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ARTICLE

Downregulation of *miR-181a* upregulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity

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Abstract
Aims/hypothesis Sirtuin-1 (SIRT1) is a potential therapeutic target to combat insulin resistance and type 2 diabetes. This study aims to identify a microRNA (miRNA) targeting SIRT1 to regulate hepatic insulin sensitivity.
Methods Luciferase assay combined with mutation and immunoblotting was used to screen and verify the bioinformatically predicted miRNAs. miRNA and mRNA levels were measured by real-time PCR. Insulin signalling was detected by immunoblotting and glycogen synthesis. Involvement of SIRT1 was studied with adenovirus, inhibitor and SIRT1-deficient hepatocytes. The role of *miR-181a* in vivo was explored with adenovirus and locked nucleic acid antisense oligonucleotides.
Results *miR-181a* targets the 3′ untranslated region (3′UTR) of *Sirt1* mRNA through a *miR-181a* binding site, and downregulates SIRT1 protein abundance at the translational level. *miR-181a* is increased in insulin-resistant cultured hepatocytes and liver, and in the serum of diabetic patients. Overexpression of *miR-181a* decreases SIRT1 protein levels and activity, and causes insulin resistance in hepatic cells. Inhibition of *miR-181a* by antisense oligonucleotides increases SIRT1 protein levels and activity, and improves insulin sensitivity in hepatocytes. Ectopic expression of *SIRT1* abrogates the effect of *miR-181a* on insulin sensitivity, and inhibition of SIRT1 activity or SIRT1 deficiency markedly attenuated the improvement in insulin sensitivity induced by antisense *miR-181a*. In addition, overexpression of *miR-181a* by adenovirus impairs hepatic insulin signalling, and intraperitoneal injection of locked nucleic acid antisense oligonucleotides for *miR-181a* improves glucose homeostasis in diet-induced obesity mice.
Conclusions/interpretation *miR-181a* regulates SIRT1 and improves hepatic insulin sensitivity. Inhibition of *miR-181a* might be a potential new strategy for treating insulin resistance and type 2 diabetes.

Keywords Hepatocytes · Insulin resistance · Locked nucleic acid · MicroRNA

Abbreviations

3′UTR	3′ Untranslated region
DIO	Diet-induced obesity
GSK3β	Glycogen synthase kinase 3β
INSR	Insulin receptor

B. Zhou and C. Li contributed equally to this study.

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LNA	Locked nucleic acid
miRNA	MicroRNA
PI3K	Phosphoinositide 3-kinase
siRNA	Small interfering RNA
SIRT1	Sirtuin-1
SHP2	SH2 domain-containing protein tyrosine phosphatase 2

Introduction

In recent years, type 2 diabetes mellitus has become a common chronic disease worldwide, a critical factor contributing to the pathogenesis of type 2 diabetes being insulin resistance [1, 2]. Sirtuin-1 (SIRT1), an NAD-dependent protein deacetylase, has been shown as a potential therapeutic target to combat insulin resistance and type 2 diabetes [3–5].

It has been reported that SIRT1 is a positive regulator of insulin signalling in skeletal muscle cells, adipocytes and hepatocytes [6–8]. Adenovirus-mediated overexpression of *SIRT1* in the liver of insulin-resistant mice attenuates hepatic steatosis and ameliorates systemic insulin resistance [9]. Moderate transgenic overexpression of *Sirt1* in *db/db* and high-fat diet-fed mice improves glucose tolerance and insulin sensitivity [10, 11]. Aged mice orally administered resveratrol, which can enhance SIRT1 activity, display increased insulin sensitivity and survival [12]. Resveratrol and its structural analogues, especially SRT1720, also protect mice against high-fat diet-induced obesity (DIO) and insulin resistance [13–15]. Further studies focused on the regulators of SIRT1 might provide potential new therapeutic approaches to combat insulin resistance and type 2 diabetes.

MicroRNAs (miRNAs) have been associated with many diseases, including type 2 diabetes [16, 17]. *miR-278* mediates energy balance by regulating insulin responsiveness in *Drosophila*, and *miR-278* mutants are insulin resistant [18]. Aberrant expression of miRNAs has been observed in the insulin-target tissues of rat and mouse models of type 2 diabetes [19–21]. In adipocytes, *miR-320* induces insulin resistance by inhibiting the insulin–phosphoinositide 3-kinase (PI3K) signalling pathway [22]. *miR-29*, which has been found to be elevated in the skeletal muscle of diabetic rats and the liver of *db/db* mice, negatively regulates insulin signalling via inhibiting the p85α subunit of PI3K [23, 24]. In muscle cells, *miR-7* downregulates IRS-1 levels as well as inhibiting insulin-stimulated Akt phosphorylation and glucose uptake [25]. However, the role of miRNAs in regulating hepatic insulin sensitivity is still largely unknown.

In the present work, we sought to identify a miRNA targeting *Sirt1* to regulate hepatic insulin sensitivity in vitro and in vivo, and to provide a potential approach for combating hepatic insulin resistance and type 2 diabetes.

Methods

Plasmid construction Plasmid construction was performed as described elsewhere (electronic supplementary material [ESM] Methods). The primer sequences are shown in ESM Table 1.

Bioinformatics analysis Human, mouse and rat *Sirt1* 3′ untranslated region (3′UTR) sequences were retrieved from the Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore). The potential miRNA targets within the conserved regions in 3′UTR of *Sirt1* were predicted by miRBase (www.mirbase.org/), TargetScan (www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and miRanda (www.microrna.org/microrna/home.do). *Sirt1* 3′UTR sequences from human, chimpanzee, dog, rat, mouse, chicken and pufferfish were aligned with each other and with the *miR-181a* seed region using PicTar.

Cell culture and treatments HEK293T and HepG2 cells were maintained in DMEM with 10% FBS. Primary cultured mouse hepatocytes were prepared from 9-week-old C57BL/6 mice or the mice with the indicated genotypes by the collagenase perfusion method as described previously [26], and cultured in DMEM with 10% FBS.

For transfection, HEK293T, HepG2 cells or primary cultured mouse hepatocytes in 12-well plates were transfected with the indicated plasmids (1.6 μg/well), miRNA mimics, antisense oligonucleotides or small interfering RNA (siRNA) at the indicated final concentrations in the culture medium using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 72 h, cells were subsequently stimulated with or without 100 nmol/l insulin (Sigma, St Louis, MO, USA) for 15 min and harvested for immunoblotting. To induce insulin resistance, HepG2 cells or primary cultured mouse hepatocytes were transfected for 54 h and then treated with 18 mmol/l glucosamine for 18 h in DMEM with 5 mmol/l glucose, before being stimulated with or without insulin for immunoblotting or measurement of glycogen synthase activity and glycogen synthesis assay. For adenovirus infection, HepG2 cells were infected with the indicated adenovirus at a multiplicity of infection of 50 for 8 h before transfection. To inhibit the activity of SIRT1, HepG2 cells were treated with 10 mmol/l nicotinamide or 50 μmol/l Sirtinol (Sigma) for an additional 12 h after transfection with antisense *miR-181a* at the indicated concentrations for 60 h. For the experiment considering insulin-induced insulin resistance, HepG2 cells were treated with 100 nmol/l insulin for 24 h. To upregulate SIRT1 protein level, HepG2 cells were treated with 0.5 mmol/l AICAR (Sigma) for 48 h.

C2C12 myoblasts were maintained in DMEM with 10% FBS and differentiated in DMEM with 2% horse serum after reaching confluence. After 4 days, the C2C12 cells were

differentiated into myotubes and transfected with *miR-181a* mimics for 72 h at the indicated concentrations.

RNA isolation and real-time PCR Total RNA was prepared using TRIzol reagent (Invitrogen). To measure mRNA levels, total RNA was reverse-transcribed and quantified by real-time PCR using Power SYBR Green (Applied Biosystems, Foster City, CA, USA). The primer sequences are shown in ESM Table 1. *miR-181a* was reverse-transcribed using a *miR-181a*-specific stem-loop primer (Applied Biosystems), and subsequently measured by real-time PCR using *miR-181a*-specific Taqman probes (Applied Biosystems). *U6* (also known as *Rnu6*) was similarly measured and used for normalisation of *miR-181a* expression.

miRNA mimics, miRNA antisense oligonucleotides, siRNA and locked nucleic acid antisense oligonucleotides miRNA-181a duplex mimics and 2′-*O*-methylated single-stranded *miR-181a* antisense oligonucleotides (anti-181a) were obtained from GenePharma (Shanghai, China). Irrelevant miRNA duplex mimics for *Caenorhabditis elegans cel-miR-239b* or 2′-*O*-methylated single-stranded *cel-miR-239b* antisense oligonucleotides were co-transfected as a mock control to equalise the total amount of miRNA or antisense oligonucleotides, respectively, for each transfection as described previously [27]. SIRT1 and its control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Locked nucleic acid (LNA) antisense oligonucleotides specific for *miR-181a* (LNA-anti-181a) and mismatched LNA oligonucleotides (LNA-control) were obtained from Exiqon (Vedbaek, Denmark).

Luciferase assay HEK293T cells in a 24-well plate were co-transfected with pSIF-GFP or the indicated plasmids expressing miRNA precursors (0.8 μg/well), pRL-*Sirt1*-3′ UTR (pRL-TK vector containing *Sirt1* 3′UTR) or pRL-*Sirt1*-3′UTRm (pRL-TK vector containing mutant *Sirt1* 3′ UTR) (0.1 μg/well) and pSV40-*β-gal* (Promega, Madison, WI, USA) (0.1 μg/well) using Lipofectamine 2000. HepG2 cells in a 24-well plate were co-transfected with the indicated miRNA mimics, pRL-*Sirt1*-3′UTR (0.1 μg/well) and pSV40-*β-gal* (0.1 μg/well) using Lipofectamine 2000. After transfection for 72 h, cells were harvested for luciferase assay as previously described [6].

Immunoblotting Immunoblotting was performed with antibodies against SIRT1 (Millipore, Billerica, MA, USA), α-tubulin (Sigma), GAPDH (Millipore), insulin receptor (INSR), Tyr1150/1151-phosphorylated INSR, Akt, Ser473-phosphorylated Akt, glycogen synthase kinase-3β (GSK3β), Ser9-phosphorylated GSK3β, SH2 domain-containing protein tyrosine phosphatase 2 (SHP2), acetyl-p53(K382), p53, acetyl-histone H3(K9), histone H3 (Cell Signaling, Beverly, MA, USA), G6Pase (Santa Cruz Biotechnology) and phosphoenolpyruvate carboxykinase (Novus, Littleton, CO, USA). Protein quantification was analysed by Quantity One software (Bio-Rad, Hercules, CA, USA), and normalised to α-tubulin or GAPDH.

Measurement of SIRT1 activity SIRT1 deacetylase activity was determined using a SIRT1 Fluorimetric Activity Assay Kit (Biomol International, New York, NY, USA). Nuclear extracts from HepG2 cells were used for measuring SIRT1

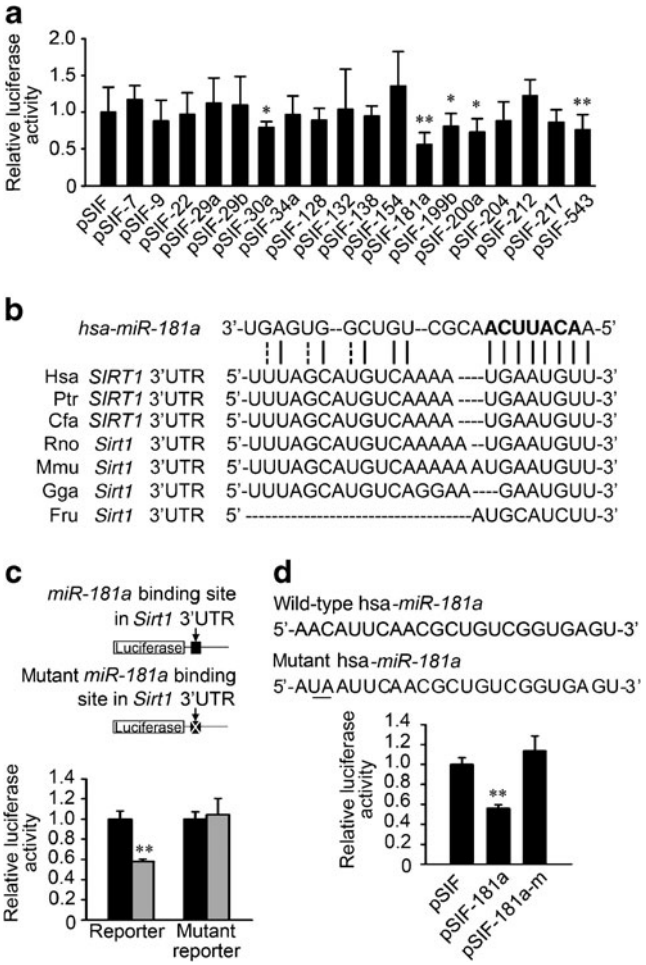


Fig. 1 *MiR-181a* targets *Sirt1*1 3′UTR. **a** Among the 18 predicted miRNAs, *miR-181a* showed the most significant repressive effect on the activity of *Sirt1* 3′UTR. After transfection with pRL-*Sirt1*-3′UTR and the indicated plasmids for 72 h, HEK293T cells were harvested for luciferase assay. *$p<0.05$, **$p<0.01$ vs pSIF-GFP (pSIF). **b** Sequence alignment of human (Hsa) *miR-181a* with the 3′UTRs of chimpanzee (Ptr), dog (Cfa), rat (Rno), mouse (Mmu), chicken (Gga) and pufferfish (Fru) *Sirt1*. The seed region of *miR-181a* is indicated in bold. **c** Mutation of the predicted *miR-181a* binding site in *Sirt1* 3′UTR abrogated the repressive effect of *miR-181a* on the activity of *Sirt1* 3′UTR when measured by luciferase assay. Black bars, pSIF; grey bars, pSIF-181a. **$p<0.01$ vs pSIF or mutant reporter. **d** Mutation of the *miR-181a* seed region abrogated the repressive effect of *miR-181a* on the activity of *Sirt1* 3′UTR. **$p<0.01$ vs pSIF or pSIF-181a-m. Error bars represent SD

deacetylase activity. Liver samples from mice fed chow or high-fat diet were lysed by radioimmunoprecipitation assay buffer, and then SIRT1 was immunoprecipitated to detect its deacetylase activity.

NAD assay Details of the NAD assay are shown in the ESM Methods.

Human study The fasting plasma of age-matched male control and diabetic participants was collected at the Centre Hospital of Xuhui District, Shanghai. Patients with fasting plasma glucose >7.0 mmol/l were considered to have diabetes, as previously described [28]. Written informed consent was obtained from each participant, and the study was approved by the Institutional Review Board of the Institute for Nutritional Sciences. Serum RNA was isolated using TRIzol LS reagent (Invitrogen).

Measurement of glycogen synthase activity and glycogen synthesis assay Glycogen synthase activity was determined using a modified method as previously described [6]. HepG2 cells were treated with or without 100 nmol/l insulin for 10 min and harvested for glycogen synthase activity assay using uridine diphosphate-[^{3}H]glucose (GE Healthcare, Piscataway, NJ, USA). Glycogen synthesis was determined as previously described [6]. After transfection with the indicated miRNA mimics or antisense oligonucleotides at the indicated final concentrations for 72 h, HepG2 cells were processed with or without 100 nmol/l insulin and 3.7×10^4 Bq/ml [^{3}H]glucose (Amersham, Van Nuys, CA, USA)

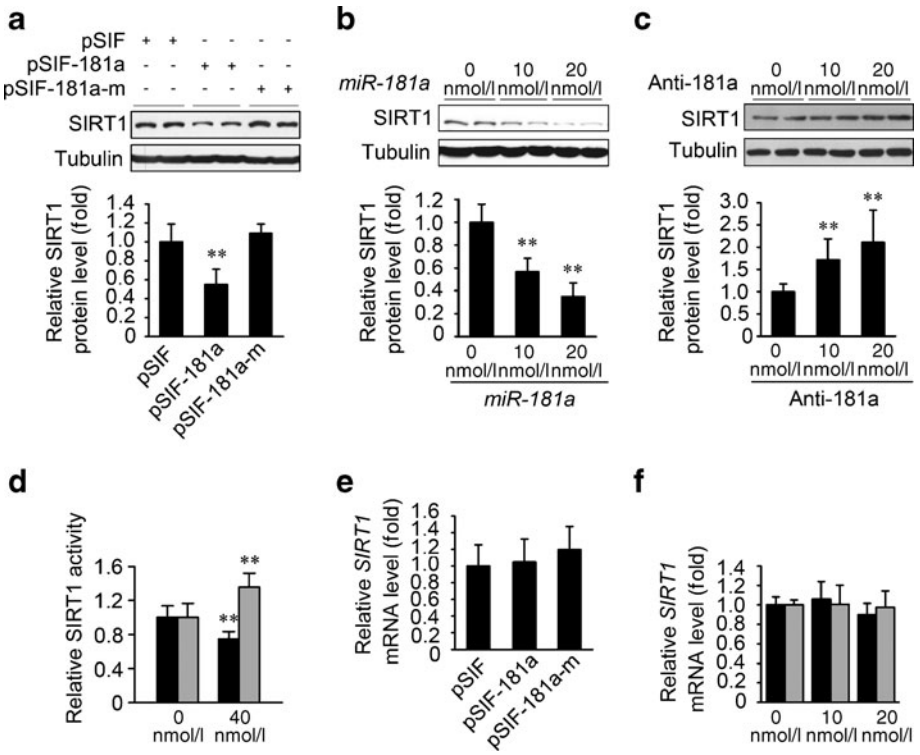


Fig. 2 *miR-181a* downregulates SIRT1 protein abundance at the translational level. **a** Overexpression of *miR-181a* but not mutant *miR-181a* decreased SIRT1 protein levels. After transfection with the indicated plasmids for 72 h, HEK293T cells were harvested for immunoblotting. $^{**}p<0.01$ vs pSIF-GFP (pSIF) or pSIF-181a-m expressing mutant *miR-181a*. **b** Overexpression of *miR-181a* dose-dependently downregulated SIRT1 protein levels. After transfection with *miR-181a* mimics at the indicated concentrations for 72 h, HEK293T cells were harvested for immunoblotting. The doses in nmol/l represent irrelevant miRNA mimics for *cel-miR-239b* or anti-*cel-miR-239b* co-transfected as a mock control to equalise the total amount of miRNA mimics or antisense oligonucleotides for each transfection; 0 nmol/l represents cells transfected with only *cel-miR-239b* mimics or anti-*cel-miR-239b*. $^{**}p<0.01$ vs cells treated with only a mock transfection. **c** Inhibition of *miR-181a* by antisense oligonucleotides dose-dependently increased SIRT1 protein levels. After transfection with anti-181a at the indicated concentrations for 72 h, HEK293T cells were harvested for immunoblotting. $^{**}p<0.01$ vs cells treated with only a mock transfection. **d** Overexpression of *miR-181a* decreased SIRT1 activity, whereas inhibition of *miR-181a* increased SIRT1 activity. After transfection with the indicated oligonucleotides for 72 h, the nuclear protein of HepG2 cells were extracted to measure SIRT1 activity. Black bars, *miR-181a*; grey bars, anti-181a. $^{**}p<0.01$ vs cells treated with only a mock transfection. **e** Overexpression of *miR-181a* had no effect on *SIRT1* mRNA levels. After transfection with the indicated plasmids for 72 h, HEK293T cells were harvested for real-time PCR. **f** Overexpression or inhibition of *miR-181a* had no effect on *SIRT1* mRNA levels. After transfection with the indicated oligonucleotides for 72 h, HEK293T cells were harvested for real-time PCR. Black bars, *miR-181a*; grey bars, anti-181a. Error bars represent SD

in DMEM with 5 mmol/l glucose for 3 h, and then harvested for glycogen synthesis assay.

Generation of recombinant adenoviruses To construct adenoviruses for overexpression of *GFP*, human *SIRT1* or mouse *miR-181a*, complementary DNAs encoding mouse pre-*miR-181a*, human *SIRT1* and *GFP* were inserted into pShuttle-CMV vector (Stratagene), and then subcloned into pAdEasy-1 adenoviral backbone vector (Stratagene). Recombinant adenoviruses were then generated following the manufacturer's protocol. Briefly, the adenoviral constructs were linearised with the restriction enzyme PacI and transfected into HEK293A cells using Lipofectamine 2000. After several rounds of propagation, the recombinant adenoviruses were purified by CsCl gradient centrifugation.

Animal experiments Details of the animal experiments are given in the ESM Methods.

Statistics Data are expressed as means±SD of at least three independent experiments. Statistical significance was assessed by Student's *t* test except where indicated. Relationships between variables were determined by the Pearson correlation coefficient test. Differences were considered statistically significant at $p<0.05$.

Results

miR-181a targets Sirt1 3′UTR through an miR-181a binding site To identify the miRNAs targeting *Sirt1* 3′UTR, the

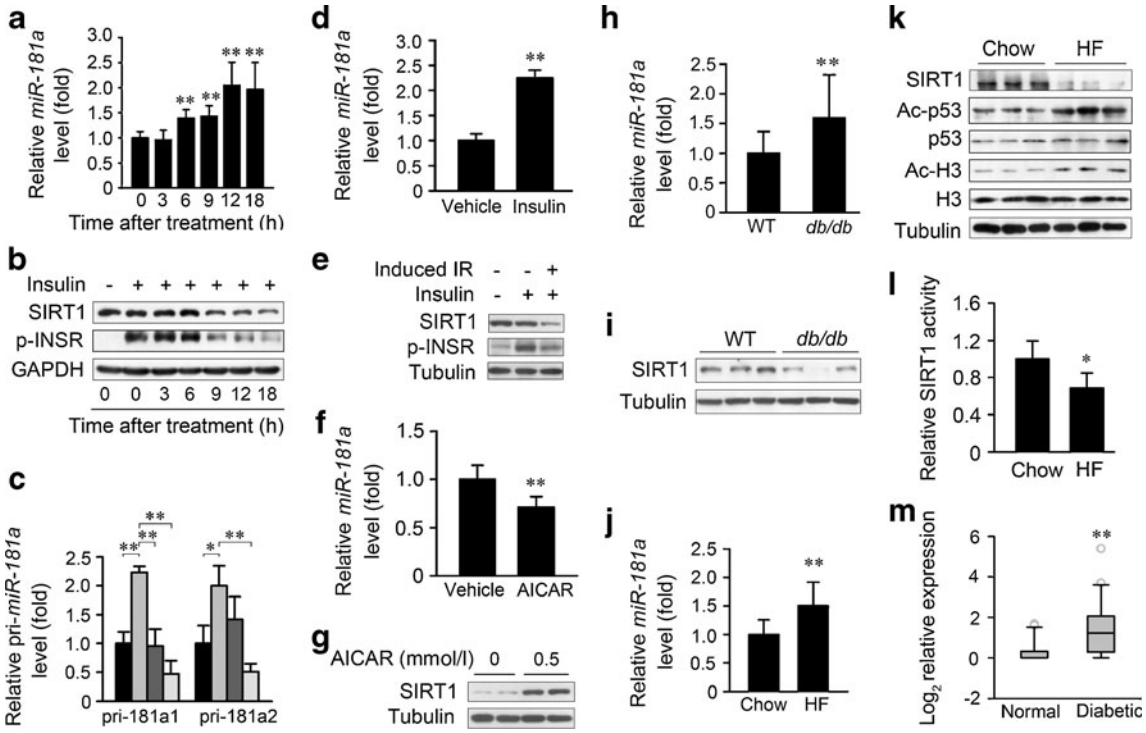


Fig. 3 *MiR-181a* is increased under insulin-resistant conditions and negatively correlated with SIRT1. **a** Glucosamine upregulated *miR-181a* level in a time-dependent manner in HepG2 cells. After treatment with glucosamine for the indicated time, HepG2 cells were harvested for real-time PCR. $^{**}p<0.01$ vs no treatment. **b** Glucosamine decreased SIRT1 protein level and induced insulin resistance in HepG2 cells in a time-dependent manner. After treatment with glucosamine for the indicated time, HepG2 cells were stimulated with or without 100 nmol/l insulin for 15 min, and then harvested for immunoblotting. **c** Glucosamine upregulated the levels of primary *miR-181a* (pri-181a), and this was significantly attenuated by transcription inhibitor actinomycin D (AMD). HepG2 cells were treated with glucosamine for 18 h to induce insulin resistance (IR) and harvested for real-time PCR. 0.5 mg/ml AMD was added 1 or 3 h before harvesting the cells. Black bars, control; mid-grey bars, glucosamine; dark grey bars, glucosamine+AMD 1 h; light grey bars, glucosamine+AMD 3 h. $^{*}p<0.05$, $^{**}p<0.01$. **d, e** Insulin-induced insulin resistance and its correlation with *miR-181a* and SIRT1 in HepG2 cells. HepG2 cells were treated with 100 nmol/l insulin for 24 h and stimulated with or without insulin for 15 min, before being harvested for real-time PCR or immunoblotting. **f, g** AICAR significantly elevated SIRT1 protein levels and decreased *miR-181a* level in HepG2 cells. HepG2 cells were treated with 0.5 mmol/l AICAR for 48 h, and then harvested for real-time PCR or immunoblotting. $^{**}p<0.01$. **h, i** *MiR-181a* was increased while SIRT1 protein levels were decreased in the liver of *db/db* mice compared with their littermates. The liver samples from 10-week-old mice with the indicated genotypes were analysed by real-time PCR and immunoblotting. $^{**}p<0.01$, $n=14$ for each genotype. **j, k, l** *MiR-181a* was increased while SIRT1 protein levels and activity were decreased in the livers of DIO mice. Tissues were collected from 16-week-old mice fed chow or a high-fat diet for 10 weeks. $^{**}p<0.01$, $^{*}p<0.05$, $n=9$ for each group. **m** *miR-181a* was increased in the serum of diabetic patients. *miR-181a* levels were detected by real-time PCR, and the relative quantification data were analysed on the log (base 2) scale. $^{**}p<0.01$, $n=20$ for each group. Error bars represent SD

miRNA target-prediction programs miRBase, miRanda, PicTar and TargetScan were used to obtain the potential miRNAs targeting *Sirt1* 3′UTR. Luciferase reporter under the control of *Sirt1* 3′UTR was used to examine the 18 selected potential miRNAs. As shown in Fig. 1a, overexpression of *miR-181a*, *miR-543*, *miR-30a*, *miR-199b* or *miR-200a* significantly downregulated the activity of the *Sirt1* 3′UTR, and *miR-181a* showed the most significant effect of all the miRNAs tested. Furthermore, we found that *miR-181a*, *miR-543* and *miR-200a* could significantly reduce the luciferase activity of *Sirt1* 3′UTR in HepG2 cells as well as in HEK293T cells (ESM Fig. 1).

To explore whether *miR-181a* directly targets *Sirt1* 3′ UTR, we analysed the *Sirt1* 3′UTRs in human, chimpanzee, dog, rat, mouse, chicken and pufferfish by PicTar, and found that the potential binding site for *miR-181a* within *Sirt1* 3′ UTRs is highly conserved from chicken to human (Fig. 1b). To test whether *miR-181a* represses the activity of *Sirt1* 3′ UTR through this conserved site, we generated a reporter with a deletion of this site (Fig. 1c, upper panel). As expected, overexpression of *miR-181a* had no effect on the mutant reporter (Fig. 1c, bottom panel). To further confirm whether *miR-181* targets *Sirt1* 3′UTR, a plasmid expressing mutant *miR-181a* with two mismatched bases in the seed region was constructed (Fig. 1d, upper panel). We found that the mutant *miR-181a* had no effect on the activity of *Sirt1* 3′

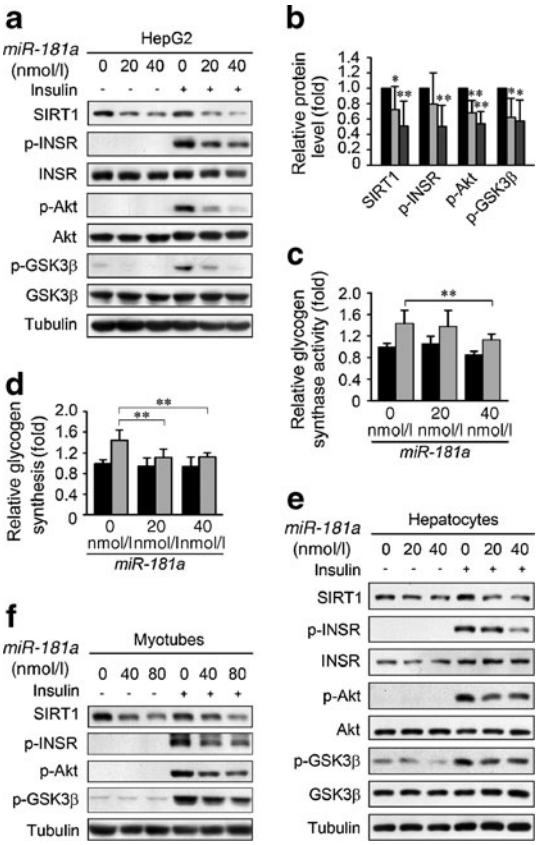


Fig. 4 Overexpression of *miR-181a* downregulates SIRT1 protein levels and induces insulin resistance. **a** Overexpression of *miR-181a* decreased SIRT1 protein levels and the insulin-stimulated phosphorylation of INSR, Akt, and GSK3β in HepG2 cells. After transfection with *miR-181a* mimics at the indicated concentrations for 72 h, HepG2 cells were stimulated with 100 nmol/l insulin for 15 min and subsequently harvested for immunoblotting. **b** Quantification of SIRT1 protein levels and insulin-stimulated phosphorylation levels of INSR, Akt and GSK3β corresponding to (**a**). Black bars, 0 nmol/l; light grey bars, 20 nmol/l; dark grey bars, 40 nmol/l. *$p<0.05$, **$p<0.01$ vs cells treated with only a mock transfection. **c**, **d** Overexpression of *miR-181a* decreased insulin-stimulated upregulation of glycogen synthase activity (**c**) and glycogen synthesis (**d**) in HepG2 cells. Black bars, control; grey bars, insulin. **$p<0.01$ vs insulin-stimulated cells treated with only a mock transfection. **e**, **f** Overexpression of *miR-181a* decreased SIRT1 protein levels and the insulin-stimulated phosphorylation of INSR, Akt and GSK3β in primary cultured hepatocytes (**e**) and C2C12 myotubes (**f**), respectively. Protein levels were analysed by immunoblotting. Error bars represent SD

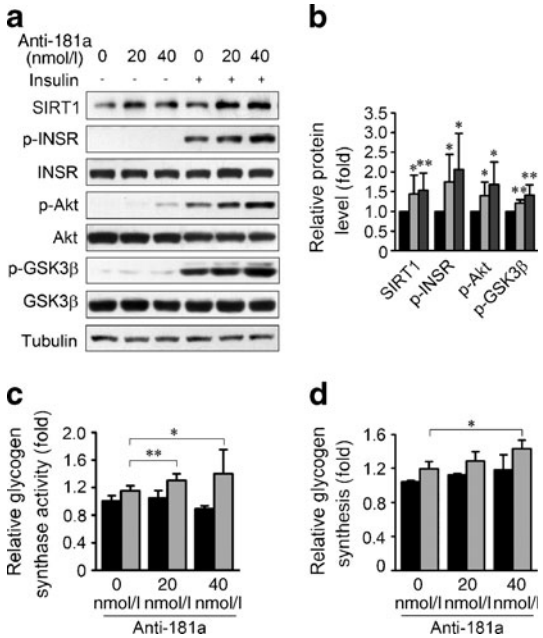


Fig. 5 Inhibition of *miR-181a* increases SIRT1 protein levels and insulin sensitivity under glucosamine-induced insulin-resistant conditions. **a** Inhibition of *miR-181a* by antisense oligonucleotides significantly increased SIRT1 protein levels and the insulin-stimulated phosphorylation of INSR, Akt and GSK3β in HepG2 cells under glucosamine-induced insulin-resistant (IR) condition. After transfection with anti-181a at the indicated concentrations for 72 h, HepG2 cells were stimulated with 100 nmol/l insulin for 15 min and harvested for immunoblotting. **b** Quantification of SIRT1 protein levels and the phosphorylation levels of INSR, Akt and GSK3β under the glucosamine-induced insulin-resistant conditions in (**a**). Black bars, 0 nmol/l; light grey bars, 20 nmol/l; dark grey bars, 40 nmol/l. *$p<0.05$, **$p<0.01$ vs cells in the same group treated only with a mock transfection. **c**, **d** Inhibition of *miR-181a* increased the insulin-stimulated upregulation of glycogen synthase activity (**c**) and glycogen synthesis (**d**) in HepG2 cells under glucosamine-induced insulin-resistant conditions. Black bars, control; grey bars, insulin. *$p<0.05$, **$p<0.01$, vs insulin-stimulated cells treated only with a mock transfection. Error bars represent SD

UTR (Fig. 1d, bottom panel). These data demonstrate that *miR-181a* targets *Sirt1* 3′UTR through its binding site within *Sirt1* 3′UTR.

miR-181a decreases SIRT1 protein abundance at the translational level To examine whether *miR-181a* downregulates SIRT1 protein level, immunoblotting was performed with HEK293T cells transfected with the indicated plasmids, miRNA mimics or miRNA antisense oligonucleotides. As shown in Fig. 2a, overexpression of *miR-181a* markedly downregulated SIRT1 protein levels, whereas overexpression of mutant *miR-181a* had no significant effect. Moreover, overexpression of *miR-181a* by its mimics dose-dependently decreased SIRT1 protein levels (Fig. 2b). Consistently, neutralisation of endogenous *miR-181a* with antisense oligonucleotides dose-dependently elevated SIRT1 protein levels (Fig. 2c). Similarly, in HepG2 cells, transfection with *miR-181a* mimics or antisense oligonucleotides had similar effects (ESM Fig. 2a,b). More importantly, overexpression of *miR-181a* markedly downregulated SIRT1 activity and increased the acetylation of two known SIRT1 targets, p53 and histone H3, in HepG2 cells, whereas neutralisation of *miR-181a* increased SIRT1 activity and decreased the acetylation of p53 and histone H3 (Fig. 2d, ESM Fig. 2a,b). Furthermore, *miR-181a* had no significant effect on the cellular NAD levels, which contributes to the endogenous deacetylase activity of SIRT1 (ESM Fig. 2d). In addition, neither overexpression nor inhibition of *miR-181a* changed *SIRT1* mRNA levels in both HEK293T cells and HepG2 cells (Fig. 2e,f, ESM Fig. 2c). These data suggest that *miR-181a* decreases SIRT1 protein levels by inhibiting its translation.

miR-181a increases under insulin-resistant conditions SIRT1, downregulated by *miR-181a*, has been suggested as a therapeutic target for the prevention of diseases related to insulin resistance [5]. We thus hypothesised that *miR-181a* levels would be correlated with insulin resistance. To investigate the correlation of *miR-181a* and insulin resistance, glucosamine-induced insulin resistance in HepG2 cells, a common model for studying insulin resistance, was used

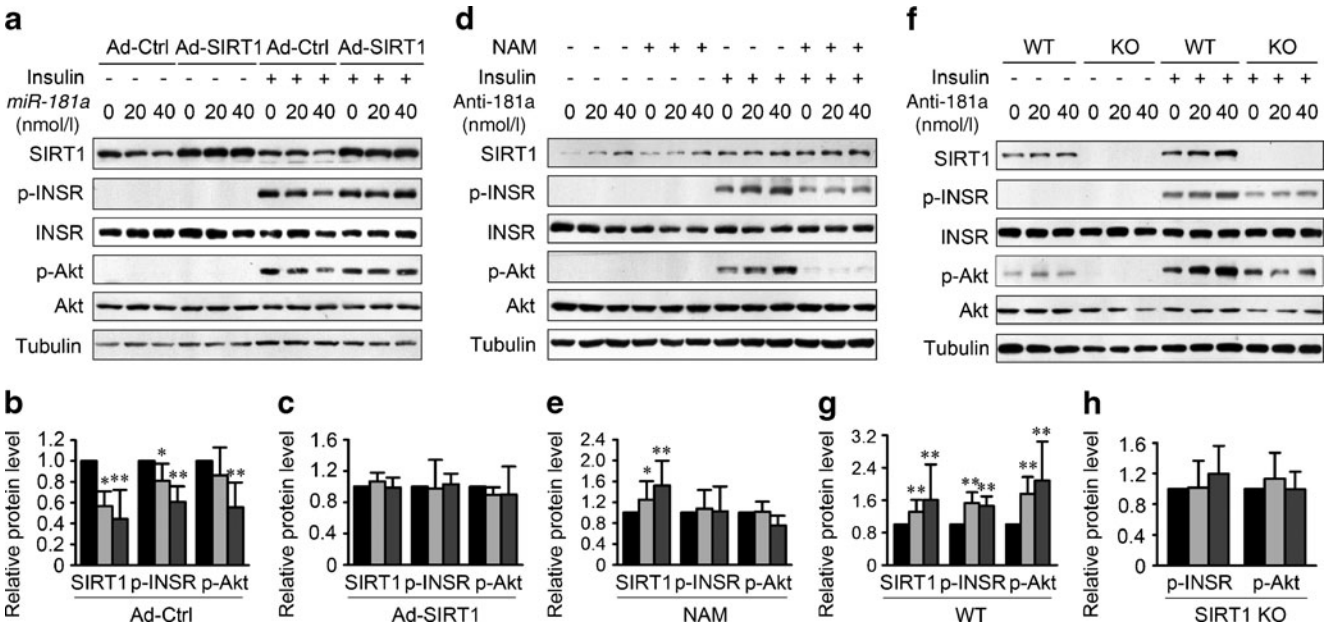


Fig. 6 Regulation of insulin signalling by *miR-181a* is significantly dependent on SIRT1. **a** Ectopic expression of *SIRT1* by adenovirus (Ad-SIRT1) abrogated the negative effect of *miR-181a* on the insulin-stimulated phosphorylation of INSR and Akt in HepG2 cells. Ad-Ctrl, control adenovirus. Protein levels were analysed by immunoblotting. **b**, **c** Quantification of SIRT1 protein levels and the insulin-stimulated phosphorylation levels of INSR and Akt in HepG2 cells infected with Ad-Ctrl (**b**) or Ad-SIRT1 (**c**) in (**a**). Black bars, 0 nmol/l; light grey bars, 20 nmol/l; dark grey bars, 40 nmol/l. *$p<0.05$, **$p<0.01$ vs cells in the same group treated with only a mock transfection. **d** Inhibition of SIRT1 activity by nicotinamide (NAM) abrogated the positive effect of anti-181a on insulin-stimulated phosphorylation of INSR and Akt in HepG2 cells under glucosamine-induced insulin-resistant conditions. Protein levels were analysed by immunoblotting. **e** Quantification of SIRT1 protein levels and the insulin-stimulated phosphorylation levels of INSR and Akt in HepG2 cells treated with NAM in (**d**). Black bars, 0 nmol/l; light grey bars, 20 nmol/l; dark grey bars, 40 nmol/l. *$p<0.05$, **$p<0.01$ vs cells in the same group treated only with a mock transfection. **f** The improvement in insulin signalling by antisense *miR-181a* was markedly abrogated in primary hepatocytes of *Sirt1* knockout (KO) mice under glucosamine-induced insulin-resistant conditions. **g**, **h** Quantification of SIRT1 protein levels and the insulin-stimulated phosphorylation levels of INSR and Akt in primary hepatocytes from wild-type (WT) mice (**g**) and *Sirt1* knockout mice (**h**) in (**f**). Black bars, 0 nmol/l; light grey bars, 20 nmol/l; dark grey bars, 40 nmol/l. **$p<0.01$ vs cells in the same group treated only with a mock transfection. Error bars represent SD

[6]. As shown in Fig. 3a,b, glucosamine induced insulin resistance and significant upregulation of *miR-181a* in HepG2 cells in a time-dependent manner, and the upregulation of *miRNA-181a* occurred earlier than the decrease in SIRT1 and the appearance of insulin resistance. In addition, we found that glucosamine increased the levels of primary *miR-181a*, which was significantly attenuated by the transcription inhibitor actinomycin D, suggesting that glucosamine upregulates *miR-181a* transcription (Fig. 3c). Moreover, under insulin-induced insulin-resistant conditions, the *miR-181a* level also increased, accompanied by a decrease in SIRT1 protein level (Fig. 3d,e). Consistently, *miR-181a* was decreased when AICAR, an activator of AMP-activated protein kinase (AMPK), elevated SIRT1 protein levels in HepG2 cells (Fig. 3f,g).

A similar increase in *miR-181a* was observed in the livers of *db/db* mice, a model of type 2 diabetes, where SIRT1 protein levels were decreased (Fig. 3h,i). In addition, *miR-181a* was also upregulated in the liver of mice fed with a high-fat diet, the content and activity of SIRT1 being decreased, which was further confirmed by the increased acetylation of p53 and histone H3 (Fig. 3j–l). Furthermore, *miR-181a* was significantly increased in the serum of patients with type 2 diabetes (Fig. 3m).

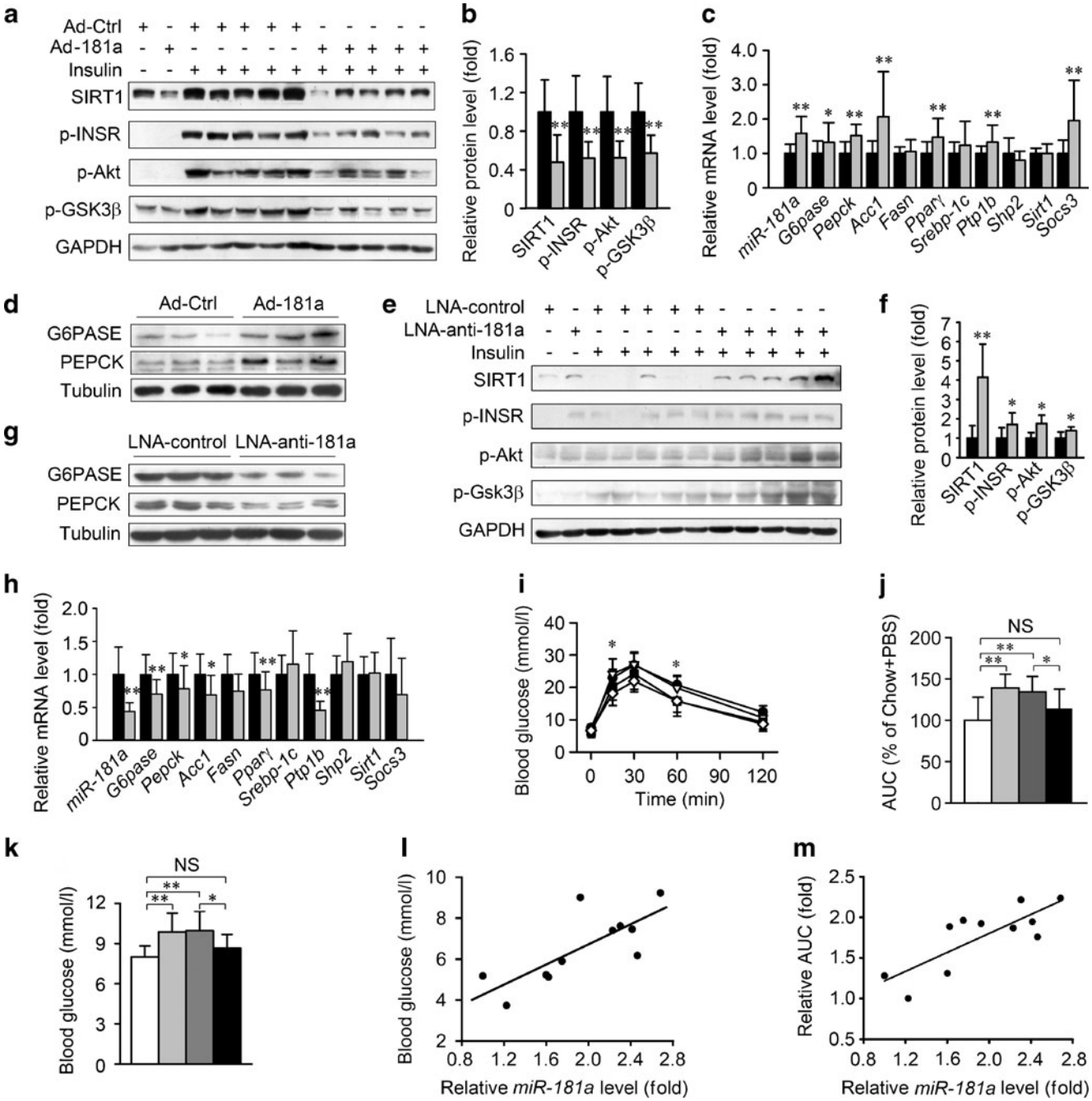
These results demonstrate that *miR-181a* increases under conditions of insulin resistance and may be associated with insulin resistance.

Overexpression of miR-181a represses SIRT1 and insulin sensitivity in hepatocytes To test whether *miR-181a* regulates insulin sensitivity, we first measured the effect of *miR-181a* on insulin signalling in hepatic cells. We found that overexpression of *miR-181a* in HepG2 cells markedly and dose-dependently decreased SIRT1 protein levels and the insulin-stimulated phosphorylation of INSR at Tyr1151, Akt at Ser473 and GSK3β at Ser9 (Fig. 4a,b). Meanwhile, the insulin-stimulated glycogen synthase activity and glycogen synthesis in HepG2 cells were significantly impaired by *miR-181a* (Fig. 4c,d). To confirm whether *miR-181a* impairs insulin signalling in other insulin-responsive cells, we overexpressed *miR-181a* in primary cultured mouse hepatocytes and differentiated C2C12 myotubes. A similar inhibitory effect of *miR-181a* on SIRT1 and insulin signalling was observed (Fig. 4e,f). In addition, the inhibitory effect of *miR-181a* on the insulin-stimulated phosphorylation of INSR, Akt, and GSK3β was similar to the effect of *Sirt1* siRNA (ESM Fig. 3). Taken together, these data show that *miR-181a* downregulates SIRT1 protein levels and insulin sensitivity in hepatocytes and C2C12 myotubes.

Inhibition of endogenous miR-181a increases SIRT1 and insulin sensitivity in hepatocytes under glucosamine-induced insulin-resistant conditions The induction of insulin resistance by *miR-181a* leads to the suggestion that inhibition of *miR-181a* might improve insulin sensitivity. We thus inhibited endogenous *miR-181a* in HepG2 cells using antisense oligonucleotides to test its effect on insulin sensitivity. Under glucosamine-induced insulin-resistant conditions, inhibition of *miR-181a* upregulated SIRT1 protein levels and the insulin-stimulated phosphorylation of INSR, Akt and GSK3β (Fig. 5a,b). Furthermore, insulin-stimulated upregulation of glycogen synthase activity and glycogen synthesis were also increased by the inhibition of *miR-181a* to HepG2 cells under glucosamine-induced insulin-resistant conditions (Fig. 5c,d). These results demonstrate that inhibition of endogenous *miR-181a* increases SIRT1 protein levels and hepatic insulin sensitivity under glucosamine-induced insulin-resistant conditions.

SIRT1 is involved in the regulation of hepatic insulin sensitivity mediated by miR-181a Next, we investigated whether SIRT1

Fig. 7 *miR-181a* regulates SIRT1 and hepatic insulin sensitivity in vivo. **a**, **b** Overexpression of *miR-181a* by adenovirus (Ad-181a) in mouse liver decreased SIRT1 levels and the insulin-stimulated phosphorylation of INSR, Akt and GSK3β. Eight-week-old male C57BL/6 mice were infected with the indicated adenovirus by tail vein injection, and were killed 12 days later to detect by immunoblotting the indicated proteins in liver stimulated with or without insulin. Black bars, control adenovirus (Ad-Ctrl); grey bars, Ad-181a. $^{**}p<0.01$ vs Ad-Ctrl, $n=9$ for each group in (**b**). **c** *miR-181a* and the mRNA levels of the genes indicated were detected by real-time PCR in mouse liver infected with Ad-181a (grey bars) or Ad-Ctrl (black bars), as described in (**a**). $^{*}p<0.05$, $^{**}p<0.01$ vs Ad-Ctrl, $n=6$–9 for each group. **d** The protein levels of G6Pase and phosphoenolpyruvate carboxykinase (PEPCK) were increased in mouse liver infected with Ad-181a when detected by immunoblotting. **e, f** Intraperitoneal injection of LNA-anti-181a increased SIRT1 protein levels and the insulin-stimulated phosphorylation of INSR, Akt, and GSK3β in the liver of DIO mice. The proteins indicated were measured by immunoblotting (**e**) and quantified by densitometry (**f**). Black bars, LNA-control; grey bars, LNA-anti-181a. $^{*}p<0.05$ vs LNA-control. **g** LNA-anti-181a decreased the protein levels of G6pase and PEPCK in the livers of DIO mice when measured by immunoblotting. **h** *MiR-181a* and mRNA levels of the genes indicated were detected in the livers of DIO mice injected with LNA-anti-181a (grey bars) or LNA-control (black bars). Real-time PCR was used to analyse these genes. $^{*}p<0.05$, $^{**}p<0.01$ vs LNA-control, $n=9$–11 for each group. **i**, **j** LNA-anti-181a improved glucose tolerance in DIO mice as determined by a glucose tolerance test (GTT) (**i**). White diamonds, chow PBS; black circles, high-fat diet+PBS; white triangles, high-fat diet+LNA-control; black squares, high-fat diet+LNA-anti-181a. The area under the curve (AUC) of GTT was also reduced by LNA-anti-181a (**j**) White bars, chow+PBS; light grey bars, high-fat diet+PBS; dark grey bars, high-fat diet+LNA-control; black bars, high-fat diet+LNA-anti-181a. $^{*}p<0.05$, $^{**}p<0.01$ vs LNA-control, $n=9$–13 for each group. **k** LNA-anti-181a decreased the 4-h fasting blood glucose levels in DIO mice. White bar, chow+PBS; light grey bar, high-fat diet+PBS; dark grey bar, high-fat diet+LNA-control; black bar, high-fat diet+NA-anti-181a. $^{*}p<0.05$, $^{**}p<0.01$ vs LNA-control, $n=9$–13 for each group. **l, m** Individual correlation between hepatic *miR-181a* levels and 4-h fasting blood glucose levels (**l**; $R^2=0.58$, $p=0.006$) or the AUC of GTT (**m**; $R^2=0.63$, $p=0.003$) in the DIO mice injected with LNA-anti-181a ($n=11$). Statistical significance was assessed by the Pearson correlation coefficient test. Error bars represent SD



is involved in the regulation of hepatic insulin sensitivity by *miR-181a*. We found that *miR-181a* successfully inhibited insulin signalling in HepG2 cells infected with control adenovirus (Fig. 6a,b). However, the repressive effect of *miR-181a* on insulin signalling was greatly attenuated in HepG2 cells infected with adenovirus expressing *SIRT1* (Fig. 6a,c). When HepG2 cells were treated with the SIRT1 inhibitor nicotinamide under glucosamine-induced insulin-resistant conditions, inhibition of *miR-181a* consistently increased SIRT1 protein levels but failed to improve insulin signalling (Fig. 6d,e). Treatment of HepG2 cells with sirtinol, a more specific SIRT1 inhibitor, showed similar results (ESM Fig. 4a). To further confirm the role of SIRT1 in the regulation of insulin sensitivity by *miR-181a*, primary cultured mouse hepatocytes from *Sirt1* knockout mice were used. The repressive effect of *miR-181a* on the insulin-stimulated phosphorylation of Akt was lost in the primary cultured hepatocytes from *Sirt1* null mice (ESM Fig. 4b). Under glucosamine-induced insulin-resistant conditions, the improvement in insulin sensitivity with antisense *miR-181a*

was also markedly attenuated when *Sirt1* was absent (Fig. 6f–h). Taken together, these findings indicate that SIRT1 participates in the regulation of hepatic insulin sensitivity mediated by *miR-181a*.

miR-181a regulates insulin sensitivity and glucose homeostasis in vivo To investigate whether overexpression of *miR-181a* also induces insulin resistance in vivo, we use adenovirus to overexpress *miR-181a* in C57/BL6 mice by tail vein injection. *miR-181a* levels were markedly upregulated in the mouse liver infected with adenovirus expressing *miR-181a* precursors (Fig. 7c). Overexpression of *miR-181a* in mouse liver significantly reduced SIRT1 protein levels and the insulin-induced phosphorylation of INSR, Akt and GSK3β (Fig. 7a,b). In addition, the mRNA levels of lipogenic genes *Acc1* and *Pparγ* (also known as *Pparg*), and the negative regulators of insulin signalling *Ptp1b* and *Socs3* were significantly increased in the mouse liver with *miR-181a* overexpression (Fig. 7c). The mRNA and protein levels of the key gluconeogenic genes *Pepck* and *G6pase* were also elevated (Fig. 7c,d).

We then detected whether inhibition of endogenous *miR-181a* could improve insulin sensitivity in vivo. After intraperitoneal injection of LNA antisense oligonucleotides for *miR-181a* (LNA-anti-181a), *miR-181a* levels in the liver of DIO mice were markedly reduced (Fig. 7h). Moreover, inhibition of *miR-181a* by LNA-anti-181a significantly increased SIRT1 protein levels and the insulin-induced phosphorylation of INSR, Akt and GsSK3β in both the liver and muscle of DIO mice (Fig. 7e,f, ESM Fig. 5a). Meanwhile, hepatic protein and mRNA levels of *Pepck* and *G6pase* were both significantly decreased in the LNA-anti-181a-treated DIO mice (Fig. 7g,h). In addition, the hepatic mRNA levels of *Acc1*, *Pparγ* and *Ptp1b* were significantly decreased in the LNA-anti-181a-treated DIO mice (Fig. 7h). In the muscle of LNA-anti-181a-treated DIO mice, mRNA levels of *Foxo1*, *Glut4* and *Pgc1α* were increased (ESM Fig. 5b). The alteration in these genes is consistent with the improved insulin signalling in liver and muscle (Fig. 7e,f, ESM Fig. 5a). In agreement with the improved insulin sensitivity in liver and muscle and the downregulation of key hepatic gluconeogenic enzyme genes induced by LNA-anti-181a, we found that LNA-anti-181a significantly downregulated 4-h fasting blood glucose levels and improved glucose tolerance in the DIO mice (Fig. 7i–k). Moreover, there was a positive correlation between the hepatic *miR-181a* levels and the fasting blood glucose, as well as the areas under the curve of glucose tolerance tests, in the DIO mice injected with LNA-anti-181a (Fig. 7l,m). Taken together, these data show that inhibition of *miR-181a* ameliorates DIO-induced hepatic insulin resistance and improves glucose homeostasis in DIO mice.

Discussion

In this study, we identified SIRT1 as a direct target of *miR-181a* and demonstrated that *miR-181a* negatively regulates SIRT1 and hepatic insulin sensitivity. These results suggest that *miR-181a* might represent a potential therapeutic target for the treatment of insulin resistance and type 2 diabetes.

Recently, a study reported that *miR-9*, *miR-135*, *miR-199b*, *miR-204*, *miR-181a* and *miR-181b* reduce endogenous SIRT1 in mouse embryonic stem cells [29]. In our study, we further demonstrate that *miR-181a* downregulates SIRT1 protein levels in hepatocytes and muscle cells. Moreover, our study also showed that *miR-181a* decreases SIRT1 protein levels in mouse liver. Previous studies have shown that SIRT1 can be regulated by multiple miRNAs, such as *miR-9*, *miR-22*, *miR-34a*, *miR-132*, *miR-199a* and *miR-217* in some different biological processes [27, 30–32]. In our experiment, we similarly observed the inhibitory effect of these miRNAs. Moreover, we also discovered that *miR-543* and *miR-30a* could significantly decrease the activity of *Sirt1* 3′UTR in HEK293T cells. *miR-30a* was found to reduce autophagic activity, which is positively regulated by SIRT1 [33, 34], suggesting that *miR-30a* may regulate autophagy by downregulating SIRT1. The distinct biological functions of these miRNAs targeting SIRT1 need to be further explored. In our study, although no obvious repressive effects of *miR-22*, *miR-34a*, *miR-132* or *miR-217* on *Sirt1* 3′UTR were observed in HEK293T cells, we did find their effects on *Sirt1* 3′UTR in HepG2 cells, suggesting that miRNAs may play different roles in different cells. Taken together, these findings suggest that SIRT1 is regulated by various miRNAs to function diversely in specific cells and tissues.

Many studies have investigated the function of *miR-181a* in tissues where it is highly expressed, such as thymus, brain and spleen [35, 36]. It has also been reported that *miR-181a* is a critical player in epithelial cell adhesion molecule (EpCAM)-positive hepatic cancer stem cells by targeting transcriptional regulators of differentiation [37]. However, the biological function of *miR-181a* in liver is still poorly understood. Here we demonstrate for the first time that *miR-181a* is an important regulator in hepatic insulin sensitivity. Overexpression of *miR-181a* induces hepatic insulin resistance, whereas inhibition of endogenous *miR-181a* improves hepatic insulin sensitivity both in vitro and in vivo. Remarkably, inhibition of *miR-181a* by LNA-anti-181a improves glucose homeostasis in DIO mice. The efficacy and safety of LNA antisense oligonucleotides in inhibiting miRNA targets have been shown in mouse and non-human primate models [38, 39]. Several different LNA drugs are currently in clinical trials against cancer and infectious diseases [40]. LNA-anti-181a might thus have potential therapeutic value for treating insulin resistance and type 2 diabetes. In addition, we found

that *miR-181a* significantly increased in the serum of patients with type 2 diabetes, implying that serum *miR-181a* is a potential diagnostic marker, similar to other miRNAs described previously [41].

Consistent with our results, knockdown of *SIRT1* impairs insulin signalling in HepG2 hepatoma cells [6]. Liver-specific *Sirt1* knockout mice display signs of hepatic insulin resistance, and exhibit significantly increased fasting glucose levels when fed with a high-fat diet [42]. Hepatic SIRT1 deficiency in mice impairs mammalian target of rapamycin complex 2 (mTORC2)/Akt signalling and results in hyperglycaemia and insulin resistance [26]. Furthermore, adenovirus-mediated overexpression of *SIRT1* in the liver of *ob/ob* mice and of LDL receptor-deficient mice fed a high-fat high-sugar diet attenuates hepatic steatosis and ameliorates systemic insulin resistance [9]. Similarly, our data show that inhibition of *miR-181a* by LNA-anti-181a is sufficient to improve hepatic insulin sensitivity accompanied by upregulation of SIRT1 protein levels in DIO mice.

Since a single vertebrate miRNA may target up to approximately 100–200 mRNAs [43], it is likely that some other *miR-181a* targets participate in the regulation of hepatic insulin sensitivity. SHP2, a tyrosine phosphatase, regulates insulin signalling positively and has been shown to be repressed by *miR-181a* in T cells [44, 45]. However, we found that protein levels of SHP2 were not changed in HepG2 cells or the liver of mice when *miR-181a* was overexpressed (data not shown). RNA-binding protein LIN-28 homologue A, another target of *miR-181a*, has also been reported as a positive regulator of insulin signalling [46, 47]. *Foxo1* and *Pgc1α* were also predicted as *miR-181a* targets by PicTar or TargetScan. Whether these genes are involved in *miR-181a*-regulated hepatic insulin sensitivity needs to be investigated. In this study, we found that inhibition of SIRT1 activity or SIRT1 deficiency abrogated the positive effect of antisense *miR-181a* on insulin signalling, and overexpression of *SIRT1* could sufficiently improve the insulin resistance induced by *miR-181a*. These data suggest that *miR-181a* regulates insulin sensitivity at least mainly through SIRT1. However, whether the effect of miR-181a on insulin sensitivity is SIRT1-dependent in vivo *also* needs to be studied.

SIRT1 has been reported as a positive regulator of insulin signalling by targeting protein tyrosine phosphatase 1B (PTP1B), AMPK, forkhead box O1 (FOXO1), peroxisome proliferator-activated receptor γ coactivator 1α (PGC1A), and so on [5]. Our in vivo experiments show that some glucose and lipid metabolism-related genes in liver or muscle, including *Ptp1b*, *G6pase*, *Pepck*, *Acc1*, *Foxo1*, *Pgc1α* and *Glut4*, are regulated by *miR-181a*. The alteration in these genes might be part of the underlying mechanisms by which *miR-181a* regulates insulin signalling through SIRT1. Besides *miR-181a*, *miR-22* and *miR-34a* were also found to be elevated in the liver of diabetic *db/db* mice [48]. Whether *miR-22* and *miR-34a* can regulate insulin sensitivity through SIRT1 needs to be investigated.

In summary, our results indicate that *miR-181a* regulates hepatic insulin sensitivity and that SIRT1 is involved in this process. Inhibition of *miR-181a* is capable of improving hepatic insulin sensitivity and glucose homeostasis, which might provide a potential new therapeutic strategy for treating insulin resistance and type 2 diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement This study was designed by BZ, CL and QWZ. The experiments were performed by BZ, CL, WQ, YZ, FZ, JXW, YNH, DMW, YL and TTY. QJ and MFL contributed to materials, methods and discussion. BZ, CL and QWZ analysed the data. QWZ supervised the project. CL, BZ and QWZ wrote the paper. All authors participated in data interpretation and revising the paper, and approved the final version of the manuscript.

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