MicroRNA-29a is involved lipid metabolism dysfunction and insulin resistance in C2C12 myotubes by targeting PPARδ

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Abstract. MicroRNA-29a (miR-29a) expression has been reported to be closely associated with skeletal muscle insulin resistance and type 2 diabetes. The present study investigated the effect of miR-29a on palmitic acid (PA)-induced lipid metabolism dysfunction and insulin resistance in C2C12 myotubes via overexpressing or silencing of miR-29a expression. Mouse C2C12 myoblasts were cultured, differentiated and transfected with miR-29a or miR-29a inhibitor lentiviral with or without subsequent palmitic acid (PA) treatment. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were performed to assess the mRNA and protein levels of related genes, respectively. PA treatment increased the expression of miR-29a in a time- and dose- dependent manner. miR-29a silencing improved insulin-induced glucose uptake and increased glucose transporter-4 (GLUT4) transportation to the plasma membrane by upregulating its target peroxisome proliferator-activated receptor δ (PPARδ). Furthermore, it was observed that miR-29a regulated the expression of genes associated with lipid metabolism, including pyruvate dehydrogenase kinase isoform, mitochondrial uncoupling protein (UCP)2, UCP3, long chain specific acyl-CoA dehydrogenase, mitochondrial and fatty acid transport protein 2. The results confirmed that silencing miR-29a induced a decrease in glucose transport and affected lipid metabolism in PA-treated C2C12 cells, and therefore may be involved in insulin resistance by targeting PPARδ in skeletal muscle. Therefore, the inhibition of miR-29a may be a potential novel strategy for treating insulin resistance and type 2 diabetes.

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Introduction

Intrauterine growth restriction (IUGR) is closely associated with an increased risk of metabolic disease development in adults, including type 2 diabetes mellitus (T2DM), hypertension, obesity, dyslipidemia and metabolic syndrome (1). Insulin resistance (IR) is the key feature of these metabolic diseases. Skeletal muscle consumes ~70% blood glucose and is responsible for the majority of insulin-stimulated glucose utilization; therefore, it is the primary tissue involved in IR in the context of metabolic disease (2). Numerous theories exist to explain the mechanisms of IR in skeletal muscle, including lipotoxicity, endocrine inflammation, oxidative stress, endoplasmic reticulum stress and mitochondrial dysfunction (3-5). Among these, lipotoxicity has become the most widely recognized theory in recent decades (3,6). However, the intrinsic molecular mechanisms have not been fully elucidated.

microRNAs (miRNAs/miRs) are approximately 19-22 nucleotides in length and are an abundant class of short endogenous noncoding RNAs. They bind to the 3' noncoding region of target genes by complementary base pairing and regulate gene expression at the post-transcriptional level through protein translation inhibition or mRNAs degradation promotion (7,8). miRNAs are widely involved in physiological process, including cell proliferation, apoptosis, differentiation, organogenesis, immunity and metabolism. In addition, they are closely associated with several pathological processes, including IR, inflammation and fibrosis (9). The involvement of miRNAs in the regulation of IR, glucose uptake and lipid metabolism in skeletal tissue and cells has been reported (10). miR-106b, miR-27a and miR-30d have been implicated in the regulation of glucose metabolism by targeting the glucose transporter type 4 (GLUT4) signaling pathway in L6 myoblast cells (11). A recent study reported that miR-1a expression significantly decreases in the soleus muscle of mice fed a high-fat diet. miR-1a may be a marker of the development of IR in skeletal muscle (12). However, the exact mechanisms and involvement of miRNAs in skeletal muscle with IR remains unknown.

It has been demonstrated that in diabetic Goto-Kakizaki rats miR-29a is predominantly expressed in three important

target tissues of insulin action: Muscle, fat and liver (13). A previous study demonstrated that the adenovirus-mediated overexpression of miR-29a, b and c in 3T3-L1 adipocytes may largely repress insulin-stimulated glucose uptake, potentially through inhibiting protein kinase B activation (13). This is in agreement with another study that revealed that the expression of miR-29a in the skeletal muscle of aged IUGR rats was associated with obesity and IR (14). It has been reported that the level of miR-29a expression in serum from patients with T2DM was almost five times higher than the expression in T2DM-susceptible individuals with normal glucose tolerance and pre-diabetes. Additionally, the expression of miR-29a gradually increased with T2DM progression (15). Furthermore, elevated urinary miR-29a expression was demonstrated to be significantly correlated with albuminuria and carotid intima-media thickness in patients with T2DM (16). Circulating miR-29b in patients also increases with T2DM and diarrhea-predominant irritable bowel syndrome (17). Taken together, these data indicate the crucial role of miR-29 in IR and T2DM.

A previous bioinformatics analysis report revealed that miR-29a has a complementary sequence to the 3' untranslated region (UTR) of peroxisome proliferator-activated receptor δ (PPARδ) using TargetScan (www.targetscan.org) and PicTar (www.pictar.org) (14). The direct interaction between miR-29a and PPARδ was further confirmed by a luciferase reporter assay (14). PPARδ is located in the chromosomal region 6p21.2-p21.1 and consists of nine exons. It is an important regulator of skeletal muscle metabolism, particularly lipid and lipoprotein metabolism, as well as lipid oxidation (18,19). Lipid metabolism dysfunction has been demonstrated to be important in the pathogenesis of obesity-associated IR (20). However, the specific role and mechanism of miR-29a in skeletal muscle IR and lipid metabolism function modulation remain largely unknown.

In the present study, mouse C2C12 myoblasts were used as the research model to understand the potential roles and molecular mechanisms of miR-29a in fatty acid-mediated lipid disturbance and IR in skeletal muscle cells. The effect of miR-29a overexpression or downregulation with palmitate intervention on insulin sensitivity, lipid metabolism and PPARδ expression was investigated. The present study aimed to obtain more experimental evidence concerning miR-29a-induced IR in skeletal muscle at the cellular level, which may contribute to provide a potential target for clinical diagnosis and treatment for IR-associated disease.

Materials and methods

Cell culture. Mouse C2C12 myoblasts were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China). Myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C with 5% CO_2 and 95% O_2. Once confluence was reached, cells were differentiated in DMEM with 2% horse serum (Gibco; Thermo Fisher Scientific, Inc.). After 4-6 days, myoblasts were differentiated into myotubes. Myotubes were treated with freshly prepared DMEM containing 0.4 mM palmitic acid (PA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h in DMEM containing 2% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) and 10% FBS to establish the insulin-resistant cellular model. Following this, C2C12 cells were transferred to a serum-free medium with 100 nmol/l insulin or PBS for 30 min prior to total or membrane protein extraction.

C2C12 cell transfection. The miR-29a lentiviral expression and empty vector were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence of miR-29a mimics comprised: 5'-TAACCGATTTCAGATGGT GCTA-3'. Following C2C12 differentiation into myotubes, C2C12 myoblasts at passage 3 with 40-50% confluence ($2x10^5$ cells per well in 6-well culture plates) were transduced with miR-29a-green fluorescent protein (GFP)-pGLPZ or GFP-pGLPZ lentivirus at a multiplicity of infection (MOI) of 100; these were set as the miR-29a and mock-transfected groups, respectively. C2C12 myoblasts that had not been transfected with lentivirus were considered the normal group.

Stable cell lines with miR-29a loss of function were generated as previously described (21). The oligonucleotides used were as follows: miR-29a inhibitor, 5'-UAACCGAUUUCA GAUGGUGCUA-3' and negative control, 5'-UUCUCCGAA CGUGUCACGUdTdT-3'. miR-29a inhibitor (sponge) and negative control sequences were synthesized and inserted into an LV2-pGLV lentiviral vector (Shanghai GenePharma). The resulting recombinant LV2-pGLV-miR-29a inhibitor sponge plasmids and LV2-pGLV-control plasmids were analyzed by restriction endonuclease and DNA sequencing. The supernatant containing the lentiviral particles was harvested to determine the virus titer and was used to infect murine C2C12 myoblasts at passage 3 with 40-50% confluence ($2x10^5$ cells per well in 6-well culture plates). The cells were infected with lentivirus at MOI of 100. At 48 h post-infection, stably transfected cell lines were selected using 2 μg/ml puromycin for 4 days to establish miR-29a inhibitor and NC cell lines.

RNA preparation. Total RNA from PA-treated C2C12 myoblasts with normal, upregulated, and downregulated miR-29a expression was obtained using TRIzol reagent (Thermo Fisher Scientific, Inc.). All RNA samples were treated with 10x DNase I (Qiagen China Co., Ltd., Shanghai, China) and heated to 60˚C for 10 min according to the manufacturer's instructions. The quality and quantity of the RNA was determined with a Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA integrity was assessed by running an aliquot of the RNA samples on 1% denaturing agarose gel, followed by staining with ethidium bromide. The ratio of 28S ribosomal (r)RNA to 18S rRNA was 2:1, indicating that RNA samples were not degraded.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The miRNA RT reactions were performed using a miRNA reverse transcriptase reaction kit (Takara Bio, Inc., Otsu, Japan) according to the user's manual. Briefly, a 7.5 μl reaction cocktail was composed of 5 μg RNA samples with RNase free H_2O, 50 nM stem-loop RT primer, RT buffer, 0.25 mM each of dNTPs, 3.33 U/ml MultiScribe reverse

transcriptase and 0.25 U/ml RNase inhibitor. The cocktail was incubated for 30 min at 16˚C, followed by 30 min at 42˚C and 5 min at 85˚C. The cDNA was subsequently used as a template for miRNA PCR quantification with the TaqMan miRNA expression assay kit (Takara Bio, Inc.) according to the manufacturer's instructions. The PCR primers used were as follows: miR-29a forward, 5'-CTGATTTCTTTTGGTGTTCA-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; PPARδ forward, 5'-ACGCACCCTTTGTCATCC-3' and reverse, 5'-GAAGAG GCTGCTGAAGTTGG-3'; PDK4 forward, 5'-ATGTGGTCC CTACAATGG-3' and reverse, 5'-CACTCAAAGGCATCT TGG-3'; UCP3 forward, 5'-TCCTGCTGCTACCTAATG-3' and reverse, 5'-GTTCCTTTGCTGCCTATG-3'; FATP2 forward, 5'-GAGGTTGGACTCTTGGTTTG-3' and reverse, 5'-CTCCGCTGTTGAAGTAGATG-3'; UCP2 forward, 5'-GGAGGTAGCAGGAAATCAG-3' and reverse, 5'-GGT ATCCAGAGGGAAAGTG-3'; LCAD forward, 5'-GTGCCA TAGCCATGACAGAG-3' and reverse, 5'-CACGACGAT CACGAGATCAC-3'; GAPDH forward, 5'-ATCACTGCC ACCCAGAAG-3' and reverse, 5'-TCCACGACGGACACA TTG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. PCR was performed in an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific, Inc.) according to the user's protocol. Aliquots of cDNA equivalent to 25 ng total RNA in a 50 μl PCR reaction mixture were initially denatured at 95˚C for 10 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. Differences in miRNA expression among the samples were converted into fold-changes using the $2^{-\Delta\Delta Cq}$ method with U6 used as the internal control, as described previously (22). All reactions were performed in triplicate.

Glucose uptake assay. C2C12 cells were cultured in 12-well plates in DMEM with regular 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) until 80% confluence was reached. Medium was subsequently replaced with 2% horse serum in DMEM for differentiation. The differentiation into myocyte-myotube formation was tracked by multinucleated cell observation under inverted phase contrast microscope (magnification, x100, Olympus Corporation, Tokyo, Japan). After the indicated treatments, C2C12 cells were pre-incubated in 0.05% glucose containing Krebs-Ringer Bicarbonate buffer (pH 7.4 with 2% BSA) at 37˚C for 30 min. Cells were subsequently stimulated with or without 100 nM insulin for 10 min, followed by 500 μM 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at 37˚C in glucose-free Krebs-Ringer Bicarbonate buffer containing 2% BSA. Following the incubation, cells were washed three times with ice-cold PBS and 2-NBDG uptake into the cells was determined by fluorescence activated cell sorting analysis with CFlow software (BD Accuri C6; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Results were determined as relative fluorescence intensity (arbitrary units) with respect to the normal group. Each sample was analyzed three times.

Western blot analysis. Cells were treated prior to direct lysis with 2x SDS sample buffer (5% glycerol, 5% β-mercaptoethanol, 3% sodium dodecyl sulfate, 0.05% bromphenol blue, 10 mM pH 6.8 Tris-HCl), sonication twice (9 sec each time; 200 W), and denaturation at 95˚C for 5 min. Plasma membrane (PM) proteins were extracted using Plasma Membrane Protein Extraction kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocols and used for detecting membrane GLUT4 with the anti-GLUT4 antibody (1:1,000; ab654). Protein concentrations was measured via a Bicinchoninic Acid assay (Pierce, Rockford, IL, USA). The proteins (30 μg) from each sample were loaded onto a 10% Tris-glycine gel, run for 120 min at 110 V and subsequently transferred onto polyvinylidene fluoride membranes for 2 h at 200 mA. Subsequently, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 1% Tween 20 for 1 h at room temperature and were probed overnight with the following primary antibodies at 4˚C: Anti-PPARδ (1:500; ab23673), GLUT4 (1:1,000; ab654), pyruvate dehydrogenase kinase isoform 4 (PDK4; 1:1,000; ab214938), mitochondrial uncoupling protein 2 (UCP2; 1:1,000; ab97931), UCP3 (1:1,000; ab10985), fatty acid transport protein 2 (FATP2; 1:1,000; ab228784), long-chain acyl-CoA dehydrogenase (LCAD; 1:1,000, ab82853) and GAPDH (1:500; ab181602). All primary mouse antibodies and the secondary antibody were purchased from Abcam (Cambridge, MA, USA). Following this, membranes were incubated a 1:5,000 dilution with a secondary goat anti-rabbit IgG-HRP antibody (ab205718) for 1 h at room temperature and chemiluminescent immunodetection was performed. The ECL_{TM} Western Blot Detection reagents (GE Healthcare, Chicago, IL, USA) were applied to the blots according to the manufacturer's protocols and they were exposed to autoradiography film. Immunopositive bands were detected and quantified using the FluorChem M system (ProteinSimple, San Jose, CA, USA). Experiments were performed at least twice using the same cell lysates.

Statistical analysis. All data are presented as the mean + standard deviation. Analysis was performed with SPSS 20.0 software (IBM Corp., Armonk, NY, USA). The statistical significance of alterations in expression patterns between two groups was determined using the two-tailed Student t-test. For alterations in the expression patterns among three or more groups, statistical significance was calculated using the Kruskal-Wallis test followed by Mann Whitney U test with Bonferroni's correction. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-29a decreases the expression of PPARδ mRNA and protein. The stable expression of miR-29a induced by lentiviral transfection in C2C12 myoblasts was verified by RT-qPCR and visualized using a fluorescence microscope (data not shown). C2C12 myoblasts developed into myotubes after 4-6 days of incubation with 2% horse serum. The overexpression (Fig. 1A) or silencing (Fig. 1B) of miR-29a resulted in significantly higher or lower levels of miR-29a expression, respectively. The effect of miR-29a overexpression or silencing on PPARδ mRNA and protein expression was examined in C2C12 myotubes by PCR and western blot analysis (Fig. 1C-F). The results revealed that overexpression of miR-29a markedly downregulated the expression of

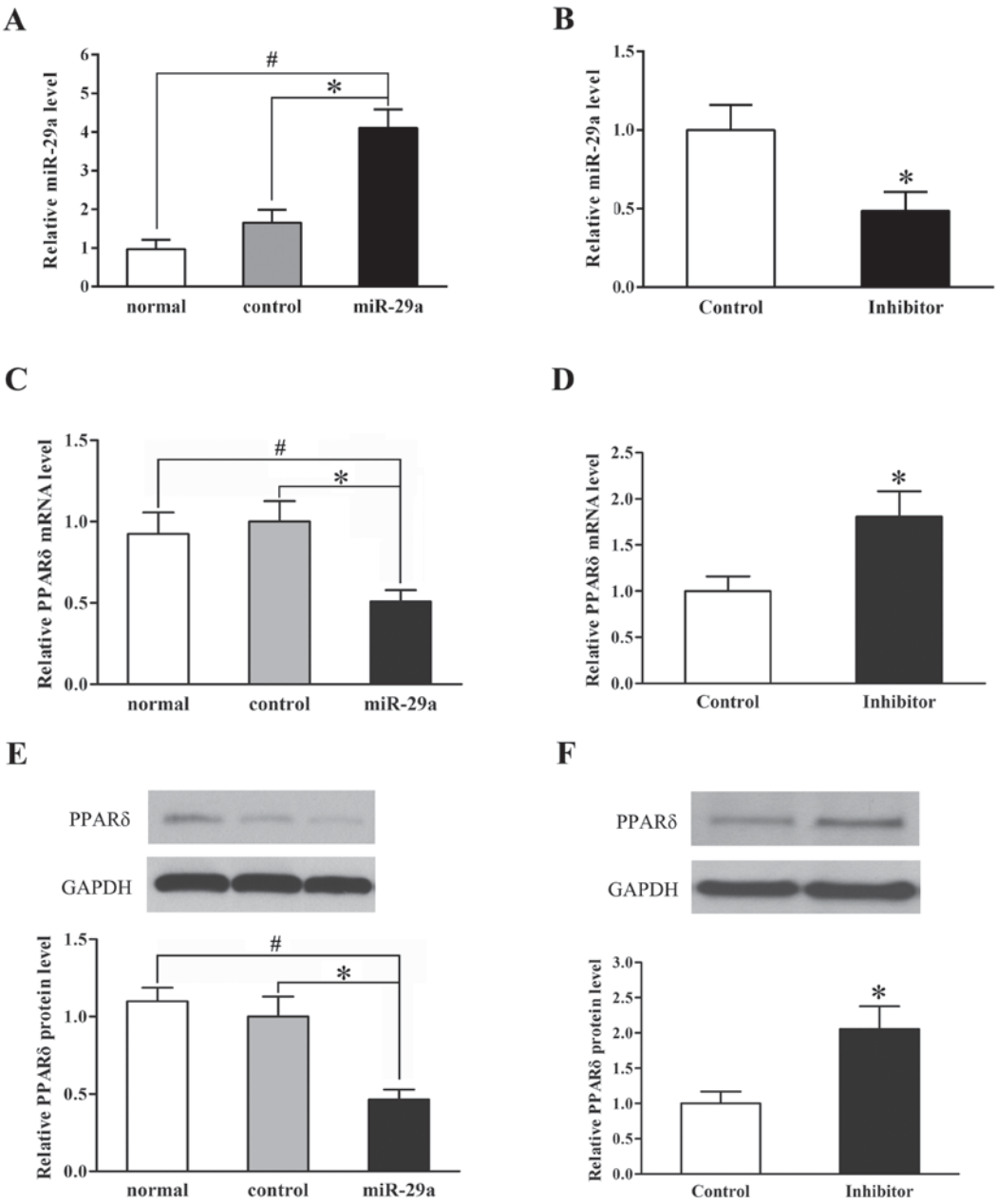

Figure 1. miR-29a overexpression and silencing in C2C12 cells mediates PPARδ expression. (A) miR-29a expression in the normal, control and miR-29a lentivirus group, and (B) miR-29a expression in cells transfected with control lentivirus or miR-29a inhibitor lentivirus were assessed by RT-qPCR. (C) RT-qPCR revealed that the overexpression of miR-29a significantly decreased the expression of PPARδ mRNA and (D) downregulation of miR-29a significantly increased the expression of PPARδ mRNA. (E) Western blot analysis demonstrated that the overexpression of miR-29a downregulated the expression of PPARδ protein and (F) miR-29a silencing upregulated PPARδ protein expression. Data are presented as the mean + standard deviation (n=3). *P<0.05 vs. control group, $^{\#}$P<0.05 vs. normal group. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-29a, microRNA-29a; Normal, untransfected group; Control, empty vector; PPARδ, peroxisome proliferator-activated receptor δ.

PPARδ mRNA (Fig. 1C) and protein (Fig. 1E). By contrast, the inhibition of miR-29a significantly increased PPARδ mRNA (Fig. 1D) and protein (Fig. 1F) expression. This data, combined with the findings of our previous bioinformatics analysis and luciferase assay (14), demonstrated that PPARδ was a target gene of miR-29a.

Expression of miR-29a increases under insulin-resistant conditions and miR-29a silencing increases insulin sensitivity and PPARδ expression. PA, a type of saturated fatty acid, is an important hyperlipidemic/dyslipidemic component that induces insulin resistance in skeletal muscle cells (21). The present study sought to determine the expression pattern of miR-29a in C2C12 cells following PA exposure. PA was demonstrated to induce the expression of miR-29a in a time- and dose-dependent manner (data not shown). Therefore, it was concluded that miR-29a may have an important role in skeletal muscle IR.

Silencing of miR-29a markedly increased insulin-induced glucose uptake, as assessed by the 2-NBDG uptake assay (Fig. 2A). PA treatment decreased the plasma membrane GLUT4 protein expression in miR-29a silencing group

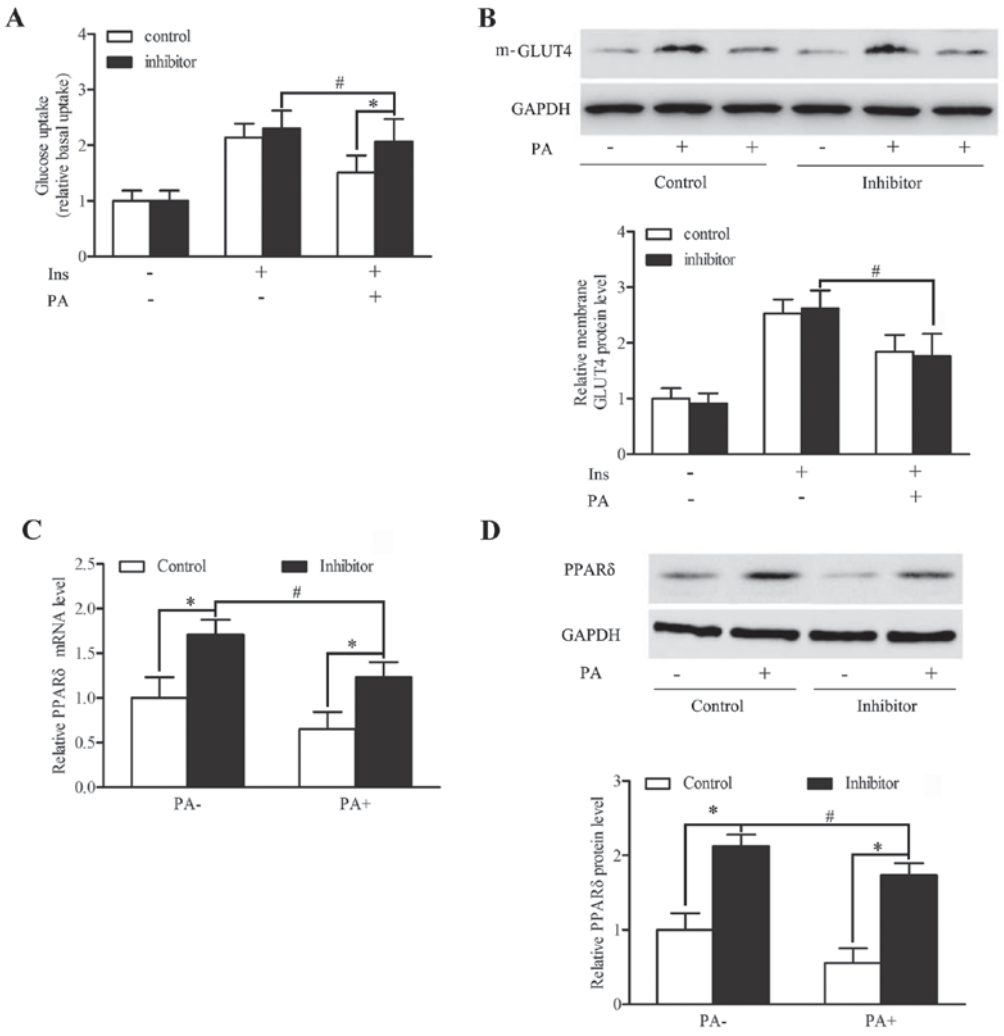

Figure 2. miR-29a silencing effects glucose uptake, the expression of plasma membrane GLUT4 and PPARδ. (A) Silencing of miR-29a expression increased insulin-induced glucose uptake. (B) PA treatment decreased the plasma membrane GLUT4 protein expression in miR-29a silencing group. (C) Silencing of miR-29a attenuated the reduction in PPARδ mRNA and (D) protein under PA treatment. Data are presented as the mean + standard deviation (n=3). *P<0.05 vs. control, #P<0.05 vs. PA-untreated inhibitor. miR-29a, microRNA-29a; Control, negative control lentivirus; Ins, insulin; PA, palmitic acid; m-GLUT4, plasma membrane glucose transporter type 4; PPARδ, peroxisome proliferator-activated receptor δ.

(Fig. 2B). In addition, PA decreased the expression of PPARδ mRNA (Fig. 2C) and protein (Fig. 2D) in C2C12 myotubes. This effect was significantly attenuated by miR-29a silencing. These results suggested that the overexpression of miR-29a, which preceded a decrease in insulin sensitivity, contributed to IR under PA treatment. Additionally, the inhibition of endogenous miR-29a expression increased insulin sensitivity and the expression of PPARδ.

Overexpression of miR-29a alters the expression of genes associated with lipid metabolism. As PPARδ is involved in lipid metabolism, the effect of miR-29a overexpression on lipid metabolism dysfunction was analyzed. The results revealed that the expression of PDK4 and FATP2 significantly decreased, while the expression of UCP3 significantly increased with the overexpression of miR-29a. No significant differences in the expression of UCP2 and LCAD were observed among the three groups (Fig. 3A). Furthermore, as determined by western blot analysis (Fig. 3B), the overexpression of miR-29a upregulated the protein expression of UCP3 and downregulated the protein expression of PDK4, FATP2 and LCAD proteins. No marked differences were observed in the expression of UCP2 protein among the three groups (Fig. 3B and C). Taken together, these data suggest that miR-29a overexpression induced IR in C2C12 myotubes by impacting on lipid metabolism.

miR-29a silencing alters the expression of genes associated with lipid metabolism. Silencing of miR-29a expression reduced the mRNA expression of UCP3 and UCP2. Additionally, decreased miR-29a expression increased the expression of PDK4 and LCAD, with no significant change in the expression of FATP2 (Fig. 4A). Furthermore, western blot analysis (Fig. 4B) revealed that the inhibition of miR-29a increased the protein expression of PDK4 and LCAD, decreased the expression of UCP3 and UCP2 proteins and had no effect on the expression of FATP2 (Fig. 4C). These data suggest that the silencing of miR-29a may have improved skeletal muscle insulin sensitivity, partially through lipid metabolism dysfunction recovery. The aforementioned results indicate that miR-29a altered the expression of genes associated with lipid

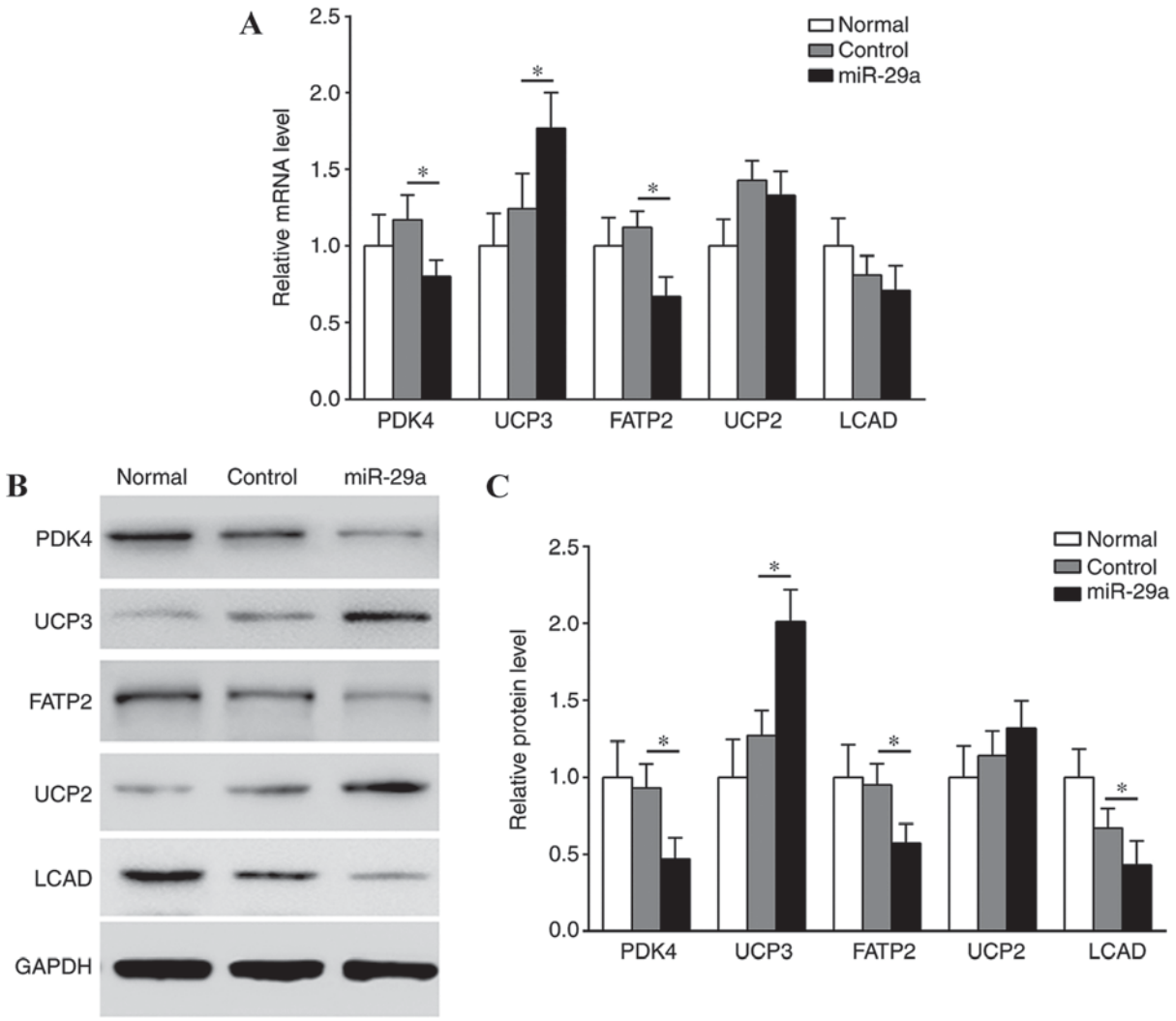

Figure 3. Overexpression of miR-29a alters the expression of genes associated with lipid metabolism in C2C12 cells. (A) Reverse transcription-quantitative polymerase chain reaction revealed the effect of miR-29a overexpression on PDK4, UCP3, FATP2, UCP2 and LCAD mRNA expression (B) Western blot analysis of lipid metabolism-associated genes. (C) The relative protein expression of PDK4, UCP3, FATP2, UCP2 and LCAD in cells overexpressing miR-29a. Data are presented as the mean + standard deviation (n=3). *P<0.05 vs. control. Normal, untransfected; Control, empty vector; miR-29a, microRNA-29a; PDK4, pyruvate dehydrogenase kinase isoform 4; UCP, mitochondrial uncoupling protein; FATP2, fatty acid transport protein 2; LCAD, long-chain specific acyl-CoA dehydrogenase, mitochondrial.

metabolism in skeletal muscle cells and silencing of miR-29a reduced IR induced by PA, and thus, may be involved in IR of skeletal muscle.

Discussion

miR-29a is one of the most abundant members of the miR-29 family that is expressed in the skeletal muscle, pancreas and liver of mice and humans. It has been identified as a potential regulator of glucose handling (23). In addition, it has been reported that miR-29a is upregulated by glucose and decreases glucose-stimulated insulin secretion in pancreatic β cells (24). The upregulation of miR-29a contributes to β cell dysfunction in pre-diabetic non-obese diabetic mice and may be characterized as a pathogenic event in type 1 diabetes (25). Our previous study demonstrated that miR-29a expression is increased in skeletal muscle from older IUGR rats characterized by IR, including increased growth rate following intrauterine growth retardation, obesity, impaired glucose tolerance (14). Furthermore, a previous report demonstrated that obesity and diet-induced IR onset are prevented in a miR-29a-deficient mouse model (23). Taken together, these results suggest that dysregulation of miR-29a expression is closely associated with IR and miR-29a may provide a potential therapeutic target for the treatment of IR-associated diseases.

A previous study demonstrated that PPARδ is a target gene of miR-29a, which downregulates PPARδ expression at the post-translational level by binding to the 3'UTR of PPARδ (14). PPARδ is a nuclear receptor that participates in a variety of physiological and pathological processes, including differentiation, inflammation, lipid metabolism, extracellular matrix remodeling and angiogenesis. PPARδ is ubiquitously expressed, with particularly high abundance in muscle tissue and macrophages (26). Increasing evidence indicates that PPARδ is an important regulator of metabolism in skeletal muscle, particularly lipid and fatty acid metabolism.

PA has been demonstrated to potently induce insulin resistance. PA impairs insulin signaling in HepG2 cells via

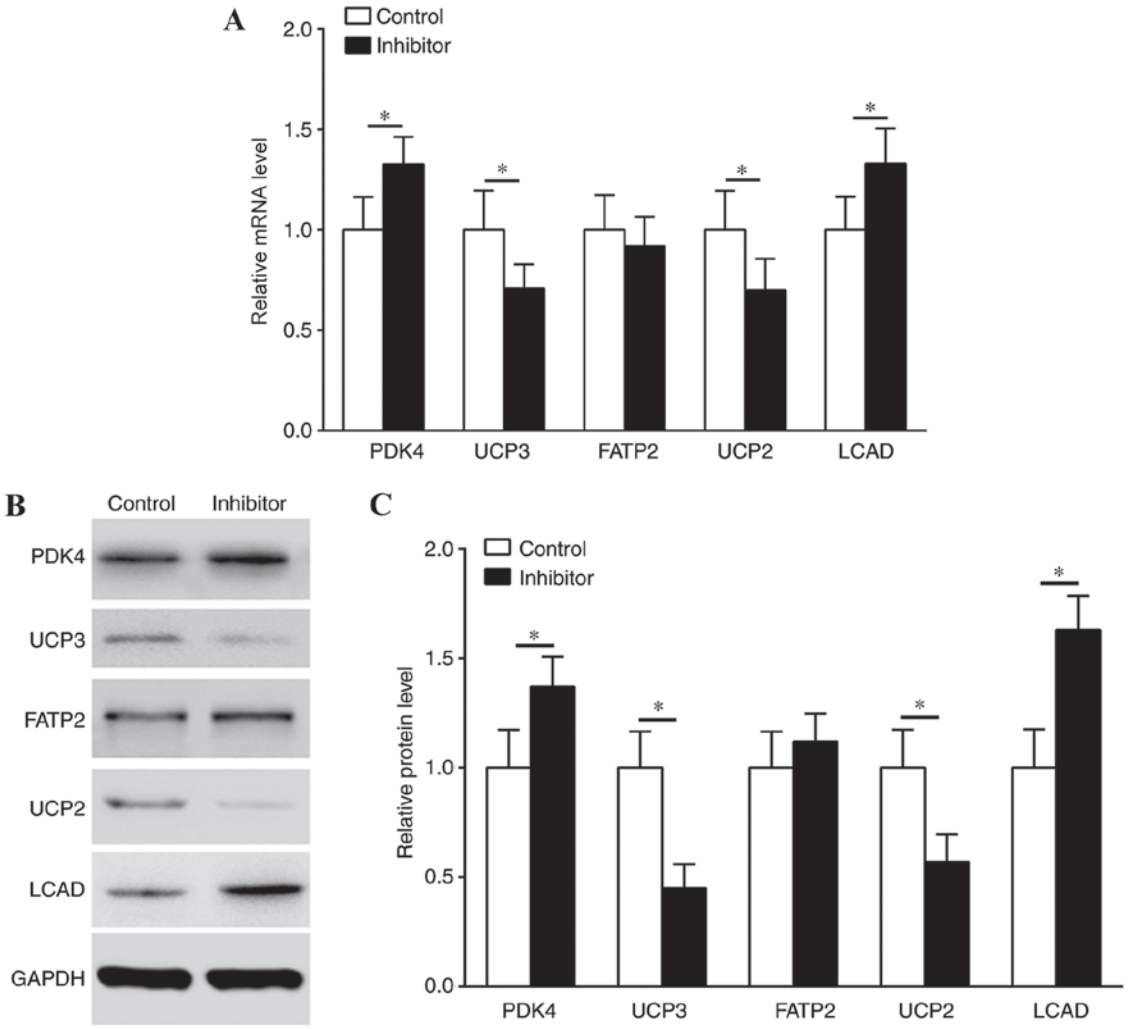

Figure 4. Silencing of miR-29a expression alters the expression of genes associated with lipid metabolism in C2C12 cells. (A) Reverse transcription-quantitative polymerase chain reaction revealed the effect of miR-29a silencing on the mRNA expression of PDK4, UCP3, FATP2, UCP2 and LCAD. (B) Western blot analysis of lipid metabolism-associated proteins. (C) The relative protein expression of PDK4, UCP3, FATP2, UCP2 and LCAD in C212 cells with silenced miR-29a expression. Data are presented as the mean + standard deviation (n=3). *P<0.05 vs. control. miR-29a, microRNA-29a; Control, negative control lentivirus; PDK4, pyruvate dehydrogenase kinase isoform 4; UCP, mitochondrial uncoupling protein; FATP2, fatty acid transport protein 2; LCAD, long-chain specific acyl-CoA dehydrogenase, mitochondrial.

a reduction in the expression of insulin receptors and insulin receptor substrate 1 (IRS-1) protein (27). Additionally, PA activates the ubiquitin-proteasome system and autophagy in human umbilical vein endothelial cells (28). Furthermore, it induces endoplasmic reticulum stress and apoptosis in insulinoma cells (29), and decreases the rate of fatty acid oxidation and tricarboxylic acid cycle in skeletal cells (30). In the present study, PA significantly upregulated the expression of miR-29a and induced IR in C2C12 myotubes, which was in line with the findings of a previous study (31). The expression of PPARδ under PA treatment was decreased at both the mRNA and protein level. These results indicated that miR-29a participated in PA-induced IR by targeting PPARδ. Our previous study had demonstrated that the overexpression or silencing of miR-29a had no significant effect on myotube morphology regulation in C2C12 cells (14).

The present study further demonstrated that in C2C12 cells, the silencing of miR-29a significantly increased insulin-stimulated glucose uptake under PA treatment and PA treatment decreased the plasma membrane GLUT4 protein expression in miR-29a silencing group. By contrast, a previous study reported that PPARδ activation promotes glucose transport in primary human skeletal myotubes, lipid cells and C2C12 cells through activating p38 mitogen-activated protein kinase and AMP-activated protein kinase (AMPK) signaling pathways, but without altering GLUT1 and GLUT4 levels (32). Kramer *et al* (33) demonstrated that the exposure of human skeletal muscle and C2C12 cells to a PPARδ agonist increases glucose uptake in a PPARδ-dependent manner. Thus, the reduced glucose uptake observed in the present study may not only be due to decreased plasma membrane GLUT4 levels, but also via the direct effects of decreased PPARδ expression.

Lipid accumulation in skeletal muscle and liver cells is considered a key factor in systemic IR development (3,34). The accumulation of triglycerides, cholesterol and free fatty acid (FFA) in diabetic animals have been previously described (35). At present, ~50 target genes of the miR-29 family have been validated experimentally from thousands of predicted target genes (36). miR-29a may also target several insulin, glucose and lipid metabolism-associated genes, including IRS-1 (31)

and lipoprotein lipase (37). Additionally, miR-29a has been reported to have an inhibitory effect on the insulin signaling pathway and lipid metabolism by targeting PPARδ in C2C12 cells (14), which is consistent with the results of the present study.

Incubation with PPARδ agonists has been demonstrated to induce AMPK phosphorylation and increase fatty acid transport, oxidation, utilization and glucose uptake in both primary cultured human muscle cells and adipocytes (33,38). PDK4 is a major isoenzyme responsible for alterations in pyruvate dehydrogenase complex activity in response to different metabolic conditions (39). It was demonstrated that PPARδ agonists specifically upregulate the expression of PDK4 mRNA in cultured human muscle cells (39). PDK4 has been reported as a key target gene of glucose and lipid metabolism in response to PPARδ activity (39). In the present study, miR-29a overexpression increased the expression of PDK4 mRNA and protein, whereas miR-29a inhibition had the opposite effect. It was therefore speculated that miR-29a may have used PPARδ as the bridge to regulate the PDK4 expression and subsequently impact lipid metabolism.

Notably, the present study demonstrated an increased expression of UCP3 in response to miR-29a overexpression, with the opposite trend observed when miR-29a expression was silenced. UCP3 is a mitochondrial inner membrane transporter that facilitates fatty acid oxidation; it increases oxidative capacity and likely contributes to decreased lipid storage in the skeletal muscle (40). Thus, alterations in UCP3 expression are a major indicator for alterations in lipid utilization as a substrate for oxidative phosphorylation in the skeletal muscle.

In the present study, a more complex picture emerged when comparing the pattern of mRNA and protein expression between C2C12 cells overexpressing miR-29a exposed to PA or with silenced miR-29a expression. For example, although the protein abundance of LCAD, an important enzyme in the dehydrogenation reaction of long-chain coenzyme A (41), decreased in the overexpression group and increased in the inhibitor group, no alteration in LCAD mRNA was detected in C2C12 cells overexpressing miR-29a, despite an upregulation in the miR-29a inhibitor group. Additionally, although a decrease in the expression of FATP2 mRNA and protein was detected in C2C12 cells with overexpressing miR-29a, the opposite change was not observed in the miR-29a silenced cells. Furthermore, decreased expression of UCP2, a key enzyme in fatty acid oxidation regulation, was not detected in C2C12 cells overexpressing miR-29a.

At present, the mechanisms through which miR-29a controls the expression of metabolic genes remain unclear. However, the variability in the findings of the present study suggests that skeletal muscle-specific functional compensation and different critical thresholds for miR-29a may exist. However, it was not clarified if the effects of miR-29a on skeletal cells also occur in other tissues, which is a limitation of the current study.

Taken together, the data revealed that the inactivation of miR-29a in C2C12 cells provided significant protection from the detrimental metabolic consequences of PA exposure on lipid deposition, accompanied by increased glucose transport, improved insulin sensitivity and lipid dysfunction, with PPARδ participating in these processes. The data were highly reciprocal to the metabolic phenotype of miR-29a-overexpressing cells (14), which further reinforced the validity of the findings. Furthermore, overexpression of miR-29a has been observed in skeletal muscle from patients with T2D compared with healthy subjects with normal glucose tolerance (42). Therefore, the present study provides evidence for the investigation of miR-29a antagonists as a potential therapeutic strategy for treating T2D and the associated metabolic complications.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

YC and YZ conceived and designed the study. PW, CJ, CC and YL performed the experiments. QW analyzed the data and drafted the manuscript. YC and YZ reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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