Decreased MicroRNA-221 is Associated with High Levels of TNF-α in Human Adipose Tissue-Derived Mesenchymal Stem Cells From Obese Woman

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\textbf{Key Words} \\
hASCs • miR-221 • TNF-α • Obesity • Adipocytes

\textbf{Abstract} \\
\textbf{Aim:} The present study aimed to investigate the regulation and involvement of miR-221 in the differentiation of human adipose tissue-derived mesenchymal stem cells (hASCs). The relationships between miR-221 and pro-inflammatory markers and adipokines were also explored. \textbf{Methods:} Eight adipose tissues were obtained from four obese (mean body mass index (BMI) =31.7 kg/m\textsuperscript{2}) and four lean (mean BMI = 21.5 kg/m\textsuperscript{2}) women. hASCs were induced to differentiate, and the related gene expression were measured in the hASC-differentiated adipocytes using real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR). \textbf{Results:} During adipogenesis, miR-221 was significantly down-regulated; furthermore, miR-221 levels were lower in hASC-differentiated adipocytes from obese subjects than in the corresponding adipocytes from lean subjects. Higher TNF-α mRNA levels were associated with lower levels of miR-221. In addition, the miR-221 levels in the adipocytes were inversely correlated with BMI. \textbf{Conclusion:} Our results support the link between miR-221 and obesity development as well as obesity related inflammatory status.
Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that bind to their target messenger RNAs (mRNAs) to induce mRNA degradation and/or to repress protein synthesis [1]. miRNAs have been implicated in many biological processes, including cell proliferation [2], fat metabolism [2], differentiation [3], and cell death [3]. MicroRNAs have been shown to play a role in tumorigenesis [1], as well as several common diseases such as cardiovascular diseases [4, 5] and diabetes [6, 7]. Recent studies have also found that miRNAs play a regulatory role in adipogenic differentiation [8, 9] and the development of obesity [10-12].

Adipose tissue contains adipose tissue-derived mesenchymal stem cells (ASCs) that can differentiate into various cell types including adipocytes, osteoblasts, myoblasts, and chondroblasts [13, 14]. Adipogenesis differentiation can be assessed by intracellular lipid accumulation and the expression of adipocyte-specific genes such as the glucose transporter type 4 (GLUT4), hormone-sensitive lipase (HSL) and peroxisome proliferator activated receptor γ (PPARG) genes [15]. Previous studies have also shown that miRNAs can influence the differentiation and the function of human ASCs (hASCs) [16, 17].

An increase of macrophage infiltration can be found in the adipose tissue of obese people. Low-grade, chronic inflammation further leads to insulin resistance in obese subjects [18, 19]. Tumor necrosis factor-alpha (TNF-α) is a macrophage-produced cytokine and is involved in chronic inflammation. An elevated TNF-α expression in the adipose tissue may contribute to metabolic dysfunction in obese subjects [20]. In addition, the plasma levels of pro-inflammatory cytokines like TNF-α [21], interleukin-6 (IL-6) [22] and interleukin-8 (IL-8) [23] are elevated in obese people. TNF-α can stimulate the secretion of inflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1) [24, 25] and several adipokines including leptin [26, 27] and thrombospondin 1 (TSP1) [28, 29]. Serum levels of leptin [30, 31] and TSP1 [32] are elevated in obese and insulin-resistant subjects. It has been shown that miR-221 can directly bind to the 3’ untranslated region (UTR) of TNF-α to cause its mRNA degradation [33]. According to the miRWalk database [34], the 3’-UTRs of leptin and TSP1 mRNAs were also predicted to have miR-221 binding sites. Taken together, miR-221 is likely to affect multiple downstream genes in the TNF-α signaling pathway in adipogenesis.

It has been reported that miR-221 is significantly down-regulated during adipogenesis in 3T3-L1 cells [10] and in human subcutaneous fat cells [35]. However, miR-221 has not been investigated in hASCs. In this study, we first aimed to clarify the role of miR-221 in hASCs adipogenic differentiation. Given that pro-inflammatory cytokines and adipokines are highly related to obesity, our second aim was to test for the influence of miR-221 on TNF-α, IL-6, IL-8, MCP-1, leptin and TSP1.

Materials and Methods

Subjects and materials

Because miR-221 is located on the X chromosome, we only investigated the hASCs derived from women. The human visceral fat tissues were obtained from 8 women who received gallbladder surgery because of clinical needs. The fat tissues surrounding the gallbladder were isolated for the current study. These subjects did not have hyperglycemia, hyperlipidemia, high blood pressure and malignant diseases on enrollment. A 1x1x1 cm³ fat tissue was obtained during surgery and stored at 4°C immediately. All protocols involved in human subjects were approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH) and all participants gave their informed consent. Eight adipose tissues were obtained from women with body mass index (BMI) between 19.6 and 33.3 kg/m². We used eight different hASC samples for each experiment and performed the technical duplicate for each sample. The participants were divided into two groups and listed in the Table 1.
**Induction of hASC differentiation and Oil Red O stain**

hASCs were isolated and induced to differentiate according to the methods described in our previous studies [36]. hASCs were propagated in the K-NAC medium (Keratinocyte-SFM medium, Asc 2-P (0.2 mM), NAC (2 mM), 5% FBS and 1% penicillin/streptomycin). To induce cell differentiation from hASCs to adipocytes, the culture medium was shifted to adipogenic medium (DMEM with high glucose (4500 mg/L), 10% FBS, dexamethasone (1 mM), insulin (0.01 mg/mL), indomethacin (0.2 mM), 3-isobutyl-1-methylxanthine (0.5 mM), 1% penicillin/streptomycin and 0.5% gentamycin) after the cells were grown into confluence. The extent of adipogenic differentiation was evaluated by Oil Red O stain, which is an indicator of intracellular lipid accumulation. After induction of adipogenic differentiation, cells were washed in PBS, fixed for 1 h in 10% formalin and washed three additional times in PBS. The cells were then stained for 30 min with Oil Red O. The phase contrast images were photographed by a digital CCD camera equipped in a microscope. The lipid content in the hASC-differentiated adipocytes, 1 ml of 100% isopropyl alcohol was added to the stained culture dish. After 5 minutes, the absorbance of the extract was assayed by an absorbance reader (BioTek Instruments, Inc, Winooski, VT) at 510 nm after dilution to a linear range.

**Total RNA extraction and real-time PCR**

Total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed by using reverse transcriptase kits (ABI, Foster City, CA, USA) according to the manufacturer’s instructions. For quantification of gene expression, real-time PCR was conducted using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA) on an Applied Biosystems 7900 real-time PCR system (Applied Biosystems). Each sample was normalized to GAPDH. The relative expression of mRNAs was quantified by 2^{-\Delta\Delta C_t} with logarithm transformation. The forward and reverse primers are shown in Table 2.

**microRNA detection**

miR-221 was quantified using a TaqMan microRNA detection assay (Applied Biosystems) and miR-221 specific primer sets were supplied by Applied Biosystems. Expression level of miR-221 in each sample was normalized to the corresponding level of U6B. The relative expression of miRNAs was quantified by 2^{-\Delta\Delta C_t} with logarithm transformation.

**microRNA Transfection**

The Ambion® Pre-miR™ miRNA Precursor (PM10337) and Pre-miR™ miRNA Precursor Molecules Negative Control (AM17110) were purchased from Applied Biosystems. They were transfected into hASCs at a final concentration of 100 nM using Oligofectamine™ transfection reagent (Life Technologies, CA, USA) according to the manufacturer’s instructions.

**Table 1. Clinical data for study participants. BMI, body mass index**

<table>
<thead>
<tr>
<th></th>
<th>Lean (n=4)</th>
<th>Obese (n=4)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (min-max)</td>
<td>48.8 (34-63)</td>
<td>52.3 (28-34)</td>
<td>0.0876</td>
</tr>
<tr>
<td>BMI (kg/m²), mean (min-max)</td>
<td>21.5 (19.6-23.7)</td>
<td>31.7 (30.2-33.3)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Waist-hip ratio, mean (min-max)</td>
<td>0.780 (0.753-0.825)</td>
<td>0.853 (0.837-0.881)</td>
<td>0.0197</td>
</tr>
</tbody>
</table>

**Table 2. The forward and reverse primers**

<table>
<thead>
<tr>
<th>primer</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18</td>
<td>Forward: TGGCCAGCCTTCTCTTGT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward: CAATCAATGCCTAGCTAC</td>
</tr>
<tr>
<td>Glut-4</td>
<td>Forward: ATGACCTGTGGCTGCTCTCT</td>
</tr>
<tr>
<td>FABP4</td>
<td>Forward: AACTGAACTCAGGGGAGACTC</td>
</tr>
<tr>
<td>HSL</td>
<td>Forward: ACCTGGCGCACAATGGACACA</td>
</tr>
<tr>
<td>PPARα</td>
<td>Forward: CAAAGAAGATCTGACACCTG</td>
</tr>
<tr>
<td>Leptin</td>
<td>Forward: GAAACCTGTGGGATTTTGT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: CGAGTGAAAGGCTGTAGC</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward: CTCAGCAACATCGACATG</td>
</tr>
<tr>
<td>TSP1</td>
<td>Forward: GTGAAAAAGATGGAGAGTCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: AACAGGGGACACCATCTCCTC</td>
</tr>
</tbody>
</table>

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Enzyme-linked immunosorbent assay (ELISA)

The protein levels of IL-6, IL-8 and MCP-1 were measured using the OptEIA human IL-6, IL-8 and MCP-1 ELISA kit (BD Biosciences Pharmingen, San Diego, USA). Briefly described, the supernatant samples were plated in duplicate wells of a 96-well plate. Freshly diluted IL-6, IL-8 and MCP-1 were used to generate a standard curve. The absorbance at 450 nm was detected by a spectrophotometric plate reader (Bio Rad Benchmark Plus). Raw data were corrected against blank wells and converted to pg/ml using the standard curve.

Statistical analysis

Data of a continuous variable was expressed as the mean ± SD. The unpaired t-test was used to compare the means between two groups. A P value less than 0.05 was considered statistically significant. Correlation coefficient (R) was performed by the JMP software (version 9). The functional annotation of a gene in relation to miR-221 was performed using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Redwood City, CA, www.ingenuity.com).

Results

Changes of miR-221 levels during adipogenic differentiation of hASCs

The entire process normally takes 12 days for hASCs to differentiate to mature adipocytes. To detect the formation of adipocytes we used Oil Red O stain for the intracellular lipid droplets. We found that miR-221 expression levels in hASCs decreased over time during the process of adipogenic differentiation regardless of whether the hASCs were derived from obese or lean subjects (Fig. 1A). However, miR-221 levels were significantly lower in the samples derived from obese subjects than from lean subjects on days 8 and 12 of differentiation (Fig. 1A). Based on the results of Oil Red O stain, the hASC-differentiated adipocytes from obese women had better differentiation than those from lean subjects on days 12 (Fig. 1B). On day 12, two differentiation markers (GLUT4 and PPARG) were checked for the relationship between the adipogenic differentiation status and miR-221 level. The results indicated strong, inverse correlations between miR-221 levels and both markers (correlation coefficient was -0.90 for GLUT4, -0.94 for PPARG, Fig. 1C).

The effect of miR-221 on lipid accumulation

Given that miR-221 levels decreased while hASCs differentiated to mature adipocytes, we further investigated the effect of miR-221 on lipid accumulation during adipocyte differentiation. Two days before the induction of differentiation, pre-miR-221 was transfected into hASCs (Fig. 2A). On day 12, the intracellular miR-221 level in the cells transfected with pre-miR-221 was still higher than that in the cells transfected with the negative control by ~100-fold. Transfection of pre-miR-221 slightly reduced lipid accumulation in hASC-differentiated adipocytes from obese subjects on day 12 as indicated by Oil Red O stain (Fig. 2B). However, pre-miR-221 had almost no effect on the lipid content of hASC-differentiated adipocytes derived from lean subjects (Fig. 2B). Using the optical density to quantify the amount of intracellular lipid, transfected pre-miR-221 was found to decrease the amount of accumulated lipid by ~20 % in the hASC-differentiated adipocytes from obese subjects (Fig. 2C). Transfection of pre-miR-221 partly caused reductions of the mRNA levels of four differentiation markers (GLUT4, HSL, PPARG and fatty acid binding protein 4 (FABP4)) by 10%~20% in obese subjects. However, only GLUT4 of the reductions reached a significant level (Fig. 2D).

miR-221 affects cytokine and adipokine levels in hASC-differentiated adipocytes from obese subjects

Since TNF-α, leptin and TSP1 were predicted to be target genes of miR-221 in the miRWalk database [34], we measured their mRNA levels in hASC-differentiated adipocytes transfected with pre-miR-221. Our experiments showed that pre-miR-221 treatment
Fig. 1. Changes of miR-221 levels during adipogenic differentiation of hASC. hASCs were induced for adipogenic differentiation by the adipogenic medium and cells were harvested on days 0, 1, 4, 8, 12 during differentiation. MiR-221 levels were measured by real-time PCR and normalized to U6B. Each hASC sample was performed in duplication. (A) The differences between cells derived from lean (n=4) and obese (n=4) females were significant on day 8 and day 12. *indicates P < 0.05 when compared to the level on day 0; #indicates P < 0.05 between hASCs derived from obese and lean subjects. (B) Lipid droplets were visualized by Oil Red-O staining from representative hASC differentiation. Lipid droplets were quantified in isopropanol extracts by using the optical density (four obese and two lean subjects). *indicates P < 0.05 when compared to the lean subjects. (C) Inverse correlations between miR-221 and GLUT4 and PPARG mRNAs during hASC differentiation of four obese women. On the X axis, miR-221 levels are expressed as $\log_{2}\Delta C_{T}$ and $\Delta C_{T} = C_{T}(\text{miR-221})-C_{T}(\text{U6B})$. The Y axis similarly represents mRNA levels as $\log_{2}\Delta C_{T}$ but $\Delta C_{T} = C_{T}(\text{gene})-C_{T}(\text{GAPDH})$.

significantly reduced TNF-α mRNA level by 50 % (Fig. 3A, left), and had a weaker effect on leptin and TSP1 (both adipokines were inhibited by approximately 20%, Fig. 3C). We then tested whether an increase of miR-221 level can influence pro-inflammatory cytokines including IL-6, IL-8 and MCP-1. Noticeably, these cytokines were not predicted to be miR-221's direct targets. Our data showed that pre-miR-221 treatment slightly decreased MCP-1 mRNA levels by ~10% (Fig. 3A, middle) but did not have any effect on IL-6 mRNA levels. Using the ELISA assay, miR-221 did not cause any change of MCP-1 protein level (Fig. 3A, right) but reduced IL-6 protein levels (Fig. 3B, left). For IL-8, miR-221 had no effect on either mRNA or protein levels (Fig. 3B, right).
Difference of miR-221 and adipokines expression levels between obese and lean subjects

We further investigated whether the relationships between miR-221 levels and adipokines are different between hASC-differentiated adipocytes from obese women (BMI>30, n=4) and those from lean women (BMI<24, n=4) (Table 1). The Figure 1A showed that miR-221 levels were significantly lower in the hASC-differentiated adipocytes from obese subjects than those from lean subjects. On the contrary, the mRNA levels of TNF-α, leptin and TSP1 were higher in the hASC-differentiated adipocytes from obese women than those from lean women (Fig. 4A). Analyzing the total of 8 samples, we found an inverse correlation between TNF-α and miR-221 levels with a correlation coefficient of -0.90 (Fig. 4B), as well as a negative relationship between miR-221 levels and BMI with a correlation coefficient of -0.75 (Fig. 4C).
**Discussion**

The present study showed that miR-221 was significantly down-regulated during adipogenesis of hASCs. Interestingly, even though the adipogenesis experiment was conducted in vitro, miR-221 levels were lower in hASC-differentiated adipocytes from obese subjects than those from lean subjects. Our studies demonstrated that over-expression of miR-221 could reduce TNF-α expression in adipocytes. A possible explanation for this phenomenon is that miR-221 directly knocks down TNF-α mRNA. The hASC-differentiated adipocytes from obese female subjects had higher TNF-α mRNA levels but lower miR-221 levels. In addition, miR-221 levels were also inversely correlated with BMI, indicating a link between the severity of obesity and miR-221 levels. Therefore, this study shows that miR-221 could potentially prevent inflammation in obese women.

The down-regulation pattern of miR-221 shown in our human samples during adipogenesis was consistent with the findings from the adipogenesis of 3T3-L1 and human subcutaneous fat cells [10, 35]. An interesting finding of the present study was that miR-221 could down-regulate the expression of TNF-α in adipocytes. TNF-α plays a crucial role in a variety of inflammatory diseases, and it also serves as a link between obesity and insulin resistance [37, 38]. An elevated TNF-α expression has been documented in human abdominal subcutaneous adipose tissues of obese persons [39]. Previous studies have shown that TNF-α treatment could enhance the expression of miR-221 in 3T3-L1 adipocytes.
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Accordingly, there may be reciprocal regulation between miR-221 and TNF-α during adipogenesis. Additionally, the present study found that over-expression of miR-221 could reduce IL-6 concentration. The data from IPA analysis suggested that miR-221 can target STAT5A, which in turn affects NFκB, estrogen receptor alpha and CREB-bind protein to cause a change of IL-6 expression level. Since both TNF-α and IL-6 have been implicated in obesity-related insulin resistance [39], over-expression of miR-221 could be a promising strategy to prevent obesity-induced insulin resistance.

The present study showed that the expression levels of miR-221 decreased more rapidly in differentiating hASCs from obese women than in those from lean women. Based on the data shown in figures 1B and 2C, the hASCs from obese women differentiated more efficiently. These results suggested that miR-221 may play a role in hASC differentiation. In addition, we showed that over-expression of miR-221 partly reduced lipid accumulation in the hASC-differentiated adipocytes from obese women but had no noticeable effect on those from lean women. Our data also suggested that miR-221 may reduce leptin and TSP1 mRNA levels because these adipokines are miR-221’s target genes. TSP-1 deficiency can reduce obesity-associated inflammation and improve insulin resistance in a diet-induced obese mouse model [40]. These data suggested that miR-221 could have beneficial effects by interfering with inflammatory status and adipokine metabolism. However, the present study used a limited number of tissue samples due to our restricted selection criteria (females with either high or low BMI) and the difficulty associated with developing hASCs. Another limitation of our study is that we only transfected miR-221 but did not transfet anti-miR-221 to hASCs before adipogenic differentiation because of limited availability of hASCs. Therefore, future studies to replicate our results are warranted.

Fig. 4. Differential expression of miR-221 in lean and obese women. (A) On day 12 of differentiation, the levels of TNF-α, leptin and TSP1 mRNA were measured by RT-qPCR analysis using GAPDH as the internal control. On the Y axis, TNF-α, leptin and TSP1 levels are expressed as log$_{10}$2$^{-\Delta Ct}$ whereas $\Delta Ct = Ct(gene) - Ct(GAPDH)$. (B) Inverse correlation between TNF-α mRNA and miR-221. On the X axis, miR-221 levels are expressed as log$_{10}$2$^{-\Delta Ct}$ and $\Delta Ct = Ct(miR-221) - Ct(U6B)$. The Y axis similarly represents mRNA levels as log$_{10}$2$^{-\Delta Ct}$ but $\Delta Ct = Ct(gene) - Ct(GAPDH)$, and (C) Inverse correlation between miR-221 and BMI. We used eight different hASC samples for each experiment and performed the duplicate experiment for each sample. * indicates $P < 0.05$. 

[10].
In summary, our data show down-regulation of miR-221 during hASC adipogenic differentiation. The decrease of miR-221 is more prominent in the hASC-differentiated adipocytes from obese women compared to those from lean women. MiR-221 levels are negatively associated with BMI and obesity related biomarkers. Therefore, miR-221 may serve as a novel target to prevent obesity and obesity-induced inflammatory diseases.

**Abbreviations**

hASCs (Human adipose tissue-derived mesenchymal stem cells); TNF-α (tumor necrosis factor-alpha); FABP4 (fatty acid binding protein 4); IL-6 (interleukin-6); GLUT4 (Glucose transporter type 4); HSL (Hormone-sensitive lipas); PPARG (peroxisome proliferator activated receptor γ); TSP1 (thrombospondin 1); BMI (body mass index).

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

**Acknowledgements**

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**References**

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