MiRNA-21 Reverses High Glucose and High Insulin Induced Insulin Resistance in 3T3-L1 Adipocytes through Targeting Phosphatase and Tensin Homologue

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Key words
• miR-21
• insulin resistance
• PTEN
• AKT

Abstract

Aims/hypothesis: Our previous study showed there was a change of microRNA (miRNA) expression profile, and miR-21 was significantly down regulated in insulin-resistant adipocytes (IR-adipocytes). Phosphatase and tensin homologs deleted on chromosome 10 (PTEN), a negative regulator of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, was identified to be a target gene of miR-21, which suggested miR-21 might be associated with insulin resistance (IR) or diabetes. However, it is not known whether miR-21 play any role in the development of IR in 3T3-L1 adipocytes.

Methods: Normal adipocytes and adipocytes transfectioned with pre-miR-21(pmiR-21) or negative control (pNeg) were treated with high glucose and high insulin for 24 h, insulin-stimulated glucose uptake was determined by 2-Deoxyglucose and high insulin for 24 h, insulin-stimulated glucose uptake was determined by 2-Deoxyglucose and high insulin for 24 h, insulin-stimulated glucose uptake was determined by 2-Deoxyglucose transport assay, miR-21 expression level was measured by using quantitative real-time RT-PCR (qRT-PCR). The protein expression levels of PTEN, Akt, phospho-Akt (Ser473), IRβ, GSK3β, phospho-GSK3β (Ser9) and GLUT4 were detected by western blotting assay.

Results: We further confirmed that miR-21 was down regulated in IR-adipocytes by qRT-PCR. Over-expression of miR-21 significantly increased insulin-induced glucose uptake and decreased PTEN protein expression, while it had no significant effect on PTEN mRNA expression in IR-adipocytes. Moreover, over-expressing miR-21 significantly increased insulin-induced phosphorylation of AKT (Ser473), GSK3β (Ser9) and the translocation of glucose transporter 4 (GLUT4) in IR-adipocytes.

Conclusions: In this study, our data demonstrate that miR-21 reverses high glucose and high insulin induced IR in 3T3-L1 adipocytes, possibly through modulating the PTEN-AKT pathway, and miR-21 may be a new therapeutic target for metabolic diseases such as T2DM and obesity.
in 3T3-L1 adipocytes. Our data validated that miR-21 markedly
down-regulated in IR adipocytes compared with normal adi-
pocytes. Over-expression of miR-21 significantly increased insu-
lin-stimulated glucose uptake in IR-adipocytes. Consistent with
its function, over-expression of miR-21 decreased the expres-
sion of PTEN, increased insulin-induced phosphorylation of Akt
(Ser473) and GSK3β (Ser9), and promoted GLUT4 translocation in
IR-adipocytes. Taken together, the current result firstly dem-
onstrated that miR-21 improved IR in 3T3-L1 adipocytes, possi-
bly through modulating PTEN-AKT pathway.

Materials and Methods

Materials
3T3-L1 pre-adipocytes were purchased from the American Type
Culture Collection (Manassas, VA), 3-isobutyl-1-methylxanthine
(IBMX), insulin and dexamethasone were obtained Sigma (St.
Louis, MO); Lipofectamine 2000, Trizol reagent and SYBR Green
I dye were obtained from Invitrogen (Carlsbad CA), pSilencer™
3.1-H1 plasmid vectors were purchased from Ambion (Austin,
TX), polyclonal rabbit against PTEN, IRβ, Akt, phospho-Akt
(Ser473), GSK3β and phospho-GSK3β (Ser9) antibodies were
purchased from Cell Signaling Technology (Beverly, MA, USA); polyclonal goat anti-GLUT4 and mouse monoclonal anti-β-actin
were purchased from Santa Cruz Biotechnology (Santa Cruz,
CA).

Cell culture and induction of insulin resistance
3T3-L1 pre-adipocytes were propagated and induced to differen-
tiation as described previously [17]. Typically, mature adi-
pocytes appeared within 9 days of differentiation. To induce IR,
3T3-L1 adipocytes were preincubated for 24 h at 37 °C with
DMEM (10% FBS) containing 5 mmol/L glucose with or without
1 μmol/L insulin, or 25 mmol/L glucose with or without 1 μmol/L
insulin.

MiR-21 plasmid construction and transient transfection
Oligonucleotides corresponding to the murine precursor
sequence of miR-21 were introduced into pSilencer™ 3.1-H1
vector to obtain pmir-21 plasmid. The introduced sequences
were as follows: sense 5′-GTACTTCTATACGACGAGTTGATTTAGATCAAGATCACATCTGTTGAAACTTCCAGCA-3′; and antisense 5′-AGCTTTTGAGCTTAATCTACGATGTTGAG-3′. pmir-21 plasmid was confirmed by DNA sequencing. Empty
plasmid pSilencer™ 3.1-H1 was used as a negative control
(pNeg). For transient transfection, mature adipocytes in six-well
plates were transfected in triplicate with pmir-21 or pNeg plas-
mid using Lipofectamine 2000 for 4 h (4 μg oligonucleotide
was used in each well).

Quantitative real-time PCR (qRT-PCR) analysis for miR-
21 and PTEN mRNA expression
For analysis of miR-21 and PTEN mRNA expression, the qRT-PCR
was performed as described previously [17]. Briefly, miRNA-
enriched total RNA was extracted from cells using an RNasey
mini kit, and miR-21 expression was determined using a Taq-
Man MicroRNA Assay kit (ABI, USA) according to the manufac-
turer’s instruction. The highly conserved snRNA U6 was used as
an internal normalizing control. For analysis of PTEN mRNA
expression, total RNA was extracted and the mRNA was quanti-
tied using SYBR green PCR master mix and a LightCycler Real
Time PCR system (Bio-Rad, Hercules, CA, USA). The sequences
of PCR the primers were as follows: (i) PTEN forward 5′-CCAG-
CATCAAAATGTTCAG-3′ and reverse 5′-AAGCCTGAGATTTGTAA-
GGAACCTC-3′ and (ii) β-actin forward 5′-GTGGCAGGATCCACAAG-
GGTTGATTCAAGAGATCAACATCAGTCTGATAAGCTATTTTT-
CTGATAAGCTATCTTGTGATATGATCTGACGTACGAAG-3′. The relative
expression ratio of miR-21 and PTEN were calculated using the
2−ΔΔCt method [18].

2-Deoxyglucose transport assay
Adipocytes in six-well plates were incubated in serum-free
medium for 2 h. Then, cells were washed 3 times with Krebs-
Ringer phosphate buffer (KRPB, consisting of 128 mmol/L NaCl,
4.7 mmol/L KCl, 5 mmol/L NaH2PO4, 5 mmol/L Na2HPO4, 1.25
mmol/L MgSO4, 1.25 mmol/L CaCl2, pH 7.4) before being
incubated for 30 min at 37 °C in the presence or absence of
100 nmol/L insulin. [1H]-2-Deoxyglucose (50 μmol/L; 9.25 KBq)
and 2-deoxyglucose (final concentration 0.1 mmol/L) were
added to each well for 10 min and cells were then washed quickly
in ice-cold PBS. 2-Deoxyglucose uptake was assayed by scintilla-
tion counting. At the same time, ice-cold containing PBS 10
μmol/L containing cytochalasin B was added to each well and
the cells were washed 3 times with ice-cold PBS for the meas-
urement of nonspecific uptake. Specific uptake, nonspecific
uptake subtracted from total uptake, was determined.

Bioinformatics analysis
3 programs, miRanda, TargetScan and PicTar, were used to pre-
dict the targets of miR-21.

Western blot analysis of total cellular lysates
Cells were washed twice in ice-cold PBS, and lysed in a buffer
containing 10 mmol/L HEPES (pH7.9), 5 mmol/L MgCl2,
10 mmol/L KCl and 0.5% NP-40. Cell lysates were collected by
centrifuging at 13 000 g for 15 min at 4 °C. Protein concentrations
in the cell lysates were determined by BCA assay. Briefly, sample
proteins (30–50 μg) were separated by 10% sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then
transferred to PVDF membranes. The membranes were blocked
for 60 min in a buffer containing 0.1% Tween-20 and 5% milk.
Antibodies against PTEN, Akt, phospho-Akt (Ser473), IRβ, GSK3β,
phospho-GSK3β (Ser9) and GLUT4 were used to identify speci-
cific proteins, which were then visualized by the ECL method. The
intensity of a protein band of interest was quantified by densit-
ometry.

Western blot analysis of total cellular membrane
GLUT4 [19]
Cells were washed twice in ice-cold HES buffer (20 mmol/L
HEPES, pH 7.5; 1 mmol/L EDTA; and 250 mmol/L sucrose) and
scraped in HES buffer in the presence of protease inhibitors.
Samples were homogenized (30 strokes in a glass Dounce
homogenizer) at 4 °C and centrifuged at 10000 × g for 5 min to
remove unbroken cells, supernatant was further centrifuged at
16000 × g for 15 min to produce a crude plasma membrane fra-
cion. The resulting pellet was resuspended in a 1.15 mol/L
sucrose cushion (HES buffer containing 1.15 mol/L sucrose), and
centrifuged at 100 000 × g for 60 min. The white
fluffy band at the interface was collected, diluted in HES, and
centrifuged again at 48 000 × g for 60 min, yielding a pellet of the
plasma membrane. The amount of GLUT4 in the plasma membrane was determined by immunoblotting using anti-GLUT4 antibody.

Statistical analysis
Data are presented as the mean ± SD. 2 groups were compared by unpaired Student’s t test and multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s test. P < 0.05 was considered significant.

Results

Establishment of insulin-resistant adipocytes
To evaluate the insulin-resistant 3T3-L1 adipocyte model, 2-deoxyglucose (2-DG) uptake was measured by radioimmunoassay to determine the insulin sensitivity of the cells. As expected after differentiation, 3T3-L1 adipocytes exhibited a marked response to an acute maximal dose of insulin (100 nmol/L) with significant increases in glucose transport above basal under all conditions studied. When cells were preincubated for 24 h in media containing 5 mmol/L glucose (normal adipocytes), acute insulin stimulation (100 nmol/L) induced a 4.4-fold increase in 2-DG transport. The addition of 1 μmol/L insulin during preincubation did not significantly affect either basal or insulin-stimulated 2-DG transport. Also, when cells were preincubated without insulin in 5 mmol/L glucose, the acute insulin response of glucose transport was not significantly different when compared with cells preincubated in 5 mmol/L glucose. However, 24 h of preincubation in media containing high glucose in the presence of 1 μmol/L insulin decreased acute insulin-stimulated glucose transport by 41.4%, when compared with cells preincubated with 25 mmol/L glucose. The data indicated that treatment of 3T3-L1 adipocytes with high glucose (25 mmol/L) and high insulin (1 μmol/L) resulted in the induction of IR (● Fig. 1).

Reduced expression of miR-21 in insulin-resistant adipocytes
We have previously shown that there was a differential expression profile of miRNAs between normal adipocytes (control group) and insulin-resistant adipocytes (IR-adipocytes) (IR group). Among them, miR-21 was the most significantly down-regulated miRNA, which was 16.5-fold reduction in the IR group compared with control group [8]. To further confirm miR-21 expression, qRT-PCR analysis was used. As shown in ● Fig. 2a, miR-21 was significantly decreased (about 5.3 folds) in the IR
group compared to control group, which was consistent with the microarray data. Together, it suggested that miR-21 expression was inversely related to IR.

Over-expression of miR-21 increased insulin-induced glucose uptake in IR-adipocytes

Results of qRT-PCR showed that miR-21 was significantly increased in the miR-21-transfected adipocytes (pmiR-21) compared with empty plasmid pSilencer™ 3.1-H1-transfected adipocytes (pNeg), suggesting miR-21 can be over-expressed by lipofectamine 2000 transfection in 3T3-L1 adipocytes (Fig. 2a). To investigate the impact of miR-21 on the formation of IR, glucose uptake was determined after pmiR-21 or pNeg were treated with high glucose and high insulin for 24 h. As shown in Fig. 2b, in the absence of insulin stimulation, there was no significant difference in glucose uptake in all groups. However, following insulin stimulation, IR adipocytes exhibited a marked reduction in insulin-induced glucose uptake (an approximate 1.7-fold reduction in the absence or presence of pNeg treatment), which was restored (46.5% recovery) following the treatment of cells with miR-21.

Fig. 3 PTEN is a target of miR-21 in 3T3-L1 adipocytes. 3T3-L1 adipocytes of control, pmiR-21 and pNeg were induced to IR-adipocytes with high glucose and high insulin for 24 h as the IR group, pmiR-21+IR group and pNeg+IR group, respectively. a Sequence complementarity between miR-21 and its target sites in the 3′ UTR of human (hsa), mouse (mmu) and rat (rno) PTEN mRNAs. b PTEN protein levels were measured by Western blot analysis. Representative immunoblots (top) and densitometric analysis (bottom) on the PTEN protein levels normalized to internal β-actin level were reported. c PTEN mRNA expression was measured using qRT-PCR, relative mRNA levels were calculated as relative change from the Control group level. Data are the mean ± SD (n = 3). ** P<0.01.
with pmiR-21. The data indicated that a negative role of miR-21 in the regulation of IR, over-expression of miR-21 significantly promoted insulin-induced glucose uptake in IR-adipocytes.

PTEN is a target of miR-21 in 3T3-L1 adipocytes
PTEN has been reported as a direct target of miR-21 in some diseases [13, 14]. Recent studies demonstrate that nearly 25% of miRNA target sites in the 3'UTR are conserved in humans and mice. Analysis of human, mouse and rat PTEN 3'UTR showed the presence of the highly conserved miR-21 recognition element (Fig. 3a). Therefore we predicted PTEN might be a potential target gene of miR-21 in 3T3-L1 adipocytes. To confirm this hypothesis, we examined the mRNA and protein expression of PTEN by qRT-PCR and Western blot analysis, respectively. Our data showed PTEN mRNA and protein levels were significantly increased in IR adipocytes compared with normal adipocytes, over-expression of miR-21 significantly decreased PTEN protein level, whereas it had no significant effect on PTEN mRNA expression in IR-adipocytes. These data indicated that PTEN was a target gene of miR-21 in 3T3-L1 adipocytes (Fig. 3b, c).

Over-expression of miR-21 led to increased insulin signaling in IR-adipocytes
PTEN is a negative regulator of PI3K-dependent signaling. Activation of PI3-K results in the activation of Akt and downstream mediators involved in insulin signaling such as GSK3. To investigate the potential involvement of PTEN in the regulation of insulin signaling by miR-21, Akt phosphorylation at Ser474 (Akt-P Ser474) was determined by Western blot analysis. As shown in Fig. 4, IR-adipocytes exhibited a significant 54.8% reduction in insulin-stimulated Akt-P Ser474 compared with normal adipocytes. This decrement was significantly recovered (58.9% recovery) by treatment of IR-adipocytes with pmir-21. To further explore whether the Akt pathway is activated, we determined the level of phospho-GSK3β (Ser9), a substrate of Akt kinase activity in adipocytes. There were about 1.8-fold decreases of phospho-GSK3β protein level in IR-adipocytes com-

![Fig. 4](image-url) Over-expression of miR-21 led to increased Akt signaling in IR-adipocytes. 3T3-L1 adipocytes of control, pmir-21 and pNeg were induced to IR-adipocytes with high glucose and high insulin for 24 h as the IR group, pmir-21+IR group and pNeg+IR group, respectively. Cell lysates were collected and subjected to western blot analysis using antibodies specific for P-Akt, P-GSK3β and IRβ. Densitometry data of Akt phosphorylation, GSK3β phosphorylation and IRβ protein levels in 3T3-L1 cells were showed, respectively b, c and d. Data are expressed as the mean ± SD (n = 3). **P < 0.01.
Insulin resistance (IR) is the pathogenic hallmark of type 2 diabetes mellitus (T2DM). Therefore, further exploration into the molecular mechanism of IR will contribute to the prevention and treatment for T2DM and its complications. Emerging evidence suggests that miRNAs play an important role in diabetes and its related complications [12,20,21]. Some studies showed mir-21 might be associated with IR or diabetes [10–12]. However, the role of miR-21 in the development of IR was not clear. In the current study, we further confirmed that the expression of mir-21 was reduced, and mir-21 significantly increased insulin-stimulated glucose uptake in IR-adipocytes (Fig. 2), suggesting that mir-21 enhanced insulin sensitivity and improved IR. What are the underlying mechanisms that mir-21 improved IR? Physiologically, insulin signals passes through a pathway involving protein kinases including, but not limited to, PI3K, AKT or protein kinase B (PKB), and GSK-3β (the PI3K/AKT/GSK-3β pathway) [22]. Emerging evidence suggests that IR can potentially be treated via modulation of the PI3K/AKT pathway by targeting its up- or downstream modulators [23]. Studies also indicated PTEN could inhibit insulin signaling and antagonized PI3K-mediated signaling, and the inhibition of PTEN may enhance insulin signaling [15,16]. Our data show that PTEN may be a target of miR-21 and miR-21 negatively regulated the process of IR by targeting PTEN. First, a search with Targetscan revealed that mir-21 is complementary to sites in the 3′ untranslated regions (3′UTR) of PTEN (Fig. 3). Second, the induction of IR significantly increased PTEN protein expression, which occurred concomitant with a 5.3-fold reduction in miR-21 expression. Third, the enhancement in PTEN protein expression was followed by treatment of IR-adipocytes with miR-21, while PTEN mRNA expression was not changed.

AKT is a downstream signal molecule of PTEN and AKT activation is a hallmark of PTEN loss [24]. To further confirm the involvement of PTEN in miR-21-mediated effects, AKT phosphorylation was determined. Our data showed that endogenous AKT protein level did not significantly change, while the phosphorylation level of AKT significantly decreased in the IR group compared with control group. Over-expression of miR-21 resulted in an increase in AKT phosphorylation and a concomitant reduction in PTEN expression (Fig. 4). Similar results were obtained concerning GSK3β (Ser9) phosphorylation level. GSK3β, a substrate of Akt kinase activity, plays an important role in the regulation of glycogen synthesis. Phosphorylation of Ser 9 in GSK3β leads to the inhibition of its activity, so, miR-21 may inhibit GSK-3β through the phosphorylation. Because the limit of experiment condition, we did not detect the PI3K activity, but we observed there was no difference that PTEN upstream signaling molecules such as IR-β (Fig. 4). Taken together, these data demonstrated the inhibition of PTEN expression led to activation of AKT-GSK3β signal pathway without perturbing PTEN upstream signaling molecules.

Akt is required for GLUT4 translocation to the cell surface following insulin stimulation, which subsequently augments glucose transport [25]. We found insulin-stimulated glucose uptake and GLUT4 translocation were significantly decreased in IR-adipocytes, and miR-21 reversed these changes (Fig. 4, 5). These results reinforce the notion that miR-21 improved IR by PTEN-AKT signaling, miR-21 could be a causal factor of the down-regulation of PTEN and activation of PI3K/AKT pathway in IR-adipocytes.
In conclusion, our study indicated that miR-21 reversed high glucose and high insulin induced IR in 3T3-L1 adipocytes through modulating the PTEN-AKT pathway, and miR-21 might be a novel potential target for prevention and therapy of IR and other metabolic diseases.

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Conflict of Interest:

None.

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