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ORIGINAL ARTICLE

Micro ribonucleic acid-363 regulates the phosphatidylinositol 3-kinase/threonine protein kinase axis by targeting NOTCH1 and forkhead box C2, leading to hepatic glucose and lipids metabolism disorder in type 2 diabetes mellitus

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Keywords
Metabolic disorder, Micro ribonucleic acid-363, Type 2 diabetes

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ABSTRACT

Aims/Introduction: Glucose metabolic disorder is the main cause for type 2 diabetes progression. Exploring the molecular mechanisms of metabolic disorder are crucial for type 2 diabetes treatment.

Materials and Methods: Micro ribonucleic acid (miR)-363, NOTCH1 and forkhead box C2 (FOXC2) expressions in high glucose (HG)-treated HepG2 cells and the livers of type 2 diabetes mellitus rats were assessed using quantitative polymerase chain reaction. Protein levels of NOTCH1, FOXC2 and phosphatidylinositol 3-kinase (PI3K)/serine/threonine protein kinase (Akt)-related proteins were evaluated using western blot. Lipid accumulation was determined using Oil Red O staining. Then glucose consumption, blood glucose level and glycogen content were detected using kits. Finally, dual luciferase reporter assay was used to verify the binding relationship between miR-363 and NOTCH1, and the binding relationship between miR-363 and FOXC2.

Results: MiR-363 was significantly upregulated in the livers of diabetic rats and HG-induced HepG2 cells, whereas NOTCH1 and FOXC2 were downregulated. In HG-induced HepG2 cells, miR-363 inhibitor markedly increased glucose consumption and uptake, and reduced accumulation of lipid droplets. Then NOTCH1 and FOXC2 were identified as downstream targets of miR-363. NOTCH1 overexpression or FOXC2 overexpression could ameliorate glucose and lipids metabolism disorder in type 2 diabetes model cells. In addition, we found that FOXC2 inhibition abolished the effect of NOTCH1 overexpression on HG-induced HepG2 cells. Finally, we proved that the PI3K/Akt pathway was the downstream pathway of FOXC2.

Conclusion: MiR-363 was considered as a key regulator of glucose and lipids metabolism in type 2 diabetes mellitus, which regulated PI3K/Akt axis by targeting NOTCH1 and FOXC2, thus leading to hepatic glucose and lipids metabolism disorder in type 2 diabetes.

INTRODUCTION

Type 2 diabetes is a very complex metabolic disorder, which is increasing rapidly in prevalence worldwide[1,2]. In the past 30 years, the number of patients with type 2 diabetes worldwide has doubled and is expected to reach 592 million by 2035[3]. Type 2 diabetes brings a huge burden to patients and society, which has become a global public health crisis, and its prevention and treatment are imminent[4]. It is widely reported that glucose metabolic disorder is the main cause of type 2 diabetes[5]. The liver has as a central role in maintaining the balance of glucose and lipids metabolism in the body[6]. In the

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liver, glucose is metabolized into pyruvate through glycolysis, and glycolytic products are used to synthesize fatty acids by de novo lipogenesis[7]. Additionally, glucose can be produced in the liver through glycogen breakdown and gluconeogenesis[8]. Therefore, exploring the molecular mechanism of hepatic glucose and lipids metabolism disorder in the liver is of great significance for the treatment of type 2 diabetes.

Micro ribonucleic acids (miRNAs) refer to endogenous noncoding RNAs with a length of 20–22 nts[9]. It is well known that miRNAs achieve biological roles through directly binding to downstream targets, thus participating in regulating development of various diseases[10]. With the in-depth study of the regulatory roles of miRNAs in the pathogenesis of diseases, a series of miRNAs have been shown to promote type 2 diabetes progression[5,11,12]. A previous study showed that miR-363 was reported to be involved in type 1 diabetes and was identified as a potential biomarker for diabetes[13]. More importantly, Wang *et al.*[14] showed that miR-363 was markedly upregulated in type 2 diabetic Goto-Kakizaki rats. However, the roles of miR-363 in regulating hepatic glucose and lipids metabolism in type 2 diabetes have not been reported.

Notch signaling is an intercellular signaling cascade, which has a major regulatory role in cell differentiation and fate determination[15]. Therefore, it is for the development and renewal of organs and tissues[16]. Much evidence has shown that NOTCH1 has an important role in regulating metabolic processes[17,18]. For instance, the expression levels of glucose-6-phosphatase was obviously elevated in the livers of liver-specific NOTCH1 knockout mice[17]. In addition, obvious diabetes and liver steatosis were observed in high-fat diet-treated NOTCH1 knockout mice[17]. At present, the specific mechanisms of NOTCH1 in regulating hepatic glucose and lipids metabolism in type 2 diabetes remain unknown.

Forkhead box C2 (FOXC2) is an important member of helix/forkhead transcription factor family[19], and plays an important role in regulating lipid metabolism and glucose metabolism[20]. Therefore, FOXC2 was considered to have the function to regulate insulin resistance[21]. More importantly, Nian *et al.*[22] showed that FOXC2 expression in visceral adipose tissue of type 2 diabetes patients was significantly lower than those of non-diabetes patients. All the aforementioned evidence suggested that FOXC2 was closely related to type 2 diabetes progression. However, there is no report about the molecular mechanisms of NOTCH1 in regulating hepatic glucose and lipids metabolism in type 2 diabetes. As we all know, insulin mainly regulates blood glucose through the phosphatidylinositol 3-kinase (PI3K)/threonine protein kinase (Akt) pathway[23]. A previous study showed that downregulation of FOXC2 promoted cell apoptosis through activation of Akt pathways in colorectal cancer[24]. However, whether FOXC2 is able to regulate hepatic glucose and lipids metabolism through the PI3K/Akt pathway in type 2 diabetes remains to be explored.

In the current study, we first explored the roles of miR-363, NOTCH1, FOXC2 and PI3K/Akt in type 2 diabetes, and further clarified the regulating relationships of the above-mentioned factors in type 2 diabetes, which is of great significance to find potential targets for type 2 diabetes mellitus treatment.

MATERIALS AND METHODS

Cell culture and model establishment

HepG2 cells were obtained from ATCC (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, MD, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY) in a humidified atmosphere of 5% CO_2 at 37°C.

Cells were incubated with 30 mmol/L glucose (Sigma-Aldrich, St. Louis, MO, USA) for 24 h as high glucose (HG) treatment. Meanwhile, cells treated with 35 mmol/L mannitol (Sigma-Aldrich) served as an osmotic pressure control group.

Cell transfection

The overexpression plasmid of NOTCH1, overexpression plasmid of FOXC2, short hairpin RNA against FOXC2 (sh-FOXC2) and miR-363 inhibitor or mimics, as well as their negative controls (NC) (including pcDNA 3.1 vector, sh-NC and inhibitor NC), were purchased from GenePharma (Shanghai, China). For *in vitro* transfection, cells were transfected with NOTCH1, FOXC2, sh-FOXC2, miR-363 inhibitor and their negative controls using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) for 24 h.

Animal experiments

Male Wistar rats (180–220 g) were purchased from SLAC (Shanghai, China). Rats were randomly divided into four groups (n = 7/group): control group, streptozocin (STZ) group, STZ + inhibitor NC group and STZ + miR-363 inhibitor group. Rats in the STZ group, STZ + inhibitor NC group and STZ + miR-363 inhibitor group were given a single intraperitoneal injection of STZ at 30 mg/kg in 0.1 mol/L acetate buffer. Rats in the control group were injected the same amount of acetate buffer intraperitoneally. Rats in the control group were fed a standard diet, whereas rats in the other groups received a high-fat diet (45 kcal% fat; Dowsontec, Shanghai, China). After 2 weeks of feeding, rats with blood glucose >16.7 mmol/L were considered to be successfully constructed type 2 diabetes models. Then, rats in STZ + inhibitor NC group and STZ + miR-363 inhibitor group were injected with lentiviruses expressing inhibitor NC and miR-363 inhibitor (1×10^9 PFU/g) obtained from GenePharma, respectively, through the tail vein. Then, all the rats were killed, and blood and livers were collected and stored at −80°C.

Glucose consumption assay

Cells were seeded into a 96-well plate (Corning Incorporated, Corning, NY, USA) at a density of 2×10^5 cells/mL. After treatment, the medium was replaced by low glucose Dulbecco's modified Eagle's medium (Gibco), and cells

were incubated with 100 nmol/L insulin (Sigma-Aldrich) for 15 min. Glucose consumption was calculated by subtracting the glucose concentration in the supernatant of the blank well from the glucose concentration in the supernatant of the well with cells. The concentration of glucose was evaluated using a glucose assay kit purchased from Nanjing Jiancheng (F006-1-1; Nanjing, China).

Glucose metabolism assay

In vivo experiments

The blood glucose level was assessed using a glucose assay kit purchased from Nanjing Jiancheng (F006-1-1). The content of glycogen in the liver was determined using a glycogen content detection kit (BC0340; Solarbio, Beijing, China). All the operations were strictly in accordance with the manufacturer's instructions.

In vitro experiments

The cellular glycogen content was evaluated using a glycogen content detection kit (BC0340; Solarbio).

Oil Red O staining

Cells were fixed with 10% polyoxymethylene (Sigma-Aldrich) for 2 h. Cells were subsequently stained with Oil Red O (Solarbio, Beijing, China) for 30 min and then washed with 60% isopropanol and phosphate-buffered saline. Cell imaging was carried out using an inverted microscope (Olympus, Southborough, MA, USA).

Western blot

The proteins were isolated from cells by using radioimmunoprecipitation assay buffer, and concentrations of protein were assessed using a bicinchoninic acid kit (Beyotime, Shanghai, China). Lysate samples were separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Then, membranes were incubated with primary antibodies including glucose transporter 2 (GLUT2; 1:1,000, ab192599; Abcam, Cambridge, UK), NOTCH1 (1:1,000, ab52627; Abcam), FOXC2 (1:1,000, ab245510; Abcam), PI3K (1:1,000, #4249; Cell Signaling Technology, Boston, USA), p-PI3K (1:1,000, #4228; Cell Signaling Technology, Boston, USA), Akt (1:1,000, ab8805; Abcam), phospho-Akt (1:1,000, #4060; Cell Signaling Technology, Boston, USA), DROSHA (1:1,000, ab242147; Abcam) and DICER (1:1,000, ab259327; Abcam). Anti-β-actin antibody (1:5,000, ab8226; Abcam) was served as a loading control. After being washed with phosphate-buffered saline-Tween 20, membranes were then incubated with the corresponding secondary antibody labeled with HRP (1:10,000, ab7090, ab6789; Abcam) for 60 min. The membranes were covered with enhanced chemiluminescence reagents (Beyotime) and the images were visualized by a gel imaging system (Bio-Rad, Hercules, CA, USA). The quantification of proteins was analyzed by the software Image J (National Institutes of Health, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cells and tissues by using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Complementary deoxyribonucleic acid was synthesized with a HiFi-Script cDNA Synthesis Kit in accordance with manufacturer's instruction (Life Technologies, CA, USA). Then, the Complementary deoxyribonucleic acid was used for real-time quantitative polymerase chain reaction assay carried out on an Eppendorf MasterCycler RealPlex4 (Eppendorf, Wesseling-Berzdorf, Germany) using an Ultra SYBR Mixture kit (Thermo Fisher Scientific). The relative expressions of miRNA and messenger RNA (mRNA) were normalized by U6 and β-actin, respectively, and calculated by the $2^{-\Delta\Delta CT}$ method. The primers used in the present study were listed as follows (5'–3'):

MiR-363 (F): GCCGGGTGGATCACGATG
MiR-363 (R): GTGCAGGGTCCGAGGT
NOTCH1 (F): GCACGTGTATTGACGACGTTG
NOTCH1 (R): GCAGACACAGGAGAAGCTCTC
FOXC2 (F): AACGAGTGCTTCGTCAAGGT
FOXC2 (R): TCTCCTTGGACACGTCCTTC
β-actin (F): CCCTGGAGAAGAGCTACGAG
β-actin (R): CGTACAGGTCTTTGCGGATG
U6 (F): CTCGCTTCGGCAGCACA
U6 (R): AACGCTTCACGAATTTGCGT

Dual luciferase reporter gene assay

The miR-363 binding sites in the 3′-UTR of NOTCH1 (CCACCCAA) and FOXC2 (UCCACCCU) were predicted using Targetscan 7.2 (http://www.targetscan.org/vert_72/). Site-directed mutagenesis of the miR-363 binding site in the 3′-UTR of NOTCH1 (position 465-471: CCACCCAA) and FOXC2 (position 756-762: UCCACCCU) were performed using a quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Wild-type (WT) and mutant-type (MUT) reporter plasmids of NOTCH1/FOXC2 3′-UTR sequences were cloned into PGL3 vector (GenePharma). Then, cells were plated onto 24-well plate and were co-transfected with NOTCH1-WT/FOXC2-WT or NOTCH1-MUT/FOXC2-MUT plasmids and miR-363 mimics, mimics NC, miR-363 inhibitor or inhibitor NC by Lipofectamine™ 3000 (Invitrogen). The luciferase activity was evaluated by a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical analysis

All data were obtained from at least three replicate experiments. Results were expressed as mean ± standard deviation. Statistical analysis was carried out using GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). Differences between normally distributed data were analyzed by Student's *t*-tests and one-way analysis of

variance (ANOVA). The *P*-values <0.05 were considered significant.

RESULTS

MiR-363 expression was elevated in HG-treated HepG2 cells, whereas NOTCH1 and FOXC2 levels were decreased

HG-treated HepG2 cells were used as type 2 diabetes model cells in this research. As shown in Figure 1a, glucose consumption and glycogen content were all markedly decreased in HG-treated cells compared with control cells or mannitol-treated cells. Increased lipid accumulation was also observed in the HG group compared with the control group or mannitol group (Figure 1c), based on Oil Red O staining. Then miR-363, NOTCH1 and FOXC2 expressions in cells were evaluated using quantitative polymerase chain reaction and western blot, and results showed that miR-363 was obviously upregulated in HG-treated HepG2 cells, whereas NOTCH1 and FOXC2 were significantly downregulated ($P < 0.05$; Figure 1c-d). Additionally, the present results showed that protein levels of GLUT2, DROSHA and DICER (proteins involved in miRNA biosynthesis) were obviously elevated in HG-treated HepG2 cells ($P < 0.05$; Figure 1d).

MiR-363 inhibition ameliorated glucose and lipids metabolism disorder *in vitro*

To probe the roles of miR-363 on metabolic disorder in HepG2 cells induced by HG, we knocked down miR-363 in HG-treated HepG2 cells. MiR-363 inhibition markedly lowered miR-363 expression in cells, suggesting the transfection was successful ($P < 0.05$; Figure 2a). Then, we probed alterations in glucose and lipids metabolism in HepG2 cells. The protein level of GLUT2 was obviously decreased in model cells after miR-363 inhibitor transfection ($P < 0.05$; Figure 2b). The decreased glucose consumption, glycogen content and the elevated lipid accumulation were also relieved by miR-363 silence ($P < 0.05$; Figure 2c-d). Taken together, the reduction of miR-363 could ameliorate glucose and lipids metabolism disorder in HepG2 cells induced by HG.

MiR-363 regulated glucose and lipids metabolism in type 2 diabetes model cells through targeting NOTCH1 and FOXC2

We subsequently probed the regulating relationships between miR-363, NOTCH1 and FOXC2 in regulating glucose and lipids metabolism. We found that miR-363 had a variety of downstream targets, we listed some downstream targets of miR-363 in Supplement Table 1. The present results showed that NOTCH1 and FOXC2 levels were markedly reduced in HG-treated HepG2 cells ($P < 0.05$; Figure 1c-d); therefore, we speculated that NOTCH1 and FOXC2 were downstream targets of miR-363 in regulating glucose and lipids metabolism in type 2 diabetes. First, we found that NOTCH1 and FOXC2 expressions were significantly elevated in model cells after miR-363 silence ($P < 0.05$; Figure 3a-b). Then, we predicted the potential binding site between miR-363 and NOTCH1 and miR-363 and FOXC2 using Targetscan 7.2 (http://www.targetscan.org/vert_72/). The results showed that miR-363 had a binding site to the region of 3′-UTR of NOTCH1 (position 465-471: CCACCCAA; Figure 3c); and miR-363 had a binding site to the region of 3′-UTR of FOXC2 (position 756-762: UCCACCCU; Figure 3e). As shown in Figure 3d and f, miR-363 mimics transfection suppressed the luciferase activity presented by NOTCH1-WT vector or FOXC2-WT vector, and miR-363 inhibitor transfection increased the luciferase activity, but both miR-363 mimics and miR-363 inhibitor had no significant effect on that of NOTCH1-MUT vector or FOXC2-MUT vector ($P < 0.05$; Figure 3d and f), suggesting that miR-363 could directly bind to NOTCH1 and FOXC2.

To further explore the function of NOTCH1 and FOXC2 in glucose and lipids metabolism, we determined the alterations in glucose and lipids metabolism after NOTCH1 or FOXC2 overexpression. Protein levels of NOTCH1 and FOXC2 were significantly increased in model cells after NOTCH1 overexpression, whereas GLUT2 were downregulated ($P < 0.05$; Figure 3g). Furthermore, the decreased glucose consumption, glycogen content and the elevated lipid accumulation were reversed by NOTCH1 overexpression ($P < 0.05$; Figure 3h-i). Similarly, the protein level of FOXC2 was markedly increased in model cells after FOXC2 overexpression, whereas GLUT2 were downregulated ($P < 0.05$; Figure 3j). The glucose consumption and glycogen content were obviously increased in model cells after FOXC2 overexpression, whereas the lipid accumulation was markedly decreased ($P < 0.05$; Figure 3k-l). Taken together, miR-363 could regulate glucose and lipids metabolism in type 2 diabetes model cells through targeting NOTCH1 and FOXC2.

NOTCH1 regulated the PI3K/Akt pathway through targeting FOXC2, thus regulating glucose and lipids metabolism in type 2 diabetes model cells

A previous study showed that FOXC2 could regulate the PI3K/Akt pathway in colorectal cancer[24]. The PI3K/Akt pathway was reported to play an important role in regulating glucose metabolism[25]. Here, we aimed to explore regulating relationships between NOTCH1, FOXC2 and the PI3K/Akt pathway in regulating glucose and lipids metabolism, so we both overexpressed NOTCH1 and knockdown FOXC2 in HG-treated HepG2 cells. As shown in Figure 4a, the levels of NOTCH1 and FOXC2 were markedly increased in model cells after NOTCH1 overexpression, whereas the phosphorylation levels of PI3K and Akt were obviously reduced. The phosphorylation levels of PI3K and Akt were significantly increased in the simultaneous overexpression of NOTCH1 and knockdown FOXC2 group compared with the overexpression NOTCH1 group, and FOXC2 expression was reversed ($P < 0.05$; Figure 4a). Furthermore, glucose consumption and glycogen content in the simultaneous overexpression of NOTCH1 and knockdown FOXC2 group were reduced, and lipid accumulation was increased compared with the overexpression NOTCH1 group ($P < 0.05$; Figure 4b-c). In total, NOTCH1

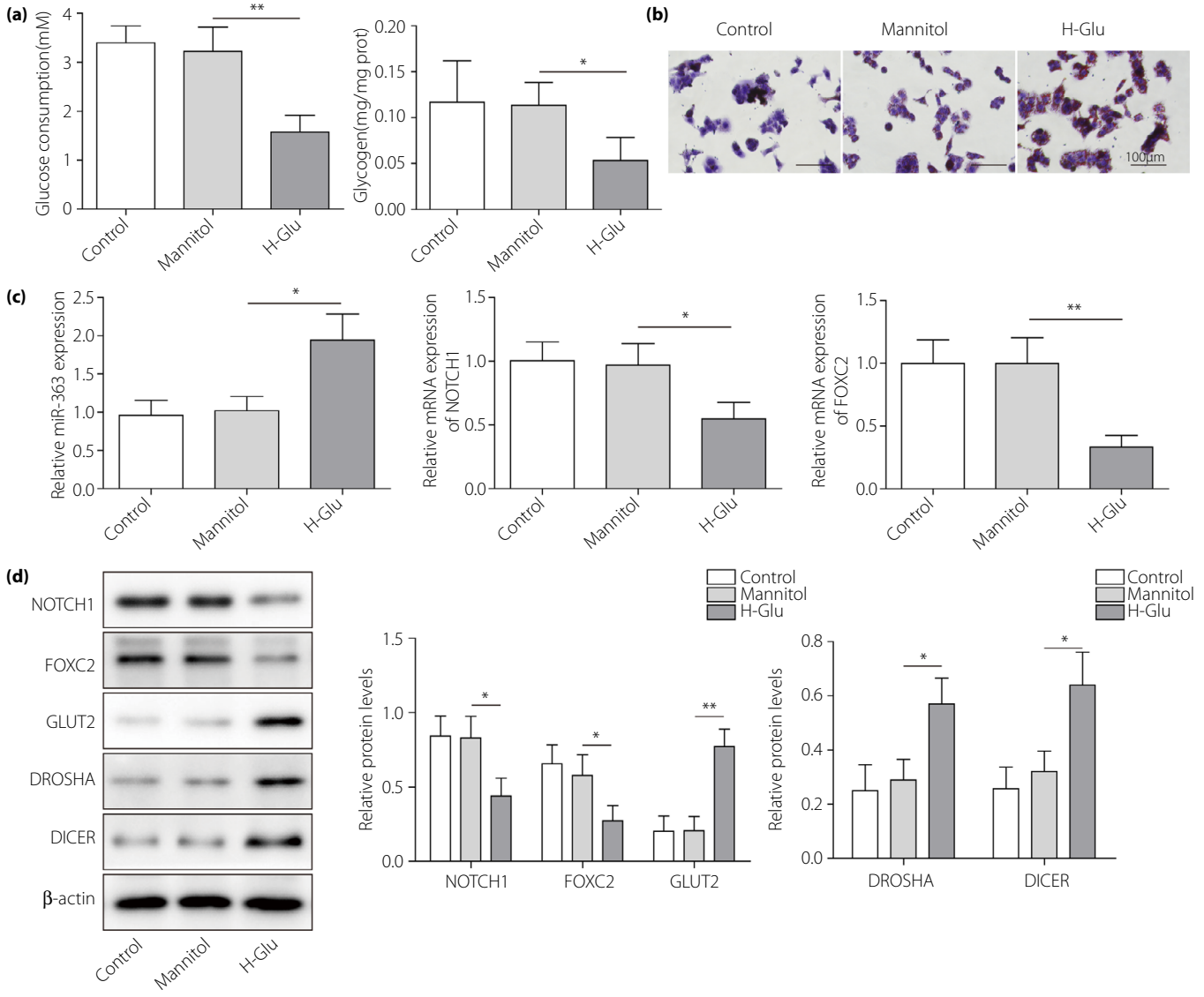

Figure 1 | MiR-363 expression was increased in high glucose (HG)-treated HepG2 cells, whereas NOTCH1 and forkhead box C2 (FOXC2) levels were decreased. HepG2 cells were treated with mannitol or high glucose. (a) Glucose consumption and glycogen content in HepG2 cells. (b) Oil Red O staining was carried out to assess lipid accumulation in HepG2 cells. (c) MiR-363, NOTCH1 and FOXC2 expressions in HepG2 cells were determined using quantitative polymerase chain reaction. (d) Protein levels of NOTCH1, FOXC2, glucose transporter 2 (GLUT2), DROSHA and DICER in HepG2 cells were assessed using western blot. The data are expressed as the mean ± standard deviation. $n = 3$. *$P < 0.05$, **$P < 0.01$. mRNA, messenger ribonucleic acid.

overexpression suppressed the PI3K/Akt pathway through targeting FOXC2, thus ameliorating glucose and lipids metabolism in type 2 diabetes model cells.

MiR-363 inhibition ameliorated glucose and lipids metabolism disorders in type 2 diabetes model rats

Although the *in vitro* results suggested that miR-363 modulated glucose and lipids metabolism in HG-treated HepG2 cells, we also investigated the effect of miR-363 on regulating glucose and lipids metabolism disorder in type 2 diabetes rats. MiR-363 expression was significantly decreased in model rats after miR-363 silence ($P < 0.05$; Figure 5a). Because of the decreased miR-363 expression resulting from miR-363 inhibitor, the hepatic expressions of NOTCH1 and FOXC2 were also markedly elevated and the levels of PI3K and Akt phosphorylation were also obviously reduced in type 2 diabetes model rats ($P < 0.05$; Figure 5b). Further testing of blood glucose and glycogen concentration in the blood and liver of rats found that

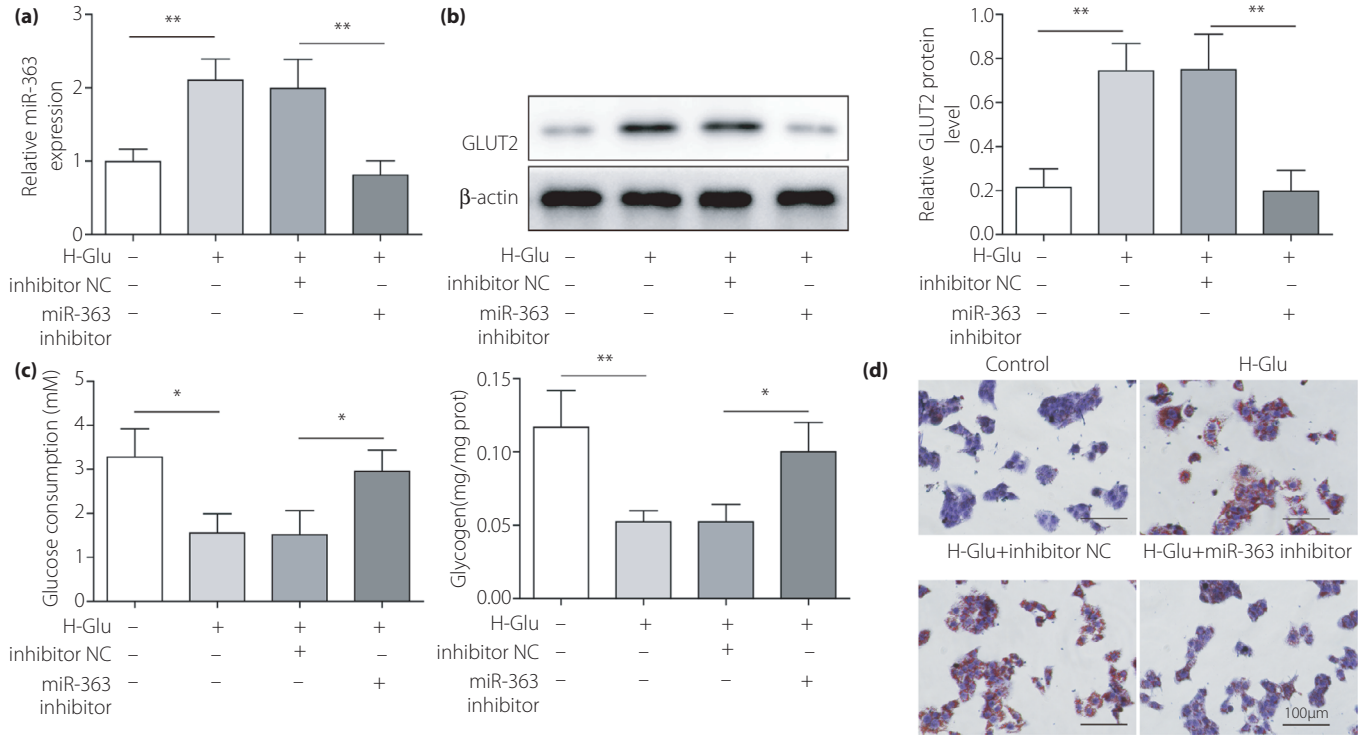

Figure 2 | MiR-363 inhibition ameliorated glucose and lipids metabolism disorder *in vitro*. High glucose (HG)-treated HepG2 cells were transfected with inhibitor negative control (NC) or miR-363 inhibitor. (a) MiR-363 expression in HepG2 cells was determined using quantitative polymerase chain reaction. (b) Western blot was used to evaluate the protein level of glucose transporter 2 (GLUT2) in HepG2 cells. (c) Glucose consumption and glycogen content in HepG2 cells. (d) Lipid accumulation in HepG2 cells was evaluated using Oil Red O staining. The data are expressed as the mean ± standard deviation. $n = 3$. *$P < 0.05$, **$P < 0.01$.

Table 1 | miR-363 partially predicts downstream target genes

Gene	Gene name	Expressed in type 2 diabetes	References
BRD4	Bromodomain-containing 4	Up	42,43
CNPY4	Canopy FGF signaling regulator 4	–	–
NOTCH1	Notch1	Down	44
SUMO2	Small ubiquitin-like modifier 2	Up	45
RSF1	Remodeling and spacing factor 1	–	–
FOXC2	Forkhead box C2 (MFH-1; mesenchyme forkhead-1)	Down	22
CBX6	Chromobox homolog 6	–	–
VSTM1	V-set and transmembrane domain-containing 1	–	–
GRIN1	Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 1	Mutation	46
SZRD1	SUZ RNA binding domain-containing 1	–	–

the blood glucose level increased and the liver glycogen content decreased in type 2 diabetes rats. After miR-363 was knocked down, the blood glucose concentration of the rats decreased, and the liver glycogen content increased ($P < 0.05$; Figure 5c). Finally, Oil Red O staining to detect the lipid content in the livers of type 2 diabetes rats that knocked down miR-363 confirmed that a large number of lipid droplets and elevated lipid accumulation were found in type 2 diabetes mellitus, and the lipids were reduced after miR-363 knockdown ($P < 0.05$; Figure 5d). As a consequence, decreased blood glucose level and increased liver glycogen content were observed in miR-363 inhibitor-treated type 2 diabetes model rats. In summary, miR-363 inhibition could ameliorate glucose and lipids metabolism disorder *in vivo*.

DISCUSSION

The pathogenesis of type 2 diabetes is complex, and a variety of miRNAs participate in regulating type 2 diabetes

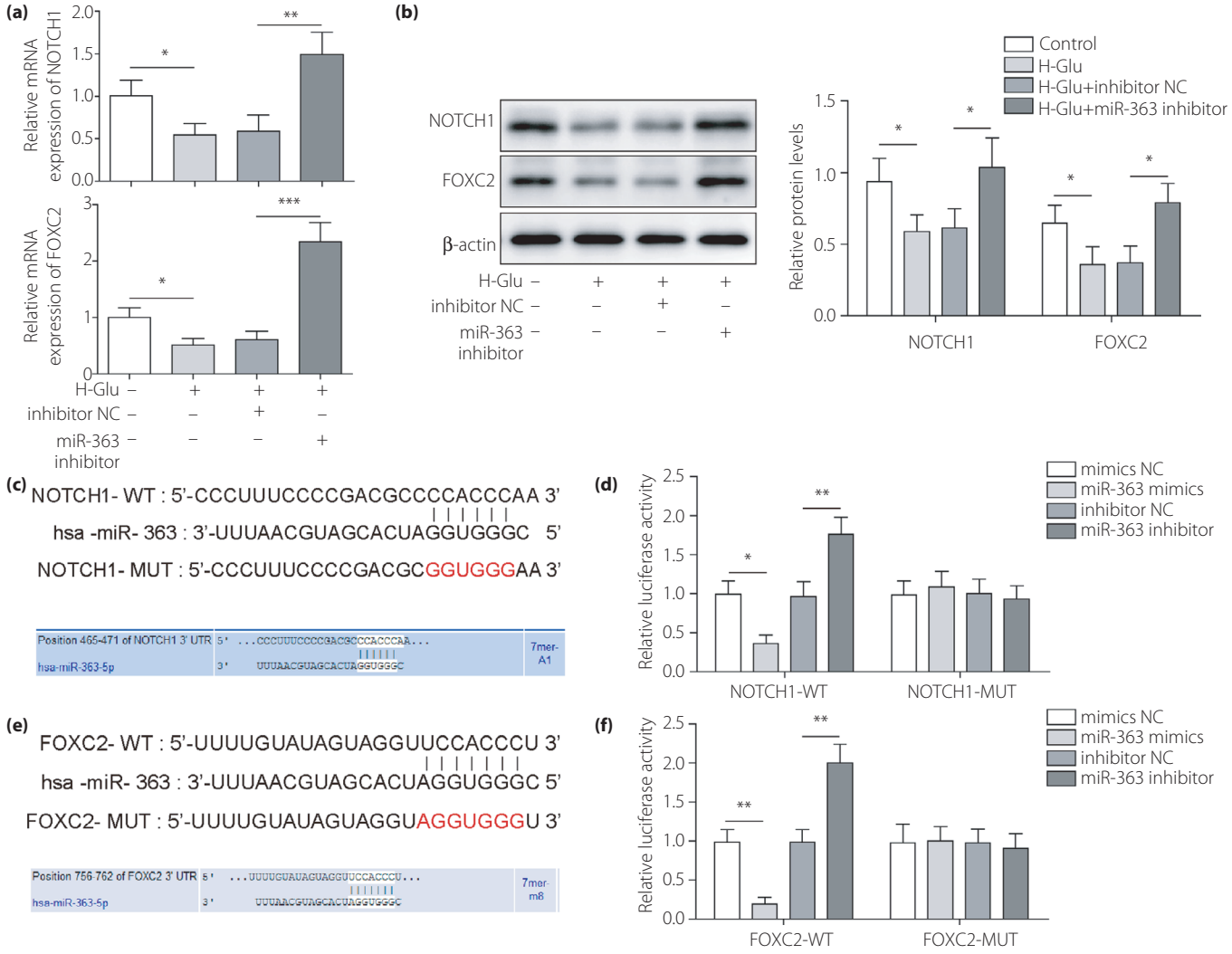

Figure 3 | MiR-363 regulated glucose and lipids metabolism in type 2 diabetes mellitus model cells through targeting NOTCH1 and forkhead box C2 (FOXC2). (a–b) The expressions of NOTCH1 and FOXC2 after miR-363 inhibition was assessed using quantitative polymerase chain reaction and western blot. (c) Bioinformatics software was applied to the binding site between miR-363 and NOTCH1. (d) Dual luciferase reporter gene assay was used to verify the regulatory relationship between miR-363 and NOTCH1. (e) Bioinformatics software was applied to the binding site between miR-363 and FOXC2. (f) Dual luciferase reporter gene assay was used to verify the regulatory relationship between miR-363 and FOXC2. High glucose (HG)-treated HepG2 cells were transfected with vector or NOTCH1. (g) Protein levels of NOTCH1, FOXC2 and glucose transporter 2 (GLUT2) in HepG2 cells were assessed using western blot. (h) Glucose consumption and glycogen content in HepG2 cells. (i) Oil Red O staining was carried out to assess lipid accumulation in HepG2 cells. (j) HG-treated HepG2 cells were transfected with vector or FOXC2. (j) Protein levels of FOXC2 and GLUT2 in HepG2 cells were assessed using western blot. (k) Glucose consumption and glycogen content in HepG2 cells. (l) Lipid accumulation in HepG2 cells was evaluated using Oil Red O staining. The data are expressed as the mean ± standard deviation. $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

development[26,27]. MiR-320 was reported to be obviously upregulated in type 2 diabetes rats, and its inhibition can markedly increase the expression of insulin-like growth factor 1[14]. In the current study, the present results confirmed that upregulated hepatic miR-363 in the glucose and lipids metabolic disorder was associated with type 2 diabetes. Our results proved that miR-363 had a huge regulatory effect on hepatic glucose and lipids metabolism disorder through targeting both NOTCH1 and FOXC2. In addition, we found that FOXC2 was also regulated by NOTCH1. All the present results clarified the specific mechanisms of miR-363, NOTCH1 and FOXC2 in regulating hepatic glucose and lipids metabolism in type 2 diabetes, which was reported for the first time.

Glucose metabolism disorder is the main reason to promote type 2 diabetes progression[5]. In type 2 diabetes, impaired insulin function results in a significant reduction in glucose uptake, glucose consumption and glycogen storage, leading to a significant increase in plasma glucose level[28,29].

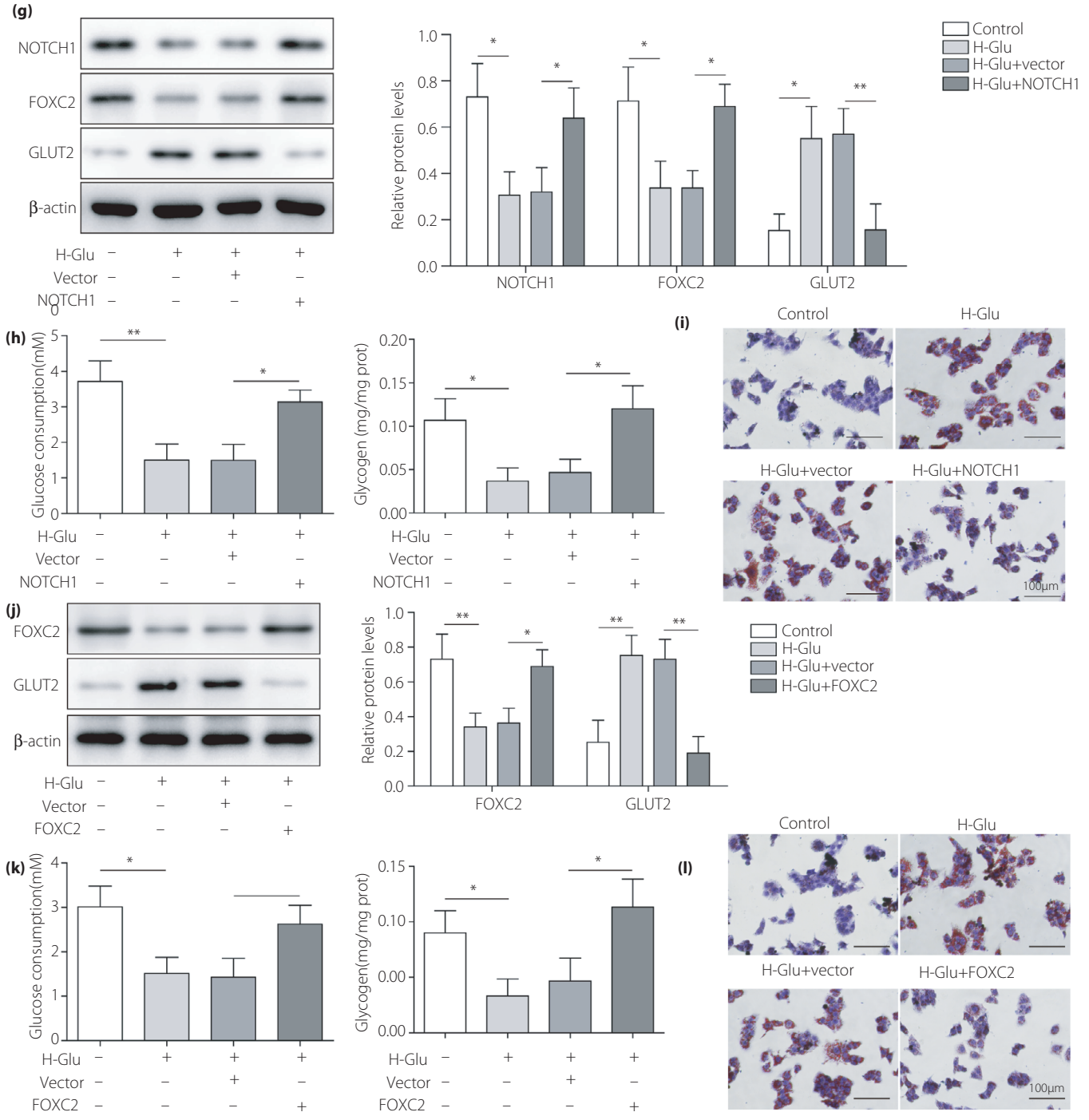

Figure 3 | Continued

In type 2 diabetes, disorders of lipid metabolism can also lead to lipid peroxidation and free radicals, leading to tissue damage, thereby aggravating the symptoms of type 2 diabetes[30]. The liver is the body's most important metabolic organ, where de novo synthesis of fatty acids, glycolysis, gluconeogenesis and glycogen decomposition occur. Improving hepatic metabolic disorder was considered to be an effective strategy for the treatment of type 2 diabetes[5]. A great deal of evidence has shown that expressions of miRNAs were abnormal in various metabolic diseases, including type 2

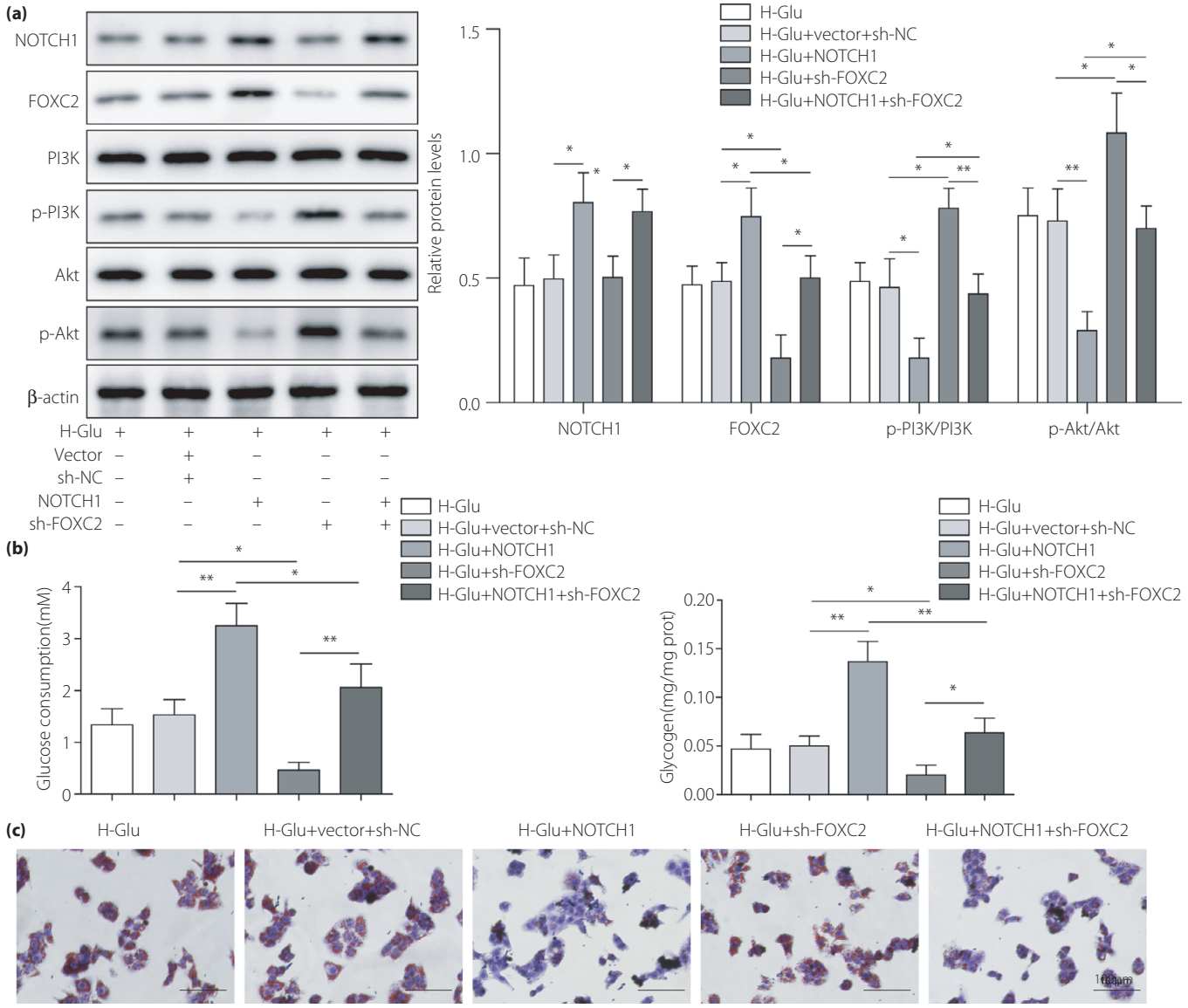

Figure 4 | NOTCH1 regulated the phosphatidylinositol 3-kinase (PI3K)/threonine protein kinase (Akt) pathway through targeting forkhead box C2 (FOXC2), thus regulating glucose and lipids metabolism in type 2 diabetes model cells. HG-treated HepG2 cells were transfected with NOTCH1, sh-FOXC2, co-transfected with sh-FOXC2 and NOTCH1. (a) Protein levels of NOTCH1, FOXC2, PI3K, phospho-PI3K (p- PI3K), Akt and phospho-Akt (p-Akt) in HepG2 cells were assessed using western blot. (b) Glucose consumption and glycogen content in HepG2 cells. (c) Oil Red O staining was carried out to assess lipid accumulation in HepG2 cells. The data were expressed as the mean ± standard deviation. $n = 3$. *$P < 0.05$, **$P < 0.01$.

diabetes. Wei *et al.*[31] showed that miR-203a-3p was remarkably downregulated in the liver of type 2 diabetes GK rats and could attenuate insulin resistance. In addition, miR-126-3p expression was markedly decreased in microparticles from type 2 diabetes patients, and the expression was related to plasma anti-oxidant capacity[32]. The aforementioned findings suggested that abnormal expressed miRNAs played a key role in type 2 diabetes progression. DROSHA and DICER are proteins involved in miRNA biosynthesis[33]. Herein, we found that DROSHA and DICER levels were markedly reduced in HG-treated HepG2 cells. The present study also showed that miR-363 was markedly upregulated in HG-treated HepG2 cells, and its inhibition could increase glucose consumption and glycogen content, and decrease lipid accumulation. To make our conclusion more credible, we also induced a type 2 diabetes rat model through STZ and HFD treatment, which is a very commonly used animal model of type 2 diabetes[34]. Blood glucose level was markedly decreased in miR-363 inhibitor-treated type 2 diabetes model rats, whereas liver glycogen content was markedly increased. These findings

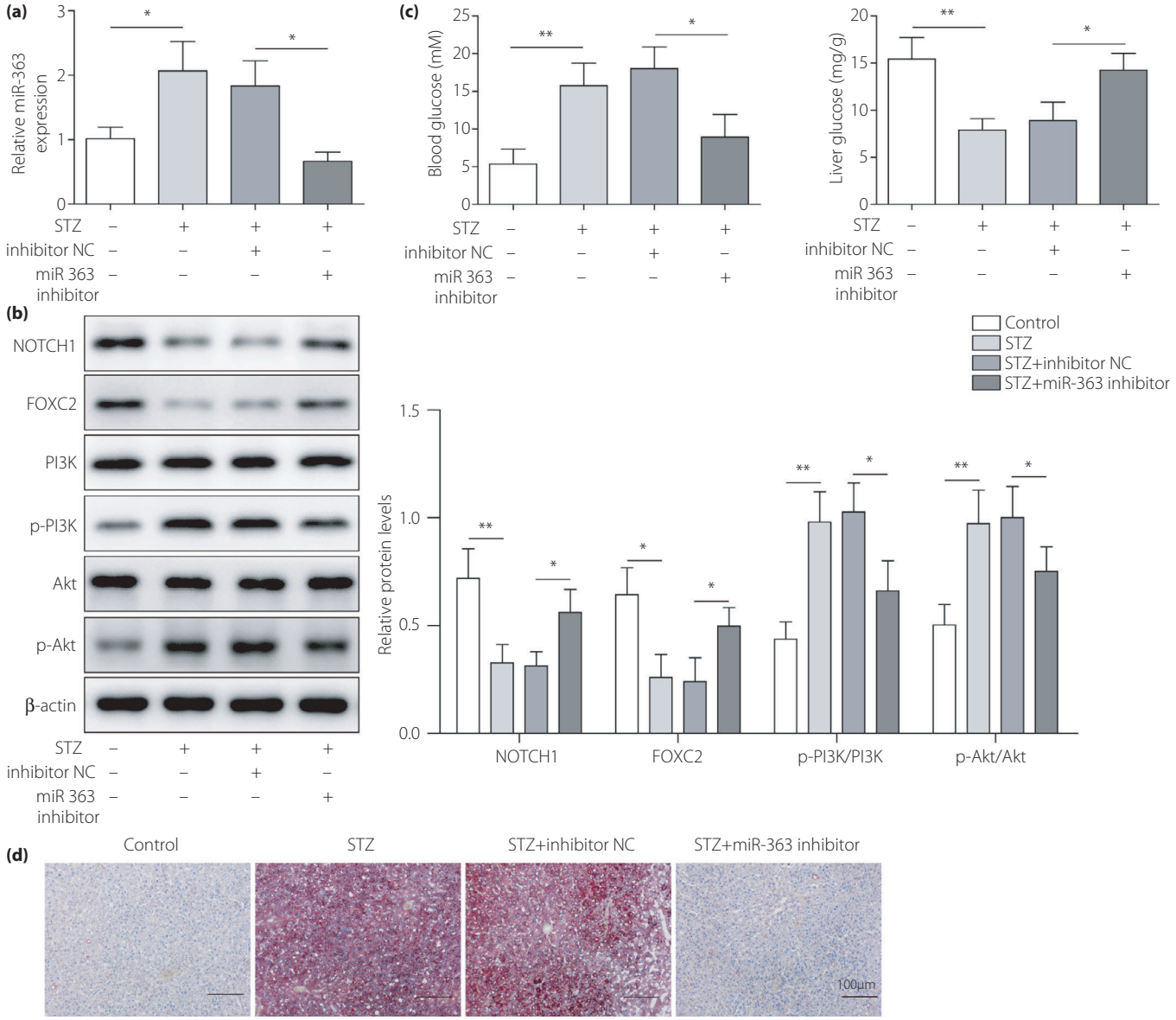

Figure 5 | MiR-363 inhibition ameliorated glucose and lipids metabolism disorder in type 2 diabetes model rats. Type 2 diabetes model rats were injected with lentiviruses expressing inhibitor NC and miR-363 inhibitor. (a) Hepatic expression of miR-363 was detected using quantitative polymerase chain reaction. (b) Protein levels of NOTCH1, forkhead box C2 (FOXC2), phosphatidylinositol 3-kinase (PI3K), phospho-PI3K (p-PI3K), threonine protein kinase (Akt) and phospho-Akt in liver tissues were determined using western blot. (c) The levels of blood glucose and liver glycogen in liver tissues. (d) Oil Red O staining was carried out to assess lipid accumulation in liver tissues. The data are expressed as the mean ± standard deviation. $n = 3$. *$P < 0.05$, **$P < 0.01$.

showed that miR-363 has a huge regulatory effect on hepatic glucose and lipids metabolism in type 2 diabetes.

MiRNAs are capable of targeting downstream mRNAs to suppress mRNA translation[35]. To further explore the specific mechanism of miR-363 in regulating glucose and lipids metabolism, bioinformatics prediction was used to identify NOTCH1 and FOXC2, which are two candidate genes in regulating the progression of type 2 diabetes[22,36], as target genes of miR-363. NOTCH signaling is considered to maintain β-cell mass in the adult pancreas[37]. Eom *et al.*[38] showed that higher glucose levels and lower insulin secretion were observed in NOTCH1 antisense transgenic mice. In addition, the NOTCH1 signaling pathway was suppressed in diabetic rats[36]. Both protein level and mRNA levels of FOXC2 were markedly reduced in type 2 diabetes patients than in healthy people[22]. Additionally, FOXC2 expression was markedly correlated with blood glucose level,

TG level and IR index[22]. As expected, the results of dual luciferase reporter gene assay confirmed that both NOTCH1 and FOXC2 could directly bind to miR-363. Furthermore, we found that miR-363 silence could dramatically increase NOTCH1 and FOXC2 expressions in HG-treated HepG2 cells.

Based on the aforementioned results, we speculated that the effects of miR-363 might be associated with regulation of NOTCH1 and FOXC2. The present results also showed that NOTCH1 and FOXC2 could regulate hepatic glucose and lipids metabolism as targets of miR-363. We observed that NOTCH1 overexpression could significantly increase the protein level of FOXC2 in model cells. In addition, NOTCH1 was reported to enhance definitive hematopoiesis in hemogenic endothelium through targeting FOXC2[39]. Therefore, we wanted to probe the regulatory relationship between NOTCH1 and FOXC2 as targets of miR-363 in type 2 diabetes. We both overexpressed NOTCH1 and knocked down FOXC2 in HG-treated HepG2 cells. Our results showed that FOXC2 silence partially reversed the positive effects of NOTCH1 overexpression in HG-treated HepG2 cells. We proved that NOTCH1 had a regulatory effect on FOXC2, and both acted as downstream targets of miR-363. A great deal of evidence suggests a crucial role for the PI3K/Akt pathway in insulin action[40,41]. In addition, Yang *et al.*[24] showed that FOXC2 could enhance cell apoptosis through regulation of the Akt pathway in colorectal cancer. We found that NOTCH1 overexpression could reduce levels of PI3K and Akt phosphorylation, whereas it was abolished by FOXC2 inhibition.

All the present results provided evidence that miR-363 regulated the PI3K/Akt axis by targeting NOTCH1 and FOXC2, leading to hepatic glucose and lipids metabolism disorder in type 2 diabetes. The present research clarified the regulatory mechanism of hepatic glucose and lipids metabolism in type 2 diabetes, which was of great significance for the treatment of type 2 diabetes.

The present research also has certain shortcomings. We predicted that miR-363 had a variety of downstream targets, whereas we just selected the targets most likely to be involved in the regulation of type 2 diabetes (NOTCH1 and FOXC2) as the research targets. In the future, we will study the roles of other miR-363 targets in diabetes and the interaction between different targets.

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DISCLOSURE

The authors declare no conflict of interest.

Approval of the research protocol: The animal experiment was approved by the research protocol.

Informed consent: Informed consent was obtained from the Ethics Committee of Shenzhen Hospital, University of Chinese Academy of Sciences.

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