

miR-338-3p mediates gluconeogenesis via targeting of PP4R1 in hepatocytes

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Abstract. Hyperglycaemia is a characteristic of type 2 diabetes. In hepatocytes, impaired insulin sensitivity leads to increased gluconeogenesis and decreased glycogenesis. MicroRNA (miR)-338-3p is associated with tumour necrosis factor (TNF)- α -induced suppression of hepatic glycogenesis via regulation of protein phosphatase 4 regulatory subunit 1 (PP4R1). However, the effect of miR-338-3p on gluconeogenesis in hepatocytes remains unknown. In a previous study, it was demonstrated that miR-338-3p is downregulated in the livers of mice and in mouse HEPA1-6 hepatocytes following treatment with TNF- α . In the present study, the effect of miR-338-3p on TNF- α -induced gluconeogenesis in hepatocytes was investigated. The levels of phosphorylated-FOXO1/FOXO1, phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator-activated receptor γ coactivator (PGC-1 α) and glucose-6-phosphatase (G6Pase) were measured by western blotting. The mRNA levels of PEPCK, PGC-1 α and G6Pase were determined by quantitative polymerase chain reaction. Pyruvate tolerance testing was used to determine the gluconeogenesis of mouse livers. The results demonstrated that treatment with TNF- α resulted in increased levels of gluconeogenesis in the livers of mice and decreased miR-338-3p expression levels in HEPA1-6 cells. Overexpression of miR-338-3p reversed TNF- α -induced glucose production via enhancement of

phosphorylated forkhead box O1 levels and downregulation of the expression levels of genes associated with gluconeogenesis, including peroxisome proliferator-activated receptor γ coactivator-1 α , phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. However, inhibition of miR-338-3p expression was revealed to enhance gluconeogenesis in the livers of mice and in HEPA1-6 cells. Furthermore, downregulation of PP4R1 was revealed to attenuate the effect on glucose production following treatment with miR-338-3p inhibitors. In conclusion, the results of the present study revealed that miR-338-3p may be involved in TNF- α -mediated gluconeogenesis via targeting of PP4R1 in hepatocytes.

Introduction

The incidence of obesity and aging has been associated with Type 2 diabetes, a disease of increasing importance in social health due to its prevalence. (1) Type 2 diabetes affects the homeostasis of glucose metabolism and leads to serious complications, including diabetic cardiomyopathy and diabetic nephropathy (2). At present, a cure for type 2 diabetes is unavailable as the underlying mechanisms of the pathogenesis of type 2 diabetes is not completely understood (3). Hyperglycaemia is characteristic of type 2 diabetes (4). Hepatic insulin resistance is a predominant cause of type 2 diabetes. In the liver, glucose metabolism homeostasis is regulated via two processes, gluconeogenesis and glycogenesis (5). Impaired insulin sensitivity in the liver leads to increased gluconeogenesis and decreased glycogenesis (6).

In the liver cells, insulin can decrease glucose production and increase uptake of blood glucose into glycogen via the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) pathway (7). Additionally, activated AKT promotes the phosphorylation of forkhead box O1 (FOXO1), which can translocate into nuclear and inhibit the expression of gluconeogenic enzymes and gluconeogenesis (8). Peroxisome proliferator-activated receptor γ coactivator (PGC-1 α) is another important regulator of gluconeogenesis in liver cells. PGC-1 α can co-activate FOXO1 and enhance the transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). PGC-1 α ,

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PEPCK and G6Pase are key regulators of gluconeogenesis in liver cells (9).

MicroRNAs (miRs) are a group of small, non-coding RNAs. miRs negatively modulate gene expression by directly binding to a partially complementary sequence in the 3'-untranslated region (UTR) of their target mRNA (10,11). Tumour necrosis factor (TNF)- α is an important mediator of insulin resistance that functions by inhibiting the PI3K/AKT pathway. Furthermore, *in vitro* and *in vivo* studies have suggested that TNF- α treatment leads to increased gluconeogenesis and decreased glycogenesis in hepatocytes (12). Our previous study demonstrated that miR-338-3p overexpression restores impaired glycogenesis induced by TNF- α by directly inhibiting protein phosphatase 4 regulatory subunit 1 (PP4R1) expression (13). However, the association between miR-338-3p and increased levels of gluconeogenesis induced by TNF- α remains unknown. In the present study, the effects of miR-338-3p on gluconeogenesis and the associated underlying mechanisms were investigated. The results suggested that miR-338-3p may have an important role in TNF- α -induced gluconeogenesis via targeting of PP4R1.

Materials and methods

Animals. In order to establish a TNF- α -induced insulin resistance model, 16-week-old male C57BL/6J mice (n=5; 30 g, Beijing HFK Bioscience Co., Ltd., Beijing, China) were fed with a standard laboratory diet and injected with 15 μ g/ml TNF- α via Alzet osmotic pumps (DURECT Corporation, Cupertino, CA, USA) for 2 weeks continuously at an infusion rate of 0.5 μ l/h. For the control group, 16-week-old male C57BL/6J mice (n=5) were injected with 0.9% NaCl via Alzet osmotic pumps for 2 weeks at the aforementioned infusion rate. Following fasting for 8 h, mice were subsequently anaesthetized via intraperitoneal injection with sodium pentobarbital (40 mg/kg body weight), and liver samples were then collected. All mice were housed in an experimental animal facility of Beijing Normal University (Beijing, China) at a temperature of 20-24°C and humidity-controlled (45-55%) environment. A 12 h light/dark cycle was maintained in the animal housing rooms. The food and water were supplied for all days. All animal experiments performed in the present study were approved by the Animal Use and Care Committee of Beijing Normal University and were performed in accordance with the Guide for Care and Use of Laboratory Animals (14).

Adenoviral vector injection. For animal experiments, 6 mice were used in each group. To investigate the effect of miR-338-3p on TNF- α -induced gluconeogenesis, after 7 days following the implantation of the TNF- α pumps, 6 C57BL/6J mice were injected with adenoviral vectors expressing miR-338-3p mimics (Ad-338m) (2×10^9 plaque-forming unit/25 g body weight). A total of 6 C57BL/6J mice were injected with adenoviral vectors expressing negative control microRNA (Ad-NC) (2×10^9 plaque-forming unit/25 g body weight). In addition, adenoviral vectors expressing miR-338-3p inhibitors (Ad-338i) or negative control inhibitors (Ad-NCI) were injected into 6 C57BL/6J mice (2×10^9 plaque-forming unit/25 g body weight). All the adenoviral vectors, including Ad-338m, Ad-NC, Ad-338i and Ad-NCI were purchased from

Shanghai GeneChem Co., Ltd. (Shanghai, China). After 7 days following injection, mice were used for following experiments. The sequences employed in the present study were as follows (5'-3'): miR-338-3p mimic, UCCAGCAUCAGUGAUUUUGU and negative control mimics, UUCUCCGAACGUGUCACG UTT; miR-338-3p inhibitor, CAACAAAACACUGAUGC UGGA and negative control inhibitors, CAGUACUUUUGU GUAGUACAA.

Pyruvate tolerance testing. After 7 days following injection, pyruvate tolerance analysis was performed. Following fasting for 14 h, mice were intraperitoneally injected with pyruvate (2 g/kg of body weight) in order to perform pyruvate tolerance testing. Blood glucose levels were measured using an ACCU-CHEK Advantage glucometer (Roche Diagnostics GmbH, Mannheim, Germany) at specific time intervals.

Cell culture. HEPA1-6 murine liver cells (American Type Culture Collection, Manassas, VA, USA) were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 units/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) and 0.1 mg/ml streptomycin (Hyclone; GE Healthcare Life Sciences) at 37°C with humidified air and 5% CO₂. In order to determine the effect of TNF- α on gluconeogenesis, the HEPA1-6 cells were treated with 10 ng/ml TNF- α for 24 h at 37°C with humidified air and 5% CO₂.

Transfection. miR-338-3p mimic and inhibitor sequences used for transfection were as follows (5'-3'): miR-338-3p mimic sense, UCCAGCAUCAGUGAUUUUGUUG and antisense, ACAAAAACACUGAUGCUGGAUU; NC sense, UUC UCCGAACGUGUCACGUTT and antisense, ACGUGACAC GUUCGGAGAATT; miR-338-3p inhibitor, CAACAAAACACUGAUGCUGGA; NCI, CAGUACUUUUGUGUAGUACAA. All miR oligos were purchased from Genepharma, Inc. (Sunnyvale, CA, USA). A day before transfection, 3.6×10^5 cells were seeded in each well of 6-well plate in 1.6 ml DMEM medium. miR-338-3p mimic and inhibitor (3.75 μ l, 20 μ M) were transfected into HEPA1-6 by using HiPerFect transfection reagent (Qiagen, Inc., Valencia, CA, USA). Subsequent experimentation was performed 48 h following transfection.

The sequences of PP4R1-specific small interfering (siRNAs) used for transfection were as follows (5'-3'): PP4R1 sense, GAACCAACTGTGAGAGCGGA; and antisense, TGG GGCAGTCAGGTCTATGA. All siRNA oligos were purchased from Genepharma, Inc. A day before transfection, 3.6×10^5 cells were planted in each well of 6-well plate in 1.6 ml DMEM. PP4R1-specific siRNAs (3.75 μ l, 20 μ M) were transfected into HEPA1-6 cells using HiPerFect transfection reagent (Qiagen, Inc.). Subsequent assays were performed 48 h following transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of HEPA1-6 cells and mouse liver cells was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1 μ g RNA was reverse transcribed into cDNA using the EasyScript First-strand cDNA Synthesis

kit (Beijing Transgen Biotech Co., Ltd., Beijing, China). The procedures of RT were described as follow: 70°C for 10 min, 42°C for 60 min, 95°C for 5 min and 4°C hold. Expression levels of PGC-1 α , PEPCK and G6Pase were determined via qPCR using the SYBR Green II kit (Takara Biotechnology Co., Ltd., Dalian, China) in accordance with the manufacturer's protocol. cDNA (1 μ g), 10 μ l SYBR-Green mixture, 0.8 μ l primers (10 μ M) and 8.2 μ l ddH₂O were mixed. The procedures of qPCR were as follows: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 20 sec. The sequences of primers used for PCR were as follows (5'-3'): G6Pase forward, TCTGTC CCGGATCTACCTTG and reverse, GTAGAATCCAAGCGC GAAA; PEPCK forward, TCTGAGATCTCTGATCCAGAC C and reverse, GAAGTCCAGACCGTTATGCAGC; PGC-1 α forward, GCCTATGAGCACGAAAGGC and reverse, TCA CACGGCGCTCTTCAATT; 18s forward, GGAAGGGCA CCACCAGGAGT and reverse, TGCAGCCCCGGACATCTA AG. The gene expression level was calculated using 2^{- $\Delta\Delta$ C_q} method (15).

Western blotting. Total protein from liver and cells was extracted with radioimmunoprecipitation (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). And the concentration of protein sample was measure by BCA kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cell protein samples (15 μ g) were separated via 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) and then blocked using 8% non-fat dry milk for 2 h at room temperature. Following this, the membranes incubated with 1:1,000 diluted primary antibodies for 12 h at 4°C. The blots were then incubated with 1:5,000 diluted horseradish peroxidase-conjugated anti-IgG (cat. no. 7074; Cell Signaling Technology, Inc. Danvers, MA, USA) for 2 h at room temperature. Proteins were then visualized using an enhanced chemiluminescent kit (EMD Millipore). Antibodies against FOXO1 (cat. no. 2880), phosphorylated FOXO1 (cat. no. 9461), PEPCK (cat. no. 6924) and GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology, Inc. Antibodies against PGC-1 α (ab54481) and G6Pase (ab83690) were purchased from Abcam (Cambridge, UK).

Luciferase reporter assay. TargetScan (<http://www.targetscan.org/>), Pictar (<http://www.pictar.org/>) and miRanda (<http://microrna.org/>) databases were used to predict that PP4R1 was a target of miR-338-3p. The miR-338-3p binding site with in the PP4R1 3'-UTR was verified and in previously study (13). The PP4R1 3'-UTR fragment, including the miR-338-3p binding site was cloned from mouse liver cells and inserted into pmirGLO vector (Promega Corporation, Madison, WI, USA). A day prior to transfection, HEPAl-6 cells were seeded at 3.6x10⁵ cells per well of 6-well plate in 1.6 ml DMEM. pmirGLO-3'UTR PP4R1 with miR-338-3p mimic or inhibitor (3.75 μ l, 20 μ M) were transfected into HEPAl-6 by using HiPerFect transfection reagent. The subsequent assays were performed after 48 h post-transfection. Luciferase activity was analyzed by using the Dual-Glo Luciferase assay system (Promega Corporation). Luciferase activity was normalized to *Renilla* luciferase activity. All the assays were repeat for 4 times.

Glucose production assay. After 24 h of TNF- α treatment, a glucose production assay was performed. Cells were washed five times with PBS and then stimulated with 10 ng/ml TNF- α , 2 mmol/l sodium pyruvate and 20 mmol/l sodium lactate in glucose- and serum-free DMEM medium for 18 h. The glucose concentration in the medium was subsequently determined using a glucose assay kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and subsequently normalized to the previously determined total protein content via a BCA kit (Sigma-Aldrich; Merck KGaA) in whole cell extracts.

Statistical analysis. All data are presented as the mean \pm standard error of the mean. The two-tailed unpaired Student's t-test was used for comparisons between two groups. In addition, One-way analysis of variance followed by Tukey's post hoc test was used for comparisons between more than two groups. Statistical analyses were performed using SPSS 3.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference. All the experiments were repeated for four times.

Results

TNF- α treatment induces gluconeogenesis in mouse livers and HEPAl-6 cells. In our previous study, it was demonstrated that treatment with TNF- α decreased miR-338-3p expression levels and suppressed the activation of the AKT/GSK pathway in the livers of mice (13). In the present study, treatment with TNF- α significantly decreased the level of p-FOXO1/FOXO1 and significantly increased the protein and mRNA expression levels of genes associated with gluconeogenesis in the livers of mice, including PGC-1 α , G6Pase and PEPCK (Fig. 1A and B). Consistently, treatment of 10 ng/ml TNF- α enhanced glucose production in HEPAl-6 cells (Fig. 1C). Furthermore, in HEPAl-6 cells following treatment with 10 ng/ml TNF- α for 24 h, the levels of p-FOXO1/FOXO1 were reduced, and the protein and mRNA expression of PGC-1 α , G6Pase and PEPCK were increased (Fig. 1D and E).

Overexpression of miR-338-3p attenuates TNF- α -induced gluconeogenesis. To determine the importance of miR-338-3p in the regulation of gluconeogenesis, an adenovirus expressing miR-338-3p mimic was injected into TNF- α -treated mice via the tail vein for 7 days. The results revealed that levels of p-FOXO1/FOXO1 were significantly increased, whereas mRNA and protein expression levels of PGC-1 α , G6Pase and PEPCK were significantly decreased, in the livers of mice injected with Ad-338m compared with the control group accompanied by ameliorated pyruvate tolerance (Fig. 2A-C). In HEPAl-6 cells, transfection with miR-338-3p mimics was demonstrated to significantly attenuate TNF- α -induced glucose production (Fig. 2D). In addition, upregulation of miR-338-3p was revealed to significantly enhanced levels of p-FOXO1/FOXO1, and significantly decreased the expression levels of PGC-1 α , G6Pase and PEPCK, compared with the NC group (Fig. 2E and F). In conclusion, these results suggested that miR-338-3p may have an important role in TNF- α -induced gluconeogenesis.

Treatment with miR-338-3p inhibitors increases glucose production in hepatocytes. The effect of treatment with

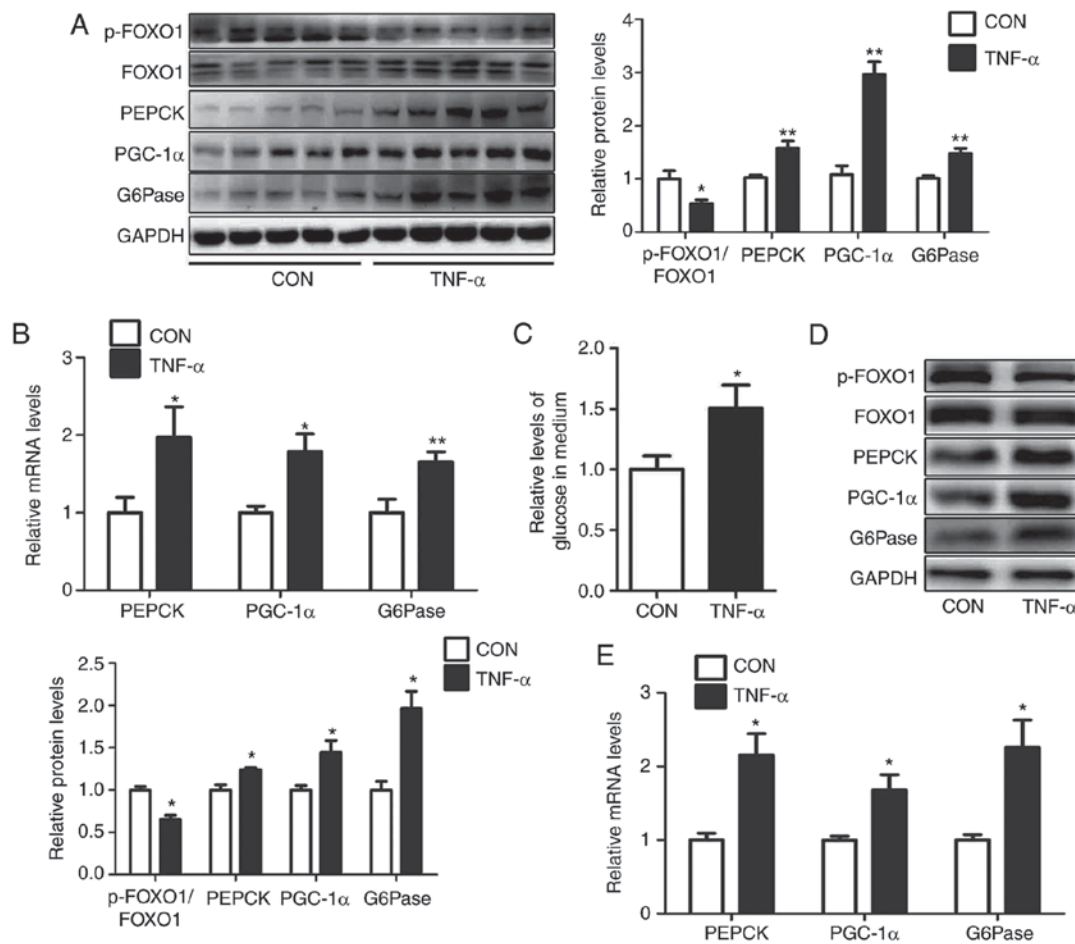


Figure 1. TNF- α treatment induces gluconeogenesis in mouse livers and HEPA1-6 hepatocytes. Levels of p-FOXO1/FOXO1, PEPCK, PGC-1 α and G6Pase in the livers of TNF- α -treated mice were analyzed via (A) western blot and (B) reverse transcription-quantitative polymerase chain reaction. (C) Relative levels of glucose in culture medium were determined, as well as (D) protein and (E) mRNA levels of p-FOXO1/FOXO1, PEPCK, PGC-1 α and G6Pase, in HEPA1-6 cells treated with TNF- α for 24 h. Data are presented as the mean \pm standard error. For animal experiments, 5 mice were used for each group. For cell experiments, $n=4$ independent experiments. * $P<0.05$ and ** $P<0.01$ vs. control. p-, phosphorylated; FOXO1, forkhead box O1; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; TNF- α , tumor necrosis factor- α ; CON, control.

miR-338-3p inhibitors on glucose production in hepatocytes was investigated in the present study. An adenovirus expressing miR-338-3p inhibitor (Ad-338i) was injected into mice via the tail vein over 7 days. Inhibition of miR-338-3p decreased the levels of p-FOXO1/FOXO1 in livers of mice; the protein and mRNA expression levels of PGC-1 α , G6Pase and PEPCK were increased in the liver, and pyruvate tolerance was impaired in mice injected with Ad-338i (Fig. 3A-C). Furthermore, HEPA1-6 hepatocytes transfected with a miR-338-3p inhibitor exhibited significantly reduced levels of p-FOXO1, increased levels of glucose production, and increased mRNA and protein expression of PGC-1 α , G6Pase and PEPCK, compared with the NC group (Fig. 3D-F). These results suggested that miR-338-3p may regulate glucose production via the AKT/FOXO1 pathway.

miR-338-3p is involved in TNF- α -mediated gluconeogenesis via targeting of PP4R1. In our previous study, PP4R1 was revealed to represent a target gene of miR-338-3p. The 3'-UTR of PP4R1 was cloned and luciferase reporter vectors were subsequently constructed (13). The relative luciferase activity was significantly decreased following transfection with miR-338-3p mimics (Fig. 4A); however, relative luciferase activity was not

markedly affected following transfection with miR-338-3p inhibitors (Fig. 4B). To verify whether miR-338-3p regulates glucose production via targeting of PP4R1, miR-338-3p inhibitors and PP4R1 siRNA were co-transfected into HEPA1-6 cells. The results revealed that downregulation of PP4R1 significantly attenuated miR-338-3p-induced increased levels of glucose production (Fig. 4C), and expression levels of genes associated with gluconeogenesis. However, transfection of PP4R1 siRNA reversed the effect of miR-338-3p inhibitor on the level of p-FOXO1/FOXO1 (Fig. 4D and E).

Furthermore, in order to determine whether PP4R1 is involved in the regulation of glucose production by TNF- α , PP4R1 siRNA was transfected into HEPA1-6 cells for 48 h, which was followed by treatment with TNF- α for 24 h. The results demonstrated that downregulation of PP4R1 significantly attenuated increased levels of glucose production following treatment with TNF- α (Fig. 4F), as well as PGC-1 α , G6Pase and PEPCK mRNA and protein expression levels. However, transfection of PP4R1 siRNA reversed the effect of TNF- α on the level of p-FOXO1/FOXO1 (Fig. 4G and H). In conclusion, these results suggested that miR-338-3p is involved in TNF- α -mediated glucose production via targeting of PP4R1.

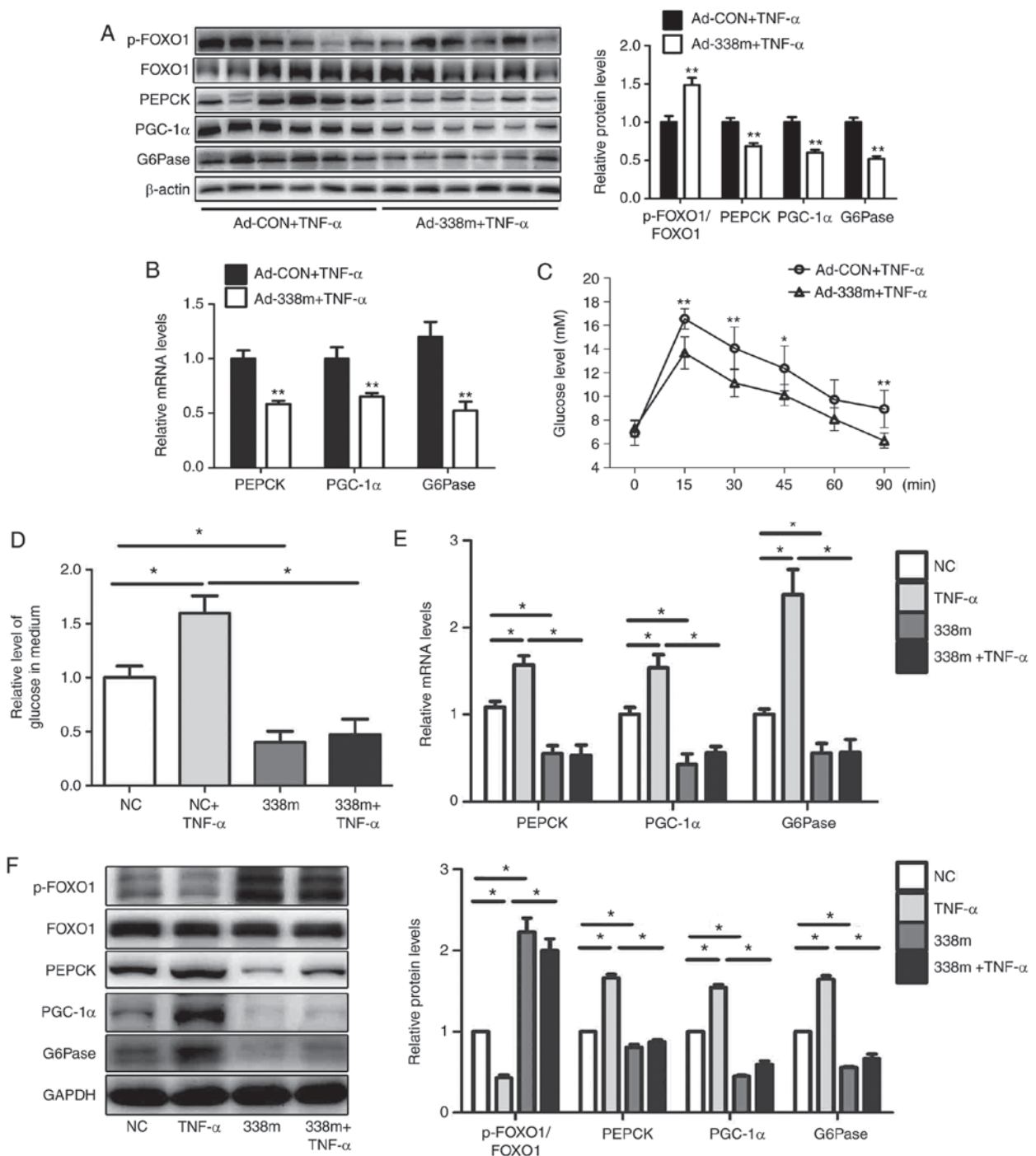


Figure 2. Overexpression of miR-338-3p attenuates TNF- α -induced gluconeogenesis. Levels of p-FOXO1/FOXO1, PEPCK, PGC-1 α and G6Pase in the livers of TNF- α -treated mice injected with Ad-338m via the tail vein for 7 days were analyzed by (A) western blot and (B) reverse transcription-quantitative polymerase chain reaction. (C) Pyruvate tolerance testing using mice was also performed. In cells, (D) relative levels of glucose in medium were determined, as well as (E) relative mRNA levels of PEPCK, PGC-1 α and G6Pase, and (F) protein levels of p-FOXO1/FOXO1, PEPCK, PGC-1 α and G6Pase, in TNF- α -treated HEPA1-6 cells transfected with miR-338-3p mimics. Data are presented as the mean \pm standard error of the mean. For animal experiments, 6 mice were used. For cell experiments, n=4 independent experiments. *P<0.05 and **P<0.01 vs. control or as indicated. miR, microRNA; p-, phosphorylated; FOXO1, forkhead box O1; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; G6Pase, glucose-6-phosphatase; Ad, adenovirus; CON, control; TNF- α , tumor necrosis factor- α ; 338m, miR-338-3p mimic; NC, mimic negative control.

Discussion

Chronic inflammation is a major factor resulting in the development of pathogenesis associated with insulin resistance (16). In addition, TNF- α promotes inflammation and attenuates insulin resistance in hepatocytes. Furthermore, *in vivo* and *in vitro* studies have suggested that treatment with

TNF- α blocks the insulin signalling pathway and results in insulin resistance (13).

In our previous study, it was demonstrated that treatment with TNF- α decreased glycogen synthesis and decreased the level of miR-338-3p in hepatocytes (13). It has been previously suggested that miR-338-3p has an important role in TNF- α -mediated glycogenesis via regulation of PP4R1 (13).

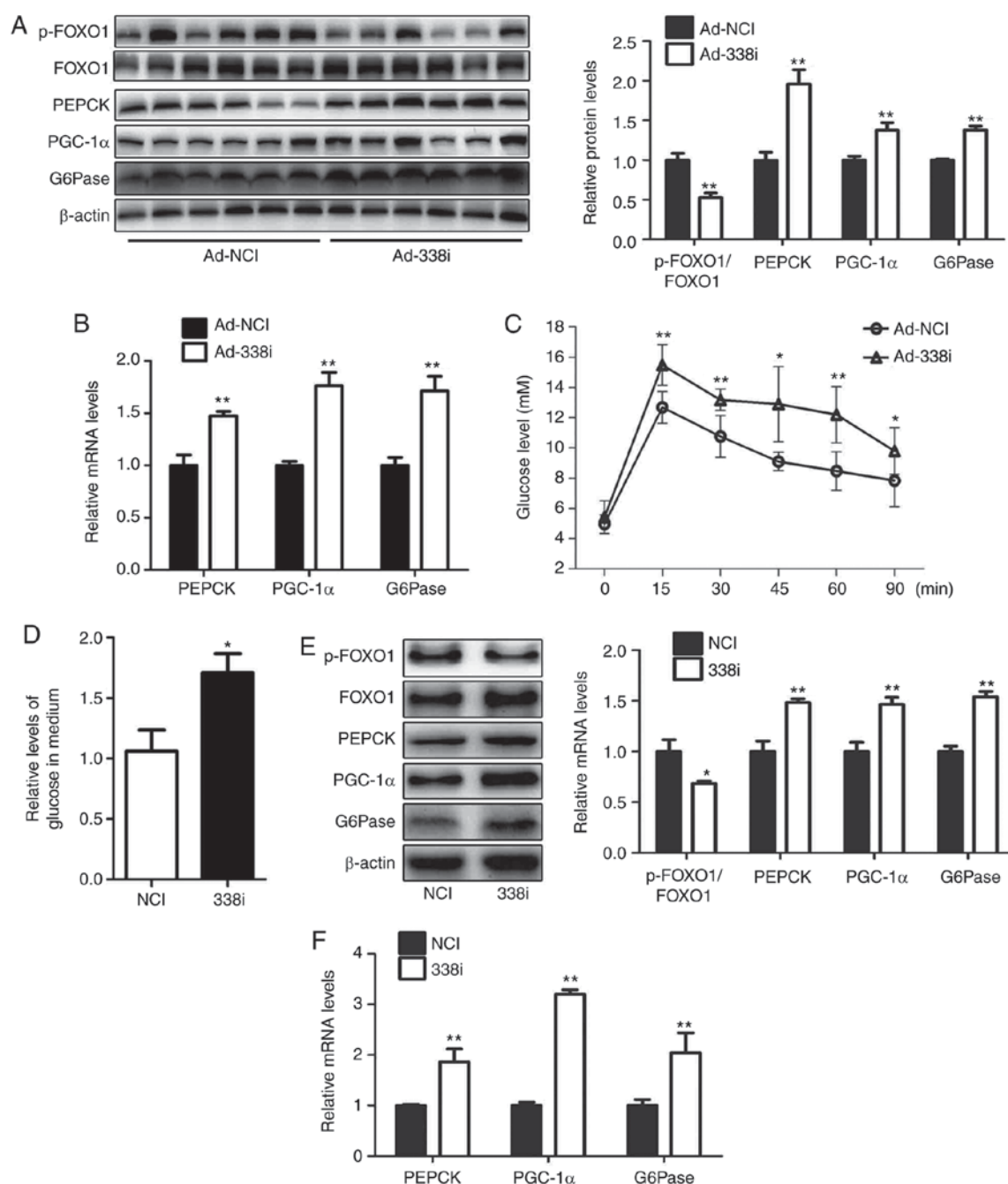


Figure 3. Downregulation of miR-338-3p enhances gluconeogenesis in hepatocytes. Levels of p-FOXO1/FOXO1, PEPCK, PGC-1 α and G6Pase in the livers of mice injected with Ad-338i via the tail vein for 7 days were analyzed by (A) western blot and (B) reverse transcription-quantitative polymerase chain reaction. (C) Pyruvate tolerance testing using mice was performed. (D) Relative levels of glucose in culture medium were also determined as well as (E) levels of p-FOXO1, FOXO1, PEPCK, PGC-1 α and G6Pase protein, and (F) PEPCK, PGC-1 α and G6Pase mRNA in HEPA1-6 cells transfected with miR-338-3p inhibitors were determined. Data are presented as the mean \pm standard error. For animal experiments, 6 mice were used. For cell experiments, n=4 independent experiments. *P<0.05 and **P<0.01 vs. control. miR, microRNA; p-, phosphorylated; FOXO1, forkhead box O1; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; G6Pase, glucose-6-phosphatase; Ad, adenovirus; NCI, negative control inhibitor; 338i, miR-338-3p inhibitor.

However, the effect of miR-338-3p on TNF- α -mediated gluconeogenesis remains unknown. In the present study, whether miR-338-3p modulates gluconeogenesis via regulation of PP4R1 expression was investigated. In our previous study, we validated the effect of miR-338-3p mimics, miR-338-3p inhibitor, Ad-338m, Ad-338i and PP4R1-targeting siRNA in HEPA1-6 cells (13). Additionally, hepatic miR-338-3p level was increased >10 fold at 7 days after the Ad-338m injection; hepatic miR-338-3p level was decreased to ~30% at

7 days after the Ad-338i injection. The results revealed that treatment with TNF- α significantly induced gluconeogenesis in mouse liver and HEPA1-6 cells. In addition, it was demonstrated that miR-338-3p overexpression significantly suppressed glucose production in the livers of TNF- α -treated mice and TNF- α -treated HEPA1-6 cells. Furthermore, the results revealed that downregulation of miR-338-3p induced gluconeogenesis in the livers of TNF- α -treated mice and TNF- α -treated HEPA1-6 cells. Finally, it was demonstrated

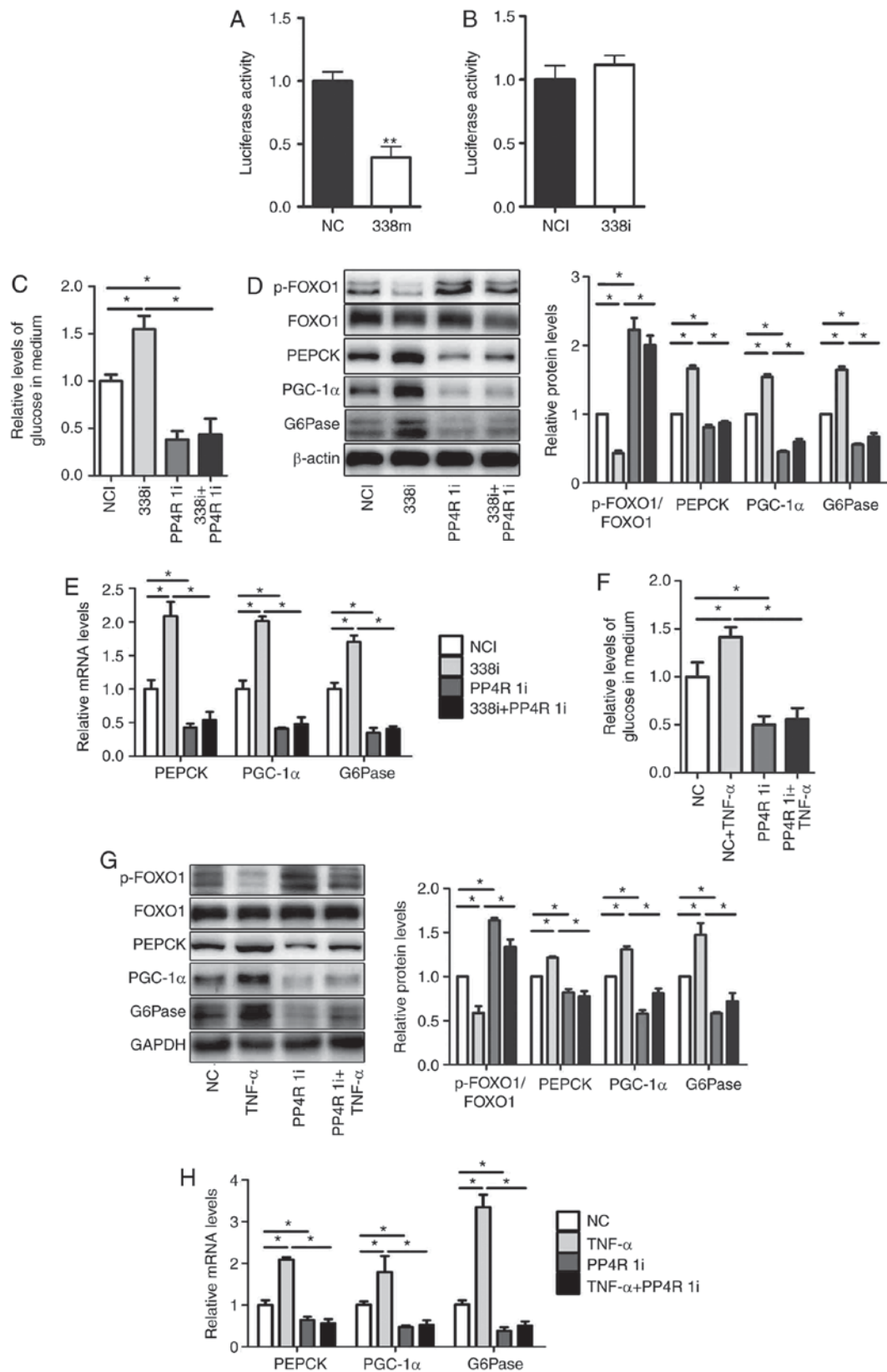


Figure 4. miR-338-3p is involved in TNF- α -mediated gluconeogenesis via targeting of PP4R1. PP4R1 is a direct target of miR-338-3p. Luciferase activity was analyzed in HEPA1-6 cells transfected with (A) miR-338 mimics (B) or inhibitors. (C) Relative levels of glucose in medium, as well as (D) levels of p-FOXO1/FOXO1, PEPCK, PGC-1 α and G6Pase protein, and (E) PEPCK, PGC-1 α and G6Pase mRNA were determined in HEPA1-6 cells co-transfected with miR-338-3p inhibitors and PP4R1 small interfering RNA. (F) Relative levels of glucose in medium, as well as (G) protein levels of p-FOXO1/FOXO1, PEPCK, PGC-1 α and G6Pase, and (H) PEPCK, PGC-1 α and G6Pase mRNA were determined in HEPA1-6 cells treated with 10 ng/ml TNF- α for 24 h followed by transfection with PP4R1 small interfering RNA for 48 h. Data are presented as the mean \pm standard error of the mean. Experiments were repeated four times. *P<0.05 and **P<0.01 vs. control or as indicated. miR, microRNA; NC, mimic negative control; 338m, miR-338-3p mimic; NCI, negative control inhibitor; 338i, miR-338-3p inhibitor; PPR41, protein phosphatase 4 regulatory subunit 1; PP4R1 i, PP4R1 siRNA; p-, phosphorylated-; FOXO1, forkhead box O1; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; G6Pase, glucose-6-phosphatase; NC, negative siRNA control; siRNA, small interfering RNA; TNF- α , tumor necrosis factor- α .

that miR-338-3p was involved in TNF- α -mediated glucose production via targeting of PP4R1.

Hepatic insulin resistance leads to increased gluconeogenesis in the liver, which may impair glucose metabolism homeostasis (17). miRs are a cluster of small, non-coding RNAs. Numerous miRs have important roles in the regulation of the activation of the insulin signalling pathway, including miR-291b-3p (18), miR-200s (19), miR-152 (20), miR-20a-5p (21), miR-19a (22) and miR-301a (23). The results of our previous study suggested that miR-338-3p regulates the AKT/GSK pathway via targeting of PP4R1, which subsequently regulates protein phosphatase 4 (PP4) expression (11). The miR-338-3p gene is located within intron 8 of the apoptosis-associated tyrosine kinase gene (24). In the present study, it was demonstrated that miR-338-3p modulates gluconeogenesis via regulation of AKT/FOXO1 pathway activation. Gluconeogenesis is an important metabolic pathway for the conversion of non-carbohydrate metabolites (lactate, glycerol and amino acids) to glucose, which provides energy for other organs during a prolonged fast; gluconeogenesis predominantly occurs in the liver and is regulated by insulin (25). Gluconeogenesis is regulated by two key enzymes in the liver: PEPCK and G6Pase. Hepatic expression of PEPCK and G6Pase is inhibited by insulin in the fed state (26). In the present study, it was revealed that hepatic overexpression of miR-338-3p induces the insulin signalling pathway, which suppresses PEPCK and G6Pase expression levels in gluconeogenesis both *in vivo* and *in vitro*.

The mechanism underlying the miR-338-3p role in TNF- α -induced gluconeogenesis was also investigated in the present study. FOXO1 co-activates PGC1 α , which subsequently induces PEPCK and G6Pase transcription. FOXO1 is the predominant modulator of insulin activity (27). Insulin suppresses FOXO1 activity via activation of the PI3K/AKT signalling pathway. Activated AKT phosphorylates FOXO1, which leads to nuclear exclusion of FOXO and subsequent suppression of FOXO-induced transcription (9). Furthermore, PGC1 α is an additional transcriptional co-activator that is activated under fasting conditions (28). Insulin suppresses PGC1 α activity via increased phosphorylation at Ser570 by activated AKT. In addition, the results of the present study revealed that miR-338-3p regulates FOXO1 phosphorylation via the PI3K/AKT pathway.

In a previous study, PP4R1 was revealed to be a direct target of miR-338-3p, which regulates AKT/glycogen synthase kinase pathway activation. PP4R1 is a regulatory subunit of PP4. PP4 has been previously reported to be an important regulator of TNF- α -induced hepatic insulin resistance (12). In the present study, it was demonstrated that PP4R1 was involved in miR-338-3p-mediated gluconeogenesis.

In conclusion, the results of the present study suggested that miR-338-3p is involved in TNF- α -induced hepatic gluconeogenesis *in vivo* and *in vitro*. miR-338-3p may be associated with TNF- α -induced gluconeogenesis via targeting of PP4R1.

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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.

Authors' contributions

SW, LL, XC and XH conducted the cell experiments, JL and XS performed the animal experiments, YZ and TS constructed the luciferase reporter vector, JG, YM and WT performed pyruvate tolerance test and glucose production assay; LD and JL made contributions to the design of the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Ethic and Animal Welfare committee College of Life Science Beijing Normal University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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