adipogenic cocktail medium was replaced with fresh original medium added with insulin only. At day 4, the medium was replaced again with fresh original medium and repeated every 2 days. At day 8, more than 90% of the cells had changed their morphology and accumulated fat droplets as evidenced by Oil Red O staining and a marked increase of gene expression of adipocyte differentiation markers, PPAR- γ (8.1-fold) and aP2 (9.9-fold). In some experiments, preadipocytes or mature adipocytes (day 8) were treated with or without 50 ng/ml mouse recombinant TNF- α (Peprotech, Rocky Hill, NJ, USA) for 24 h.

2.5. Transfection of a miR-1934 mimic in 3T3-L1 adipocytes

Synthetic double-stranded oligonucleotide mimicking mature endogenous miR-1934-5p (miR-Mimic, 50 nM) or miR-mimic negative control (All Stars negative control, 50 nM) (all from Qia-gen) was delivered into 3T3-L1 preadipocytes or mature adipocytes (d 7) (Ge et al., 2012), which had been previously cultured over-night in an antibiotic-free medium. Delivery was performed using HiPerFect Transfection Reagent (Qiagen) according to the manufacturers' protocols. The transfection efficiency was confirmed by a ~1000-fold overexpression of miR-1934 in mimic-transfected cells when compared to control cells. The medium was replaced with a fresh medium 24 h after transfection, and then cells were challenged with or without 50 ng/ml TNF- α for an additional 24 h (Ge et al., 2012, Meerson et al., 2013). This concentration of TNF- α was used based on the results of preliminary tests in which 50 ng/mL TNF- α significantly suppressed expression of miR-1934 while stimulated gene expression of IL-6 and MCP-1 (Supplementary Fig. 1).

2.6. Overexpression of miR-1934 in de novo formed fat tissue

The 3T3-L1 preadipocytes were infected with a lentivirus containing the miR-1934 sequence or a lentivirus containing a scrambled control sequence (GeneChem, Shanghai, China) using Lipofectamine 2000 (Invitrogen). For details of the lentivirus see <http://www.genechem.com.cn/Zaiti.aspx?zt=GV369>. Stably infected cells were selected by 1000

ng/ml Puromycin (Sigma–Aldrich, Bornem, Belgium). A total of 10^7 infected 3T3-L1 preadipocytes was resuspended in 200 μ l PBS and injected sc in the back of BALB/c nude mice (miR-1934-expressing cells were injected on the right side and control cells on the left side of each mouse). Four weeks post implantation, the *de novo* formed tissue was removed. A piece of the isolated samples was fixed in 4% formaldehyde for 24 h and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin-eosin-safran (HES). Adipocyte areas were measured by an imaging software (NIS-Elements D; Nikon, USA).

2.7. ELISA

Levels of ApN in human serum and levels of IL-6 and MCP-1 in culture medium were measured by commercial ELISA kits according to the manufacturer's instructions (ApN, R&D, Minnesota, USA; IL-6, Usn Life Science, Wuhan, China; MCP-1, Huamei, Wuhan, China). For ApN, human serum was diluted 10,000 times before detection; for IL-6, culture medium was diluted 10 times, while for MCP-1, no dilutions were performed before determination. All samples were detected in duplicate. Briefly, assay diluent was added to each well, following with standard, control and sample dilutions. The plate was incubated at room temperature for 2 h and being washed by each well for several times. Then conjugate was added and incubated for another 2 h, following by washes. After 30 min of substrate incubation, stop solution was added, and the OD values were read at 450 nm immediately.

2.8. Statistical analysis

Data were presented as means $\pm$ SEM for the indicated numbers of mice or subjects or independent cultures. Comparisons between two groups were carried out using two-tailed unpaired Student's t-test. Comparisons of at least 3 groups were performed by one-way ANOVA followed by the Newman–Keuls' test. The relationships between miR-1934 and clinical variables were evaluated by Pearson analysis. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Downregulation of miR-1934 expression in adipose tissue of obese mouse

We first examined the expression of miR-1934 in mouse AT. At the age of 14 weeks, mice fed a high-fat diet showed much greater increases in body weight (25.30 ± 1.38 g vs. 34.51 ± 1.55 g, $P < 0.01$), inguinal fat pad weight (0.19 ± 0.02 g vs. 0.53 ± 0.09 g, $P < 0.01$) and epididymal fat pad weight (0.48 ± 0.06 g vs. 1.55 ± 0.31 g, $P < 0.01$) than mice fed a chow diet (see Supplementary Table 2 for more characteristics). The mRNA levels of the pro-inflammatory adipokines (IL-6, CD11c and MCP-1) were significantly increased and the mRNA levels of the anti-inflammatory one (ApN) were significantly decreased in epididymal AT of HFD mice compared with their respective levels in chow diet-fed control mice (Fig. 1A). Moreover, miR-143, reported to be upregulated in AT of HFD-induced obese mice (Takanabe et al., 2008), also showed a reasonable upregulation in our obese mice (Supplementary Fig. 2). As noted, expression of miR-1934 was downregulated by 43.6% in epididymal AT and by 67.8% in inguinal AT of HFD mice when compared to control mice (Fig. 1B).

3.2. Downregulation of miR-1934 expression in adipose tissue of obese human

We then examined the expression of miR-1934 in omental AT, known to play a central role in the pathogenesis of the metabolic syndrome (Alexopoulos et al., 2014), miR-1934 of lean subjects (BMI: $21.17 \pm 0.41 \text{ kg/m}^2$) and obese (BMI: $28.40 \pm 1.43 \text{ kg/m}^2$) age-($49.45 \pm 9.69\text{yr}$ vs. $54.38 \pm 10.68\text{yr}$) and sex-matched subjects (10/11 vs. 7/7 men/women per group) (see Supplementary Table 3 for more characteristics). Compared to the lean subjects, obese subjects displayed apparent AT inflammation as evidenced by an increase in the expression of pro-inflammatory adipokines (IL-6 and TNF- α) and a decrease of the expression of anti-inflammatory adipokines [Interleukin-10 (IL-10) and ApN] (Fig. 2A). In addition, the expression of miR-1934 was downregulated in AT of obese subjects (-49.7% , $P < 0.05$ vs. lean, Fig. 2B).

3.3. Downregulation of miR-1934 in circulation of obese human

Given that miRNAs can be secreted into circulation (Chen et al., 2012), we further compared the circulating levels of miR-1934 between obese and lean subjects. As shown by Table 1, the two groups of participants were also well-matched in age and sex. Compared to the lean subjects, the obese subjects were associated with a worse metabolic profile (elevated levels of BP, FPG, 2hPG, HbA1c, FINS, TG, and reduced levels of HDL), as well as a higher proinflammatory state (elevated levels of hs-CRP and reduced levels of ApN). Circulating levels of miR-1934 were downregulated in obese subjects (-32.5% , $P < 0.05$ vs. lean, Fig. 3). It correlated negatively with BMI ($r = -0.362$, $P = 0.016$), WC ($r = -0.394$, $P = 0.008$), FINS ($r = -0.443$, $P = 0.008$), HOMA-IR ($r = -0.447$, $P = 0.008$), LDL-c ($r = -0.342$, $P = 0.041$) and hs-CRP ($r = -0.403$, $P = 0.020$), while correlated positively with HOMA-IS ($r = 0.430$, $P = 0.010$). No correlations were found between miR-1934 and other clinical variables including ApN.

3.4. Downregulation of miR-1934 in adipocytes by TNF- α treatment

To gain the insight into whether the downregulation of miR-1934 in obesity is resulted from low-grade inflammation, we examined the expression of miR-1934 in 3T3-L1 adipocytes upon treatment with TNF- α . As expected, the expression of miR-1934 was downregulated by TNF- α treatment in both preadipocytes (Fig. 4A) and differentiated mature adipocytes (Fig. 4B).

3.5. In vitro effects of miR-1934 overexpression on adipocyte inflammatory responses

To determine whether the decreased miR-1934 was involved in adipocyte inflammatory responses in the same condition above, overexpression of miR-1934 was performed by mimic transfection, and the cells were challenged with 50 ng/ml TNF- α . In pre-adipocytes, TNF- α markedly stimulated gene expression of both IL-6 and MCP-1, miR-1934 mimic *per se* did not affect the basal expression of IL-6 or MCP-1. However, miR-1934 mimic inhibited TNF- α -induced expression of these two cytokines (Fig. 5A). Similar results were obtained in differentiated mature adipocytes (Fig. 5B). Moreover, the protein levels of IL-6 and MCP-1 in culture medium were consistent with their mRNA levels (Fig. 6).

3.6. In vivo effects of miR-1934 overexpression on adipocyte inflammation

We next examined whether miR-1934 plays a role *in vivo* on the inflammatory status. 3T3-L1 preadipocytes were infected with a lentivirus expressing miR-1934 or a scrambled control sequence, and then injected sc into the back of nude mice (see Fig. 7). *De novo* tissue was formed from *in vivo* differentiation of these infected cells. Four weeks after transplantation, only those mice that had the *de novo* formed tissues on both sides of back were used. Histological analyses showed similar presence of normal adipocytes in the center of *de novo* formed tissues from both groups (Fig. 7A) with comparable adipocyte size ($74.79 \pm 5.08 \mu\text{m}^2$ for control vs. $86.89 \pm 8.67 \mu\text{m}^2$ for miR-1934, $n = 4/\text{group}$). As expected, miR-1934 was abundantly overexpressed (7.9-fold) in the *de novo* tissue formed from cells expressing miR-1934 compared to the control (Fig. 7B). The *de novo* tissue formed from cells expressing miR-1934 displayed reduced pro-inflammatory status than the control group as shown by a 48% decrease of IL-6, 32% decrease of TNF- α and 67% decrease of IL-1 β mRNA levels, as well as a 2.2 fold increase of IL-10 mRNA levels (Fig. 7C).

4. Discussion

Ge et al. have shown that miR-1934 was upregulated in the transgenic mice overexpressing ApN specifically in white AT. Remarkably, its expression fell to almost undetectable levels in the ApN-knockout mice, indicating that ApN was a pre-requisite for its expression *in vivo* (Ge et al., 2012). Yet, the biological function of this novel miRNA has never been described. The aim of the present work was to explore whether this miRNA may represent novel mechanism for controlling AT inflammation.

To date, only a very small part of miRNAs has been confirmed to be differentially expressed in white AT between lean and obese human. Less than 10 upregulated miRNAs and ~30 downregulated miRNAs in white AT of obese human have been described (Arner and Kulyte, 2015). Our results for the first time showed that expression of miR-1934 was downregulated in visceral AT from both obese mice and humans. The changes may be linked to the local low-grade inflammatory environment, since a similar decrease in miR-1934 expression was observed by TNF- α (a pro-inflammatory adipokine, largely induced in AT by obesity (2)) treatment in both pre- and mature 3T3-L1 adipocytes. Apart from visceral AT, subcutaneous AT was also generally known to play an important role in obesity-induced inflammation. Thus it would be of great value to investigate the expression of miR-1934 in subcutaneous AT of obese human for further researches. Interestingly, according to existing reports, most of the downregulated miRNAs in obesity showed a protective role in AT inflammation (Arner and Kulyte, 2015). Arner et al. overexpressed 11 individual miRNAs in human adipocytes. Nine of these miRNAs significantly reduced the secretion of pro-inflammatory chemokine (C-C motif) ligand 2 (CCL2), which was in line with the observation that they were downregulated in AT in human obesity (Arner et al., 2012). More-over three of these downregulated miRNAs were recently found to increase ApN secretion in conditioned media (Belarbi et al., 2015). The previous study of Ge et al. also identified that miR-883b-5p, which was upregulated by ApN and downregulated in omental AT of obese human repressed the lipopolysaccharide-binding protein (LBP) and TLR4

signaling (Ge et al., 2012). Thus the downregulation of miR-1934 in obese AT highly suggested its protective importance in obesity-induced inflammation.

Recent studies have demonstrated that miRNAs can be secreted into circulation. The circulating miRNAs can reside in microvesicles (exosomes, microparticles, apoptotic bodies) and in protein/lipoprotein complexes [high density lipoprotein (HDL) and Argonaute 2]; these formations make the circulating miRNAs remarkable stable and resistant to RNase activity and degradation (Ge et al., 2014; Fernandez-Messina et al., 2015; Vickers et al., 2011; Valadi et al., 2007; Thery et al., 2009; Deng et al., 2009). A growing body of evidence demonstrates that a profiling of circulating miRNAs are dysregulated in subjects with obesity or T2DM, and some of these dysregulated miRNAs are closely associated with obesity measures (e.g. BMI and fat mass), markers of inflammation (e.g. blood lymphocyte count and LBP), and diabetes measures (e.g. FPG and HbA1c), suggesting a diagnostic role of these miRNAs in the detection and classification of morbid obesity and T2DM (Heneghan et al., 2011; Ortega et al., 2013; 2014). Our results also showed that the circulating levels of miR-1934, which were decreased in obese subjects, were correlated reversely with BMI, WC, LDL-c, insulin resistance and inflammatory marker. This fact supported an important association of miR-1934 with obesity and obesity-linked inflammation. However, it should be noted that no correlation was found between miR-1934 and ApN in serum from humans. Importantly, the regulation of miR-1934 by ApN was established in AT of mouse, while this regulation was not directly proven in human by our present work and may be distinct due to differences between species. On the other hand, our preliminary experiment has detected the expression of miR-1934 in several tissues besides AT, including liver, kidney, muscle, brain, brown AT and spleen (data not shown). Thus the overall secretion of miR-1934 by these tissues may mask the expected repercussions especially on AT.

We further investigated the function of miR-1934 *in vitro* and *in vivo*. As expected, overexpression of miR-1934 in 3T3-L1 adipocytes repressed TNF- α -induced IL-6 and MCP-1 gene expression. So far, targeting certain miRNAs in white AT is poorly achieved because fat cells are insusceptible to selective transfer of exogenous nucleotide due to the containing of lipid droplets. In recent years, local injection of preadipocytes in nude mice has been shown to be representative for *de novo* adipogenesis and adipose tissue formation (Lijnen et al., 2006; Bauters et al., 2015; Neels et al., 2004). Thus we chose to deliver lentivirus-mediated miR-1934 oligonucleotides in preadipocytes cells and implanted subcutaneously in the back of nude mice. It turned out that overexpressing miR-1934 also exhibited lower levels of pro-inflammatory cytokines like IL-6, TNF- α , IL-1 β and higher anti-inflammatory one like IL-10. It is widely accepted that apart from adipocytes, macrophage also contributed to AT inflammation. Whether miR-1934 overexpression affected the macrophage infiltration and secretion of inflammatory cytokines is worth being clarified. However, the low mass of the *de novo* fat pad in the nude mice model might preclude the isolation of macrophage from tissue (Scroyen et al., 2009). This disadvantage called for future study in other mice model, such as a transgenic mouse, in which the systemic effect of miR-1934 could also be investigated. In addition, the *in vivo* differentiated adipocyte size in our *de novo* formed AT was smaller than that in a previous study performed in the same way, which may result from the use of different cell lines. The 3T3-

F442A cells used previously are generally regarded as more advanced in the adipose differentiation process than the 3T3-L1 cells (Gregoire et al., 1998).

Finally, some limitations in our present study should be mentioned. First, the plasma from subjects underwent laparoscopic cholecystectomy in cohort 1 was not available, we collected the plasma from another cohort to check the circulating miR1934. Thus, no correlation in miR1934 expression between AT and circulation could be observed. Plus, we have no mechanistic explanation for how miR-1934 regulates adipocyte inflammation. Unraveling the gene targets of miR-1934 may better understand the mechanisms underlying its anti-inflammatory function. At present, the gene targets of miR-1934 can be predicted by the online computational databases, miRDB (<http://www.mirdb.org/miRDB/>) and Targetscan (<http://www.targetscan.org/>). There is an overlap of 65 predicted targets of miR-1934 in these two databases (see Supplementary Table 4). Experiments *in vitro* are ongoing in our department to validate the targets of miR-1934.

Taken together, our present work showed that miR-1934 was downregulated in mouse and human obesity. The circulating miR-1934 levels were associated reversely with obesity, insulin resistance and inflammatory marker. Furthermore, miR-1934 turned out to play an anti-inflammatory role in adipocytes. This miRNA may represent a novel mechanism for controlling AT inflammation.

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Dr. Qian Ge is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis. Lulu Liu researched data, contributed to discussion, reviewed/edited manuscript. Qifu Li contributed to discussion, reviewed/edited manuscript. Chaodong Wu and Xiao-qi Xiao contributed to discussion, reviewed/edited manuscript. Rufe Gao, Danting Li and Wenlong Zhang researched data. Tingting Du and Yue Wang collected human adipose tissue samples. Chuan Peng, Shumin Yang and Qianna Zhen reviewed manuscript. Qian Ge analyzed data, wrote manuscript, contributed to discussion, reviewed/edited manuscript. All authors read and approved the final manuscript.

References

- Alexopoulos N, Katrasis D, Raggi P. Visceral adipose tissue as a source of inflammation and promoter of atherosclerosis. *Atherosclerosis*. 2014; 233:104–112. [PubMed: 24529130]
- Arner P, Kulyte A. microRNA regulatory networks in human adipose tissue and obesity. *Nat. Rev. Endocrinol*. 2015; 11:276–288. [PubMed: 25732520]
- Arner E, Mejhert N, Kulyte A, Balwierz PJ, Pachkov M, Cormont M, Lorente-Cebrian S, Ehrlund A, Laurencikienė J, Heden P, Dahlman-Wright K, Tanti JF, Hayashizaki Y, Ryden M, Dahlman I, van Nimwegen E, Daub CO, Arner P. Adipose tissue microRNAs as regulators of CCL2 production in human obesity. *Diabetes*. 2012; 61:1986–1993. [PubMed: 22688341]
- Bauche IB, El Mkaem SA, Pottier AM, Senou M, Many MC, Rezsosazy R, Penicaud L, Maeda N, Funahashi T, Brichard SM. Overexpression of adiponectin targeted to adipose tissue in transgenic mice: impaired adipocyte differentiation. *Endocrinology*. 2007; 148:1539–1549. [PubMed: 17204560]
- Bauters D, Scroyen I, Van Hul M, Lijnen HR. Gelatinase A (MMP-2) promotes murine adipogenesis. *Biochim. Biophys. Acta*. 2015; 1850:1449–1456. [PubMed: 25869489]

- Belarbi Y, Mejhert N, Lorente-Cebrian S, Dahlman I, Arner P, Ryden M, Kulyte A. microRNA-193b controls adiponectin production in human white adipose tissue. *J. Clin. Endocrinol. Metab.* 2015; 100:E1084–E1088. [PubMed: 26020766]
- Chartoumpekis DV, Zaravinos A, Ziros PG, Iskrenova RP, Psyrogiannis AI, Kyriazopoulou VE, Habeos IG. Differential expression of microRNAs in adipose tissue after long-term high-fat diet-induced obesity in mice. *PLoS One.* 2012; 7:e34872. [PubMed: 22496873]
- Chen X, Liang H, Zhang J, Zen K, Zhang CY. Secreted microRNAs: a new form of intercellular communication. *Trends Cell Biol.* 2012; 22:125–132. [PubMed: 22260888]
- Deng ZB, Poliakov A, Hardy RW, Clements R, Liu C, Liu Y, Wang J, Xiang X, Zhang S, Zhuang X, Shah SV, Sun D, Michalek S, Grizzle WE, Garvey T, Mobley J, Zhang HG. Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes.* 2009; 58:2498–2505. [PubMed: 19675137]
- Fernandez-Messina L, Gutierrez-Vazquez C, Rivas-Garcia E, Sanchez-Madrid F, de la Fuente H. Immunomodulatory role of microRNAs transferred by extracellular vesicles. *Biol. Cell.* 2015; 107:61–77. [PubMed: 25564937]
- Ge Q, Ryken L, Noel L, Maury E, Brichard SM. Adipokines identified as new downstream targets for adiponectin: lessons from adiponectin-overexpressing or -deficient mice. *Am. J. Physiol. Endocrinol. Metab.* 2011; 301:E326–E335. [PubMed: 21540448]
- Ge Q, Gerard J, Noel L, Scroyen I, Brichard SM. MicroRNAs regulated by adiponectin as novel targets for controlling adipose tissue inflammation. *Endocrinology.* 2012; 153:5285–5296. [PubMed: 23015294]
- Ge Q, Brichard S, Yi X, Li Q. microRNAs as a new mechanism regulating adipose tissue inflammation in obesity and as a novel therapeutic strategy in the metabolic syndrome. *J. Immunol. Res.* 2014; 2014:987285. [PubMed: 24741638]
- Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. *Physiol. Rev.* 1998; 78:783–809. [PubMed: 9674695]
- Heneghan HM, Miller N, McAnena OJ, O'Brien T, Kerin MJ. Differential miRNA expression in omental adipose tissue and in the circulation of obese patients identifies novel metabolic biomarkers. *J. Clin. Endocrinol. Metab.* 2011; 96:E846–E850. [PubMed: 21367929]
- Hotamisligil GS. Inflammation and metabolic disorders. *Nature.* 2006; 444:860–867. [PubMed: 17167474]
- Kloting N, Berthold S, Kovacs P, Schon MR, Fasshauer M, Ruschke K, Stumvoll M, Bluher M. MicroRNA expression in human omental and subcutaneous adipose tissue. *PLoS One.* 2009; 4:e4699. [PubMed: 19259271]
- Lijnen HR, Christiaens V, Scroyen I, Voros G, Tjwa M, Carmeliet P, Collen D. Impaired adipose tissue development in mice with inactivation of placental growth factor function. *Diabetes.* 2006; 55:2698–2704. [PubMed: 17003333]
- Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat. Med.* 2002; 8:731–737. [PubMed: 12068289]
- Maury E, Brichard SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol. Cell Endocrinol.* 2010; 314:1–16. [PubMed: 19682539]
- Meerson A, Traurig M, Ossowski V, Fleming JM, Mullins M, Baier LJ. Human adipose microRNA-221 is upregulated in obesity and affects fat metabolism downstream of leptin and TNF- α . *Diabetologia.* 2013; 56:1971–1979. [PubMed: 23756832]
- Muller G, Schneider M, Biemer-Daub G, Wied S. Microvesicles released from rat adipocytes and harboring glycosylphosphatidylinositol-anchored proteins transfer RNA stimulating lipid synthesis. *Cell Signal.* 2011; 23:1207–1223. [PubMed: 21435393]
- Neels JG, Thinnes T, Loskutoff DJ. Angiogenesis in an in vivo model of adipose tissue development. *Faseb J.* 2004; 18:983–985. [PubMed: 15084517]
- Ogawa R, Tanaka C, Sato M, Nagasaki H, Sugimura K, Okumura K, Nakagawa Y, Aoki N. Adipocyte-derived microvesicles contain RNA that is transported into macrophages and might be secreted

into blood circulation. *Biochem. Biophys. Res. Commun.* 2010; 398:723–729. [PubMed: 20621060]

Ortega FJ, Moreno-Navarrete JM, Pardo G, Sabater M, Hummel M, Ferrer A, Rodriguez-Hermosa JJ, Ruiz B, Ricart W, Peral B, Fernandez-Real JM. miRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. *PLoS One.* 2010; 5:e9022. [PubMed: 20126310]

Ortega FJ, Mercader JM, Catalan V, Moreno-Navarrete JM, Pueyo N, Sabater M, Gomez-Ambrosi J, Anglada R, Fernandez-Formoso JA, Ricart W, Fruhbeck G, Fernandez-Real JM. Targeting the circulating microRNA signature of obesity. *Clin. Chem.* 2013; 59:781–792. [PubMed: 23396142]

Ortega FJ, Mercader JM, Moreno-Navarrete JM, Rovira O, Guerra E, Esteve E, Xifra G, Martinez C, Ricart W, Rieusset J, Rome S, Karczewska-Kupczewska M, Strackowski M, Fernandez-Real JM. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care.* 2014; 37:1375–1383. [PubMed: 24478399]

Ortega FJ, Moreno M, Mercader JM, Moreno-Navarrete JM, Fuentes-Batllevell N, Sabater M, Ricart W, Fernandez-Real JM. Inflammation triggers specific microRNA profiles in human adipocytes and macrophages and in their supernatants. *Clin. Epigenetics.* 2015; 7:49. [PubMed: 25926893]

Ouchi N, Walsh K. Adiponectin as an anti-inflammatory factor. *Clin. Chim. Acta.* 2007; 380:24–30. [PubMed: 17343838]

Parra P, Serra F, Palou A. Expression of adipose microRNAs is sensitive to dietary conjugated linoleic acid treatment in mice. *PLoS One.* 2010; 5:e13005. [PubMed: 20886002]

Scroyen I, Cosemans L, Lijnen HR. Effect of tissue inhibitor of matrix metalloproteinases-1 on in vitro and in vivo adipocyte differentiation. *Thromb. Res.* 2009; 124:578–583. [PubMed: 19608218]

Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, Alford A, Waters KM. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. *Mol. Endocrinol.* 2009; 23:1876–1884. [PubMed: 19819989]

Takanabe R, Ono K, Abe Y, Takaya T, Horie T, Wada H, Kita T, Satoh N, Shimatsu A, Hasegawa K. Up-regulated expression of microRNA-143 in association with obesity in adipose tissue of mice fed high-fat diet. *Biochem. Biophys. Res. Commun.* 2008; 376:728–732. [PubMed: 18809385]

Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* 2009; 9:581–593. [PubMed: 19498381]

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 2007; 9:654–659. [PubMed: 17486113]

Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. microRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* 2011; 13:423–433. [PubMed: 21423178]

Weisell RC. Body mass index as an indicator of obesity. *Asia Pac J. Clin. Nutr.* 2002; 8:S681–S684.

Xie H, Lim B, Lodish HF. microRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes.* 2009; 58:1050–1057. [PubMed: 19188425]

Yang S, Luo T, Zhou H, Lv Q, Liu L, Zhang W, Gao R, Chen S, Xia W, Luo M, Cheng Q, Li Q. Rosiglitazone inhibits expression and secretion of PEDF in adipose tissue and liver of male SD rats via a PPAR-gamma independent mechanism. *Endocrinology.* 2014; 155:941–950. [PubMed: 24424059]

Zhuang G, Meng C, Guo X, Cheruku PS, Shi L, Xu H, Li H, Wang G, Evans AR, Safe S, Wu C, Zhou B. A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation. *Circulation.* 2012; 125:2892–2903. [PubMed: 22580331]

Fig. 1. Expression of inflammatory adipokines (A) and miR-1934 (B) in adipose tissue of C57L/6J mice

C57L/6J mice were fed a chow diet (CD) or a high-fat diet (HFD) for 8 weeks. Expression of adipokines [Interleukin-6 (IL-6), Monocyte Chemotactic Protein-1 (MCP-1), CD11c, Adiponectin (ApN)] and miR-1934 were quantified by RT-qPCR. Values were normalized to cyclophilin (for adipokines) or U6 (for miR-1934) and presented as relative expression compared to CD mice. Data are means $\pm$ SEM for 8 mice per group. * $P < 0.05$ vs. CD.

Fig. 2. Expression of inflammatory adipokines (A) and miR-1934 (B) in human omental AT Omental AT was obtained from 21 lean (Lean) and 14 obese subjects (Obese) undergoing laparoscopic cholecystectomy. Expression of adipokines [IL-6, TNF- α , Interleukin-10 (IL-10), ApN] and miR-1934 were quantified by RT-qPCR. Values were normalized to GAPDH (for adipokines) or U6 (for miR-1934) and presented as relative expression compared to lean subjects. Data are means $\pm$ SEM. * P < 0.05 vs. Lean, ** P < 0.01 vs. Lean.

Fig. 3. Expression of miR-1934 in human serum

Serum was obtained from 25 lean (Lean) and 24 obese subjects (Obese). Expression of miR-1934 in serum was quantified by RT-qPCR. Values were normalized to U6 and presented as relative expression compared to lean subjects. Data are means $\pm$ SEM. * P < 0.05 vs. Lean.

Fig. 4. Downregulation of miR-1934 by TNF- α treatment in 3T3-L1 preadipocytes (A) and differentiated mature adipocytes (B)

Pre- or differentiated adipocytes were treated with or without 50 ng/ml TNF- α for 24 h. Expression of miR-1934 was quantified by RT-qPCR. Values were normalized to U6 and presented as relative expression compared to control conditions (CTRL; i.e. without TNF- α). * $P < 0.05$ vs. CTRL.

Fig. 5. Overexpression of miR-1934 inhibited TNF- α -induced gene expression of inflammatory cytokines in 3T3-L1 preadipocytes (A) and differentiated mature adipocytes (B)

Pre- or differentiated adipocytes were transfected with 50 nM miR-1934 mimic (MIC) or negative control (CTRL). 24 h post-transfection, the medium was renewed, then cells were incubated with 50 ng/ml TNF- α (TNF/TNF + MIC) or without it for another 24 h.

Expression of IL-6 and MCP-1 were measured by RT-qPCR. Values were normalized to cyclophilin and presented as relative expression compared to CTRL. Data are means $\pm$ SEM for three repeated experiments. * P < 0.05 vs. CTRL, # P < 0.05 vs. TNF.

Fig. 6. Overexpression of miR-1934 inhibited TNF- α -induced protein expression of inflammatory cytokines in 3T3-L1 preadipocytes (A) and differentiated mature adipocytes (B) Pre- or differentiated adipocytes were cultured and challenged as described in Fig. 5. Protein levels of IL-6 and MCP-1 in medium were measured by ELISA. * $P < 0.05$ vs. CTRL, # $P < 0.05$ vs. TNF.

Fig. 7. In vivo effects of miR-1934 overexpression on the *de novo* formed fat tissue
 3T3-L1 preadipocytes were transfected with a lentivirus expressing miR-1934 (MIR) or a scrambled control sequence (CTRL) and then injected sc into the back of nude mice (the cells expressing miR-1934 were injected on the right side and the control cells on the left side of each mouse). These mice were fed high-fat diet and only those who had the *de novo* formed tissues on both sides of back were sacrificed 4 weeks later. **(A)** HES staining showed the presence of normal adipocytes in the center of the *de novo* formed tissue (magnification, $\times 100$). **(B)** miR-1934 was overexpressed in the *de novo* tissue formed from cells expressing miR-1934 (MIR) compared to CTRL. Expression of miR-1934 was quantified by RT-qPCR. Values were normalized to U6 and presented as relative expression compared to CTRL. **(C)** mRNA levels of IL-6 and TNF- α were decreased in the *de novo* tissue formed from cells expressing miR-1934 (MIR) compared to the CTRL. Expression of IL-6 and TNF- α were quantified by RT-qPCR. Values were normalized to β -actin and presented as relative expression compared to CTRL. Data are means $\pm$ SEM for four mice per group. * $P < 0.05$ vs. CTRL, *** $P < 0.001$ vs. CTRL.

Table 1

The anthropometric and biochemical characteristics of subjects.

	Lean (N = 25)	Obese (N = 24)	P Value
Age (years)	51.12 ± 2.02	46.96 ± 2.18	0.167
Sex (men/women)	12/13	12/12	0.889
BMI (kg/m ²)	21.94 ± 0.22	30.81 ± 0.46	<0.001
WC (cm)	81.96 ± 6.25	98.39 ± 7.23	<0.001
SBP (mmHg)	112.24 ± 2.82	123.67 ± 3.94	0.022
DBP (mmHg)	71.60 ± 2.16	79.79 ± 2.27	0.012
TC (mmol/L)	4.53 ± 0.17	4.17 ± 0.21	0.886
TG (mmol/L)	1.55 ± 0.16	2.16 ± 0.30	0.031
HDL-C (mmol/L)	1.35 ± 0.08	0.87 ± 0.05	<0.001
LDL-C (mmol/L)	2.59 ± 0.10	2.62 ± 0.15	0.142
HbA1c (%)	5.71 ± 0.07	6.07 ± 0.13	0.017
FPG (mmol/L)	5.26 ± 0.10	5.61 ± 0.09	0.012
FINS(mIU/L)	5.49 ± 0.77	12.06 ± 1.14	<0.001
HOMA-IR	1.14 ± 0.16	3.06 ± 0.31	<0.001
HOMA-IS	0.05 ± 0.01	0.02 ± 0.00	<0.001
2hPG (mmol/L)	6.78 ± 0.14	8.83 ± 0.27	<0.001
ApN (ug/ml)	18.79 ± 2.70	12.09 ± 1.22	0.032
hs-CRP (mg/L)	0.53 ± 0.12	1.99 ± 0.51	0.004

Data are presented as means ± SEM or number.

P values for comparisons between two groups are based on the two-tailed unpaired Student's t-test or χ^2 test.

BMI: body mass index; DBP: diastolic blood pressure; FINS: fasting insulin; FPG: fasting plasma glucose; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment for insulin resistance; HOMA-IS: homeostasis model assessment for insulin sensitivity; hs-CRP: high-sensitivity C-reactive protein; LDL-C: low-density lipoprotein cholesterol; SBP: systolic blood pressure; TC: total cholesterol; TG: triglycerides; 2hPG: 2-h post-load plasma glucose; WC: waist circumference.