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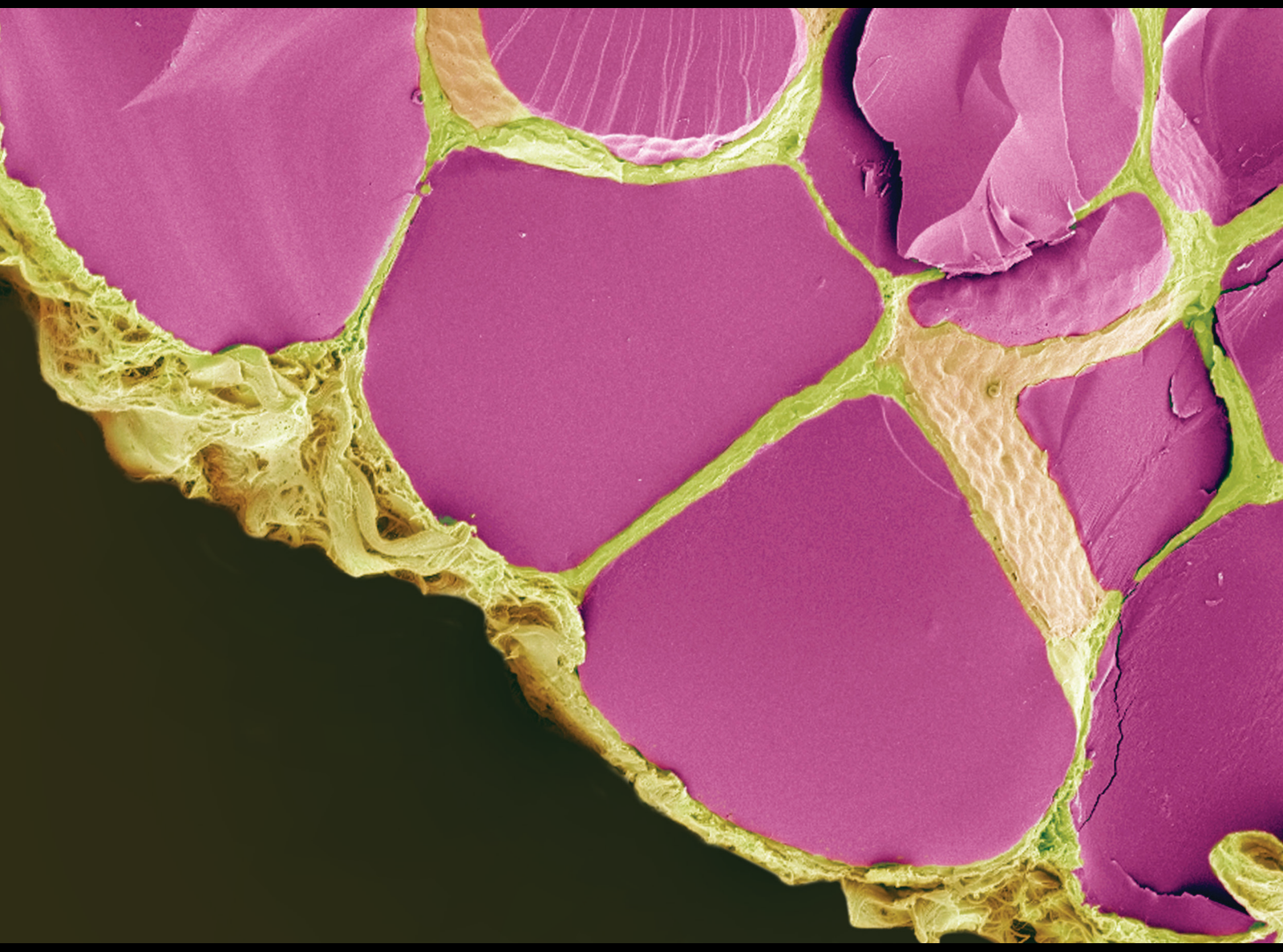

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Research Article

The Phosphatase PHLPP2 Plays a Key Role in the Regulation of Pancreatic Beta-Cell Survival

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Guest Editor: Jan Skupien

Currently available antidiabetic treatments fail to halt, and may even exacerbate, pancreatic β-cell exhaustion, a key feature of type 2 diabetes pathogenesis; thus, strategies to prevent, or reverse, β-cell failure should be actively sought. The serine threonine kinase Akt has a key role in the regulation of β-cell homeostasis; among Akt modulators, a central role is played by pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) family. Here, taking advantage of an in vitro model of chronic exposure to high glucose, we demonstrated that PHLPPs, particularly the second family member called PHLPP2, are implicated in the ability of pancreatic β cells to deal with glucose toxicity. We observed that INS-1 rat pancreatic β cell line maintained for 12–15 passages at high (30 mM) glucose concentrations (INS-1 HG) showed increased expression of PHLPP2 and PHLPP1 both at mRNA and protein level as compared to INS-1 maintained for the same number of passages in the presence of normal glucose levels (INS-1 NG). These changes were paralleled by decreased phosphorylation of Akt and by increased expression of apoptotic and autophagic markers. To investigate if PHLPPs had a casual role in the alteration of INS-1 homeostasis observed upon chronic exposure to high glucose concentrations, we took advantage of shRNA technology to specifically knock-down PHLPPs. We obtained proof-of-concept evidence that modulating PHLPPs expression may help to restore a healthy β cell mass, as the reduced expression of PHLPP2/1 was accompanied by a recovered balance between pro- and antiapoptotic factor levels. In conclusion, our data provide initial support for future studies aimed to identify pharmacological PHLPPs modulator to treat beta-cell survival impairment. They also contribute to shed some light on β-cell dysfunction, a complex and unsatisfactorily characterized phenomenon that has a central causative role in the pathogenesis of type 2 diabetes.

1. Introduction

Type 2 diabetes (T2D) is a complex disease, brought about by the combination of abnormalities in both the production and the function of the pancreatic hormone insulin [1]. Although classically these two defects were seen as separate entities, in the last decades, it has become evident that they share common pathogenetic mechanisms, with insulin regulating not only glucose utilization from peripheral target tissues, but also its own synthesis and secretion as well as the maintenance of an adequate β-cell mass [1–3]. Notably, while impaired insulin action in peripheral tissue—the so-called "insulin resistance"—remains fairly constant as the disease progresses, β-cell function worsens continuously with time in diabetic patients, as a consequence of the persisting exposure to damaging factors, such as high glucose concentrations (glucose toxicity), increased levels of circulating free fatty acids (lipotoxicity), and proinflammatory cytokines (chronic inflammation) [2–5]. Furthermore, currently available antidiabetic treatments fail to

halt, and may even exacerbate, pancreatic β-cell exhaustion; thus, despite promising observations with molecules belonging to the more recently introduced therapeutic classes [6], strategies to prevent, or reverse, β-cell failure should still be actively sought. There are two primary components to β-cell dysfunction in T2D: impaired insulin secretion and reduced β-cell mass. In adult humans, the rate of new β-cell formation is low, and the maintenance of an adequate mass is achieved mainly throughout a tight regulation of apoptotic rates [7]. The serine threonine kinase Akt, also known as protein kinase B (PKB), has a key role in the regulation of β-cell homeostasis. Akt exists in three isoforms that are considered indistinguishable in their domain architecture and upstream regulation but are nonredundant in their expression patterns and biological functions [8, 9]. Specifically, all three isoforms have been detected in pancreatic β-cells; with studies in knock-out mouse models suggesting that Akt1 regulates mainly β-cell survival, Akt2 is required to modulate the insulin secretory response, while Akt3 loss does not appear to significantly alter either β-cell mass or function [9]. The three Akt isoforms are activated by sequential phosphorylation at two key sites; the phosphorylation of the first residue, located in a segment called the activation loop (Threonine 308/309/307 in Akt1/2/3, respectively), triggers the phosphorylation of a site located in the carboxyl-terminal domain, termed the hydrophobic phosphorylation motif (Serine 473/474/472 in Akt1/2/3 respectively) [8]. We and others have reported decreased Akt activation upon exposure to glucotoxicity, lipotoxicity, and/or chronic inflammation [4, 9–14]. Akt inhibition is mediated by dephosphorylation by two protein phosphatases: protein phosphatase 2A that acts on the threonine residue [15] and pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) family targeting the serine residue [16–18]. PHLPP proteins appear to be ubiquitously expressed, with particularly high levels in the brain, and their increased expression has been reported in numerous cancer cell lines, including cancers of the brain, breast, lung, prostate, and ovary. The PHLPP family comprises two members: PHLPP1, which in turn exists in two splice variants, (α and β), and PHLPP2 (also known as PHLPPL). The three isozymes share very similar domain structure but have a certain degree of substrate specificity, with PHLPP1 preferentially targeting Akt2 and 3 and PHLPP2 showing a higher affinity toward Akt1 and 3 [18]. A few years ago, we have shown increased PHLPP1 expression in adipose tissue and skeletal muscle biopsies from obese, insulin-resistant subjects and hypothesized that PHLPPs may represent an additional player in insulin resistance [14]. Here, we investigated if PHLPPs may be implicated in the ability of pancreatic β cells to deal with chronic exposure to high glucose concentrations in an *in vitro* model. We also aimed to obtain proof-of-concept evidence that modulating PHLPPs expression may help to restore a healthy β cell mass.

2. Methods

2.1. Cell Culture and Adenoviral Infection. Rat pancreatic β-cells lines were maintained at 37°C with 5% CO_2 for 12–15 passages in RPMI 1640 cell medium (Sigma-Aldrich, Milan, Italy), supplemented with 50 μM β-mercaptoethanol, 10% (vol/vol) fetal bovine serum, and 1% (vol/vol) penicillin/streptomycin [19], and containing 11.1 mM (INS-1 NG) or 30 mM (INS-1 HG) glucose. Adenoviral infection was carried out, as previously described [20] incubating 50–60% confluent INS-1 HG with increasing quantities of a 1 : 1 mixture containing Ad-U6-rat-PHLPP1-shRNA and Ad-U6-rat-PHLPP2-shRNA (viral titer 3.7×10^{10} PFU/ml, Vector Biosystems, Malvern, PA, USA) or Ad-U6 scrambled-shRNA (mock-infected control cells) for 7 hrs and 30 minutes. Cells were then washed to remove virus and serum-starved or maintained in fresh complete growth medium for 48 hrs, depending on the specific experimental requirements.

2.2. Total RNA Extraction and Real-Time Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted from INS-1 NG and INS-1 HG using Trizol (Life Technologies, Gaithersburg, MD), reverse transcribed and analyzed by RT-qPCR using a Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Results were normalized to β-actin levels according to the Livak method, as previously described [21–23]. Primers sequences are available upon request.

2.3. Insulin Stimulation and Western Blot Analysis. To assess insulin-stimulated protein phosphorylation, INS-1 were serum-starved for 48 hrs with FBS-free medium containing bovine serum albumin and glucose at the appropriate concentrations; human insulin (10^{-7} M) was then added, when indicated, for 7 minutes before cell lysis in a buffer containing 1.5% NP-40. Cell lysates were processed and analyzed by Western Blot, according to previously established methods [14, 20]. A home-made primary antibody generated and validated by our research group [14] was employed to detect PHLPP1 levels; anti-PHLPP2 antibody was obtained from Abcam (Cambridge, United Kingdom). The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): anti-Bad, anti-Bcl-xL, anti-cleaved caspase-3, anti-LC3-II, anti-total and phospho Akt, anti-total and phospho FoxO1, and anti-total and phospho mTor. Equal protein loading was confirmed by reblotting the membranes with monoclonal antibody against β-actin (Sigma-Aldrich; Milan, Italy); p-Akt/Akt, p-FoxO1/FoxO1, and pmTor/mTor ratios were calculated to analyze the relative phosphorylation levels. Densitometric analysis was performed using a ImageJ software (NIH, USA).

2.4. Insulin Secretion Assay. INS-1 cells were seeded in 24-multiwell plates at a density of 10^5 cells/well in growth medium containing 11.1 mM or 30 mM glucose, as appropriate. Twenty hours before the insulin secretion assay, cells were switched to a medium containing 5 mM glucose; the medium was then replaced with a glucose-free Krebs phosphate buffer for 2 hrs. INS-1 was then incubated in the presence of increasing concentrations of glucose in fresh

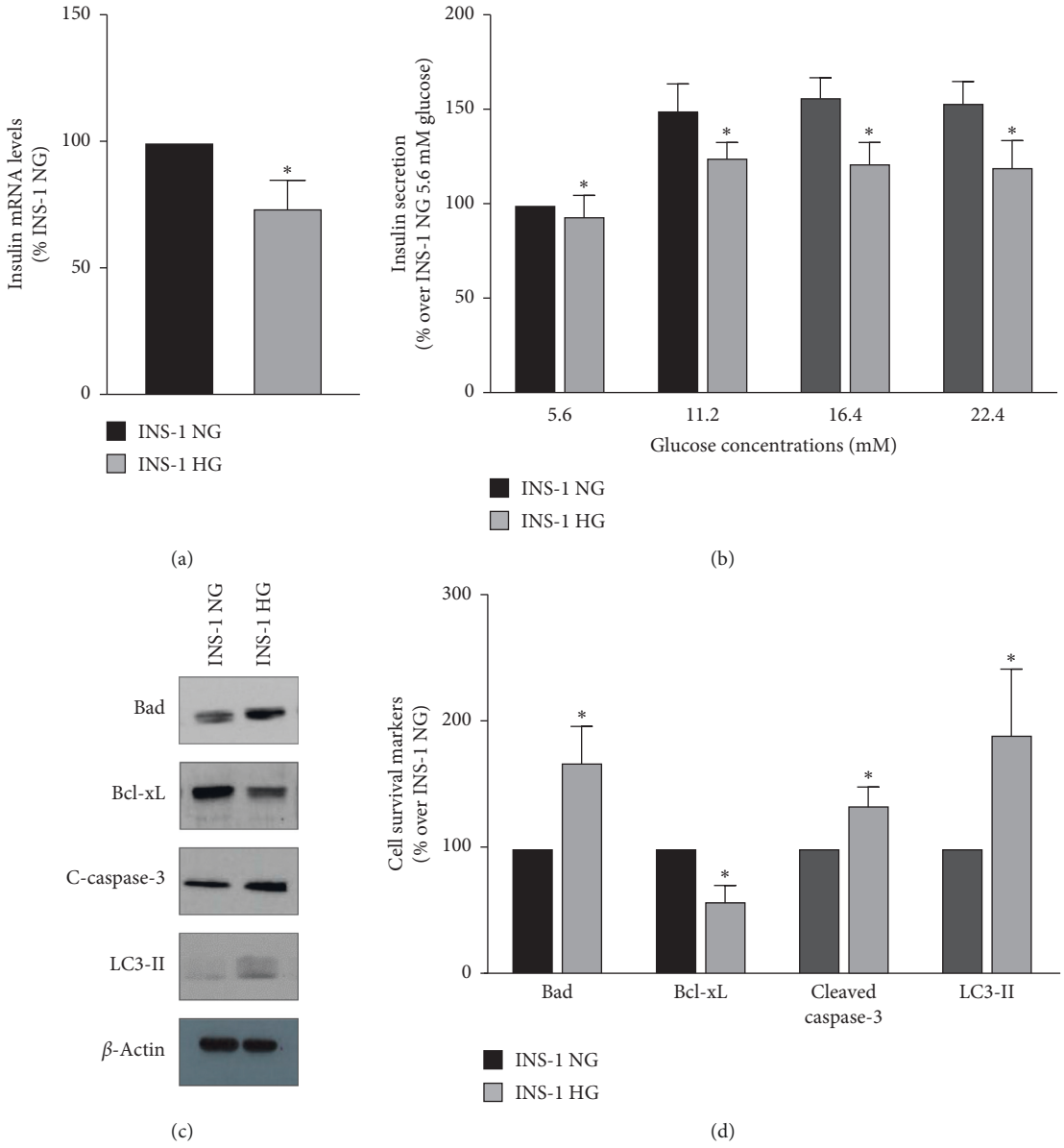

Figure 1: INS-1 cells maintained for 12–15 passages at 30 mM glucose concentrations show reduced insulin synthesis and secretion and altered expression of prosurvival markers. INS-1 was maintained at high (30 mM, INS-1 HG) or normal (11.2 mM, INS-1 NG) glucose concentrations for 12–15 passages. (a) Insulin mRNA levels were assessed by real-time RT-PCR; $n = 6$, *indicates significant ($p < 0.05$) differences for INS-1 NG vs INS-1 HG. (b) Glucose-stimulated insulin secretion was assessed by measuring with a specific Elisa kit insulin concentration in medium obtained from INS-1 NG and INS-1 HG exposed to increasing glucose concentrations (5.6, 11.2, 15.6, and 22.4 mM) for 20 minutes; $n = 4$, *indicates significant ($p < 0.05$) differences for INS-1 NG vs INS-1 HG. (c) Representative western blot images of cell survival markers levels in INS-1 HG and INS-1 NG: Bad (upper panel), Bcl-xL (middle-upper panel), cleaved caspase-3 (middle-lower panel), and LC3-II (lower panel). (d) Graph of the mean changes of densitometric values of cell survival markers in INS-1 NG and INS-1 HG; $n = 5$–10, *indicates significant ($p < 0.05$) differences for INS-1 NG vs INS-1 HG.

Krebs buffer for 20 minutes. Cell media were removed and diluted to assess insulin concentration with a specific rat insulin Elisa assay (Mercodia, Uppsala, Sweden).

2.5. Statistical Analysis. All results were calculated as mean fold variation (±SD) over the appropriate control point. Statistical differences were assessed by Student's t test or ANOVA as indicated. A p value ≤0.05 was considered statistically significant. Analyses were performed with GraphPad Prism version 8.2.0 software (San Diego, CA, USA).

3. Results

3.1. Chronic Exposure to High Glucose Concentrations Results in a Significant Increase of PHLPP2 and PHLPP1 Expression. To mimic chronic exposure to high glucose levels, we cultured INS-1 rat pancreatic β-cells at 30 mM glucose for

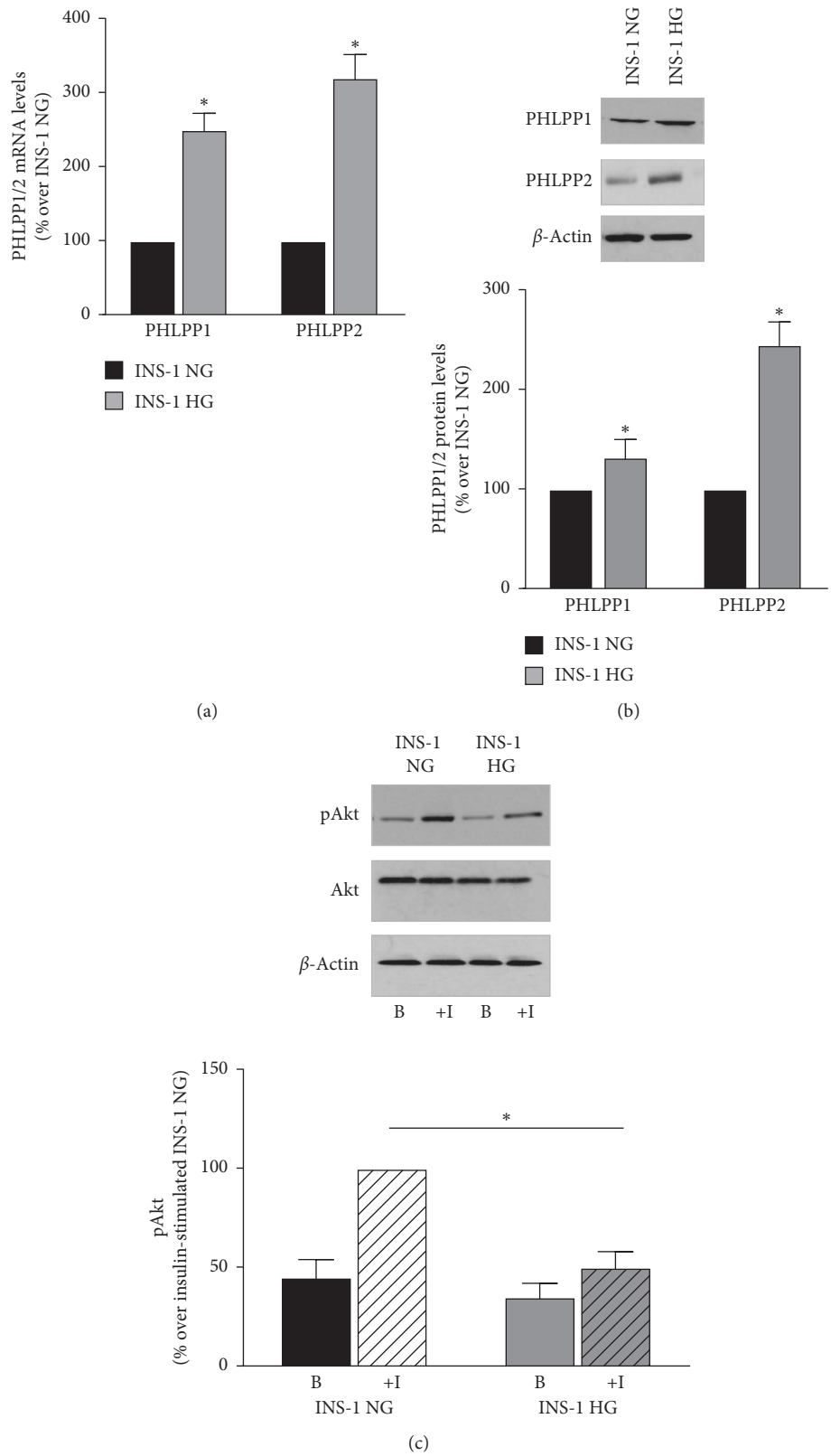

Figure 2: PHLPP1 and PHLPP2 expression is increased and Akt phosphorylation is reduced in INS-1 HG. (a) PHLPP1 and PHLPP2 mRNA levels were assessed by real-time RT-PCR (A), $n = 5$, *indicates significant ($p < 0.05$) differences for INS-1 NG vs INS-1 HG; (b) representative Western Blot images of PHLPP1 (upper panel) and PHLPP2 (lower panel) levels in INS-1 NG and INS-1 HG. Graphs of the mean changes over INS-1 NG values of the densitometric values of PHLPP1/2 expression obtained in 4 independent experiments and normalized for β-actin levels; *indicates significant ($p < 0.05$) differences for INS-1 NG vs INS-1 HG. (c) Representative Western Blot images of Akt phosphorylation on the Serine 473 residue (upper panel) and of total Akt levels (middle panel) in INS-1 NG and INS-1 HG stimulated (+I) or not (b) with 10^{-7} M insulin. Graphs of the mean changes over insulin-stimulated INS-1 NG values of the densitometric values of pAkt obtained in 4 independent experiments and normalized for total Akt levels. *indicates significant ($p < 0.05$) differences for insulin-stimulated INS-1 HG vs insulin-stimulated INS-1 NG.

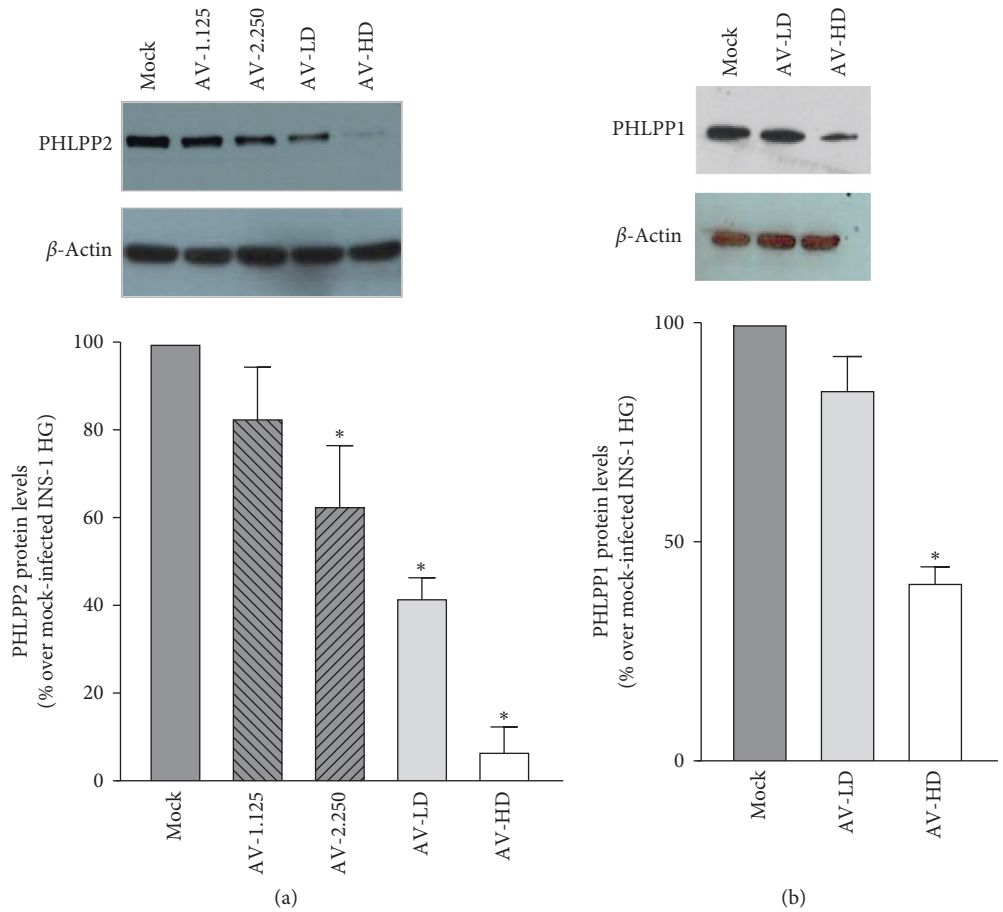

Figure 3: Infection with shRNA constructs against PHLPP2/1 results in a significant knock-down of the expression of both isoenzymes. (a) Representative Western Blot images of PHLPP2 levels in INS-1 HG infected with increasing concentrations of the adenoviral vectors encoding for shRNA against PHLPP1/2 : 1.125×10^5 PFU (AV1.125); 2.250×10^5 PFU (AV2.250); 4.5×10^5 PFU (AV-LD); and 9×10^5 PFU (AV-HD). Control mock-infected cells were infected with empty AV constructs. Graphs of the mean changes over mock-infected INS-1 HG values of the densitometric values of PHLPP2 expression obtained in 4 independent experiments and normalized for β-actin levels; *indicates significant ($p < 0.05$) differences for AV-infected INS-1 HG vs mock-infected INS-1 HG. (b) Representative Western Blot images of PHLPP1 levels in INS-1 HG infected with 4.5×10^5 PFU (AV-LD) or 9×10^5 PFU (AV-HD) of the of the adenoviral vectors encoding for shRNA against PHLPP1/2 as compared to control mock-infected INS-1 HG cells. Graphs of the mean changes over mock-infected INS-1 HG values of the densitometric values of PHLPP1 expression obtained in 4 independent experiments and normalized for β-actin levels; *indicates significant ($p < 0.05$) differences for AV-infected INS-1 HG vs mock-infected INS-1 HG.

12–15 passages (INS-1 HG). This glucose concentration has been previously shown to efficaciously induce glucose toxicity in pancreatic β-cell lines that require culture media containing 11.2 mM glucose for their normal growth [24]. As compared to INS-1 cells maintained for the same number of passages at normal glucose concentrations (INS-1 NG), the functionality of INS-1 HG was significantly impaired, as demonstrated by the decreased insulin mRNA levels (Figure 1(a), $n = 6$, $p = 0.0001$, INS-1 HG vs INS-1 NG) and the hampered glucose-stimulated insulin secretion (Figure 1(b), $n = 4$, $p = 0.0001$, by 2-way ANOVA for the secretion curve of INS-1 HG vs INS-1 HG). Furthermore, the balance between pro- and antiapoptotic Bcl family protein expression appeared altered in INS-1 HG (Figures 1(c) and 1(d), $n = 4$, $p = 0.04$, INS-1 HG vs INS-1 NG). The activation of apoptotic pathways in INS-1 HG was suggested also by the increased levels of cleaved caspase-3 (Figures 1(c) and 1(d), $n = 10$, $p = 0.0001$, for INS-1 HG vs INS-1 NG). In addition, we observed a two-fold increase in the amount of LC3-II protein, which hinted to a higher activation of autophagic pathways in INS-1 HG cells as compared to INS-1 NG (Figures 1(c) and 1(d), $n = 6$, $p = 0.0001$, for INS-1 HG vs INS-1 NG).

These functional changes were paralleled by a significant increase in PHLPP2 expression at both mRNA (Figure 2(a), $+320 \pm 35\%$, $p = 0.0001$, for INS-1 HG vs INS-1 NG $n = 5$) and protein level (Figure 2(b) $+245 \pm 25\%$, $p = 0.0001$, for INS-1 HG vs INS-1 NG, $n = 4$) in INS-1 HG as compared to INS-1 NG. Similar, but less evident, changes were observed for PHLPP1 expression, which showed a $250 \pm 22\%$ increase in mRNA levels (Figure 2(a), $p = 0.0001$ for INS-1 HG vs INS-1 NG, $n = 5$) and $132 \pm 20\%$ increase in protein levels (Figure 2(b), $p = 0.047$ for INS-1 HG vs INS-1 NG, $n = 4$).

Since PHLPP dephosphorylates and inactivates Akt kinase [16–18], we assessed Akt phosphorylation on the activation loop residue, Ser473, which is directly targeted by

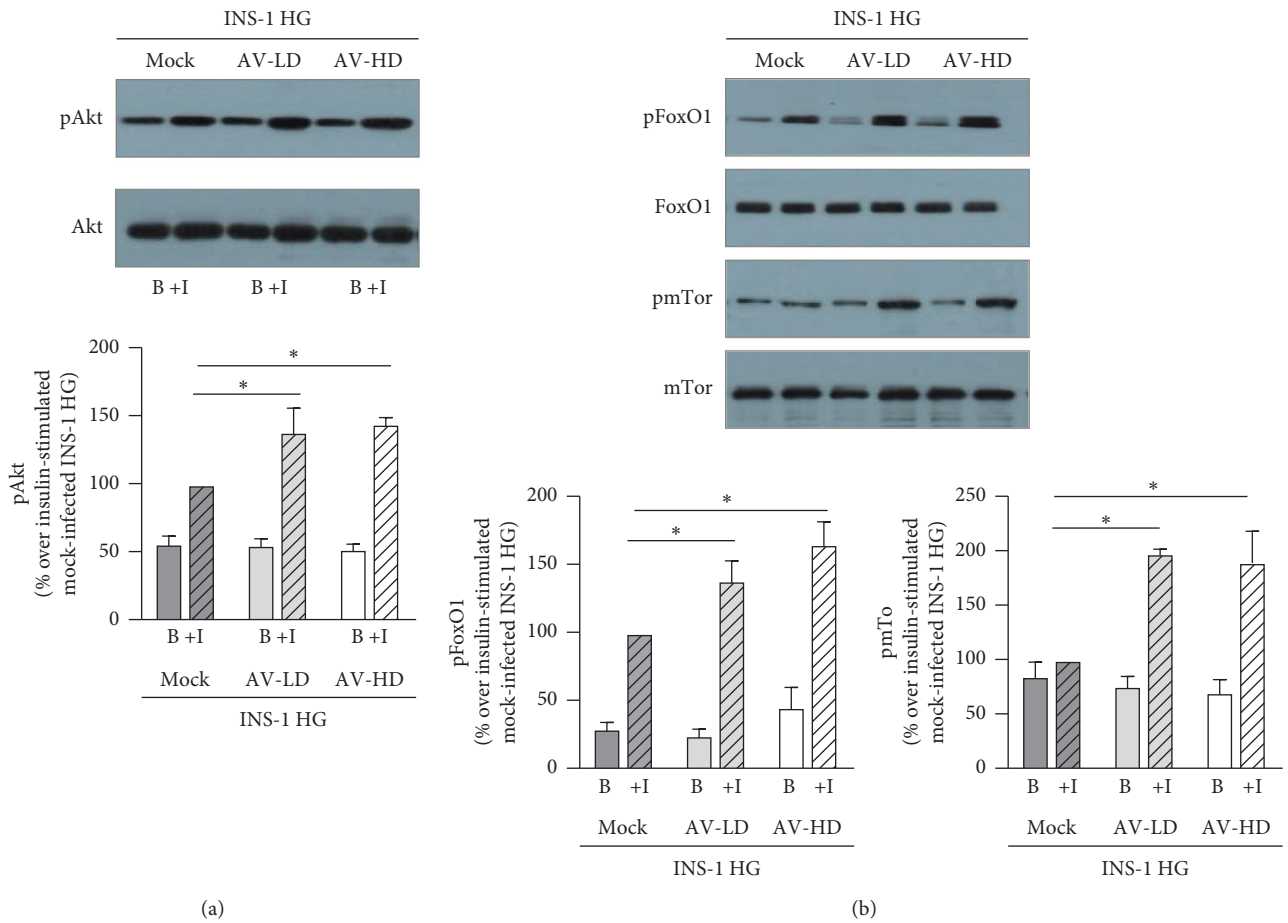

Figure 4: PHLPP2/1 knock-down results in a significant improvement of insulin-stimulated phosphorylation of Akt, FoxO1, and mTor. (a) Representative western blot images of Akt phosphorylation on the Serine 473 residue (upper panel) and of total Akt levels (middle panel) in mock-infected INS-1 HG, INS-1 HG AV-LD, INS-1 HG AV-HD-stimulated (+I), or not (b) with 10^{-7} M insulin. Graphs of the mean changes over insulin-stimulated mock-infected INS-1 HG values INS-1 NG values of the densitometric values of pAkt obtained in 3 independent experiments and normalized for total Akt levels; *indicates significant ($p < 0.05$) differences for AV-infected INS-1 HG vs mock-infected INS-1 HG. (b) Representative Western Blot images of FoxO1 phosphorylation (upper panel), total FoxO1 levels (upper-middle panel), mTor phosphorylation (lower-middle panel), total mTor levels (lower panel) in mock-infected INS-1 HG, INS-1 HG AV-LD, INS-1 HG AV-HD-stimulated (+I), or not (b) with 10^{-7} M insulin. Graphs of the mean changes over insulin-stimulated mock-infected INS-1 HG values and INS-1 NG values of the densitometric values of pFoxO1 or pmTor obtained in 3–5 independent experiments and normalized for total levels of the unphosphorylated protein; *indicates significant ($p < 0.05$) differences for AV-infected INS-1 HG vs mock-infected INS-1 HG.

PHLPPs. As shown in Figure 2(c), basal and insulin-stimulated Akt phosphorylation on Ser473 was reduced in INS-1 HG as compared to INS-1 NG (upper panel, $p = 0.049$ for basal INS-1 HG vs basal INS-1 NG; $p = 0.0001$ for insulin-stimulated INS-1 HG vs insulin-stimulated INS-1 NG, $n = 6$). Interestingly, we observed a slight, not significant, decrease of total Akt expression in INS-1 HG (Figure 2(c), middle panel). The reduction of insulin-stimulated Akt phosphorylation was statistically significant when normalized taking into account the reduced total Akt levels (Figure 2(c), $p = 0.0001$, for insulin-stimulated INS-1 HG vs insulin-stimulated INS-1 NG, $n = 6$).

3.2. Infection with shRNA Constructs Against PHLPP2/1 Resulted in a Significant Knock-Down of the Expression of Both Isoenzymes. To investigate if PHLPPs had a casual role in the alteration of INS-1 homeostasis observed upon chronic exposure to high glucose concentrations, we took advantage of shRNA technology to specifically knock-down PHLPPs, employing a previously validated protocol [20]. We choose to concurrently downregulate both PHLPP family member, since the expression of both appeared increased upon chronic exposure to high glucose concentrations, and specifically reducing only one isoenzyme may have induced compensatory overexpression of the cognate protein, confounding the data interpretation. To this end, we employed a mixture containing two adenoviral vectors encoding for a shRNA against PHLPP1 or against PHLPP2 in a 1 : 1 ratio. We initially performed a dose-response curve and observed that a dose of at least 4.5×10^5 PFU was required to consistently reduce PHLPP2 levels; doubling this dose, we obtained an almost complete PHLPP2 knock-down (Figure 3(a), $p = 0.0001$, as compared to mock-infected INS-

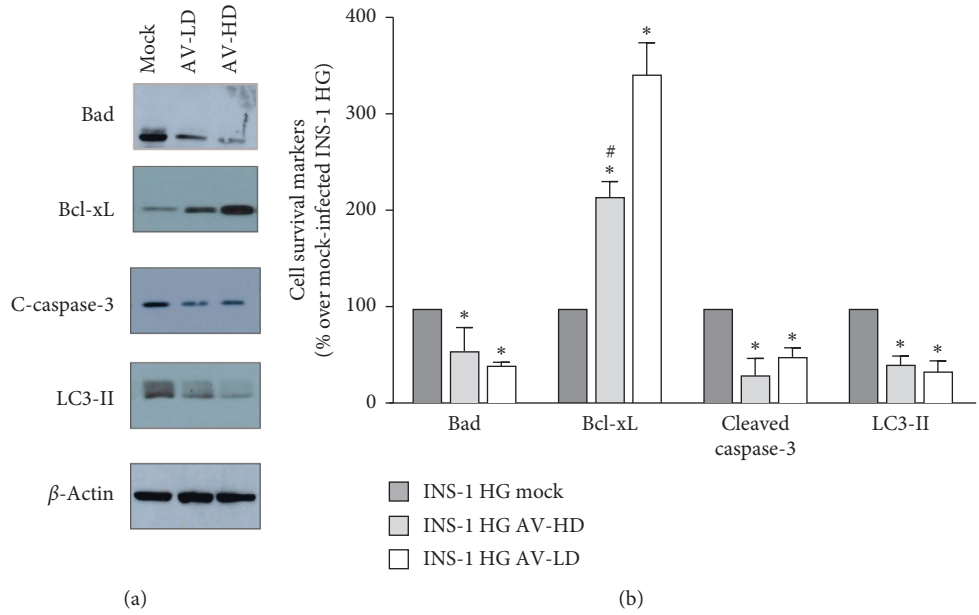

FIGURE 5: PHLPP2/1 knock-down results in a significant improvement of cell survival markers expression profile. (a) Representative western blot images of cell survival markers levels in mock-infected INS-1 HG, INS-1 HG AV-LD, and INS-1 HG AV-HD: bad (upper panel), Bcl-xL (middle-upper panel), cleaved caspase-3 (middle-lower panel), and LC3-II (lower panel). (b) Graph of the mean changes of densitometric values of cell survival markers levels in mock-infected INS-1 HG, INS-1 HG AV-LD, and INS-1 HG AV-HD; $n = 3$–5; *indicates significant ($p < 0.05$) differences for AV-infected INS-1 HG vs mock-infected INS-1 HG, $^{\#}$indicates significant ($p < 0.05$) differences for INS-1 HG AV-LD vs INS-1 HG AV-HD.

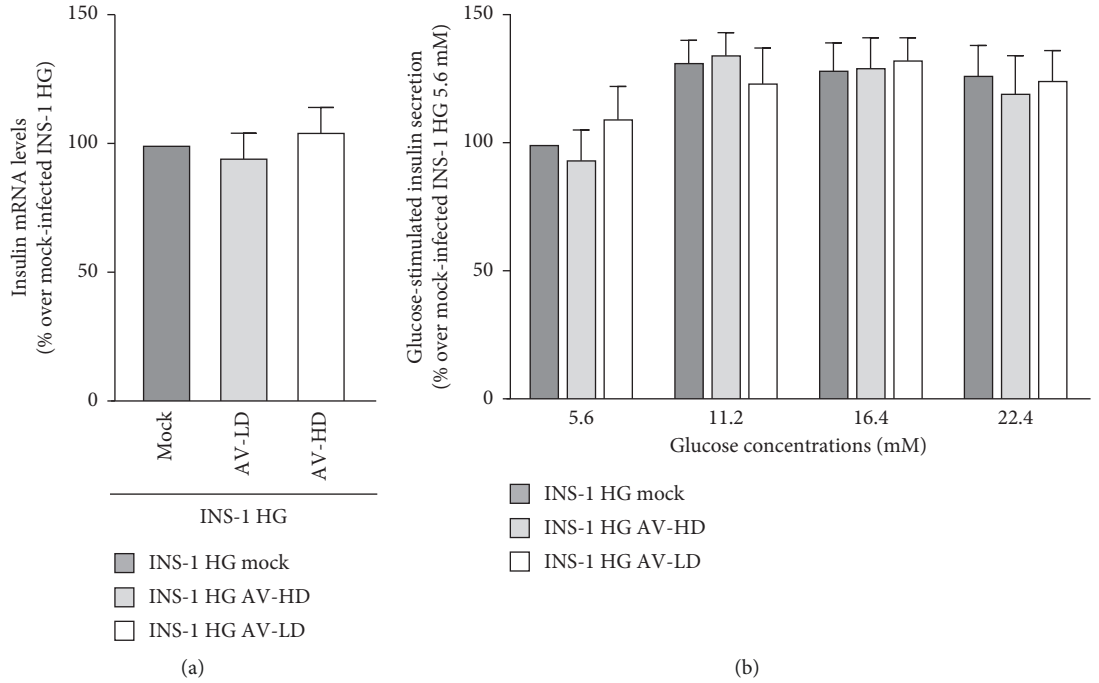

FIGURE 6: PHLPP2/1 knock-down does not restore insulin synthesis and secretion in INS-1 HG. (a) Insulin mRNA levels were assessed by real-time RT-PCR in mock-infected INS-1 HG, INS-1 HG AV-LD, and INS-1 HG AV-HD: $n = 4$. (b) Glucose-stimulated insulin secretion was assessed by measuring with a specific Elisa kit insulin concentration in medium obtained mock-infected INS-1 HG, INS-1 HG AV-LD, and INS-1 HG AV-HD exposed to increasing glucose concentrations (5.6, 11.2, 15.6, and 22.4 mM) for 20 minutes; $n = 4$.

1 HG cells). We then tested if these two concentrations resulted in PHLPP1 expression inhibition and observed a 15% reduction of PHLPP1 levels with the 4.5×10^5 PFU ($p = 0.04$) and a 85% decrease with the 9×10^5 PFU dose ($p = 0.0001$) (Figure 3(b)). Higher viral doses resulted in a notable and rapid decrease in cell viability; all subsequent

experiments were thus carried out with the above-mentioned quantities that will be referred to as low dose (AV-LD) and high dose (AV-HD).

3.3. shRNA-Mediated PHLPP2 and PHLPP1 Knock-Down Restores Insulin Signaling in INS-1 HG. Next, we evaluated if in INS-1 HG infected with either AV-LD or AV-HD, the reduction of PHLPPs expression resulted in a restored activation of Akt signaling pathway upon insulin stimulation. We observed that insulin-stimulated Akt phosphorylation levels were 1.39 and 1.45 fold higher in INS-1 HG AV-LD and INS-1 HG AV-HD, respectively, as compared to mock-infected control INS-1 HG ($p = 0.0022$, for INS-1 HG AV-LD vs mock-infected INS-1 HG and $p = 0.0003$ for INS-1 HG AV-HD vs mock-infected INS-1 HG, $n = 4$, Figure 4(a)). The increased Akt phosphorylation levels were mirrored by significantly increased insulin-stimulated phosphorylation of two Akt substrates that have been suggested to play key roles in the regulation of specific β-cell function: FoxO1 transcription factor ($p = 0.0018$ for INS-1 HG AV-LD vs mock-infected INS-1 HG and $p = 0.0001$ for INS-1 HG AV-HD vs mock-infected INS-1 HG, $n = 3$, Figure 4(b)), and mTor kinase ($p = 0.0008$ for INS-1 HG AV-LD vs mock-infected INS-1 HG and $p = 0.0001$ for INS-1 HG AV-HD vs mock-infected INS-1 HG, $n = 35$ (Figure 4(b)).

shRNA-mediated PHLPP2 and PHLPP1 knock-down reestablishes the balance of apoptotic factor and decreases the activation of autophagic pathways.

We thus assessed if the reduced PHLPPs levels and the restored activation of Akt signaling pathway resulted in improved cell viability. To this end, we measured Bad and Bcl-xL expression and observed that the pro/antiapoptotic factor balance was repristinated in both INS-1 AV-LD and INS-1 HG AV-HD as compared to mock-infected INS-1 HG Bad: ($p = 0.0007$ for INS-1 HG AV-LD vs mock-infected INS-1 HG and $p = 0.0001$ for INS-1 HG AV-HD vs mock-infected INS-1 HG, $n = 4$, Bcl-xL $p = 0.0008$ for both INS-1 HG AV-LD and INS-1 HG AV-HD vs mock-infected INS-1 HG, $n = 3$, Figure 5(a)). In keeping with these results, we observed also a significantly decreased caspase-3 cleavage in INS-1 HG infected with either AV-LD or AV-HD ($p = 0.0001$ for INS-1 HG AV-LD vs mock-infected INS-1 HG and $p = 0.0002$ for INS-1 HG AV-HD vs mock-infected INS-1 HG, $n = 4$). Notably, while the effects obtained with the higher AV dose were of a higher magnitude as far as Bcl proteins expression was concerned, INS-1 HG AV-LD showed a lower caspase activity than cells infected with the higher AV dose; this may reflect a PHLPP-independent effect and be related to the AV itself (Figure 5(b)). The restored prosurvival profile was paralleled by decreased expression of the LC3-II ($p = 0.0001$ for both INS-1 HG AV-LD and INS-1 HG AV-HD vs mock-infected INS-1 HG, $n = 5$, Figures 5(a) and 5(b)).

shRNA-mediated PHLPP2 and PHLPP1 knock-down was not sufficient to restore insulin mRNA levels or glucose-stimulated insulin secretion.

We assessed if PHLPPs knock-down also resulted in a recovered INS-1 HG functionality. We thus measured INS-1 mRNA expression levels by real-time RT-PCR and observed no significant increase in cells infected with neither AV dose as compared to mock-infected cells (Figure 6(a), $n = 3$). Similarly, glucose-stimulated insulin secretion remained impaired in knocked down cells as the amount of insulin released in response to increasing glucose concentration was no different between INS-1 HG AV-LD, INS-1 HG AV-HD, and mock-infected INS-1 HG (Figure 6(b), $n = 4$).

4. Conclusions

In the present study, we report that the altered pancreatic β-cell homeostasis observed upon chronic exposure to 30 mM glucose is paralleled by increased expression of PHLPPs, with a consequent reduction of the phosphorylation levels of their primary target, the serine threonine kinase Akt. Interestingly, knocking-down PHLPPs, throughout adenoviral-mediated shRNA delivery, we were able to restore a prosurvival profile in INS-1 HG cells chronically exposed to high glucose levels. Specifically, the ratio between the proapoptotic factor Bad and its prosurvival counterpart Bcl-xL went back to the value measured in healthy INS-1 NG cells. Bad levels have been suggested to be directly regulated by Akt pathway [25], and indeed, we observed significantly increased phosphorylation of Akt and of its major antiapoptotic effector FoxO1 [26] in INS-1 HG infected with the adenoviral constructs encoding for specific shRNA sequences against PHLPP2 and PHLPP1. The insulin-stimulated activation of another Akt substrate mTor was also significantly improved when PHLPPs expression was knocked down. mTor senses nutrient availability and regulates cell homeostasis, and it has been suggested that its loss impairs β-cells homeostasis as well as insulin sensitivity in peripheral tissues [27]. Among the intracellular processes controlled by mTor, autophagy as gained attention as a possible player in the survival of pancreatic β-cells with conflicting data showing, in different experimental models, pro- or antiapoptotic effects of an increased activation of autophagic pathways [28–30]. In our model, the recovered prosurvival profile was paralleled by decreased expression of the autophagic marker LC3-II, supporting the hypothesis of a negative impact of a disproportionated activation of autophagic pathways on cell survival.

At odds with the restored cell survival profile, efficient glucose-stimulated insulin secretion and synthesis were not recovered by INS-1 HG infected with adenoviral vectors carrying the shRNA sequences against PHLPP2/1. It has been reported that Akt isoforms have different roles in pancreatic β-cells, with Akt1 mainly controlling cell survival and Akt2 mostly involved in the regulation of insulin secretion [9], and it has also been suggested that PHLPP family members possess a selective preferences toward Akt isoforms, as PHLPP2 seems to prefer Akt1 and PHLPP1 favors Akt2, even if this may depend on the predominantly expressed substrate isoform [17, 18]. Since we obtained an almost complete silencing of PHLPP2 in INS-1 cells infected with the highest adenoviral concentration (INS-1 HG AV-HD), while the maximal reduction of PHLPP1 achieved was around 65%, it may be possible to

hypothesize that Akt2 function, and consequently insulin secretion and synthesis, was less efficiently restored. However, the observation that no significant difference was observed when comparing INS-1 HG AV-HD, expressing 45% of PHLPP1 with respect to mock-infected INS-1 HG, with cells infected with a lower adenoviral titer with PHLPP1 levels around 85% of those observed in mock INS-1 HG (INS-1 HG AV-LD), renders this explanation quite unlikely and rather points to the possibility that glucose toxicity may more profoundly damage β-cell function than β-cell survival, causing a depletion of insulin deposits that may not be restored with a short-term improvement of Akt activation [31, 32]. Indeed, we believe that any attempt to restore β-cell homeostasis in a *in vivo* setting should not overlook the important difference among the mechanisms regulating β-cell mass and those regulating specific β-cell functions such as insulin synthesis and secretion. The lack of significant differences between INS-1 HG AV-LD and INS-1 HG AV-HD cells for the majority of functional and molecular read-out analyzed also suggests that PHLPP2 may play a more pivotal role than the cognate protein, PHLPP1, in the dysregulation of beta-cell survival. Our data thus contribute a small piece of knowledge to the comprehension of the specific functions of PHLPP family members and to the less explored mechanisms regulating their own expressions [18, 33]. Interestingly, a few years ago, we and others showed a specific increase of PHLPP1, with unaltered PHLPP2 levels, in adipose tissue and skeletal muscle biopsies of obese, insulin-resistant individuals [14, 34]; these data have been more recently confirmed by Behera et al. in a high-fat fed animal model [35]. Here, we report a significant increase of both isoenzymes upon exposure to high glucose concentrations; however, PHLPP2 showed the larger and more statistical sound changes. The results of the shRNA-mediated inhibition experiments confirm a preeminent role of PHLPP2 in the regulation of pancreatic β-cells homeostasis.

Furthermore, in the older study, we did not observe any direct correlation between PHLPP1 expression and glucose levels, while the agent of the increased expression appeared to be insulin levels. In contrast in the present study, PHLPPs levels were increased in response to high glucose concentration, even if our data do not allow to establish if glucose is able to directly promote PHLPP transcription or transduction or if the increase in PHLPP protein levels was mediated by indirect mechanisms [14]. Nonetheless our results underlie that PHLPPs possess a specificity that has not been fully explored to date and may be differentially regulated in different tissue. Clarifying this point is mandatory in order to exploit these phosphatases as possible pharmacological targets.

In conclusion, our data provide initial support for future studies aimed to identify pharmacological PHLPPs modulator to treat beta-cell survival impairment. They also contribute to shed some light on β-cell dysfunction, a complex and unsatisfactorily characterized phenomenon that has a central causative role in the pathogenesis of type 2 diabetes.

Data Availability

All data used to support the findings of the study are included within the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

TG : HDL-C Ratio Is a Good Marker to Identify Children Affected by Obesity with Increased Cardiometabolic Risk and Insulin Resistance

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Metabolic syndrome (MetS) is an important predictor of cardiovascular diseases in adulthood. This study aims to examine the clinical utility of triglyceride to high-density lipoprotein ratio (TG : HDL-C) in identifying cardiometabolic risk and insulin resistance (IR) among children with obesity, in comparison with MetS as defined by the International Diabetes Federation (IDF). Data of 232 children with obesity aged 10–16 years were obtained from our study, MyBFF@school study, conducted between January and December 2014. Children were divided into tertiles of TG : HDL-C ratio. The minimum value of the highest tertile was 1.11. Thus, elevated TG : HDL-C ratio was defined as TG : HDL-C ≥1.11. Children with MetS were categorized based on the definition established by the IDF. Out of 232 children, 23 (9.9%) had MetS, out of which 5.6% were boys. Almost twofold of boys and girls had elevated TG : HDL-C ratio compared to MetS: 13.8% vs. 5.6% and 13.8% vs. 4.3%, respectively. Children with elevated TG : HDL-C ratio had lower fasting glucose compared to children with MetS (boys = 5.15 ± 0.4 vs. 6.34 ± 2.85 mmol/l, $p = 0.02$; girls = 5.17 ± 0.28 vs. 6.8 ± 4.3 mmol/l, $p = 0.03$). Additionally, boys with elevated TG : HDL-C ratio had a higher HDL-C level compared to those with MetS (1.08 ± 0.18 vs. 0.96 ± 0.1 mmol/l, $p = 0.03$). There was no significant difference across other MetS-associated risk factors. Overall, TG : HDL-C ratio demonstrated higher sensitivity (42.7% vs. 12.9%) but lower specificity (74.8% vs. 93.2%) than MetS in identifying IR, either in HOMA-IR ≥2.6 for prepubertal children or HOMA-IR ≥4 for pubertal children. TG : HDL-C ratio in children with obesity is thus as useful as the diagnosis of MetS. It should be considered an additional component to MetS, especially as a surrogate marker for IR.

1. Introduction

Metabolic syndrome (MetS) is defined as the clustering of risk factors for cardiovascular diseases (CVDs) and type 2 diabetes (T2D), which include obesity, dyslipidemia, hypertension, and glucose intolerance [1]. With the increasing prevalence of overweight and obesity among children and youths, the "pediatric metabolic syndrome" has become a global public health concern. Children and adolescents with MetS are at greater risks of developing cardiovascular complications early, during the most productive years of their adult life [2].

Early identification and intervention are therefore crucial [3]. However, cutoffs and individual components used to diagnose MetS in children have not been standardized and need further elucidation. Among the most common

definitions used for MetS is the one proposed by the International Diabetes Federation (IDF) [4], with fixed cutoffs for blood pressure, lipids, glycemia, and abdominal circumference points assessed by percentile. For children aged 10 years or older, the IDF proposed that the diagnosis of MetS should be based on waist circumference ≥90th percentile and the presence of two or more clinical features: elevated triglycerides, low HDL cholesterol, high blood pressure, or increased plasma glucose. Using this definition, a study involving 1014 Malaysian children aged 13 years showed that 258 (25.4%) were overweight/obese, out of which 10% had MetS [5].

Insulin resistance (IR) and central obesity are among the factors contributing to the anthropometric, physiological, and biochemical abnormalities in those with MetS [6, 7]. Nevertheless, due to the invasiveness and difficulty of measuring IR, the current definition of MetS in pediatric population regards elevated fasting glucose as a marker for glucose intolerance. Although the ability of the IDF criteria to predict CVD events has been established [8–10], the diagnosis of MetS is often made at the clinic or hospital level. Given the increasing burden of obesity among children, there is a need for an alternative: a simple method or tool with good sensitivity/specificity to identify children at risk of cardiometabolic diseases and IR in the community.

Current research has demonstrated the usefulness of triglyceride to high-density lipoprotein (TG : HDL-C) ratio in predicting cardiometabolic risk and IR [11–15]. However, evidence also indicated that there are ethnic variations [16, 17] and that genetic background is important in determining the presence of MetS among obese children [18]. Our study contributes to the existing literature by reporting the use of TG : HDL-C ratio in identifying cardiometabolic risk and IR among obese Malay school children, in comparison with MetS as defined by the IDF. In an earlier report, we showed that a significantly higher number of obese ethnic Malay school children at a higher tertile of TG : HDL-C ratio had acanthosis nigricans (AN) and MetS [11].

2. Materials and Methods

2.1. Study Design and Population. This study employed a cross-sectional design. Data were obtained from the MyBFF@school study conducted between January and December 2014 in Malaysia. MyBFF@school was a school-based lifestyle intervention program that included nutritional, physical activity, and psychology modules specifically designed for children with obesity. Detailed descriptions of the methodology have been previously published [11]. Ethical approval was granted by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia. Written informed consent was obtained from parents or guardians, and every child was required to sign an assent form. All tests were performed in accordance with the approved guidelines.

2.2. Health and Physical Examination. Prior to health and physical examination, children were asked to fast overnight for at least 8 hours. Anthropometric measurements were performed by trained personnel, and health examinations were performed by medical officers and pediatricians. Standing height was measured without shoes to the nearest 0.1 cm using a calibrated stadiometer (Seca 217, Germany). Body weight and body fat mass were measured in light clothing without shoes and socks to the nearest 0.1 kg using a precalibrated body impedance analyzer (InBody 720, Korea). Waist circumference was measured two times to the nearest 0.1 cm over the skin midway between the tenth rib and the iliac crest at the end of normal expiration, using a nonextensible tape (Seca 201, Germany), and the mean was recorded. Two readings of blood pressure were measured after 5 minutes of rest using a mercury sphygmomanometer (Accoson, UK) in a seated position with the arm supported at the heart level, and the mean was recorded. Pubertal status was assessed (self-administered) using the Tanner staging scale [19, 20]. Children were also examined—by pediatricians—for the presence of acanthosis nigricans (AN) over the neck [21].

2.3. Biochemical Parameters. Venepuncture was performed by nurses and doctors. Blood samples were transported cold to the central laboratory at the Institute for Medical Research within two hours of collection and processed on the same day. Aliquots of serum/plasma samples were kept at −20°C prior to analysis. The HbA1c level was determined by cationic exchanged high-performance liquid chromatography (Adams A1c HA-8160, Arkray Inc., Japan) following the National Glycohemoglobin Standardization Programme guidelines. Fasting plasma glucose, triglycerides, total cholesterol, HDL, LDL, and liver enzymes (ALT, AST, and GGT) were analyzed using an automated analyzer (Dirui CS-400, China) with reagents purchased from Randox Laboratories (Antrim, UK), and the AST : ALT ratio was calculated. Fasting insulin concentration was measured using an automated enzyme immunoassay analyzer (TOSOH AIA-360, Japan). The interassay coefficient of variability (CV) for insulin at 8.7, 44.4, and 143.2 μU/ml was 2.5%, 2.6%, and 2.4%, respectively.

2.4. Measures. Overweight and obesity were defined as the BMI z-score above 1 and 2 standard deviation, respectively, for age and sex, according to the WHO BMI chart (2008) [22]. Tanner staging was assessed by showing a standardized Tanner staging picture to the child. Stage 1 external genitalia development and breast development for boys and girls were classified as prepubertal, while stage 2 and above were defined as pubertal. AN was determined based on Burke's quantitative dichotomous score [21]. The IR status was based on the homeostasis model assessment (HOMA), calculated by multiplying the value of fasting plasma insulin and fasting plasma glucose and then dividing by 22.5 [21]. The pubertal transition from Tanner stage I to Tanner stage III or IV was associated with IR [23]. For prepubertal children, a score of HOMA ≥2.6 [24] was classified as IR, while a score less than 2.6 was classified as insulin sensitive. For pubertal children, a score of HOMA ≥4.0 was categorized as IR, while a score less than 4.0 was categorized as insulin sensitive [25]. MetS was

established based on the definition proposed by the IDF [4]. It was considered present if the waist circumference measurement was ≥90th percentile of the Malaysian children waist circumference chart [26] with the presence of at least two of the following criteria: triglycerides ≥ 1.7 mmol/L, HDL cholesterol < 1.03 mmol/L, systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, or fasting plasma glucose ≥ 5.6 mmol/L [4]. The AST : ALT ratio less than 1 was categorized as a high risk of NAFLD, while a ratio more than 1 was categorized as a low risk of NAFLD [27].

3. Statistical Analysis

The normality test for continuous data was determined using the Kolmogorov–Smirnov test. Means and standard deviations (SDs) were calculated for continuous variables. Comparison of means between two groups was conducted using the independent-sample *t*-test, while categorical comparisons were made using the chi-square test. Statistical significance was set at 0.05. Sensitivity was calculated as the number of cases with elevated TG : HDL-C ratio or MetS who are IR by the HOMA-IR cutoff, divided by the total number of IR cases. Specificity was calculated as the total number of cases with normal TG : HDL-C ratio or absence of MetS that were insulin sensitive, divided by the total number of insulin-sensitive cases. Analyses were run using IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp., and StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.

4. Results

A total of 425 children with obesity (overweight/obese) participated in the MyBFF@school study, while 274 (65%) children consented for taking blood. Out of these 274 children, 232 were older than 10 years and had complete data of waist circumference and blood samples which were assayed for triglyceride, glucose, high-density lipoprotein (HDL-C), and liver aminotransferases (AST and ALT) and GGT.

Table 1 presents the characteristics of 232 children included in this study according to the sex group. Of 232 children, 23 (9.9%) were found to have MetS, out of whom 5.6% were boys. Pubertal status was comparable between girls and boys, despite the former being slightly older than the latter (13.3 ± 1.98 vs. 12.7 ± 2.0, $p = 0.002$). More girls had AN than boys, but both groups had a similar IR status. Table 2 describes the anthropometric and biochemical characteristics of study children. Even though boys showed higher BMI *z*-scores and larger waist circumference, girls had a higher percentage of body fat (BF) (42.5 ± 4.7 vs. $38.8 \pm 6.7\%$, $p < 0.001$). There was no significant difference in other blood parameters except for liver enzymes. Boys had significantly higher AST, ALT, and GGT levels compared to girls.

Children were divided into tertiles of TG : HDL-C ratio to determine the cut-off values separating the highest tertile from the other two lower tertiles. The minimum value of the highest tertile was 1.11 (Table 3). Table 4 compares the MetS-associated risk factors for girls and boys, divided into those with elevated TG : HDL-C ratio (≥1.11) versus those with a diagnosis of MetS. It was shown that almost twofold of boys and girls had elevated TG : HDL-C ratio compared to MetS (boys = 13.8% vs. 5.6%, girls = 13.8% vs. 4.3%). Children with elevated TG : HDL-C ratio had lower fasting glucose compared to children with MetS (boys = 5.15 ± 0.4 vs. 6.34 ± 2.85, $p = 0.02$; girls = 5.17 ± 0.28 vs. 6.8 ± 4.3 mmol/l, $p = 0.03$) (Figure 1(a)). Additionally, boys with elevated TG : HDL-C ratio had a higher HDL-C level compared to those with MetS (1.08 ± 0.18 vs. 0.96 ± 0.1 mmol/l, $p = 0.03$) (Figure 1(b)). There was no significant difference across other MetS-associated risk factors in either gender/group including the triglyceride level (Figure 1(c)), waist circumference (Figure 1(d)), and blood pressure.

The sensitivity and specificity of TG : HDL-C ratio and MetS in identifying children with either HOMA-IR ≥2.6 or HOMA-IR ≥4 are shown in Table 5. Overall, TG : HDL-C ratio showed higher sensitivity (42.7% vs. 12.9%) but lower specificity (74.8% vs. 93.2%) than MetS in identifying children with either HOMA-IR ≥2.6 or HOMA-IR ≥4.

5. Discussion

The utility of TG : HDL-C ratio in predicting IR among children with obesity has been reported in prior studies [11, 14, 28, 29]. Similarly, studies have also shown the use of TG : HDL-C ratio in identifying children at risk for MetS [14, 30, 31]. In this study, we found that TG : HDL-C ratio ≥1.11 (sensitivity = 42.7%, specificity = 74.8%) separated children with the highest tertile of TG : HDL-C ratio from the remaining two-thirds. To date, no specific TG : HDL-C cutoff has been established for children. We decided to use tertiles as they are a robust estimate and comparable to other studies [28, 30, 32]. Our cut-off value for TG : HDL-C ratio to identify MetS and IR among obese Malay school children is slightly lower than the cut-off value of 1.25 (sensitivity = 80%, specificity = 75%) established for obese Chinese children [30] and 2.0 (sensitivity = 55.6%, specificity = 72.9%) for overweight Korean children [28]. These differences could be attributed to ethnic and genetic variations, which have been said to influence the relationship between TG : HDL-C ratio and MetS across populations [16, 33]. For instance, Asians were reported to be more prone to abdominal obesity than Caucasians [34], so different cutoffs for TG : HDL-C ratio were used to identify IR and MetS.

We found no difference between elevated TG : HDL-C ratio and diagnosis of MetS with respect to all MetS risk markers, except for the glucose level. However, more children were identified at increased risks of cardiovascular diseases and T2D than did a diagnosis of MetS. In addition, we compared liver enzymes levels between those with elevated TG : HDL-C ratio and MetS, as elevated hepatic enzymes in adults were associated with obesity, IR, and T2D [35]. Like other risk factors, there was no difference in liver enzymes between the two groups. This indicates that TG : HDL-C ratio is as useful as the MetS diagnostic criterion in

Table 1: Clinical characteristics of 232 children by gender.

	Boys ($n = 114$, 49.2%)	Girls ($n = 118$, 50.8%)	X^2	p value	All
Age (mean ± SD)	12.7 ± 2.0	13.3 ± 1.98	NA	0.02[a]	12.6 ± 2.01
Pubertal status					
Prepubertal	42 (37.2)	29 (25.4)	3.632	0.07	71 (31.3)
Pubertal (Tanner stage ≥ 2)	71 (62.8)	85 (74.6)			156 (68.7)
Abdominal obesity					
WC < 90th centile	11 (9.6)	15 (12.7)	0.547	0.46	26 (11.2)
WC ≥ 90th centile	103 (90.4)	103 (87.3)			206 (88.8)
BMI z-score >1 or 2 SD					
Overweight	17 (14.9)	33 (28)	5.884	0.02	50 (21.6)
Obese	97 (85.1)	85 (72)			182 (78.4)
Acanthosis nigricans					
Presence	47 (42)	74 (65.5)	12.52	<0.001	121 (53.8)
Absence	65 (58)	39 (34.5)			104 (46.2)
Insulin resistance					
Prepubertal					
HOMA-IR < 2.6	15 (35.7)	9 (31)	0.168	0.68	24 (33.8)
HOMA-IR ≥ 2.6	27 (64.3)	20 (68.9)			47 (66.2)
Pubertal					
HOMA-IR < 4	35 (49.2)	44 (54.3)	0.094	0.76	79 (50.6)
HOMA-IR ≥ 4	36 (50.8)	41 (45.7)			77 (49.4)
Liver enzyme test					
Low risk (ALT : AST >1)	97 (85.1)	104 (88.1)	0.465	0.495	201 (86.6)
High risk (ALT : AST≤1)	17 (14.9)	14 (11.9)			31 (13.4)
Metabolic syndrome					
Nonmetabolic syndrome	101 (88.6)	108 (91.5)	0.557	0.455	209 (90.1)
With metabolic syndrome	13 (11.4)	10 (8.5)			23 (9.9)

[a]Independent-group t-test; NA: not available.

Table 2: Anthropometric and biochemical characteristics of 232 children.

Cardiovascular risk factors	Boys ($n = 114$)	Girls ($n = 118$)	p value	All
Obesity				
Mean BMI z-score	2.8 ± 0.9	2.4 ± 0.7	<0.001	1.04 ± 0.82
Mean waist circumference (cm)	91.1 ± 10.9	85.6 ± 8.3	<0.001	88.34 ± 10.1
Mean body fat (%)	38.8 ± 6.7	42.5 ± 4.7	<0.001	40.7 ± 6
Blood lipids				
Mean total cholesterol (mmol/l)	4.62 ± 0.76	4.56 ± 0.8	0.6	4.59 ± 0.79
Mean TG (mmol/l)	1.1 ± 0.5	1.1 ± 0.5	0.23	1.1 ± 0.46
Mean HDL-C (mmol/l)	1.1 ± 0.2	1.17 ± 0.2	0.32	1.2 ± 0.2
Mean LDL-C (mmol/l)	3.2 ± 0.86	3.2 ± 0.82	0.9	3.2 ± 0.84
Blood pressure				
Mean systolic blood pressure (mmHg)	111 ± 11	108 ± 10	0.11	110 ± 10
Mean diastolic blood pressure (mmHg)	73 ± 9	69 ± 9	0.01	70 ± 9
Insulin resistance markers				
Mean TG : HDL-C	0.95 ± 0.47	1.04 ± 0.53	0.16	0.99 ± 0.5
Mean fasting glucose (mmol/l)	5.4 ± 1.35	5.4 ± 1.1	0.23	5.39 ± 1.22
Mean insulin	18.4 ± 12.2	21.59 ± 26.8	0.243	
Liver enzymes/NAFLD blood markers				
Mean ALT (U/l)	19.1 ± 18.1	13.3 ± 11.7	0.004	16.1 ± 15
Mean AST (U/l)	27.6 ± 21.67	20.06 ± 12.55	0.001	23.7 ± 18
Mean GGT (U/l)	31.9 ± 34.35	20.8 ± 13.6	0.001	26.2 ± 26.5
Mean AST : ALT	2.07 ± 1.9	1.89 ± 1.8	0.447	1.9 ± 1.8

p value was obtained from comparison of means using the independent-sample t-test.

Table 3: Minimum, mean, and maximum of the plasma TG : HDL-C concentration ratio tertiles by gender.

	Boys	Girls	All
Tertiles 1	(0.33, 0.57, 0.7)	(0.27, 0.55.0.71)	(0.27, 0.56, 0.71)
Tertiles 2	(0.72, 0.9, 1.1)	(0.71, 0.88, 1.1)	(0.71, 0.89, 1.1)
Tertiles 3	(1.11, 1.58, 3.21)	(1.11, 1.48, 2.75)	(1.11, 1.54, 3.21)

Table 4: Comparison of metabolic syndrome-associated risk factors between children with metabolic syndrome (MetS) and elevated TG : HDL-C ratio (TG : HDL-C ≥ 1.11).

Biochemical/clinical parameters	Boys ($n = 45$)			Girls ($n = 42$)		
	Elevated TG : HDL-C ≥1.11 ($n = 32$, 71.1%)	MetS ($n = 13$, 28.9%)	p value	TG : HDL-C ≥1.11 ($n = 32$, 75%)	MetS ($n = 10$, 25%)	p value
Age	12.6 ± 1.97	13.1 ± 1.26	0.482	12.6 ± 1.9	13.2 ± 1.5	0.46
Cardiovascular risk factors						
Obesity						
Mean BMI z-score	2.9 ± 0.9	2.88 ± 0.6	0.95	2.6 ± 0.7	2.5 ± 0.5	0.78
Mean body fat (%)	38.5 ± 7.3	39.7 ± 4.9	0.623	42.6 ± 4.9	43.9 ± 3.7	0.337
Blood lipids						
Mean total cholesterol (mmol/l)	4.68 ± 0.78	4.37 ± 0.9	0.253	4.5 ± 0.91	4.67 ± 0.9	0.623
Mean LDL-C (mmol/l)	3.33 ± 0.84	3.2 ± 1.14	0.636	3.2 ± 0.98	3.4 ± 0.74	0.439
Blood pressure						
Mean systolic blood pressure (mmHg)	112 ± 9	116 ± 11	0.26	108 ± 12	111 ± 9	0.476
Mean diastolic blood pressure (mmHg)	73 ± 8	75 ± 8	0.468	69 ± 10	70 ± 7	0.885
Insulin resistance markers						
Mean TG : HDL-C	1.44 ± 0.33	1.69 ± 0.83	0.17	1.34 ± 0.25	1.66 ± 0.77	0.045
Mean insulin	21.68 ± 13.44	22.54 ± 22.54	0.844	20.6 ± 11.9	26.7 ± 23.27	0.28
Liver enzymes/NAFLD blood markers						
Mean ALT (U/l)	22.6 ± 17.3	19.23 ± 19.4	0.57	14.8 ± 10.9	22.2 ± 27.2	0.229
Mean AST (U/l)	32 ± 28.24	27.7 ± 24.76	0.634	23.3 ± 16.4	25.1 ± 21.6	0.80
Mean GGT (U/l)	34.0 ± 25.3	43.23 ± 64.9	0.507	21.2 ± 13.2	30.7 ± 19.9	0.09
Mean AST : ALT	1.64 ± 1.1	1.64 ± 0.62	0.98	2.36 ± 3.14	1.7 ± 1.1	0.64

p value was obtained from comparison of means using the independent-group t-test; NA: not available.

providing clinical information. Compared to MetS, TG : HDL-C ratio offered higher sensitivity (42.7% vs. 12.9%) but lower specificity (74.8% vs. 93.2%) in identifying children with IR among Malay children with obesity. Similar specificity (≈74%) but greater sensitivity (55.6%) of TG : HDL-C ratio were reported in a study of Korean children and adolescents with obesity [28]. However, the study used a lower value of HOMA-IR (≥3) and a higher TG : HDL-C ratio cutoff (≥2) to identify subjects with IR. Currently, there is no consensus on the HOMA-IR cut-off value which ranged between 1.14 and 5.56 [36, 37]. Despite the discrepancies of the HOMA-IR cutoffs, our findings suggested that TG : HDL-C ratio offers a slightly improved diagnostic value to screen for comorbidity, especially IR, among children with obesity. The International Society for Pediatric and Adolescent Diabetes (ISPAD) in its 2018 guidelines suggested that nonfasting lipid profiles are adequate for the screening of comorbidities among T2D pediatric population [38]. Thus, TG : HDL-C ratio can be an alternative, simple screening tool for comorbidities at the community or public health level.

We found that girls had higher body fat (BF) percentage despite showing lower BMI z-scores and smaller waist circumference compared to boys (Table 2). This is in line with an earlier study finding that the relationships between BMI and BF percentage were heterogeneous and varied between sexes and ethnic groups and across age groups [39]. There is a need, therefore, to reevaluate BF-based definition of obesity among the pediatric population. On the contrary, our results showed that boys had higher liver enzyme concentration than girls. This is consistent with the findings of Bussler et al. on the effects of gender on liver enzyme concentration [40] Differences between the two sexes are due to interactions between sex hormones and metabolic processes [41, 42], and it has been suggested that estrogen signaling in women protects against the development of IR and nonalcoholic fatty liver disease [43].

The application of TG : HDL-C ratio could be extended to distinguish metabolically healthy obese (MHO) individuals from unhealthy obese individuals. Despite meeting traditional BMI criteria for obesity, there appeared to be a normal/healthy metabolic profile that sets MHO individuals apart from their metabolically unhealthy obese counterparts [44]. To date, there is no universal definition for MHO, and research into the pediatric population is relatively lacking. However, for adults, MHO is most often defined using IR [45]. It was reported that, among other parameters (BMI, waist circumference, and apolipoprotein-B), MHO subjects showed lower TG : HDL-C ratio compared to those affected by obesity with cardiometabolic risk [46]. Another study showed that MHO individuals had lower levels of liver enzymes (such as ALT, AST, and GGT) and less fat in the liver [46]. Hence, TG : HDL-C ratio can be considered an additional marker to distinguish between MHO and metabolically unhealthy obese subjects. As shown in our study, those with elevated TG : HDL-C ratio had comparable risk to those with MetS. Having said this, the usefulness of TG : HDL-C ratio as a marker to distinguish between MHO and metabolically unhealthy obesity is beyond the scope of this paper.

Our study has a number of limitations: First, the prevalence of MetS reported might differ if a different

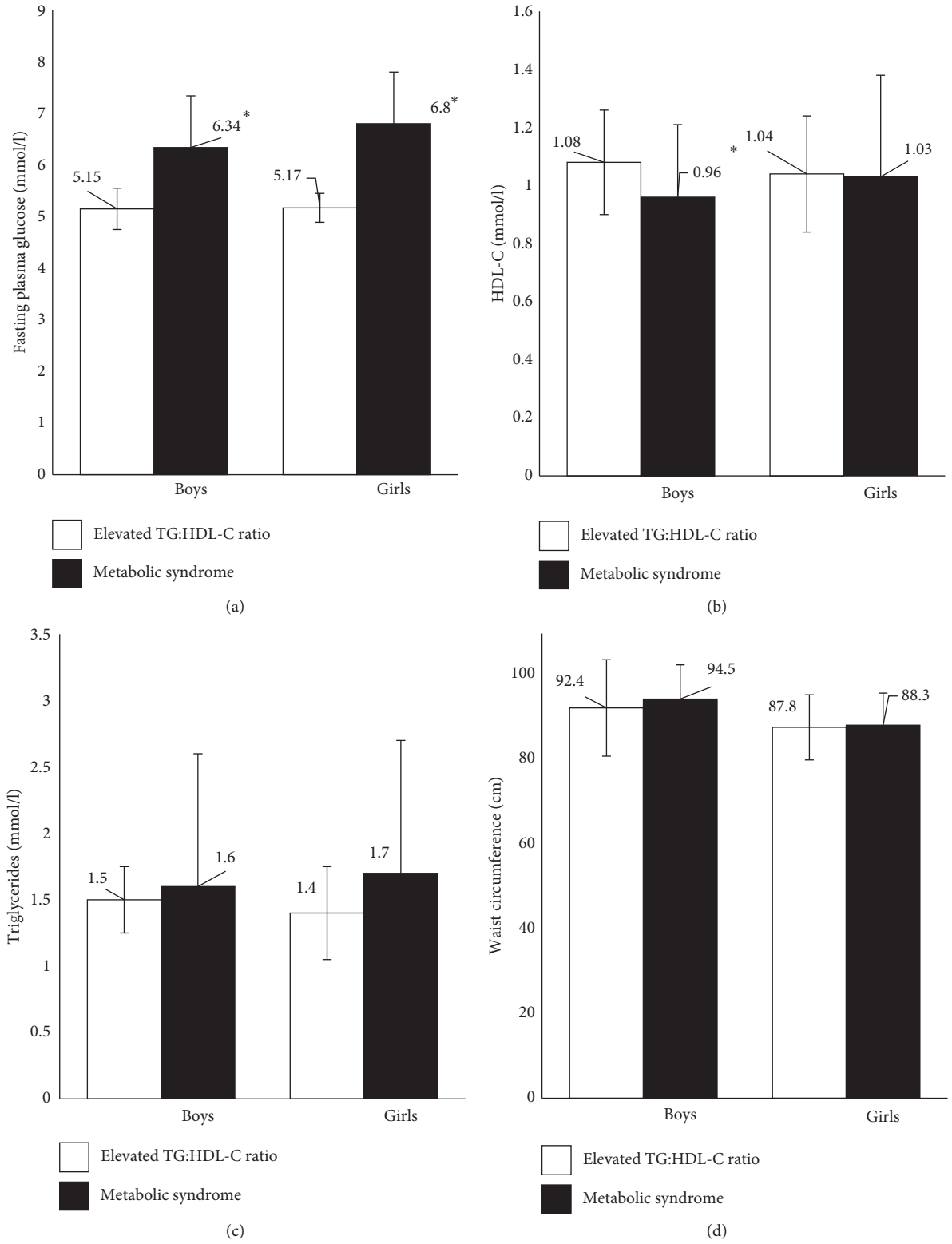

Figure 1: Comparison of (a) fasting plasma glucose, (b) HDL-C, (c) triglycerides, and (d) waist circumference between children with metabolic syndrome and elevated TG : HDL-C ratio. * p value < 0.005 by the independent-group t-test.

definition of MetS was used. Our definition of MetS was based on the IDF, with fixed cutoffs for blood pressure, lipids, glucose, and abdominal circumference assessed by percentile and according to age groups. Although this is convenient, some children with MetS might have been excluded since the IDF requires central/abdominal obesity—measured by

Table 5: % sensitivity (true positives/all positives) and specificity (true negatives/all negatives) of HOMA-IR ≥4 (pubertal) and HOMA-IR ≥ 2.6 (prepubertal) for TG : HDL-C ratio ≥ 1.11 and metabolic syndrome.

	TG : HDL-C ratio ≥ 1.11		Metabolic syndrome	
	Sensitivity	Specificity	Sensitivity	Specificity
HOMA-IR ≥2.6 or≥4	42.7 (53/124)	74.8 (69/103)	12.9 (16/124)	92 (96/103)

waist circumference—as a prerequisite for diagnosing MetS [47]. Second, our study subjects are restricted to ethnic Malay children. This does not represent the true multiracial population in Malaysia. It has long been established that the predictability of TG : HDL-C ratio as a surrogate marker for IR is influenced by ethnic and genetic variations [16, 33]. Our future studies thus will attempt to address this limitation—by obtaining adequate representation of all ethnic groups—in order to evaluate the use of TG : HDL-C ratio as a measure of IR among Malaysian children.

6. Conclusion

In conclusion, the determination of TG : HDL-C ratio among obese Malay children provided equally useful clinical information to MetS. TG : HDL-C ratio should be considered an additional component to MetS, as a surrogate marker for IR. Additionally, the advantage of TG : HDL-C ratio is its routine measurement in children with obesity that can be done in a nonfasting state and conveniently measured using a portable analyzer with good precision at low cost. Given that existing studies on TG : HDL-C ratio are mostly cross-sectional in nature, there is a need to carry out longitudinal studies across different ethnic groups to evaluate the utility of TG : HDL-C ratio in identifying those at risk for T2D and CVDs.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

miR-98-5p Alleviated Epithelial-to-Mesenchymal Transition and Renal Fibrosis via Targeting Hmga2 in Diabetic Nephropathy

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Recently, microRNAs have been recognized as crucial regulators of diabetic nephropathy (DN) development. Epithelial-to-mesenchymal transition (EMT) can play a significant role in tubulointerstitial fibrosis, and it is a hallmark of diabetic nephropathy progression. Nevertheless, the function of miR-98-5p in the modulation of EMT and renal fibrosis during DN remains barely investigated. Hence, identifying the mechanisms of miR-98-5p in regulating EMT and fibrosis is of huge significance. In our present research, decreased miR-98-5p was demonstrated in db/db mice and mice mesangial cells treated with the high dose of glucose. Meanwhile, activated EMT and increased fibrosis was accompanied with the decrease of miR-98-5p *in vitro* and *in vivo*. Additionally, to further find out the roles of miR-98-5p in DN development, overexpression of miR-98-5p was applied. Firstly, *in vivo* investigation exhibited that elevation of miR-98-5p restrained proteinuria, serum creatinine, BUN, the EMT process, and fibrosis. Furthermore, high glucose was able to promote mice mesangial cell proliferation, EMT process, and induced renal fibrosis, which could be prevented by overexpression of miR-98-5p. Moreover, high mobility group A (HMGA2) can exhibit an important role in diverse biological processes. Here, HMGA2 was investigated as a target of miR-98-5p currently. Luciferase reporter assay was conducted and the correlation of miR-98-5p and HMGA2 was validated. Moreover, it was displayed that HMGA2 was remarkably elevated in db/db mice and mice mesangial cells. Furthermore, miR-98-5p strongly depressed HMGA2 protein and mRNA levels in mice mesangial cells. Overall, these revealed miR-98-5p could suppress the EMT process and renal fibrosis through targeting HMGA2 in DN.

1. Introduction

DN is a common complication of diabetes, which can contribute to ESRD all over the world [1, 2]. Increasing evidences have demonstrated that inflammatory process, oxidative stress, and autophagy are responsible for the progression of DN [3–5]. As a frequent complication of diabetes, DN still cannot be treated effectively, and it is urgent to develop more effective methods to repress its progression [6].

As well known, EMT is a kind of biological course, and epithelial cells can transdifferentiate into mesenchymal cells in this process. This process is able to contribute a lot to pathological fibrosis and cancer development [7, 8]. As reported, in the period of EMT, epithelial cells will drop the apical-basal polarity and junctions, which leads to the phenotypes of mesenchymal cells. The mesenchymal cells have increased migratory and invasive ability, which results in the accumulation of ECM components [9]. For example, epithelial cells going through the EMT process are involved in kidney fibrosis [10]. Moreover, in human renal tissues, mesenchymal indicator-positive TECs is related with the upregulated serum creatinine levels [11]. These studies have revealed a crucial role of EMT and renal fibrosis in DN pathogenesis.

MicroRNAs are small noncoding RNAs involved in multiple processes [12]. They can regulate protein expression via mRNA degradation or translational repression [13–15].

In recent studies, they have reported that increasing number of microRNAs can regulate the progression of DN [16]. For instance, miR-146a exerts an anti-inflammation role in DN pathogenesis [17]. miR-27a induces podocyte injury through activation of β-catenin in DN [18]. Besides these, via targeting PTEN and SMAD7, miR-21 promotes renal fibrosis in DN [19]. Up to now, the biological function of miR-98-5p in the EMT process and renal fibrosis of DN progression remains barely investigated. Hence, we concentrated on the mechanisms of miR-98-5p in DN development.

Currently, we hypothesized that miR-98-5p was involved in DN via the EMT process. In our present study, it was revealed miR-98-5p was a significant modulator of EMT and renal fibrosis in DN via targeting HMGA2. Enhancement of miR-98-5p attenuated mice mesangial cells proliferation and the progression of EMT and renal fibrosis.

2. Materials and Methods

2.1. Animals. Animal experiments were based on the standards of the NIH Instructions for the Care and Use of Laboratory Animals. The study was approved by the ethics committee of Qingpu Branch of Zhongshan Hospital affiliated to Fudan University. Male db/db mice with the background of C57BL/Ks and the control C57BL/Ks mice were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). Mice were kept in the center with 12-hour light and 12-hour dark periods, and they were provided water and food without limitations. Db/db mice were divided into two groups (LV-NC and LV-miR-98-5p, $n = 8$ for every group) at random. At the age of 12 weeks, the mice were injected with LV-miR-98-5p or LV-NC via the tail vein. Subsequently, anaesthetization was carried out by a xylazine-ketamine mixture intraperitoneal injection before all the mice were sacrificed at 24 weeks.

2.2. Blood and Urine Determination. Blood glucose was tested using the Glucose LiquiColor Test (Stanbio Laboratory, Boerne, TX, USA). 24-hour urine was obtained using the metabolic cage every four weeks. Serum creatinine, BUN level, and urinary creatinine level were examined on an AEROSET clinical chemistry system (Abbott Laboratories, Chicago, IL, USA). Urine albumin concentration was detected using the mice albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA).

2.3. Cell Culture. Mouse mesangial cells and HEK-293T cells were purchased from the ATCC (Manassas, VA, USA). DMEM medium with 10% FBS (Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) was employed to maintain all the cells. Cells were grown at a 5% CO2 atmosphere at 37°C.

2.4. CCK8 Assay. Cells were plated in a 96-well plate for a whole night. Then, cell viability was tested using the CCK-8 method (CCK8, Dojindo, Japan).

2.5. EdU Assay. EdU experiment was conducted using a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Shanghai, PR, China). After treating with 50 mM EdU for two hours, 4% paraformaldehyde was used to fix the cells. Then, the cells were stained by Apollo Dye Solution with the nucleic acid stained by Hoechst-33342. Images were observed using an Olympus FSX100 microscope.

2.6. qRT-PCR. Total RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed using the first-strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Detection of the expression of miR-98-5p and mRNA expression of E-cadherin, N-cadherin, HMGA2, TGF-β1, COL4A1, and qPCR was carried out using a SYBR Premix Ex Taq II (TaKaRaBio Technology, Dalian, China) on the 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Primers were synthesized by GenePharma (Shanghai, China). The primers used are listed in Table 1. U6 RNA was employed as an internal microRNA control. GAPDH acted as an internal mRNA control. Fold change was calculated using $2^{-\Delta\Delta Ct}$.

2.7. Western Blot. Cells were harvested, and the lysis buffer was used to extract the cell proteins. Protein extracts were boiled, and cell extracts were isolated on 10% SDS-PAGE gels. Then, the protein bands were transferred onto PVDF membranes. The primary antibodies E-cadherin, N-cadherin, HMGA2, TGF-β1, COL4A1, and GAPDH were incubated with the membranes for a whole night at 4°C. The next day, secondary antibodies linked by HRP were used. The immunoreactive bands were exposed using ECL-PLUS/Kit (GE Healthcare, Piscataway, NJ, USA).

2.8. Dual Luciferase Assay. The binding sites between HMGA2 and miR-98-5p were predicted by TargetScan (http://www.targetscan.org/vert_71/). The 3′-untranslated region (UTR) of HMGA2 was amplified from cDNA of HEK-293T cells and inserted into pMIR (Promega Corporation, Madison, WI, USA). pMIR-HMGA2 3′-UTR or pMIR-HMGA2 3′-UTR mutant was transfected into HEK-293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 hours, the activity of luciferase and *Renilla* activity were detected using a Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

2.9. Statistical Analysis. Data were manifested as mean ± SD and analyzed by SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The Student's *t*-test and ANOVA were carried out among different groups. Differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. miR-98-5p was Downregulated in DN. Firstly, to investigate the role of miR-98-5p in DN development, db/db mice with C57BL/Ks background were employed in our study. During the period, we observed that the blood glucose

Table 1: Primers used for real-time PCR.

Genes	Forward (5′–3′)	Reverse (5′–3′)
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG
miR-98-5p	ATCCAGTGCGTGTCGTG	TGCTTGAGGTAGTAAGTTG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
E-cadherin	CAATCTCAAGCTCATGG	CCATTCGTTCAAGTAGTC
N-cadherin	GTGCATGAAGGACAGCCTCT	CCACCTTAAAATCTGCAGGC
HMGA2	TGGGAGGAGCGAAATCTAA	GGTGAACTCAAGCCGAAG
TGF-β1	TATTGAGCACCTTGGGCACT	ACCTCTCTGGGCTTGTTTCC
COL4A1	CTCTGGCTGTGGCAAATGTG	CCTCAGGTCCTTGCATTCCA

and the urine ACR in db/db mice were increased from 12 weeks progressively (Figures 1(a) and 1(b)). Additionally, at 24 weeks, the serum creatinine and BUN were examined and as exhibited, they were remarkably upregulated in db/db mice (Figures 1(c) and 1(d), $n = 8$ for each group). Furthermore, as shown in Figure 1(e), in the kidney tissues of db/db mice, miR-98-5p was greatly decreased ($n = 8$ for each group).

3.2. miR-98-5p Reduced the Renal Dysfunction of db/db Mice. Then, to find out whether miR-98-5p can modulate renal dysfunction of db/db mice, the level of miR-98-5p in db/db mice was modulated by the lentivirus system. By LV-miR-98-5p delivery, as exhibited, miR-98-5p was efficiently increased in the renal cortex after 3 months (Figure 2(a), $n = 8$ for each group). In Figure 2(b), increase of miR-98-5p reduced hyperglycemia development. Besides, LV-miR-98-5p treatment attenuated the urinary protein excretion compared with the control mice (Figure 2(c)). Additionally, overexpression of miR-98-5p repressed creatinine of the serum and BUN level (Figures 2(d) and 2(e), $n = 8$ for each group).

3.3. EMT and Renal Fibrosis Was Induced in db/db Mice. Next, the expression of EMT biomarkers (E-cadherin and N-cadherin) was determined using qRT-PCR and western blot assays in the renal cortex. The results in Figures 3(a) and 3(b) showed that E-cadherin mRNA expression was remarkably reduced in db/db mice, while N-cadherin was enhanced ($n = 8$ for each group). Consistent results were observed in western blot experiments. Figures 3(c)–3(e) exhibit that EMT was obviously triggered in db/db mice ($n = 8$ for each group). Meanwhile, renal fibrosis biomarkers (TGF-β1 and COL4A1) were tested using qRT-PCR and western blots. The results in Figures 3(f) and 3(g) indicated that TGF-β1 and COL4A1 mRNA expression were obviously elevated in db/db mice ($n = 8$ for each group). In addition, TGF-β1 and COL4A1 protein expression were also increased in db/db mice (Figures 3(h)–3(j), $n = 8$ for each group).

3.4. EMT and Renal Fibrosis Were Repressed by miR-98-5p in db/db Mice. Moreover, after miR-98-5p was greatly overexpressed, shown in Figures 4(a) and 4(b), E-cadherin mRNA level was obviously increased with a decrease of N-cadherin mRNA expression ($n = 8$ for each group). Apart from these, western blot assays shown in Figures 4(c)–4(e) manifested that E-cadherin protein was induced and N-cadherin was reduced by miR-98-5p upregulation in db/db mice ($n = 8$ for each group). Meanwhile, renal fibrosis biomarkers (TGF-β1 and COL4A1) were tested using qRT-PCR and western blot assays. Furthermore, we found that TGF-β1 and COL4A1 mRNA expression were obviously repressed by overexpression of miR-98-5p *in vivo* (Figures 4(f) and 4(g), $n = 8$ for each group). Consistently, TGF-β1 and COL4A1 protein expression were restrained by miR-98-5p in db/db mice (Figures 4(h)–4(j), $n = 8$ for each group).

3.5. miR-98-5p Suppressed Mouse Mesangial Cell Proliferation, EMT, and Renal Fibrosis. Furthermore, as indicated in Figure 5(a), miR-98-5p expression was upregulated in mouse mesangial cells treated with HG (25 mM glucose). To study whether miR-98-5p regulated mouse mesangial cell proliferation, LV-miR-98-5p was infected into mouse mesangial cells and it was successfully increased (Figure 5(b)). CCK-8 assay was conducted, and as displayed in Figure 5(c), LV-miR-98-5p greatly repressed cell growth. Additionally, EDU assay (Figures 5(d) and 5(e)) implied that cell proliferation was triggered by high glucose and miR-98-5p reversed this process. Besides these, results of western blot assays shown in Figures 5(f)–5(h) suggested that E-cadherin protein was increased and N-cadherin was inhibited by miR-98-5p *in vitro*. Additionally, in Figures 5(i)–5(k), western blot data revealed that TGF-β1 and COL4A1 protein expression were retarded by miR-98-5p.

3.6. miR-98-5p Targeted HMGA2. Subsequently, HMGA2 was searched as the target of miR-98-5p. Luciferase reporter plasmids of WT-HMGA2 and MUT-HMGA2 binding sites were manifested in Figure 6(a). Cotransfection of WT-HMGA2 with miR-98-5p mimics suppressed the reporter activity in HEK-293T cells (Figure 6(b)). Figures 6(c) and 6(d) show that HMGA2 was strongly induced in the kidney tissues of db/db mice. Then, we found HMGA2 expression was elevated in mouse mesangial cells indicating high glucose, as shown in Figures 6(e) and 6(f). HMGA2 was strongly inhibited by miR-98-5p overexpression in mouse mesangial cells, as shown in Figures 6(g) and 6(h).

4. Discussion

MicroRNAs can participate in various cardiovascular processes, which are closely correlated with numerous cardiovascular diseases, such as coronary heart disease,

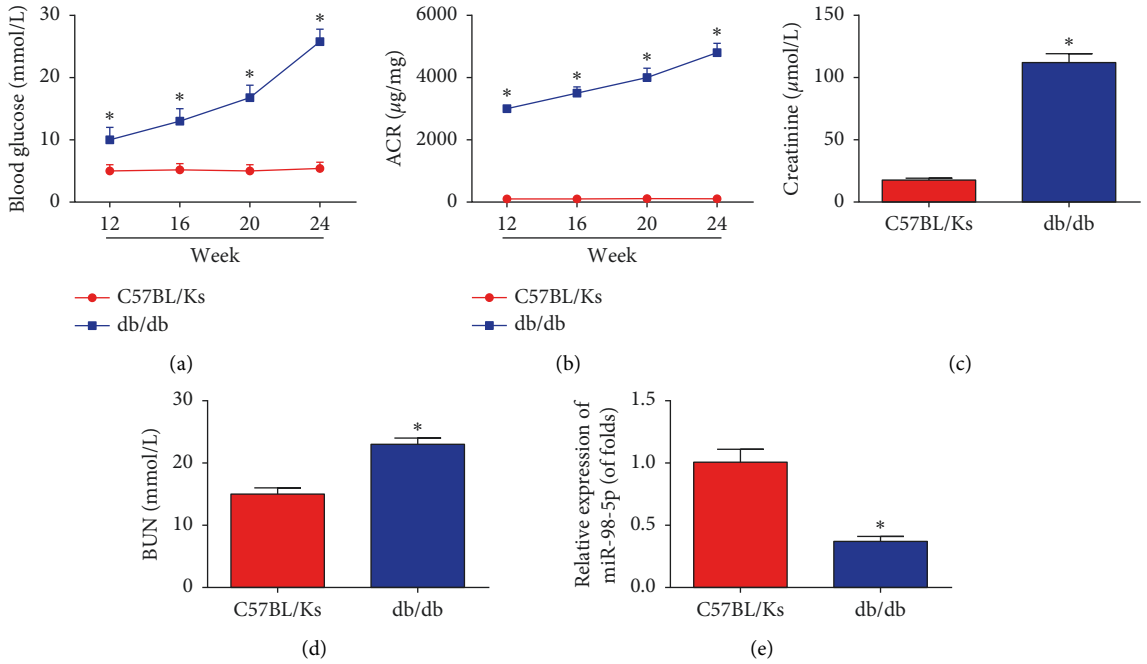


FIGURE 1: miR-98-5p was decreased in db/db mice. (a) Blood glucose was determined every 4 weeks since the age of 12 weeks. (b) Urine ACR was detected every 4 weeks since the age of 12 weeks. (c) Serum creatinine was examined at the age of 24 weeks. (d) BUN was determined at the age of 24 weeks. (e) miR-98-5p expression in the renal cortex was measured by real-time PCR. $n = 8$ for each group. Data are representative of three experiments. Error bars stand for the mean ± SD of at least triplicate experiments. $^*P < 0.05$.

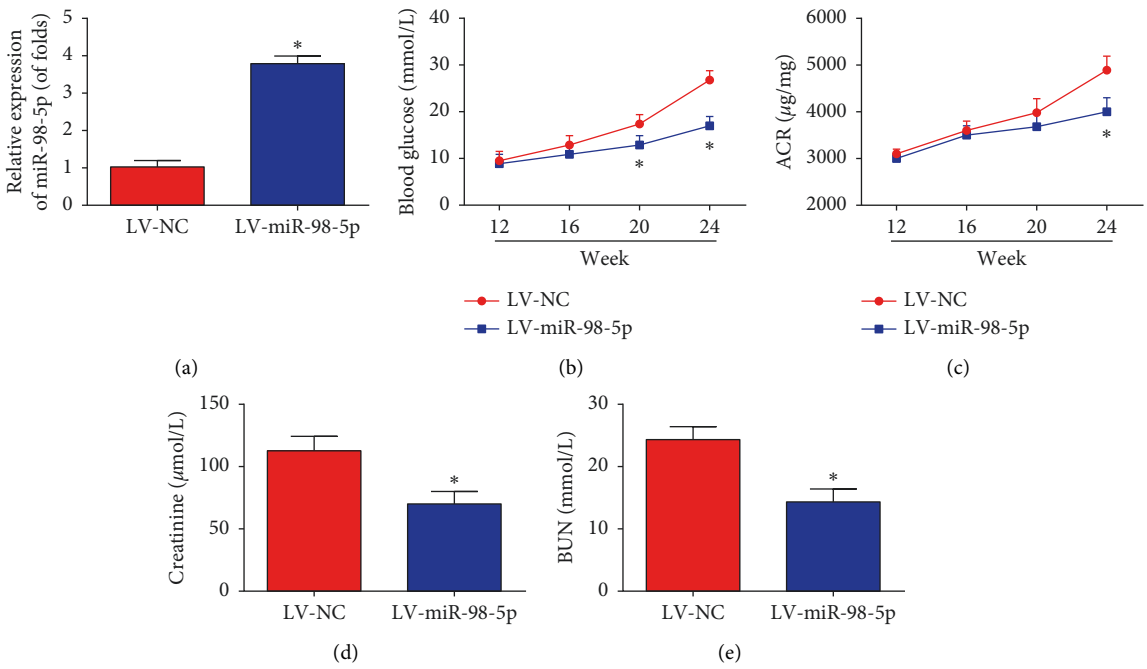


FIGURE 2: Overexpression of miR-98-5p attenuated renal dysfunction in db/db mice. (a) miR-98-5p expression in the renal cortex was tested using real-time PCR. (b) Blood glucose was determined every 4 weeks. (c) Urine ACR was detected every 4 weeks. (d) Serum creatinine was examined at the age of 24 weeks. (e) BUN was determined at the age of 24 weeks. $n = 8$ for each group. Data are representative of three experiments. Error bars stand for the mean ± SD of at least triplicate experiments. $^*P < 0.05$.

hypertension, and myocardial infarction [20]. Accumulating studies have indicated that microRNAs are dysregulated in DN progression [21–23]. Here, miR-98-5p/HMGA2 axis was identified as a novel mechanism in development of DN. As exhibited, miR-98-5p was decreased in db/db mice and mice mesangial cells indicated with high glucose, whereas

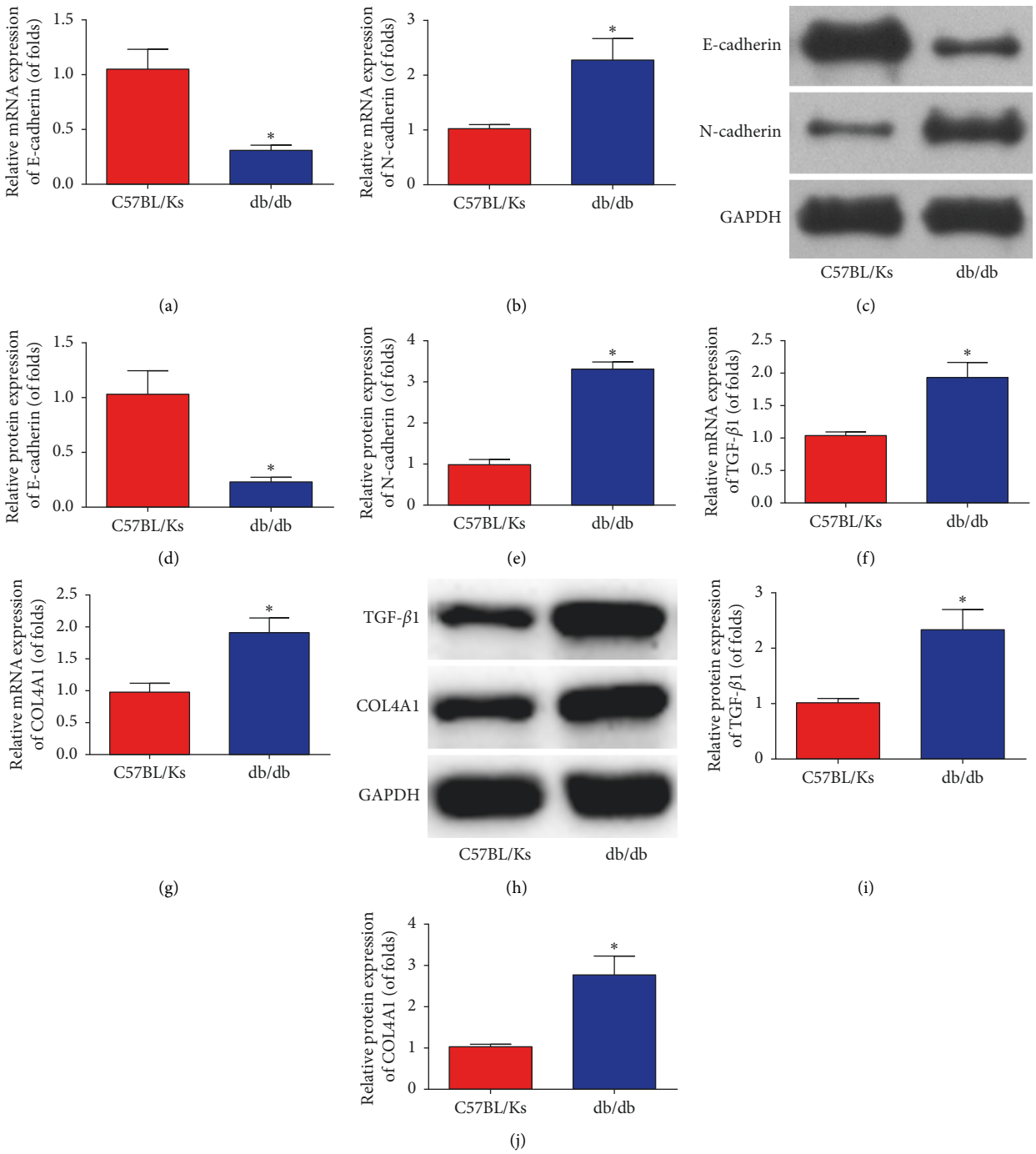


Figure 3: EMT and renal fibrosis was induced in db/db mice. mRNA expression of E-cadherin (a) and N-cadherin (b). The western blot images of E-cadherin and N-cadherin (c). Quantification of the western blot images of E-cadherin and N-cadherin (d and e). mRNA expression of TGF-β1 (f) and COL4A1 (g). The western blot images of TGF-β1 and COL4A1 (h). Quantification of the western blot images of TGF-β1 (i) and COL4A1 (j). $n = 8$ for each group. Data are representative of three experiments. Error bars stand for the mean ± SD of at least triplicate experiments. $^*P < 0.05$.

HMGA2 was greatly increased. Moreover, mouse mesangial cell proliferation and EMT process were strongly prevented by miR-98-5p overexpression.

miR-98 is a member of a highly conserved small RNA family. It acts as a crucial tumor suppressor in several tumors. For example, miR-98 can inhibit HCC progression via EZH2 and inactivating Wnt/β-catenin [24]. By targeting ITGB3, miR-98 inhibits the progression of NSCLC [25]. Additionally, another study indicates that high glucose concentration can trigger proliferation of endothelial cells by regulating miR-98 [26]. Here, we studied that miR-98-5p was decreased in db/db mice and mice mesangial cells, which are treated with high glucose. In addition, we observed that overexpression of miR-98-5p reduced hyperglycemia

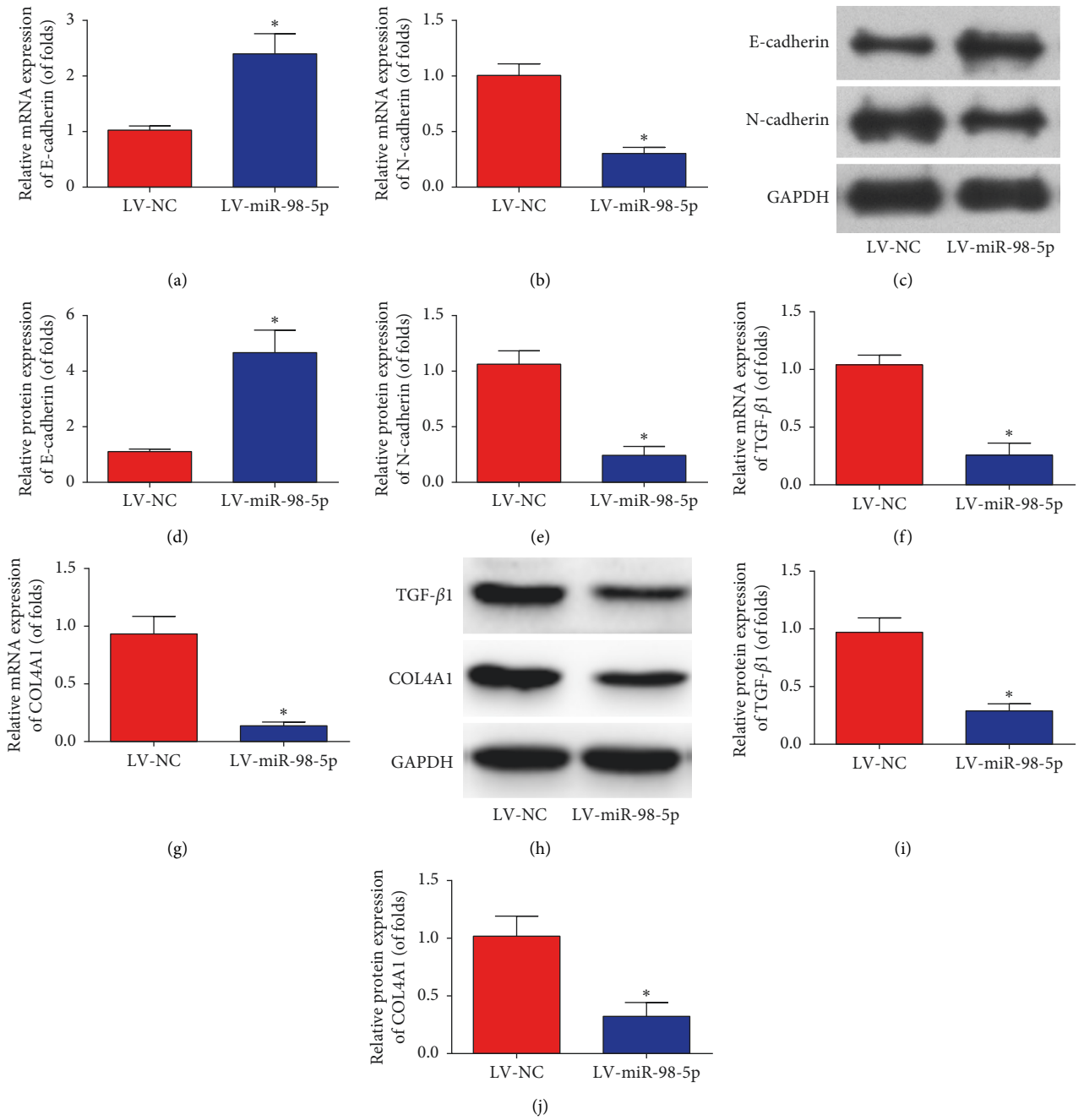
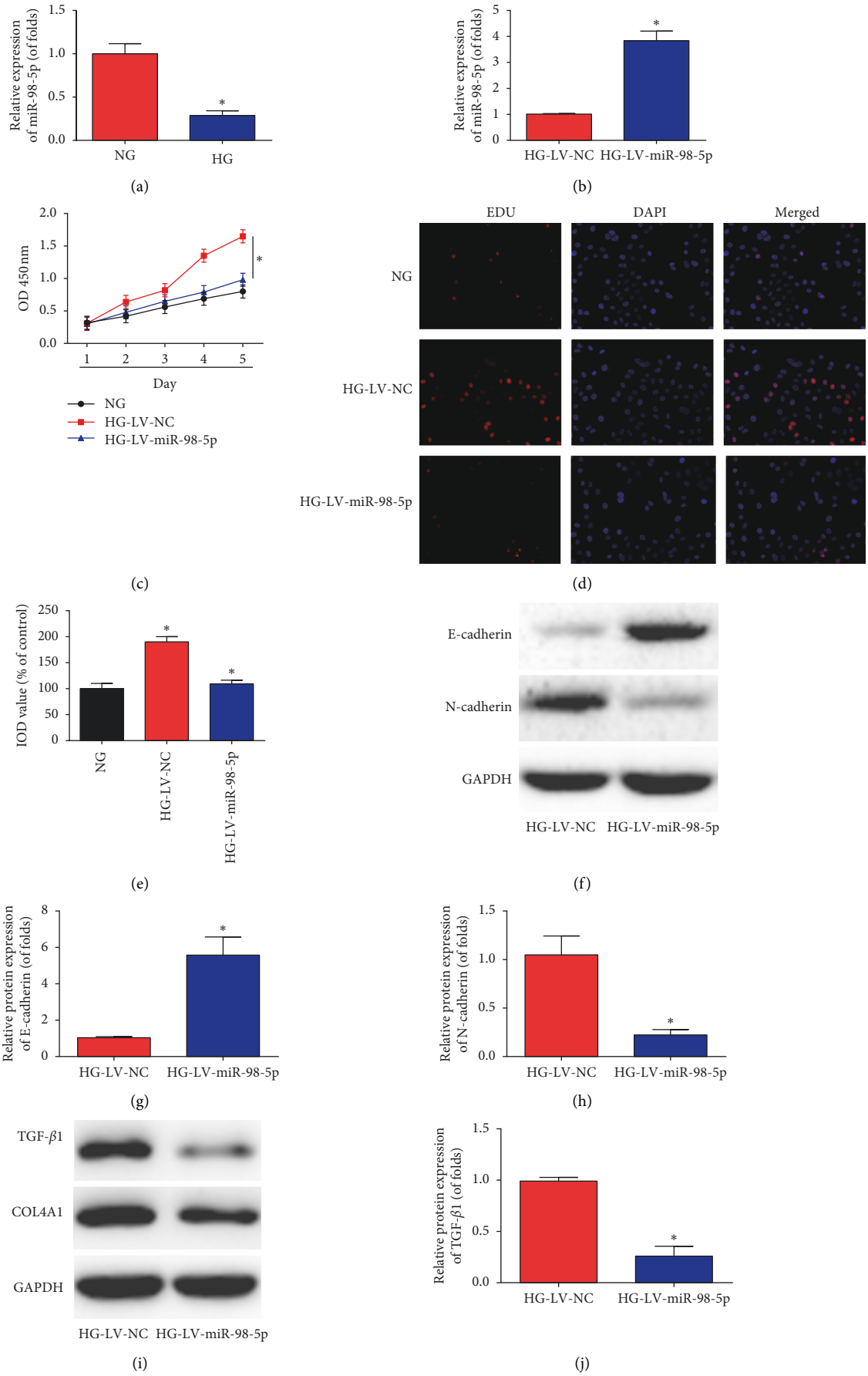


Figure 4: EMT and renal fibrosis were restrained by overexpression of miR-98-5p in db/db mice. mRNA expression of E-cadherin (a) and N-cadherin (b). Western blot images of E-cadherin and N-cadherin (c). Quantification of the western blot images of E-cadherin and N-cadherin (d and e). mRNA expression of TGF-β1 (f) and COL4A1 (g). The western blot images of TGF-β1 and COL4A1 (h). Quantification of the western blot images of TGF-β1 (i) and COL4A1 (j). $n = 8$ for each group. Data are representative of three experiments. Error bars stand for the mean ± SD of at least triplicate experiments. $^*P < 0.05$.

development and the renal dysfunction of db/db mice. The improvement of kidney functions might be due to the improvement of hyperglycemia. In our future study, we would like to investigate this.

EMT plays a crucial role in the progression of DN [27]. For instance, miR-30c can protect DN by suppressing EMT *in vivo* [28]. miR-23b can function as an EMT suppressor in DN through inactivating PI3K-AKT pathway activation [10]. In addition, miR-130b can protect renal tubulointerstitial fibrosis via repressing EMT in DN [29]. Furthermore, previous research studies have indicated that miR-98 can inhibit the EMT process in several cancers. miR-98 can repress TWIST expression to prevent the progression of NSCLC [30]. MiR-98 prevents the invasion capacity and EMT of HCC [31]. Here, in our study, it was observed that EMT was greatly triggered in db/db mice and



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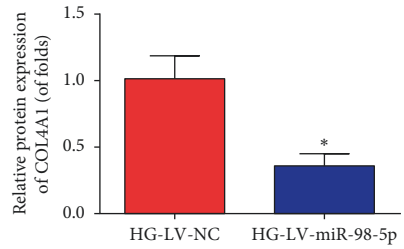
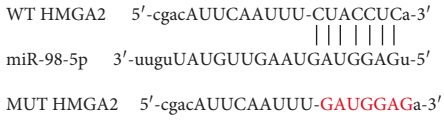


(k)

Figure 5: Overexpression of miR-98-5p alleviated high glucose-induced cell proliferation, EMT, and renal fibrosis in cultured mice mesangial cells. (a) Real-time qPCR analysis showing miR-98-5p expression in mice mesangial cells treated with high glucose (HG, 25 mM) as compared with the cells treated with normal glucose (NG, 5.6 mM). (b) Real-time qPCR analysis showing miR-98-5p expression in mice mesangial cells infected with LV-miR-98. (c) Result of CCK-8 assay in mouse mesangial cells. (d and e) Result of EDU assay in mouse mesangial cells. Western blot images of E-cadherin and N-cadherin (f). Quantification of the western blot images of E-cadherin and N-cadherin (g and h). The western blot images of TGF-β1 and COL4A1 (i). Quantification of the western blot images of TGF-β1 (j) and COL4A1 (k). $n = 8$ for each group. Data are representative of three experiments. Error bars stand for the mean ± SD of at least triplicate experiments. $^*P < 0.05$.



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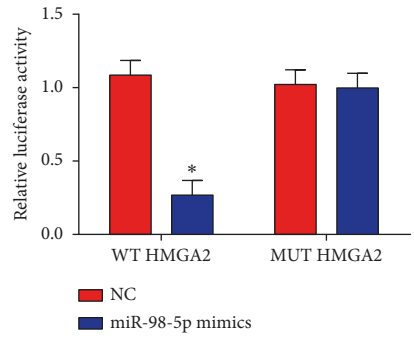

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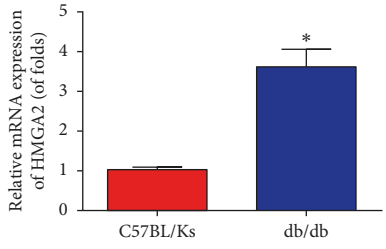

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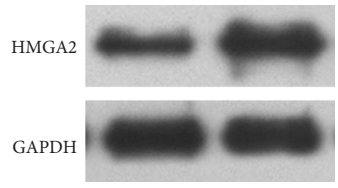

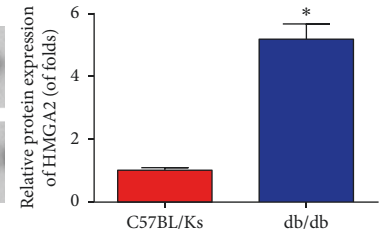

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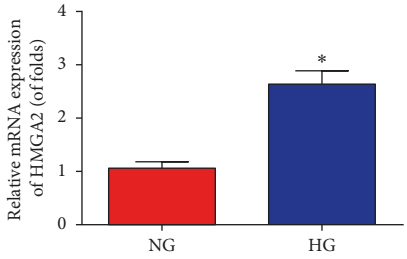

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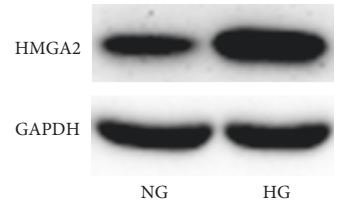

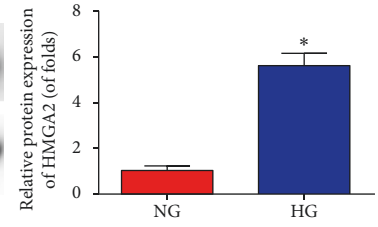

(f)

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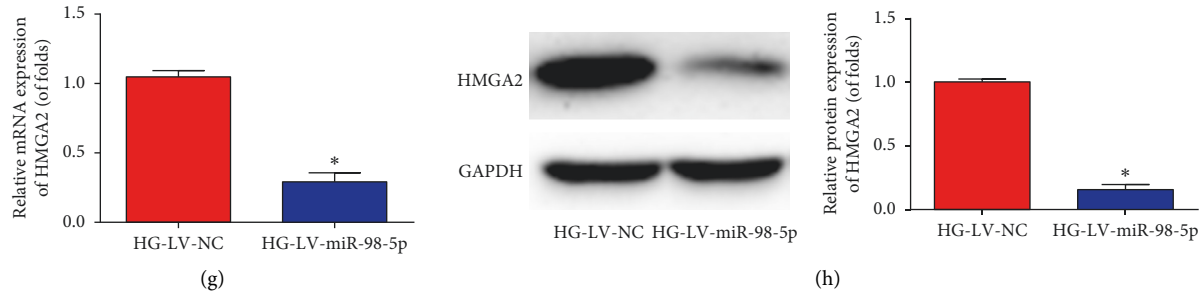

Figure 6: HMGA2 was a direct target of miR-98-5p. (a) The luciferase reporter constructs containing the wild type (WT-HMGA2) or mutant HMGA2 (MUT-HMGA2) sequence. (b) WT-HMGA2 or MUT-HMGA2 was cotransfected into HEK-293T cells with miR-98-5p mimics or their corresponding negative controls. (c) mRNA expression of HMGA2 in db/db mice. (d) Protein expression of HMGA2 in db/db mice. (e) mRNA expression of HMGA2 in mouse mesangial cells. (f) Protein expression of HMGA2 in mouse mesangial cells. (g) mRNA expression of HMGA2 in mouse mesangial cells transfected with LV-miR-98-5p. (h) Protein expression of HMGA2 in mouse mesangial cells transfected with LV-miR-98-5p. Data are representative of three experiments. Error bars stand for the mean ± SD of at least triplicate experiments. $^*P < 0.05$.

overexpression of miR-98-5p was able to restrain EMT process in DN. As exhibited, E-cadherin expression was induced by LV-miR-98-5p, whereas N-cadherin was suppressed by overexpression of miR-98-5p. In addition, we observed that renal fibrosis biomarkers TGF-β1 and COL4A1 were greatly induced in db/db mice. Overexpression of miR-98-5p remarkably inhibited TGF-β1 and COL4A1 *in vitro* and *in vivo*.

HMGA2 has been documented to regulate adipogenesis and mesenchymal differentiation and promote benign mesenchymal tumors [32, 33]. Recent studies have demonstrated that HMGA2 exerts a critical role in DN progression. Let-7d can prevent the EMT process triggered by TGF-β1 and renal fibrogenesis via regulating HMGA2 expression [34]. Meanwhile, loss of HMGA2 weakens EMT in tubular epithelial cells [35]. Common variation of HMGA2 gene can greatly enhance nephropathy of type 2 diabetic patients [36]. In our research, it was displayed that HMGA2 was a target of miR-98-5p. We proved that HMGA2 was elevated in DN models. Upregulation of miR-98-5p repressed HMGA2 levels in mice mesangial cells.

5. Conclusions

In conclusion, a novel role of miR-98-5p/HMGA2 axis was implied in DN progression in our research. miR-98-5p might act as a meaningful biomarker for DN.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

YZ and SB contributed to the study design; JX, WL, JL, LF, PZ, and TJ performed the experiment; YZ, JX, WL, and TJ collected the data; YZ, JX, and JL analyzed the results; YZ and JX drafted the manuscript; SB revised the manuscript; and all authors approved the final proof.

Acknowledgments

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Review Article

Effects of Uric Acid on Diabetes Mellitus and Its Chronic Complications

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With the deepening of the researches on uric acid, especially in the study of metabolic diseases, uric acid has been found to be closely related to obesity, metabolic syndrome, nonalcoholic fatty liver disease, diabetes, and other metabolic diseases. Uric acid causes a series of pathophysiological changes through inflammation, oxidative stress, vascular endothelial injury, and so on and thus subsequently promotes the occurrence and development of diseases. This review confirmed the positive correlation between uric acid and diabetes mellitus and its chronic complications through the pathogenesis and clinical studies aspects.

1. Introduction

In recent years, human intake of foods such as those with the umami flavor (rich in purines), high added sugar (sucrose), and high fructose corn syrup have increased dramatically [1]. Fructose is the main component of added sugar. Unlike other sugars, fructose can cause mitochondrial oxidative stress [2, 3] and inhibits AMPK [4], and the subsequent intracellular ATP depletion [5] and nucleotide turnover lead to a significant increase in serum uric acid [6]. In addition to causing gout, many studies have shown that hyperuricemia is also closely related to cardiovascular diseases, metabolic syndrome, insulin resistance, and diabetes [7, 8]. However, its function is a matter of debate [9]. Here, we reviewed the effects of hyperuricemia on diabetes and its complications and concluded that high levels of uric acid is closely related to diabetes and its chronic complications.

1.1. Uric Acid Formation. In the human body, uric acid is the ultimate product of purine metabolism (Figure 1 [10]). It is generated in the liver. Purine nucleotides decompose to hypoxanthine and guanine, some of which can be recycled and phosphorylated into hypoxanthine nucleotides, while the remaining part is metabolized by xanthine dehydrogenase/oxidase (XDH/XO) enzymatic reaction to the terminal product uric acid. XDH/XO is mainly expressed in the parenchymal cells of the liver and small intestine. XDH has low reactivity and can be converted to XO. Uric acid production primarily depends on the amount of substrate and the activity of XO [11]. In the end, XDH/XO promotes the final steps in purine metabolism which convert hypoxanthine to xanthine and xanthine to UA [11]. The kidney also plays an important role in the regulation of blood uric acid levels. The circulating uric acid is easily filtered from the glomeruli into the renal tubule. About 90% of filtered UA is reabsorbed by the middle of the proximal convoluted tubule mainly by urate transporter 1 (URAT1) and glucose transporter 9 (GLUT9) [12], and the remaining excreted 10% is responsible for 60–70% of total body uric acid excretion [13, 14]. A small amount of uric acid secreted in the intestine is responsible for 30–40% [14]. The production and excretion rate of uric acid is relatively constant in healthy people. Changes in the uric acid content in body fluids can reflect the state of metabolism, immunity, and other functions of the human body. If the body produces too much uric acid or the excretion mechanism is degraded, the body will retain excessive uric acid. Hyperuricemia was defined as the circulating uric acid levels of more than 5.7 mg/dl for women and 7.0 mg/dl for men [15]. When the blood uric acid concentration exceeds the norm, the human body fluid

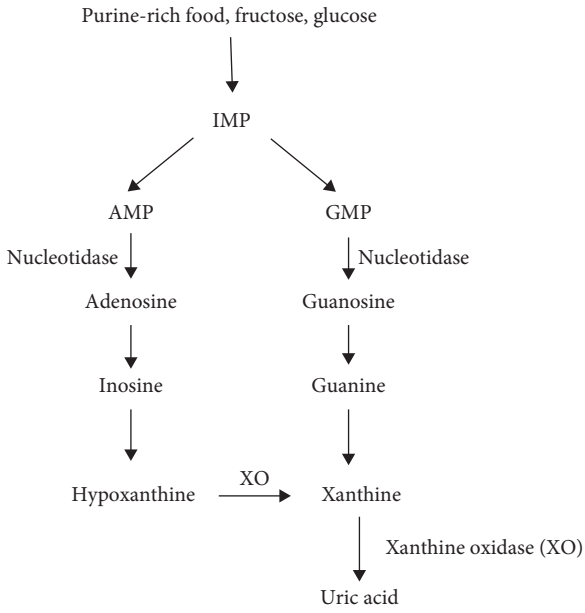

Figure 1: Process of purine metabolism in humans.

becomes acidic, which affects the normal function of the human cells, subsequently leading to metabolic disease in the long term [16–18].

2. Pathological Mechanism of Uric Acid on Diabetes and Its Chronic Complications

2.1. Uric Acid and Diabetes. At present, many studies have shown that the relevant pathological mechanisms include some aspects as follows (Figure 2):

(1) *Inflammation*. Increased uric acid levels in the blood promoted the expression of interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) [19], and CRP production [20]. In animal studies, the activation of inflammation induced by UA decreases insulin sensitivity in mice [21], and infusion of UA into mice can increase TNF-α levels and activate the classical inflammatory pathway [22]. In human studies, serum UA was positively associated with TNF-α, interleukin-6 and C-reactive protein in healthy people [23].

(2) *Oxidative Stress*. Excessive uric acid will lead to an increase in reactive oxygen species (ROS) production, which leads to inflammation and dysfunction in the vessel [24]. UA is a powerful antioxidant that can remove superoxide and hydroxyl radicals in plasma, and UA has prooxidant effects in vascular tissue by increasing ROS production, such as H_2O_2 [24]. UA-mediated oxidative stress-induced lipid peroxidation, DNA damage, and activation of inflammatory factors finally lead to cellular damage [24]. Oxidative stress also can affect the expression of insulin gene, causing a decrease in insulin secretion [25].

(3) *Endothelial Dysfunction*. Endothelial dysfunction is characterized by deficiencies in the synthesis and/or bioavailability of endothelium-derived NO [26]. In addition, UA reduces endothelial NO bioavailability in humans [27]. Uric acid inhibits proliferation and migration of endothelial cells and NO secretion [20]. UA can react with NO to form 6-aminouracil, UA-dependent ROS reacts with NO to form peroxynitrite, and UA can hold back L-arginine uptake and stimulate L-arginine degradation [6]. As a result of the effects of hyperglycemia and neurohormonal activation, UA levels are independently associated with endothelial dysfunction in animals and humans, thereby promoting hypertension [28].

(4) *Inhibiting Insulin Pathway*. UA directly inhibits the trigger of insulin signaling pathway by an ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) recruitment at the receptor level [29].

All factors interference with glucose homeostasis and insulin sensitivity promotes the development of diabetes [30–32].

2.2. Uric Acid and Diabetic Chronic Complications. The aforementioned changes to diabetes are also directly related to the metabolic disorder: desulfation of glycosaminoglycans (GAGs) and formation of advanced glycation end products (AGE) and receptors (RAGE) [33]. It is widely believed that polyol bypass, protein kinase C, hexosamine activation, advanced glycosylation products (AGEs), increased hyperglycemia-induced mitochondria production of reactive oxygen species (ROS), inflammation, and endothelial dysfunction are the common pathogenic characteristics of chronic complications of diabetes mellitus [10,33–39], which mainly include macroangiopathy, microangiopathy, and neuropathy. Two other mechanisms are associated with chronic complications as follows (Figure 2):

(1) *Activation of RAAS*. Uric acid can lead to the activation of the renin-angiotensin-aldosterone System (RAAS), through increasing the production of juxtaglomerular renin [40]. UA-induced ROS stimulated the increase of plasma angiotensin II which induced aldosterone release, leading to activation of RAAS [24, 41]. RAAS activation induced afferent renal arteriolopathy and tubulointerstitial fibrosis in rodent models [42]. In diabetes, RAAS activation causes a range of pathological changes including vascular dysfunction, high intraglomerular pressure, inflammation, and so on, leading to cardiovascular and renal complications [43].

(2) *Thrombus*. Uric acid seems to trigger platelet adhesion and aggregation, thus favoring vascular thrombosis [44].

3. Epidemiology Studies

3.1. Uric Acid and Diabetes. The relationship between uric acid and diabetes has gradually become a hot topic of

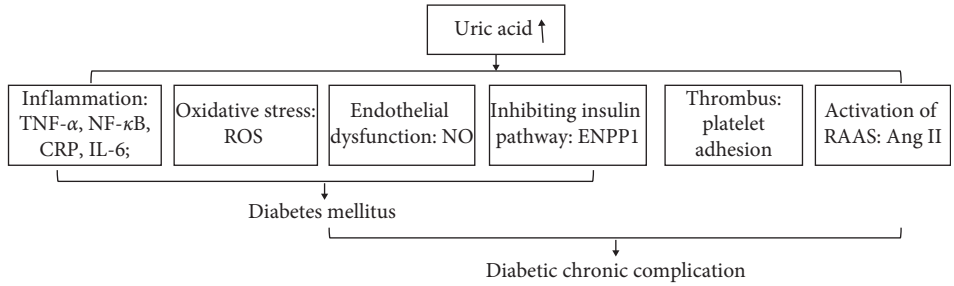

Figure 2: Metabolism of uric acid leading to diabetes mellitus and its chronic complication.

research, but controversy still exists. On the one hand, some study reported uric acid was not associated with diabetes. For example, Sluijs et al. [45] used a genetic score of 24 uric acid-related sites for Mendelian randomization studies, in the European prospective survey data—Cancer and Nutrition (EPIC) study, which was an interactive case-cohort study of vast number of subjects from eight European countries. In EPIC, after a mean of 10 years of follow-up, the results suggested that hyperuricemia was not salient associated with a higher risk of diabetes after adjusting for interference factors when their participant number was increased from 10,576 to 41,508. Similarly, a large prospective cohort study was performed by Li [46] who followed up 4412 nondiabetic patients for 4.7 years to study urate changes in glucose metabolism. They found the uric acid concentration was not related to an increased risk of type 2 diabetes mellitus (T2DM).

On the opposite hand, more clinical trials demonstrated uric acid was significantly associated with diabetes. For example, Bombelli et al. [47, 48] randomly selected 3,200 northern Italian residents between the ages of 25 and 74 and found that increased uric acid resulted in an increased risk of impaired fasting glucose (IFG), and people with higher median UA levels may also develop metabolic syndrome and diabetes. In women, serum uric acid (SUA) levels in the normal range were associated with an increased risk of new-onset diabetes compared with women with low-normal values [49]. Older adults with high levels of uric acid (6.0 mg/dl for men and 5.5 mg/dl for women) were more susceptible to metabolic syndrome and T2DM, especially in the 75–84 years age group [50]. Serum UA was an important predictor of risk of metabolic syndrome, diabetes, and hypertension in adult males [51]. However, the relationship between blood UA and decreased insulin sensitivity in patients with type 1 diabetes mellitus is weaker than in healthy subjects [52].

Through reading a large number of literature and studies, we believe that uric acid is closely related to diabetes. Poor lipid metabolism in individuals with higher UA levels may lead to increased fasting and postprandial insulin levels, high-sensitivity C-reactive protein, hepatic insulin resistance index, and decreased glomerular filtration rate and skeletal muscle insulin sensitivity; high levels of SUA may impair liver insulin sensitivity and insulin clearance [53]. Perticone F [54] was documented when hypertensive NGT $\geq$ 155 mmHg, and UA is closely related to 1-h postload glucose during an oral glucose tolerance. We [55] analyzed the clinical characteristics and islet function index of 403 newly diagnosed patients with T2DM (mean age, 50.21 $\pm$ 13.34 years old; 62.5% male) and analyzed the SUA levels according to gender. Multivariate linear regression analysis showed that SUA had an independent effect on insulin secretion in female patients; the islet β-cell function of male was also affected by SUA, age, body mass index (BMI), and blood lipids; SUA correlated positively with insulin secretion and the insulin resistance index in male patients.

In terms of gestational diabetes, Leng [56] found that the SUA level is positively related with the risk of T2DM and prediabetes in the Tianjin region of China gestational diabetes mellitus (GDM) prevention planning data. In the group with GDM and impaired glucose tolerance (IGT), the mean SUA level was significantly increased in early pregnancy, and a UA level of 3.95 mg/dl could predict GDM with 60% specificity and 100% sensitivity [57].

3.2. Uric Acid and Diabetic Chronic Complications

3.2.1. Uric Acid and Diabetic Macrovascular Disease. Diabetic macroangiopathy refers to atherosclerosis of blood vessels such as the aorta, coronary artery, basilar artery, renal artery, and peripheral arteries, especially in the heart and cerebrovascular diseases, which is caused by dysfunction of endothelial cells, advanced glycation end product (AGEs/RAGEs) system, the hexosamine pathway, inflammation, oxidant stress, protein kinase (PKC), and polyol [34–37]. Some clinical studies have shown a positive correlation between uric acid and diabetic macroangiopathy. Yan et al. [58] used Mendelian randomized analysis to determine whether there is a causal relationship between UA and diabetic macrovascular disease and found that the prevalence of diabetic macrovascular disease was significantly higher in the hyperuricemia group than in the healthy population, suggesting that UA and diabetic macrovascular disease are related. Indeed, the link between female-weighted genetic risk score (GRS) and diabetic macrovascular disease was greater than expected. Hyperuricemia was also observed to be associated with an increased incidence of atrial fibrillation in hospitalized patients with T2DM [59]. Hyperuricemia can increase the risk of sudden atrial fibrillation by approximately four-fold [60] and is associated with cardiovascular mortality [61]. Cardiovascular and cerebrovascular diseases are mainly caused by ischemia and hypoxia resulting from coronary atherosclerosis. Du et al. [62] performed a meta-analysis of patients with T2DM to determine whether SUA

levels were associated with cerebral infarction and calculated the ratio of means (RoM) for SUA and the average cerebral infarction or average diabetes control ratio of individual studies and then compared it with the calculated 95% confidence intervals. The results showed that higher SUA levels might lead to cerebral infarction in patients with T2DM. Wang et al. [63] used the "Comprehensive Diabetes Prevention and Control Study (CRPCD)" data to explore the relationship between SUA and ischemic stroke in patients with T2DM in China. A total of 19,442 participants were enrolled in a cross-sectional study. The SUA level was significantly higher in patients over 60 years of age than in people under 60 years of age. Serum UA levels were independently and positively correlated with ischemic stroke in patients under 60 years of age, and it was characterized by U-type association in patients over 60 years of age. We speculated that the incidence of other established stroke risk factors such as hypertension, dyslipidemia, and chronic kidney disease increased with age would made it difficult to establish UA as an independent role in stroke.

Diabetic hyperglycemia causes metabolic abnormalities, which can affect systemic organs. Diabetic foot is caused by peripheral vascular disease, peripheral (motor, sensory, and autonomic) neuropathy, and excessive mechanical stress (repetitive external or minor trauma) in diabetic patients, leading to the destruction and deformity of the soft tissue and bone joint system of the foot [64]. The pathogenesis is partly the same as diabetic vascular and neuropathy complications [65]. Uric acid can be used as an independent risk factor to assess the development of diabetic foot [66].

3.2.2. Uric Acid and Diabetic Microangiopathy. Diabetic microangiopathy is a specific complication of diabetes. The typical changes comprise microcirculatory disorders and microvascular basement membrane thickening, which mainly lead to diabetic nephropathy (DN) [51] and diabetic retinopathy (DR) [67–70].

(1) Uric Acid and Diabetic Nephropathy. Diabetic nephropathy is a long-standing microvascular complication of diabetes and is the leading cause of end-stage renal disease in developed countries [10, 71]. As an inflammatory factor, UA increases oxidative stress and promotes the activation of the renin-angiotensin-aldosterone system (RAAS) [21, 41]. Therefore, UA levels are associated with the occurrence and development of DN and are independent risk factors for early kidney disease [72, 73], which help to predict microalbuminuria progression [74]. Serum UA and microalbuminuria levels were significantly positively correlated with renal disease in patients with T2DM [75]. Patients with higher SUA levels have poorer renal function, independent of glycated hemoglobin (HbA1c) or the duration of diabetes [76]. In T2DM, there is an independent and significant positive association between higher blood UA and an increased risk of a reduced glomerular filtration rate (eGFR) [77]. Blood UA levels greater than 5.5 mg/dl can predict chronic kidney disease of stage 3 and above in T2DM [78]. The level of SUA that protects against progression of type 2 diabetic nephropathy (diabetic kidney disease (DKD)) is lower than the current normal value. The optimal cut-off value is 377.5 μmol/l (6.3 mg/dl) for men and 309.0 μmol/l (5.2 mg/dl) for women [79]. In Chinese patients with T2DM, UA-related alleles such as *SLC2A9* rs11722228 (solute carrier family 2 member 9), *SLC2A9* rs3775948, and *ABCG2* rs2231142 (ATP binding cassette subfamily G member 2) may affect susceptibility to DKD [80]. Contrast-enhanced ultrasound (CEUS) was used to show renal microvascular hyperperfusion, with a decreased glomerular filtration rate and reduced UA excretion in patients with DKD [81]. Xanthine oxidase (XO) is a very important enzyme that is responsible for the conversion of sulfhydryl groups to UA. Elevation of UA by 1 μmol/l enhanced the probability of albuminuria by 1.5%, and a rise in XO activity of 1 U/l also increased the probability of albuminuria by 1.5%. In diabetes, both XO and uric acid are independently associated with albuminuria [82].

In patients with type 1 diabetes without complications, higher UA levels are associated with lower GFR, which is due to UA-mediated increased resistance in afferent renal arteriole promoting the renal microcirculation ischemia [83, 84].

In type 1 diabetes, kidney damage is more common in men whose SUA and creatinine concentrations and the albumin excretion rate are higher than those in female patients. Indeed, hyperglycemia adversely affects the activity of estrogen receptors (ER) and this may be gender-specific. The progression of renal disease in men with T1D is associated with a decline in free estradiol levels [85], and 17β-estradiol shows antioxidant, antiapoptotic, and anti-inflammatory properties [86]. The SUA level in boys but not girls with T1D was positively correlated with subclinical inflammation marker levels (CRP, IL-6, TNF-α), renal function indicators (albumin excretion rate, cystatin-C level), and blood pressure; it was negatively correlated with anti-inflammatory IL-10 [87].

(2) Uric Acid and Diabetic Retinopathy. Diabetic retinopathy (DR) is a specific fundus lesion that is the main cause of blindness in patients with diabetes [88]. Based on the changes of haemodynamics or vascular geometry, vascular injury is considered to be the prime motivator for the initiation and progression of DR, including pericytosis, platelet aggregation, thickening of basement membrane, and neuroglial damage [89]. The blood retinal barrier, as precondition to vision acuity, is vulnerable to injury during the progression of DR. This is a consequence of the interplay of AGE, hexosamine, polyol, inflammation, NO decline, oxidative stress, PKC, and RAS [38]. Uric acid is closely related to these pathological changes. Clinically, DR is classified into nonproliferative diabetic retinopathy (NPDR) (also known as simple type or background type) and proliferative diabetic retinopathy (PDR), according to whether or not retinal neovascularization occurs [90]. In Chinese patients with T2DM, reduction in urinary uric acid excretion (UUAE) is an independent risk factor for DR [91]. Elevated SUA levels are significantly associated with albuminuria and DR severity [92], but not with the retinal nerve fibre layer or

macular thickness [93]. A study reported that increased SUA levels were associated with an increased severity of DR in Taiwan [94]. Kuwata [95] analyzed data from 1839 patients with T2DM in Japan by gender stratification and found that higher SUA levels were associated with an increased risk of DR in men, but not in women. The results showed sex hormones play an important role in the metabolism of uric acid, which deserved to discuss the specific mechanism further.

3.2.3. Uric Acid and Diabetic Peripheral Neuropathy. Diabetic neuropathy is one of the most common chronic complication of diabetes [96], characterized by damage to nerve glial cells, axons, and endothelial cells, and the morbidity from 30% to 50% in T2DM [97]. Diabetic peripheral neuropathy (DPN) is the main clinical manifestation of sensory and autonomic nerve symptoms, distal symmetry polyneuropathy, and motor neuropathy are the most common types of DPN [98]. The pathophysiology changes conclude polyol pathway, PKC activity, increased AGEs, oxidative stress (ROS), inflammation (IL-1β, IL-6, TNFα, and COX-2), microvascular alterations (endothelial dysfunction), nerve degeneration and regrowth (MMPs, Schwann cells and ECM), and the changes of the blood-nerve barrier [39, 99, 100]. Lin et al. [101] observed significant differences in the ratio of motor and sensory nerve amplitude and conduction velocity (CV) parameters between groups with different blood UA levels (both $P < 0.05$). Blood UA levels were negatively correlated with the ratio of motor and sensory nerve amplitude and CV. Blood UA at 9 mg/dl and total cholesterol of 5.2 mmol/l were significantly associated with DPN in patients who had suffered from T2DM for more than 10 years. Yu et al. [102] performed a meta-analysis of 1388 patients with T2DM with peripheral neuropathy and in 4746 patients without peripheral neuropathy and showed that SUA levels were significantly elevated in patients with diabetes complicated with peripheral neuropathy and that increased hyperuricemia was related with increased risk of peripheral neuropathy.

4. Conclusion

Complex genetic and environmental factors contribute to causing diabetes, and chronic complications of diabetes may occur throughout the body. The pathogenesis of T2DM is complex, involving various interacting factors. Its increased incidence rate is a great concern worldwide. Hyperuricemia is closely related to the development of diabetes and its chronic complications. Many animal and human experiments have confirmed that UA mainly affects diabetes and its complications through inflammation, oxidative stress, endothelial function damage, and other effects. We call for further researches to explore the molecular mechanism, especially in the direct effect of uric acid on insulin secretion.

Conflicts of Interest

The authors declare that there are no conflicts of interest related with the publication of this paper.

Authors' Contributions

Qing Xiong and Jie Liu contributed equally to this work.

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Research Article

Serum Fibroblast Growth Factor 21 Levels Are Positively Associated with Metabolic Syndrome in Patients with Type 2 Diabetes

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Background. Fibroblast growth factor 21 (FGF21) acts as a potent metabolic regulator. Serum FGF21 levels were significantly higher in obesity and type 2 diabetes mellitus (T2DM) populations. The aim of this study was to evaluate the relationship between serum FGF21 levels and metabolic syndrome (MetS) in T2DM patients. *Methods*. Fasting blood samples were obtained from 126 T2DM patients. MetS and its components were defined according to the diagnostic criteria from the International Diabetes Federation. Serum FGF21 concentrations were measured using a commercially available enzyme-linked immunosorbent assay. *Results*. Among these patients, 84 (66.7%) had MetS. Female gender, hypertension, systolic blood pressure (SBP), diastolic blood pressure (DBP), waist circumference (WC), body weight (BW), body mass index (BMI), body fat mass, fasting glucose, glycated hemoglobin level (HbA1c), triglyceride level (TG), urine albumin-to-creatinine ratio (UACR), insulin level, homeostasis model assessment of insulin resistance (HOMA-IR), and FGF21 levels were higher, whereas high-density lipoprotein cholesterol level (HDL-C) and estimated glomerular filtration rate (eGFR) were lower in DM patients with MetS. Univariate linear analysis revealed that hypertension, BMI, WC, body fat mass, SBP, DBP, logarithmically transformed TG (log-TG), low-density lipoprotein cholesterol (LDL-C) level, log-glucose, log-creatinine, log-UACR, log-insulin, and log-HOMA-IR positively correlated, whereas HDL-C and eGFR negatively correlated with serum FGF21 levels in T2DM patients. Multivariate forward stepwise linear regression analysis revealed that body fat mass (adjusted R^2 change = 0.218; $P = 0.008$) and log-TG (adjusted R^2 change = 0.036; $P < 0.001$) positively correlated, whereas eGFR (adjusted R^2 change = 0.033; $P = 0.013$) negatively correlated with serum FGF21 levels in T2DM patients. *Conclusions*. This study showed that higher serum FGF21 levels were positively associated with MetS in T2DM patients and significantly positively related to body fat mass and TG but negatively related to eGFR in these subjects.

1. Introduction

Type 2 diabetes mellitus (T2DM), a chronic metabolic disease characterized by hyperglycemia and insulin resistance, is a significant health problem and global burden, with an increasing prevalence worldwide [1]. According to the data from the International Diabetes Federation, 336 million people were diagnosed with T2DM globally in 2011, and the figure is expected to elevate to 552 million by 2030 [2]. Metabolic syndrome (MetS), with a prevalence rate of 23.6% among adults in the European country according to the National Cholesterol Education Program Adult Treatment Panel III definition, is an independent risk factor for T2DM and cardiovascular disease (CVD) [2, 3]. The MetS population is expected to have a two- to five-fold risk of developing DM and heart disease over the following 5–10 years than people without MetS [4].

Fibroblast growth factor 21 (FGF21) is a polypeptide with 210 amino acids from a human gene located on chromosome 19 at the 5′ region of the 1,2-fucosyltransferase. It is produced

preferentially in the liver [5] and has been identified as an endocrine and metabolic hormone because of its potent effect on lipid and glucose metabolism and on insulin sensitivity and energy balance [6]. An animal study revealed that FGF21 had favorable effects of lowering serum glucose and triglyceride (TG) levels and improving lipoprotein profiles in genetic compromised FGF transgenic mice and primates [7, 8]. However, pieces of evidence of FGF21 as a potential disease marker for human metabolic-related illness are growing. Epidemiology studies revealed that higher serum FGF21 is an independent predictor of the MetS in Asian individuals and FGF21 levels elevated significantly among prediabetic and diabetic patients and can predict the diabetes development in a Chinese population [9, 10]. Eto et al. and Bobbert et al. also represented that the circulating FGF21 concentrations have a positive association with parameters in T2DM Japanese patients and the occurrence of MetS and T2DM in Caucasian patients, respectively [11, 12]. Taken together, FGF21, from physiological and clinical perspectives, is a potential biomarker for the early detection of human metabolic disorder.

Although emerging studies have evaluated the relationships of this hepatokine to obesity-related disease, whether or not FGF21 predicts MetS in T2DM patients, the interrelationships of FGF21 with the metabolic parameters among these populations have not been described in detail. Therefore, we investigated how circulating FGF21 levels are correlated with metabolic parameters in T2DM Taiwanese patients with MetS.

2. Materials and Methods

2.1. Participants. This study was approved by the Protection of the Human Subjects Institutional Review Board of Tzu Chi University and Hospital and was conducted in accordance with the Declaration of Helsinki. Diabetes mellitus (DM) was diagnosed as the fasting plasma glucose was either ≥126 mg/dL or if the 2 h glucose during an oral glucose tolerance test was ≥200 mg/dL or using oral hypoglycemic medications or insulin [13]. Written informed consent was obtained from all participants prior to enrolling in this study. Finally, from November 2014 to March 2015, a total of 126 patients with T2DM follow-up in the metabolic outpatient department at Buddhist Tzu Chi General Hospital, Hualien, Taiwan were enrolled. After the participant was seated for at least 10 min, blood pressure (BP) was measured in the morning using standard mercury sphygmomanometers with appropriate cuff sizes. Systolic BP (SBP) and diastolic BP (DBP) were taken three times at 5 min intervals and were averaged for analysis. Hypertensive patients were diagnosed based on SBP ≥140 mmHg and/or DBP ≥90 mmHg or taking any antihypertensive medication in the past 2 weeks. If patients had an acute infection, heart failure, and malignancy at the time of blood sampling, or if they refused to provide informed consent for the study, they were excluded.

2.2. Anthropometric Analysis. In light clothing and without shoes, the body weight (BW) and body height of each participant were measured to the nearest 0.5 kg and 0.5 cm, respectively. With the hands on the hips, waist circumference (WC) was assessed using a tape around the waist from the point between the lowest ribs and the hip bones. Body mass index (BMI) was calculated as weight in kilograms divided by height in square meters. Bioimpedance measurements of body fat mass were performed at the bedside according to the standard tetrapolar whole body (hand-foot) technique using a single-frequency (50 kHz) analyzer (Biodynamic-450; Biodynamics Corporation, Seattle, USA). All measurements were performed by the same operator [14–16].

2.3. Biochemical Investigations. Following an overnight fast, approximately 5 mL blood samples of all participants were immediately centrifuged at 3000 g for 10 min. Serum concentrations for blood urea nitrogen (BUN), creatinine, fasting glucose, glycated hemoglobin (HbA1c), TG, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using an autoanalyzer (Siemens Advia 1800; Siemens Healthcare GmbH, Henkestr, Germany) [14–16]. Urine albumin-to-creatinine ratio (UACR) was measured using a random spot urine test. Serum FGF21 (Phoenix Pharmaceuticals, Inc. Burlingame, CA, USA) concentrations were measured using commercially available enzyme immunoassay kits, and serum insulin (Labor Diagnostika Nord, Nordhorn, Germany) concentrations were determined using a commercially available enzyme-linked immunosorbent assay [14–16]. Insulin resistance was evaluated using homeostasis model assessment of insulin resistance (HOMA-IR) as follows: HOMA-IR = fasting serum insulin (μU/mL) × fasting plasma glucose (mg/dL)/405 [14–16]. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation in this study.

2.4. MetS and Its Components. The prevalence of MetS was defined according to the International Diabetes Federation definition [17]. People had central obesity with a WC ≥90 cm in men or ≥80 cm in women (Chinese criteria) and matched two or more of the following criteria: fasting serum glucose ≥100 mg/dL, TGs ≥150 mg/dL, HDL-C level <40 mg/dL in men or <50 mg/dL in women, or BP ≥130/85 mmHg were classified as having MetS. The use of antihypertensive drugs was considered as high BP in this analysis. T2DM was determined using the World Health Organization criteria [13]. A patient was considered as having DM if the fasting plasma glucose was ≥126 mg/dL or if he or she was undergoing an antidiabetic therapy.

2.5. Statistical Analysis. Data were tested for normal distribution using the Kolmogorov–Smirnov test. Normally distributed data were expressed as mean ± standard deviation, and comparisons between patients were performed using the Student's independent *t*-test (two-tailed). Data not normally distributed were expressed as medians and interquartile ranges, and comparisons between patients were

performed using the Mann–Whitney U test (TG, fasting glucose, HbA1c, BUN, creatinine, insulin, HOMA-IR, and FGF21). Data expressed as the number of patients were analyzed by the χ^2 test. FGF21 levels were tested for independency associated with MetS by the multivariate logistic regression analysis. Because TG, fasting glucose, HbA1c, BUN, creatinine, insulin, HOMA-IR, and FGF21 levels were not normally distributed, they underwent base 10 logarithmic transformations to achieve normality. Clinical variables that correlated with serum FGF21 levels in patients with T2DM were evaluated using univariate linear regression analysis. Variables that were significantly associated with FGF21 levels in patients with T2DM were tested for independency by multivariate forward stepwise regression analysis. Data were analyzed using SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered as statistically significant.

3. Results

Table 1 shows the laboratory and clinical characteristics of the 126 enrolled T2DM patients. A total of 84 patients (66.7%) had MetS. Patients who had MetS had significantly higher serum FGF21 levels than those without MetS ($P < 0.001$). Compared with DM patients without MetS, those with MetS showed a much higher proportion of women ($P = 0.008$) and as expected more hypertension ($P < 0.001$); higher SBP ($P < 0.001$) and DBP ($P < 0.001$); higher WC ($P < 0.001$); higher BW ($P < 0.001$), BMI ($P < 0.001$), and body fat mass ($P < 0.001$); higher fasting glucose ($P = 0.004$), HbA1c level ($P = 0.005$), UACR ($P < 0.001$), TG ($P < 0.001$), insulin level ($P < 0.001$), and HOMA-IR ($P < 0.001$); and lower HDL-C concentrations ($P = 0.003$) and eGFR ($P = 0.003$). No statistically significant differences in MetS were found in terms of use of statins, fibrates, or antidiabetic drugs.

The unadjusted and multivariate logistic regression analysis of FGF21 levels with other factors associated with MetS is presented in Table 2. The unadjusted serum FGF21 levels with MetS showed that FGF21 increased per 1 pg/mL (odds ratio (OR): 1.008, 95% CI: 1.003–1.012, $P = 0.001$) increased the 0.8% risk of MetS in patients with T2DM. Multivariate logistic regression analysis adjusted for age and gender revealed a 0.7% increase in the risk of MetS (adjusted OR 1.007, 95% CI: 1.002–1.011, $P = 0.004$) for every 1 pg/mL increase in FGF21 (Model 1). After multivariate logistic regression analysis with Model 1 added with eGFR and UACR, an increased 0.5% risk of the MetS (adjusted OR 1.005, 95% CI 1.001–1.010, $P = 0.027$) was observed for every 1 pg/mL increase in FGF21 (Model 2). Multivariate logistic regression analysis using Model 2 with added serum insulin level and HOMA-IR also revealed an increased 0.5% risk of MetS (adjusted OR 1.005, 95% CI: 1.000–1.010, $P = 0.035$) for every 1 pg/mL increase in FGF21 (Model 3). Each of these analyses confirmed that serum FGF21 level is positively associated with MetS in patients with T2DM.

The univariate and multivariate linear regression analyses of the clinical variables associated with fasting serum FGF21 levels in patients with T2DM are presented in Table 3. Hypertension ($r = 0.201$, $P = 0.024$), BMI ($r = 0.259$, $P = 0.003$), WC ($r = 0.301$, $P = 0.001$), body fat mass ($r = 0.359$, $P < 0.001$), SBP ($r = 0.191$, $P = 0.032$), DBP ($r = 0.180$, $P = 0.043$), logarithmically transformed TG (log-TG; $r = 0.499$, $P < 0.001$), LDL-C level ($r = 0.176$, $P = 0.049$), log-glucose ($r = 0.187$, $P = 0.036$), log-creatinine ($r = 0.194$, $P = 0.029$), log-UACR ($r = 0.198$, $P = 0.031$), log-insulin ($r = 0.334$, $P < 0.001$), and log-HOMA-IR ($r = 0.358$, $P < 0.001$) positively correlated, whereas HDL-C ($r = -0.219$, $P = 0.014$) and eGFR ($r = -0.325$, $P < 0.001$) negatively correlated with serum FGF21 levels in patients with T2DM. Multivariate forward stepwise linear regression analysis of the variables significantly associated with fasting serum FGF21 levels revealed that body fat mass (adjusted R^2 change $= 0.218$, $P = 0.008$) and log-TG (adjusted R^2 change $= 0.036$, $P < 0.001$) positively correlated, whereas eGFR (adjusted R^2 change $= 0.033$; $P = 0.013$) negatively correlated with serum FGF21 levels in patients with T2DM.

4. Discussion

The major findings of our present study are summarized as follows. T2DM patients with MetS have significantly elevated FGF21 concentrations accompanied with a higher proportion of women; higher prevalence of hypertension and elevated BP values; elevated body adiposity items; unfavorable lipid, glucose, and renal function profiles; and increased insulin resistance parameters in comparison with non-MetS individuals with T2DM. FGF21 values have a positive correlation with body fat mass and serum TG level and are negatively correlated with eGFR in T2DM population.

The cluster of interrelated risk factors including hypertension, hyperglycemia, dyslipidemia, and visceral obesity indicates that MetS has cross talk with many cardiometabolic diseases. As expected, our study reveals that T2DM patients with MetS have significantly higher BW, BMI, WC, body fat mass, and SBP and DBP values; higher prevalence of hypertension; elevated TG, fasting glucose, and HbA1c concentrations; and lower HDL-C level than T2DM patients without MetS. Previous epidemiological studies have shown that MetS is closely related to the prevalence of chronic kidney disease (CKD) [18–20]. A systematic review and meta-analysis revealed that MetS and its components have been associated with impaired renal function and microalbuminuria or overt proteinuria [21]. Not surprised, MetS has been associated with increased risks for DM and CVD occurrence, and established cardiovascular risk factors have promoted the development of CKD [22]. Our study also confirmed that significant impaired renal function with elevated UACR and decreased eGFR values is noted in T2DM subjects with MetS than those without MetS.

FGF21, which is primarily secreted by the liver and expressed to a lesser extent in adipocyte, skeletal muscle, pancreas, and thymus, is a hepatokine response to the metabolic imbalance deterioration and has been implicated as a potential biomarker for early detection of these

Table 1: Clinical variables of the 126 type 2 diabetes mellitus patients with or without metabolic syndrome.

Variables	All participants ($n = 126$)	No metabolic syndrome ($n = 42$)	Metabolic syndrome ($n = 84$)	P value
Age (years)	62.43 ± 12.32	60.83 ± 14.11	63.23 ± 11.32	0.306
Height (cm)	161.48 ± 8.51	162.86 ± 7.70	160.80 ± 8.85	0.201
Body weight (kg)	70.11 ± 13.51	62.94 ± 8.75	73.69 ± 14.07	<0.001*
Body mass index (kg/m^2)	26.77 ± 3.94	23.68 ± 2.45	28.32 ± 3.63	<0.001*
Waist circumference (cm)	89.98 ± 9.57	82.25 ± 7.45	93.85 ± 8.07	<0.001*
Body fat mass (%)	31.74 ± 7.56	25.48 ± 6.13	34.88 ± 6.13	<0.001*
Systolic blood pressure (mmHg)	141.65 ± 20.38	128.76 ± 15.09	148.10 ± 19.66	<0.001*
Diastolic blood pressure (mmHg)	82.73 ± 10.92	76.33 ± 8.91	85.93 ± 10.45	<0.001*
Total cholesterol (mg/dL)	163.19 ± 30.10	158.40 ± 25.99	165.58 ± 31.83	0.208
Triglyceride (mg/dL)	121.00 (85.00–183.75)	89.50 (58.50–112.25)	136.50 (101.75–217.00)	<0.001*
HDL-C (mg/dL)	46.56 ± 12.16	51.10 ± 13.46	44.30 ± 10.83	0.003*
LDL-C (mg/dL)	101.18 ± 26.71	96.50 ± 23.50	103.52 ± 28.02	0.165
Fasting glucose (mg/dL)	137.50 (121.00–173.50)	124.00 (115.50–151.75)	142.00 (127.00–182.75)	0.004*
Glycated hemoglobin (%)	7.40 (6.60–8.90)	6.90 (6.30–7.80)	7.85 (6.80–9.15)	0.005*
Blood urea nitrogen (mg/dL)	16.00 (12.00–18.00)	15.00 (12.00–18.00)	16.00 (12.00–18.75)	0.175
Creatinine (mg/dL)	0. 90 (0.70–1.00)	0.90 (0.70–1.00)	0.90 (0.70–1.00)	0.466
eGFR (mL/min)	85.47 ± 25.64	94.80 ± 28.59	80.80 ± 22.80	0.003*
UACR (mg/g)	15.81 (7.15–105.63)	7.95 (4.66–18.33)	25.31 (9.81–190.83)	<0.001*
Insulin (μIU/mL)	7.01 (3.25–13.62)	3.65 (1.81–6.17)	9.78 (5.08–18.02)	<0.001*
HOMA-IR	2.40 (1.14–4.98)	1.18 (0.77–1.95)	3.63 (1.91–6.77)	<0.001*
FGF21 (pg/mL)	192.69 (109.82–283.48)	141.45 (66.16–227.56)	218.95 (139.80–325.63)	<0.001*
Women (n, %)	47 (37.3)	12 (28.6)	45 (53.6)	0.008*
Hypertension (n, %)	66 (52.4)	11 (26.2)	55 (65.5)	<0.001*
Statin use (n, %)	60 (47.6)	15 (35.7)	45 (53.6)	0.058
Fibrate use (n, %)	8 (6.3)	1 (2.4)	7 (8.3)	0.196
Metformin use (n, %)	69 (54.8)	19 (45.2)	50 (59.5)	0.126
Sulfonylureas use (n, %)	70 (55.6)	21 (50.0)	49 (58.3)	0.375
DDP-4 inhibitor use (n, %)	77 (61.1)	23 (54.8)	54 (64.3)	0.301
Thiazolidinedione use (n, %)	5 (4.0)	2 (4.8)	3 (3.6)	0.747
Insulin use (n, %)	30 (23.8)	12 (28.6)	18 (21.4)	0.375

Values for continuous variables are given as means ± standard deviation and are tested by Student's t-test. Variables that are not normally distributed are given as medians and interquartile range and are tested by Mann–Whitney U test. Values are presented as number (%), and analysis was done using the chi-square test. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; UACR, urine albumin-to-creatinine ratio; HOMA-IR, homeostasis model assessment of insulin resistance; FGF21, fibroblast growth factor 21; DDP-4, dipeptidyl peptidase 4. *$P < 0.05$ was considered statistically significant.

Table 2: Odds ratio for metabolic syndrome by multivariable logistic regression analysis of fibroblast growth factor 21 levels among the 126 patients with type 2 diabetes mellitus.

FGF21 (pg/mL)	Unadjusted		Model 1		Model 2		Model 3	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Per 1 pg/mL FGF21 increase	1.008 (1.003–1.012)	0.001*	1.007 (1.002–1.011)	0.004*	1.005 (1.001–1.010)	0.027*	1.005 (1.000–1.010)	0.035*

Model 1 is adjusted for age and gender. Model 2 is adjusted for the Model 1 variables and for estimated glomerular filtration rate and urine albumin-to-creatinine ratio. Model 3 is adjusted for the Model 2 variables and for insulin level and homeostasis model assessment of insulin resistance. *$P < 0.05$ by multivariate logistic regression analysis. FGF21, fibroblast growth factor 21; OR, odds ratio; CI, confidence interval.

cardiometabolic diseases [23, 24]. FGF21 is initially recognized as a "favorable" cytokine involved in metabolic regulation of insulin-independent glucose transport in cells. An animal study revealed that FGF21 specifically upregulates the glucose transporter 1 (GLUT1) with greater expression of GLUT1 mRNA at the adipocyte cellular membrane and then induces noninsulin-dependent glucose uptake in the insulin resistance model and obesity (ob/ob mice) [6]. Systematically administered FGF21 can lower serum TG and glucose levels and improve lipoprotein profiles significantly in genetically compromised diabetic monkeys [8]. Nevertheless, studies had revealed that serum FGF21 levels are significantly higher in obese patients with MetS components than that in healthy controls, progressively elevated with worsening dysglycemia from normal glucose tolerance to prediabetes and diabetes, and prominently increased in human cardiometabolic diseases such as obesity, MetS, T2DM, coronary artery disease, and nonalcoholic fatty liver disease [9, 10, 23, 25]. A recent study reported that serum FGF21 concentrations were significantly associated with SBP, DBP, BMI, serum TG, and fasting glucose levels in a Japanese adult population without metabolic disorders medication, suggesting that an FGF21 compensatory response to metabolic stress or resistance is associated with "metabolic imbalance" [26].

Table 3: Correlation between serum fibroblast growth factor 21 levels and clinical variables among the 126 patients with type 2 diabetes mellitus.

Variables	Logarithmically transformed fibroblast growth factor 21 (pg/mL)				
	Univariate		Multivariate		
	R	P value	Beta	Adjusted R^2 change	P value
Women	0.172	0.055	—	—	—
Hypertension	0.201	0.024*	—	—	—
Age (years)	0.121	0.179	—	—	—
Height (cm)	–0.059	0.513	—	—	—
Body weight (kg)	0.168	0.060	—	—	—
Body mass index (kg/m^2)	0.259	0.003*	—	—	—
Waist circumference (cm)	0.301	0.001*	—	—	—
Body fat mass (%)	0.359	<0.001*	0.218	0.218	0.008*
SBP (mmHg)	0.191	0.032*	—	—	—
DBP (mmHg)	0.180	0.043*	—	—	—
Total cholesterol (mg/dL)	0.163	0.068	—	—	—
Log-triglyceride (mg/dL)	0.499	<0.001*	0.357	0.036	<0.001*
HDL-C (mg/dL)	–0.219	0.014*	—	—	—
LDL-C (mg/dL)	0.176	0.049*	—	—	—
Log-glucose (mg/dL)	0.187	0.036*	—	—	—
Log-HbA1c (%)	0.059	0.517	—	—	—
Log-BUN (mg/dL)	0.001	0.996	—	—	—
Log-creatinine (mg/dL)	0.194	0.029*	—	—	—
eGFR (mL/min)	–0.325	<0.001*	–0.205	0.033	0.013*
Log-UACR (mg/g)	0.198	0.031*	—	—	—
Log-insulin (μIU/mL)	0.334	<0.001*	—	—	—
Log-HOMA-IR	0.358	<0.001*	—	—	—

Data of triglyceride, glucose, HbA1c, BUN, creatinine, UACR, insulin, and HOMA-IR levels showed skewed distribution and therefore were log-transformed before analysis. Analysis of data was done using the univariate linear regression analysis or multivariate stepwise linear regression analysis (adapted factors were hypertension, body mass index, waist circumference, body fat mass, SBP, DBP, log-triglyceride, HDL-C, LDL-C, log-glucose, log-creatinine, eGFR, log-UACR, insulin, and HOMA-IR). SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, glycated hemoglobin; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; UACR, urine albumin-to-creatinine ratio; HOMA-IR, homeostasis model assessment of insulin resistance. *$P < 0.05$ was considered statistically significant.

The mechanism by which FGF21 affects the pathogenesis of the MetS and T2DM is likely to be multifactorial. FGF21 needs to bind to β-Klotho complex, an FGF co-receptor expressed prominently in metabolically active tissues such as the liver, adipocyte, and pancreas, to activate FGF receptor–mediated signaling [24]. In the adipocyte, FGF21 promotes white adipose tissue (WAT) uptake of glucose and converts WAT into brown adipose tissue, which also stimulates glucose uptake [27]. In addition, FGF21 can reduce glucolipotoxicity by protecting the pancreas β-cells from apoptosis possibly due to its lowering lipid and glucose effects [28]. A previous study suggested that obesity-related adipocyte inflammatory condition can suppress β-Klotho expression by tumor necrosis factor-alpha and impair FGF21 function in adipose tissue causing glucose intolerance [29]. Similar actions may also lead to FGF21 resistance in subclinical inflammation such as MetS and T2DM [23]. Accumulating evidence suggests that dyslipidemia has a strong association with inflammatory processes [30, 31]. A study revealed that the circulating FGF21 level has a positive association with high-sensitive C-reactive protein, a parameter of inflammation, in T2DM patients. Besides, high-sensitive C-reactive protein is an independent determinant for the serum FGF21 value in T2DM population [32]. A positive correlation between FGF21 levels and obesity-related parameters such as WC, waist-to-hip ratio, BMI, and body fat percentage were found even after adjusting for age. Furthermore, FGF21 levels progressively elevated when the number of MetS components increased [9].

Our present study revealed that the FGF21 value has correlation with obesity and dyslipidemia parameters such as BMI, WC, body fat mass, and serum TG, HDL-C, and LDL-C levels in the univariate linear regression analysis in T2DM patients and that body fat mass and serum log-TG levels still had positive association with serum FGF21 level even after performing multivariate linear regression analysis, indicating the phenomenon of FGF21 resistance exists in T2DM populations. Tyynismaa et al. reported that liver fat and serum TG levels are the most proximal correlates of circulating FGF21 levels in healthy young adult twins [33]. Novotny et al. have also demonstrated that FGF21 had a positive association with WC and serum TG in MetS population in line with our study [24]. Genetic variations have a moderate role to influence the differences in FGF21 concentrations and may explain the weak relationships between the serum log-TG levels with FGF21 in our study population [33].

Compensatory hyperinsulinemia due to insulin resistance plays a central role in MetS [34]. In line with previous studies, our present study revealed that T2DM patients with MetS have a higher serum insulin level and

HOMA-IR value than that of individuals without MetS [14, 34]. Under lipid heparin infusion–induced supraphysiological level of free fatty acid (FFA), the serum FGF21 level was elevated, accompanied with hyperinsulinemia in a recent study. This situation suggested that higher FFA, which is often observed in T2DM populations and presumably secondary to the increased lipolysis, may be one of the main stimulators to increase serum FGF21 in MetS patients with T2DM [35]. As mentioned earlier, high serum FGF21 levels were observed in obesity-related disorders and insulin-resistant patients [9, 10], suggesting FGF21 resistance leading to its compensatory upregulation. This scenario is comparable with hyperinsulinemia and hyperleptinemia in MetS and T2DM [12].

Serum FGF21 levels might also be regulated by renal function. Studies revealed that compared with control volunteers, serum FGF21 levels were eight-fold higher in nondiabetic patients receiving peritoneal dialysis [36]. Median circulating FGF21 values were more than 15-fold higher in hemodialysis patients than in patients with an eGFR >50 mL/min. Furthermore, serum creatinine positively and eGFR negatively predicted FGF21 concentrations in multiple regression analysis in control patients [37]. Our present study, which is also in line with previous studies, revealed that FGF21 value has a negative correlation with eGFR values after multivariate forward stepwise linear regression analysis.

There are some limitations to the current study. First, this was a cross-sectional study with a potential selection bias, and therefore, further longitudinal studies are needed before a cause–effect relationship between serum FGF21 and MetS can be established in the T2DM population. Second, the sample size might have been too small, and some confounders (e.g., smoking) were not included and may have influenced the predictive power in our study [38]. Third, previous study demonstrated that serum FGF21 levels exhibit a major nocturnal rise occurring between midnight and early morning in non-DM patients. Lu et al. also reported that the peak FGF21 levels were observed in the fasting state (8 am.) between participants with and without T2DM [39]. Our present study investigated circulating FGF21 levels only used fasting blood sample as most human studies reported before and may overestimate the serum FGF21 concentrations due to the diurnal rhythm of T2DM patients. Further larger sample studies measuring the 24 hour profile of FGF21 in T2DM individuals are needed to confirm this observation. However, little information is available to determine the interrelationships of FGF21 with the MetS in the T2DM population. This study is the first to examine the relationship between serum FGF21 levels among MetS patients with T2DM. Further animal or clinical studies are needed to determine whether serum FGF21 plays a causal role directly in mediating with MetS in patients with T2DM.

5. Conclusions

Serum FGF21, a significant biomarker associated with MetS, levels are positively associated with body fat mass and serum TG level and negatively associated with eGFR values in patients with T2DM.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The funding source had no role in the conception and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Bang-Gee Hsu, Du-An Wu, and Ming-Chun Chen conceived and designed the experiments. Ruo-Yao Gao and Jia-Sian Hou performed the experiments. Bang-Gee Hsu, Du-An Wu, and Ming-Chun Chen contributed reagents and analyzed the data. Ruo-Yao Gao, Bang-Gee Hsu, and Ming-Chun Chen wrote the manuscript. All authors read and approved the final manuscript. Ruo-Yao Gao, Bang-Gee Hsu, and Du-An Wu contributed equally to this study.

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Research Article

miR-29a Negatively Affects Glucose-Stimulated Insulin Secretion and MIN6 Cell Proliferation via Cdc42/β-Catenin Signaling

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Background. Diabetes is a progressive metabolic disease characterized by hyperglycemia. Functional impairment of islet β cells can occur to varying degrees. This impairment can initially be compensated for by proliferation and metabolic changes of β cells. Cell division control protein 42 (Cdc42) and the microRNA (miRNA) miR-29 have important roles in β-cell proliferation and glucose-stimulated insulin secretion (GSIS), which we further explored using the mouse insulinoma cell line MIN6. *Methods*. Upregulation and downregulation of miR-29a and Cdc42 were accomplished using transient transfection. miR-29a and Cdc42 expression was detected by real-time PCR and western blotting. MIN6 proliferation was detected using a cell counting kit assay. GSIS under high-glucose (20.0 mM) or basal-glucose (5.0 mM) stimulation was detected by enzyme-linked immunosorbent assay. The miR-29a binding site in the Cdc42 mRNA 3′-untranslated region (UTR) was determined using bioinformatics and luciferase reporter assays. *Results*. miR-29a overexpression inhibited proliferation ($P < 0.01$) and GSIS under high-glucose stimulation ($P < 0.01$). Cdc42 overexpression promoted proliferation ($P < 0.05$) and GSIS under high-glucose stimulation ($P < 0.05$). miR-29a overexpression decreased Cdc42 expression ($P < 0.01$), whereas miR-29a downregulation increased Cdc42 expression ($P < 0.01$). The results showed that the Cdc42 mRNA 3′-UTR is a direct target of miR-29a *in vitro*. Additionally, Cdc42 reversed miR-29a-mediated inhibition of proliferation and GSIS ($P < 0.01$). Furthermore, miR-29a inhibited β-catenin expression ($P < 0.01$), whereas Cdc42 promoted β-catenin expression ($P < 0.01$). *Conclusion*. By negatively regulating Cdc42 and the downstream molecule β-catenin, miR-29a inhibits MIN6 proliferation and insulin secretion.

1. Introduction

Diabetes is a progressive metabolic disease characterized by hyperglycemia, and it is the third most common chronic disease worldwide, after cancer and cardiovascular disease [1, 2]. Based on the pathogenesis of diabetes, it can be divided into type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) [3]. T1DM is characterized by autoimmune-induced loss of β cells in the pancreas, which leads to insufficient insulin secretion or complete insulin deficiency [4]. T2DM is caused by genetic, environmental, behavioral, and other risk factors, and it is characterized by hyperglycemia, insulin resistance, and relative insulin deficiency [5]. During the development of both T1DM and T2DM, functional impairment of islet β cells can occur to varying degrees [6]. This impairment can initially be compensated for by β-cell proliferation and changes in metabolism. However, as the disease progresses, the islet β-cell proliferation is reduced and insulin secretion continues to decline, eventually leading to irreversible functional failure. Therefore, studying islet β-cell proliferation and insulin secretion is of great significance.

It has been reported that both islet β cells self-replication under elevated blood glucose conditions and transformation of islet α cells to β cells may increase the number of islet β cells [7–9]. In β cells, glucose can regulate insulin secretion

in a process known as glucose-stimulated insulin secretion (GSIS) [10]. GSIS can maintain blood glucose levels within the physiological range, which involves transportation of glucose into β cells through the plasma membrane glucose transporters, followed by transformation of glucose to glucose-6-phosphate and the subsequent rises of Ca^{2+} and metabolic coupling factors such as ATP, glutamate, NADPH, and monoacylglycerol from glycolytic or mitochondrial metabolism [11, 12]. GSIS is composed of two phases: a rapid and transient first phase and a slow and lasting second phase. Both phases involve active mobilization of insulin secretory granules from the cytoplasm to the plasma membrane, requiring small GTP-binding proteins known as small GTPases-mediated actin cytoskeletal remodeling [12–14].

MicroRNAs (miRNAs) are short non-coding RNAs of approximately 22-nt in length, which are recognized as important regulators of gene expression after transcription [15]. To date, the human genome has been shown to encode more than 2000 miRNAs, which are involved in a wide variety of biological and pathological processes [16]. miRNAs act as negative regulators by repressing mRNA translation or causing mRNA degradation after transcription, so abnormal miRNA expression interferes with many physiological and pathological processes [17]. Many miRNAs have been found to be involved in the pathogenesis of diabetes and insulin resistance, and they affect the function of islet β cells [18, 19]. miR-29a is one of the most abundant miRNAs expressed in the β cells of the mouse and human pancreas, and many studies have shown upregulation of miR-29a in diabetic models [20–22]. It belongs to the miR-29 family, which is composed of three closely related precursors: miR-29a, miR-29b1, and miR-29b2 (which are identical but encoded by two distinct precursor stem sequences), and miR-29c [23]. The sequences of mature miR-29 family members are conserved in humans, rats, and mice, and the seed sequence that regulates gene expression by binding to target mRNAs, AGCACC, is also identical [20]. miR-29a has been reported to play a negative regulatory role in insulin secretion by human and mouse islet β cells, and miR-29a overexpression reduced GSIS levels *in vitro* [24]. Conversely, it has also been reported that miR-29a positively regulates insulin secretion *in vivo* [20]. Therefore, the role of miR-29a in GSIS warrants further study.

Cell division control protein 42 (Cdc42) is a member of the Rho family of small GTPases [25], and it plays an important role in the second phase of GSIS [26, 27]. It has been confirmed that Cdc42 can be found in cloned islet β cells, normal mouse islet cells, and normal human islet cells, and it is localized to insulin secretory granules [28]. Under physiological conditions, glucose regulates actin cytoskeleton rearrangement and stimulates insulin secretion by mediating the transformation between Cdc42-GDP (inactive) and Cdc42-GTP (active) [29]. Salunkhe et al. found that phosphorylation of focal adhesion kinase (FAK), which phosphorylates Cdc42 under glucose stimulation, disrupts the F-actin barrier, allowing insulin secretory granules to redistribute in islet β cells and thereby promoting insulin secretion [30]. It has also been reported that Cdc42 mediates insulin secretory granule transportation and insulin secretion via the PAK1-Raf-1/MEK/ERK pathway [31]. Additionally, Cdc42-PAK1-Rac1 has been shown to play a regulatory role in insulin exocytosis and may also play a role in actin remodeling and insulin granule mobilization [32]. These studies suggest that Cdc42 has a significant role in GSIS.

β-Catenin is a transcription factor, mostly known as a key component of the canonical Wnt signaling pathway to regulate cell proliferation [33, 34]. Activated Wnt signaling inhibits ubiquitin-mediated proteasomal degradation of β-catenin, thus causing β-catenin to accumulate. Subsequently, β-catenin translocates to the nucleus to form a transcriptionally active complex with T-cell factor (TCF) and lymphoid enhancer factor and promotes transcription of proliferation-related genes, such as c-Myc [35, 36]. β-Catenin can also regulate cell-cell adhesion between pancreatic β cells via forming complexes with cadherins, which is important for correct regulation of insulin release [37–39]. Emerging evidence has shown upregulation of β-catenin protein under diabetic conditions, and hyperglycemia can promote translocation of β-catenin [40, 41].

In a study on human non-small cell lung cancer, miR-29a overexpression led to significant inhibition of Cdc42 protein expression, whereas Cdc42 mRNA expression was unchanged [42]. In gastric cancer, miR-29a inhibits Cdc42 expression at both the protein and RNA levels [43]. Additionally, miR-29a inhibits glioma invasion by targeting Cdc42 [44]. Furthermore, in breast cancer, Cdc42 negatively regulates p53, and miR-29a positively regulates p53 by targeting Cdc42 and, notably, miR-29a inhibits insulin secretion by negatively regulating Cdc42 and P85 [45]. Cdc42 has also been identified as a direct target of miR-29a in mouse osteoclasts using a luciferase reporter assay [46]. And many studies have confirmed that β-catenin can be regulated as a downstream molecule of Cdc42 [47–49]. Therefore, the role of miR-29a in islet β-cell proliferation and GSIS may be achieved through interaction with Cdc42/β-catenin signaling.

The aim of the current study was to explore the effects of miR-29a and Cdc42 on islet β-cell proliferation and GSIS using MIN6 cells, and to identify the effect of the miR-29a/Cdc42/β-catenin signaling cascade in these cells. The results indicate that miR-29a plays a negative regulatory role in GSIS and MIN6 cell proliferation, whereas Cdc42 plays a positive regulatory role. And miR-29a negatively affects GSIS and MIN6 cell proliferation via inhibiting Cdc42/β-catenin signaling pathway.

2. Materials and Methods

2.1. Cell Line and Culture. The mouse insulinoma cell line MIN6 was obtained from BoGu Biotechnology Co. Ltd. (Shanghai, China). High-glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Cromwell, CT, USA). The MIN6 cells were maintained in high-glucose DMEM supplemented with 12% FBS, 10 μl/L β-mercaptoethanol

(Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg streptomycin mixture (Solarbio, Beijing, China) at 37°C in 5% CO_2.

2.2. Transient Transfection. 5.5×10^5 MIN6 cells were inoculated in 6-well plate and incubated for 24 hours in DMEM medium. The 20 μM final concentration of miR-29a mimic, inhibitor, negative control (NC; miR-29a NC), and Cdc42-pcDNA3.1 was synthesized by Gemma Co. Ltd. (Shanghai, China). siRNA fragments (siRNA-497, siRNA-569, and siRNA-643) and an NC-siRNA fragment were also obtained from Gemma Co. Ltd. Oligonucleotide and plasmid transfection was conducted using Lipofectamine 2000 (Gemma Co. Ltd.). Opti-MEM was purchased from Gibco company (Grand Island, NY, USA). Firstly, 100 pmol of siRNA was added to 200 μl Opti-MEM and blended gently. Secondly, 200 μl Opti-MEM was used to dilute 5 μl lip2000 reagent. This was maintained for 5 minutes at room temperature after mixing. The lip2000 reagent diluent was then added to the siRNA diluent at room temperature for 20 minutes to form the siRNA-lip2000 complex. The medium was replaced by serum-free medium, and siRNA-lip2000 complex was added into the pore containing cells and medium. The fluorescence and cell status were observed after 6 hours. The serum-free medium was extracted and medium was added. The sequences of the oligonucleotides are shown in Table 1. After 24–48 h of transfection, MIN6 cells were used for the following experiments.

2.3. Real-Time Polymerase Chain Reaction (RT-PCR). When the cell confluency reached 75%, the miR-29a mimic, miR-29a inhibitor, and miR-29a-NC, NC-siRNA, siRNA-497, siRNA-569 and siRNA-643 were separately transiently transfected into MIN6 cells. After 36 h, total RNA was extracted from the MIN6 cells using the total RNA isolation reagent (Omega, Norcross, GA, USA) according to the manufacturer's instructions. 2 μl PrimeScript buffer, 0.5 μl Random 6 mers, 0.5 μl Oligo dT Primer, 0.5 μl 1 × PrimeScript RT Enzyme Mix I, 0.5 μl gene-specific primers, up to 10 μl RNase free ddH_2O, and 500 ng total RNA were added to prepare for reverse transcription system. Then, reverse transcription was performed at 37°C for 15 minutes, and after 5 seconds at 85°C, the machine was maintained at 4°C (when using gene-specific primers, the first step of reverse transcription reaction condition was changed to 42°C 15 minutes). RT-PCR was performed with an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). 0.4 μl of forward primer, 0.4 μl of reverse primer, 10 μl of TB Green Premix Ex Taq, 0.4 μl 50 × ROX reference dye, 2 μl of template, 6.8 μl of ddH_2O were added to form 20 μl of the total reaction system. After mixing, 18 μl total reaction system and 2 μl cDNA were added to each pore. The reaction mixture was incubated at 95°C for 30 s followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative difference in gene expression.

Table 1: The sequences of miR-29a mimic and Cdc42-mus.

Name	Sequences (5′-3′)
miRNA-29a mimic	UAGCACCAUCUGAAAUCGGUUA
	ACCGAUUUCAGAUGGUGCUAUU
Cdc42-mus-643	UCACACAGAAAGGCCUAAATT
	UUUAGGCCUUUCUGUGUGATT
Cdc42-mus-569	GCCUAUUACUCCAGAGACUTT
	AGUCUCUGGAGUAAUAGGCTT
Cdc42-mus-497	GCUUGUUGGGACCCAAAUUTT
	AAUUUGGGUCCCAACAAGCTT

2.4. Western Blot Analysis. Transient transfection was performed when the MIN6 cell confluency reached 75%, and protein extraction was carried out after 40 h. Cdc42-pcDNA3.1, Cdc42-siRNAs, miR-29a mimic, miR-29a inhibitor, and miR-29a-NC were transiently transfected into cells. Protein was electrophoresed on SDS-polyacrylamide gel consisting of 5% stacking gel and 12% separating gel (Solarbio). First, 15 μg of protein was added to each slot. After the proteins were separated, they were transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA) under 200 mA for 1 hour and 15 minutes. Next, 5% nonfat milk (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) was used for 2 h blocking. Then, the PVDF membrane was incubated overnight at 4°C with diluted (1 : 1000) primary antibodies (Cdc42 antibody, Abcam, Cambridge, MA, USA; β-catenin, Affinity Biologicals, Shanghai, China; and β-actin, Zhongshanjinqiao Company, Beijing, China). Subsequently, the membrane was incubated for 1.5 h at room temperature with diluted (1 : 5000) secondary antibodies (HPR-labeled anti-rabbit IgG of goat, HPR-labeled anti-rat IgG of goat; both were purchased from Zhongshanjinqiao Company). Lastly, the proteins were detected using an EasySee Western Blot Kit (TransGen Biotech, Beijing, China) with a Gel Imaging System (Bio-Rad, Hercules, CA, USA).

2.5. Cell Proliferation Assay. A cell proliferation assay was performed using a cell counting kit (CCK; TransGen) after Cdc42-pcDNA3.1, Cdc42-siRNA-643, miR-29a inhibitor + Cdc42-siRNA-643, and miR-29a mimic + Cdc42-pcDNA3.1 were separately transiently transfected into MIN6 cells. MIN6 cells were incubated in 96-well plates for 24 h. Next, 10 μl CCK solution and 90 μl high-glucose (4500 mg/L) DMEM were added to the cells. The cells were then placed in an incubator at 37°C for 1 h before assessment. The optical density at 450 nm (OD_{450}) at 24, 48, and 72 h was measured using an SpectraMax Paradigm enzyme labelling apparatus (Molecular Devices LLC, Sunnyvale, CA, USA), and corresponding cell growth curves were plotted.

2.6. Insulin Secretion Assay. After 40 h of transfection, the medium was removed and cells in each group were divided into two subgroups. Next, 1 ml Krebs-Ringer bicarbonate HEPES (KRBH, PanEra, Guangzhou, China) buffer was added to each well and the mixture was incubated for 1 h.

Thereafter, the KRBH buffer was removed and 1 ml KRBH containing 5.0 or 20.0 mM glucose (Solarbio) was added into subgroups separately for 1 h. The levels of insulin were detected by enzyme-linked immunosorbent assay (ELISA) using an ELISA Kit for Insulin (Cloud-Clone Corp., Wuhan, China) according to the manufacturer's instructions.

2.7. Luciferase Reporter Assays. The miR-29a binding site in the Cdc42 mRNA 3′-UTR was identified in a bioinformatics analysis (Gemma Co. Ltd.), and a luciferase reporter assay was performed. First, the entire nonmutated 3′-untranslated region (UTR) of the Cdc42 gene was cloned into a pGL3-Basic vector (Gemma Co. Ltd.) at a site immediately downstream of the luciferase gene. Second, the Cdc42 3′-UTR was mutated with a mutagenesis kit (Promega, Madison, WI, USA) and similarly cloned into a pGL3-Basic vector. 1×10^5 MIN6 cells were seeded into 6-well plates and cultured for 24 h. Next, the cells were cotransfected with 2.5 μg of either of the pGL3-Basic vectors, and 2.5 μg of either miR-29a or miR-29a-NC using Lipofectamine 2000 (Gemma Co. Ltd.). At 48 h after transfection, cell lysates were prepared using Luciferase Assay Buffer II, and the luciferase activity was measured using a Luciferase Assay System (Promega). The experiment was performed in triplicate.

2.8. Statistical Analysis. Statistical analysis was performed using Prism 6 (GraphPad Software, San Diago, CA, USA) or SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Student's *t*-test were used to assess the differences between groups. $P < 0.05$ and $P < 0.01$ were considered to be statistically significant and highly statistically significant, respectively.

3. Results

3.1. Effects of miR-29a on MIN6 Cells

3.1.1. Transfection Efficiency of miR-29a Mimic and Inhibitor. To ensure the validity of subsequent miR-29a-related experiments, we determined the transfection efficiency of the miR-29a mimic and inhibitor. The miR-29a mRNA transcription level significantly increased in the miR-29a mimic group compared with the miR-29a-NC group ($P < 0.01$) and significantly decreased in the miR-29a inhibitor group ($P < 0.01$) (Figure 1(a)). These results indicated successful transfection.

3.1.2. miR-29a Negatively Effects MIN6 Cell Proliferation. To determine the effect of miR-29a on MIN6 cell proliferation, we increased and decreased miR-29a expression using the miR-29a mimic and inhibitor, respectively, and detected the proliferation rate at 24, 48, and 72 h. The CCK results showed that there were no significant differences in the proliferation rate between the miR-29a NC group and the miR-29a mimic and inhibitor groups after 24 h. In contrast, the proliferation rate of the miR-29a mimic group significantly decreased after 48 h ($P < 0.01$) and 72 h ($P < 0.01$), and the proliferation rate in the miR-29a inhibitor group significantly increased after 48 h ($P < 0.01$) and 72 h ($P < 0.01$) (Figure 1(b)). These results indicated that miR-29a negatively effects MIN6 cell proliferation.

3.1.3. miR-29a Negatively Effects Insulin Secretion by MIN6 Cells. To identify the effect of miR-29a on insulin secretion by MIN6 cells, we increased and decreased miR-29a expression using the miR-29a mimic and inhibitor, respectively, and detected the level of insulin secretion after stimulation with 5.0 and 20.0 mM glucose. The ELISA results showed that miR-29a overexpression inhibited insulin secretion under high-glucose stimulation ($P < 0.01$) (Figure 1(c)), and miR-29a downregulation promoted insulin secretion under high-glucose stimulation ($P < 0.01$) (Figure 1(d)). Regardless of whether miR-29a was up- or downregulated, there was no effect on insulin secretion under basal-glucose stimulation (Figures 1(c) and 1(d)). These results indicated that miR-29a plays a negative regulatory role in GSIS, but not in insulin secretion at physiological blood glucose levels.

3.2. Effects of Cdc42 on MIN6 Cells

3.2.1. Transfection Efficiency of Cdc42-pcDNA3.1. To ensure the validity of subsequent Cdc42-related experiments, Cdc42-pcDNA3.1 was transiently transfected into MIN6 cells when the cell confluency reached 75%, and protein extraction was carried out after 40 h. The western blot results showed that Cdc42 expression increased after transfection with Cdc42-pcDNA3.1 compared with the expression in the pcDNA3.1 group ($P < 0.01$) (Figure 2(a)). This result indicated successful transfection.

3.2.2. Screening of Cdc42 Small Interfering RNA (siRNA) Fragments. To effectively reduce the Cdc42 expression, we screened three siRNA fragments (siRNA-497, siRNA-569, and siRNA-643) to identify which one was the most effective. When the cell confluency reached 75%, NC-siRNA, siRNA-497, siRNA-569, and siRNA-643 were transiently transfected into MIN6 cells. In each group, total RNA was extracted after 36 h. The RT-PCR results showed that Cdc42 mRNA expression significantly decreased after transfection with siRNA-497 ($P < 0.05$) and siRNA-643 ($P < 0.01$) compared with the expression in the siRNA-NC group. Among the four groups, the siRNA-643 group had the lowest Cdc42 mRNA expression (Figure 2(b)). The results indicated that siRNA-497 and siRNA-643 could more effectively reduce Cdc42 mRNA expression than siRNA-569, and siRNA-643 may have the optimal interference effect.

To determine whether siRNA-643 was the optimal siRNA fragment, when the cell confluency reached 75%, the siRNAs were transiently transfected into MIN6 cells, and protein extraction was carried out after 40 h. The western blot results showed that Cdc42 protein expression in the siRNA-569 and siRNA-643 groups significantly decreased ($P < 0.01$) compared with the expression in the siRNA-NC group. Among

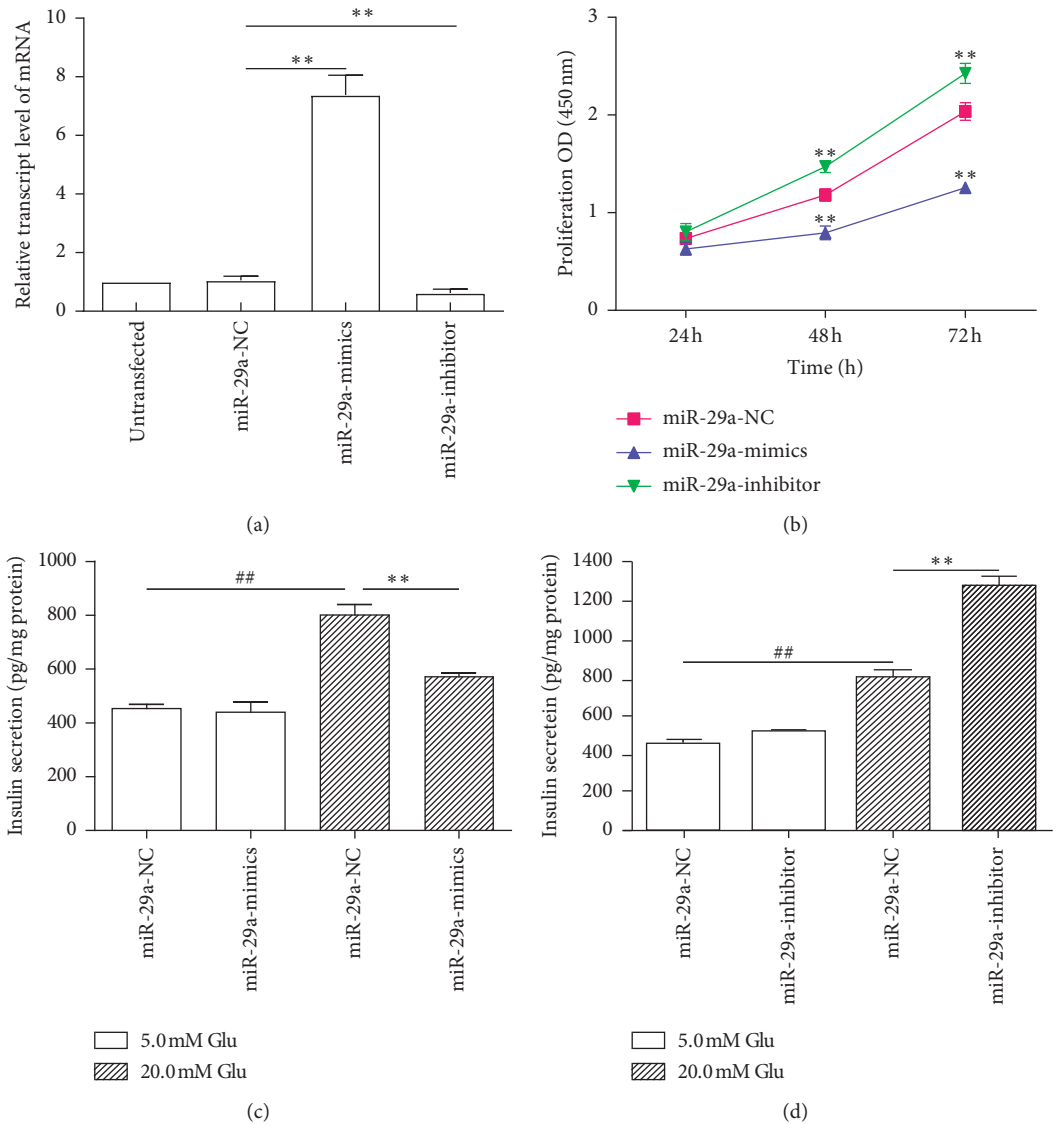

Figure 1: Effects of miR-29a on MIN6 cells. (a) Effects of miR-29a mimic and inhibitor on miR-29a mRNA. RT-PCR to detect miR-29a mRNA expression after transient transfection with miR-29a mimic and inhibitor ($n = 3$). $^{**}P < 0.01$, compared with miR-29a NC group, as assessed by paired Student's t-test. (b) Effects of miR-29a on MIN6 cell proliferation. CCK assay to detect proliferation after transfection with miR-29a mimic and inhibitor ($n = 3$). $^{**}P < 0.01$, compared with miR-29a NC group, as assessed by one-way ANOVA, followed by Fisher's least significant difference test. (c and d) Effects of miR-29a on insulin secretion by MIN6 cells. ELISA to detect insulin secretion levels in MIN6 cells after transient transfection with miR-29a mimic and inhibitor under basal-glucose (5.0 mM) and high-glucose (20.0 mM) stimulation ($n = 3$). $^{\#\#}P < 0.01$, compared with 5.0 mM glucose group, and $^{**}P < 0.01$, compared with miR-29a-NC group, as assessed by paired Student's t-test. Data are shown as mean ± SD. NC: negative control.

the four groups, the siRNA-643 group had the lowest Cdc42 protein expression (Figure 2(c)). Based on the Cdc42 mRNA and protein expression levels, we selected siRNA-643 as the Cdc42-siRNA fragment to use in subsequent experiments.

3.2.3. Cdc42 Positively Effects MIN6 Cell Proliferation. To identify the effect of Cdc42 on MIN6 cells proliferation, the absorbance at 450 nm was measured at 24, 48 and 72 h after transient transfection of MIN6 cells with Cdc42-pcDNA3.1 and Cdc42-siRNA-643, and corresponding cell growth curves were plotted. The CCK results showed that there were no significant differences in the proliferation rate between the Cdc42-pcDNA3.1 and pcDNA3.1 groups, or between the Cdc42-siRNA-643 and siRNA-NC groups, after 24 h. In contrast, the proliferation rate in the Cdc42-siRNA-643 group was significantly decreased after 48 h ($P < 0.01$) and 72 h ($P < 0.01$), and the proliferation rate in the Cdc42-pcDNA3.1 group was significantly increased after 48 h ($P < 0.01$) and 72 h ($P < 0.01$) (Figure 2(d)). These results indicated that Cdc42 positively effects the proliferation rate of MIN6 cells.

3.2.4. Cdc42 Positively Effects Insulin Secretion by MIN6 Cells. To identify the effect of Cdc42 on insulin secretion by

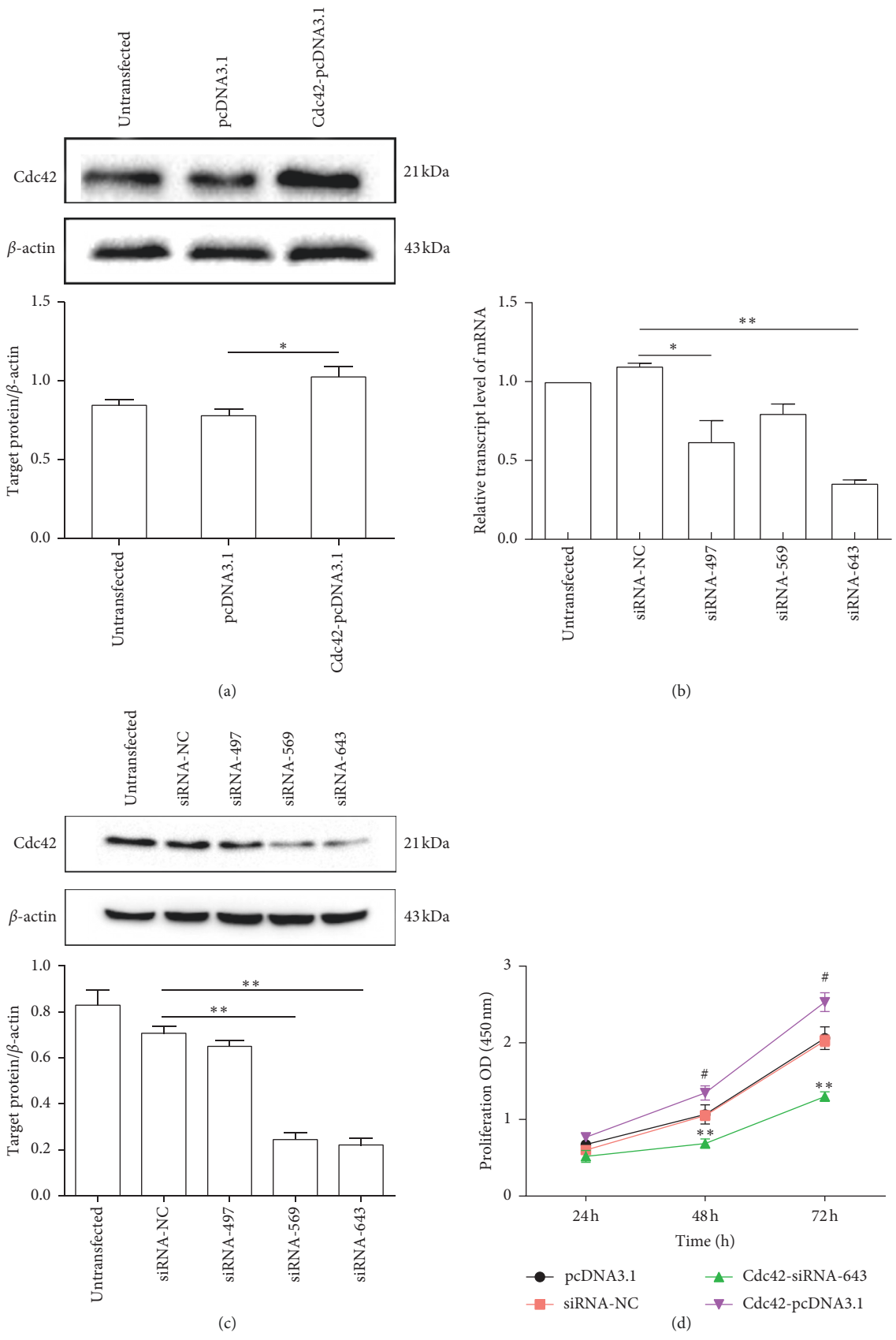

Figure 2: Continued.

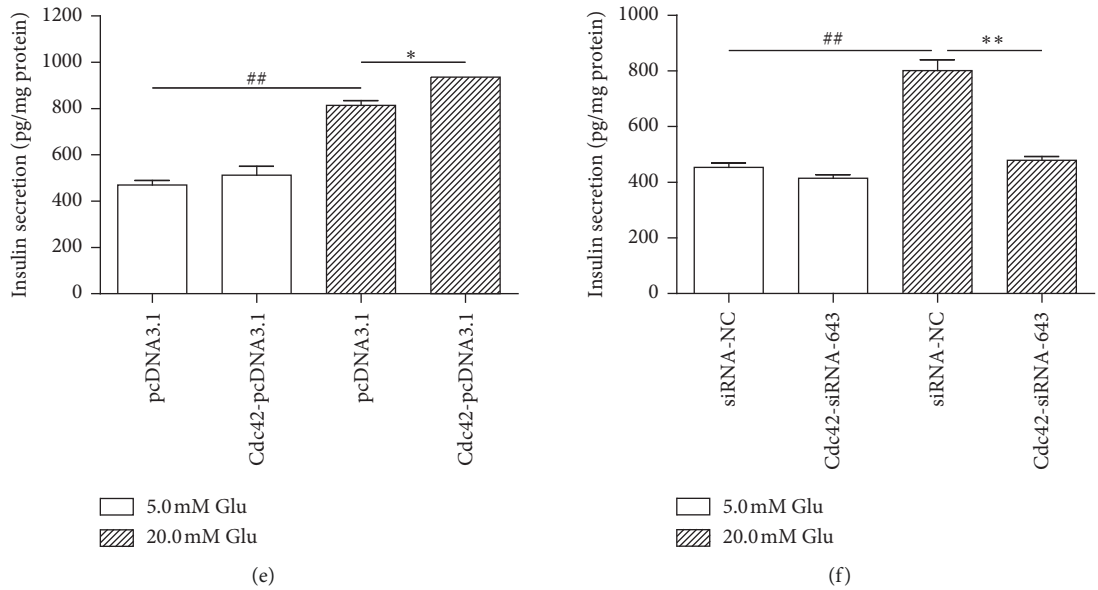

Figure 2: Effects of Cdc42 on MIN6 cells. (a) Effects of Cdc42-pcDNA3.1 on Cdc42 expression in MIN6 cells. Western blot to detect Cdc42 protein expression after transfection with Cdc42-pcDNA3.1 ($n = 3$). $^{*}P < 0.01$, compared with pcDNA3.1 group, as assessed by paired Student's t-test. (b and c) Screening of Cdc42-siRNA fragments. (b) RT-PCR to detect Cdc42 mRNA expression after transient transfection with different siRNA fragments ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with siRNA-NC group, as assessed by paired Student's t-test. (c) Western blot to detect Cdc42 protein expression after transient transfection with different siRNA fragments ($n = 3$). $^{**}P < 0.01$, compared with siRNA-NC group, as assessed by paired Student's t-test. (d) Effects of Cdc42 on MIN6 cell proliferation. CCK assay to detect MIN6 cell proliferation after transfection with Cdc42-siRNA and Cdc42-pcDNA3.1 ($n = 3$). $^{**}P < 0.01$, compared with siRNA-NC group, $^{\#}P < 0.01$, compared with pcDNA3.1 group, as assessed by one-way ANOVA, followed by Fisher's least significant difference test. (e and f) Effects of Cdc42 on insulin secretion by MIN6 cells. ELISA to detect insulin secretion levels in MIN6 cells after transient transfection with Cdc42-pcDNA3.1 and Cdc42-siRNA under basal-glucose (5.0 mM) and high-glucose (20.0 mM) stimulation ($n = 3$). $^{*}P < 0.05$, compared with pcDNA3.1 group, $^{**}P < 0.01$, compared with siRNA-NC group, and $^{\#\#}P < 0.01$, compared with 5.0 mM glucose group, as assessed by paired Student's t-test. Data are shown as mean ± SD. NC: negative control.

MIN6 cells, we increased and decreased Cdc42 expression using Cdc42-pcDNA3.1 and Cdc42-siRNA-643, respectively, and detected the level of insulin secretion under 5.0 and 20.0 mM glucose stimulation by measuring the amount of secreted insulin in the supernatant. The ELISA results showed that Cdc42 overexpression promoted insulin secretion under high-glucose stimulation ($P < 0.05$) (Figure 2(e)), and Cdc42 downregulation inhibited insulin secretion under high-glucose stimulation ($P < 0.01$) (Figure 2(f)). Regardless of whether Cdc42 was up- or downregulated, there were no effects on insulin secretion under basal-glucose stimulation (Figures 2(e) and 2(f)). These results indicated that Cdc42 plays a positive regulatory role in GSIS, but not in insulin secretion at physiological blood glucose levels.

3.3. Effects of miR-29a/Cdc42 on MIN6 Cells

3.3.1. miR-29a Negatively Effects Cdc42 Protein Expression. Many studies have indicated that Cdc42 mRNA is a direct target of miR-29a in cancer progression [42–46]. Thus, we hypothesized that miR-29a can affect the expression of Cdc42 during diabetes progression. To identify the effect of miR-29a on Cdc42 protein expression, we transiently transfected the miR-29a mimic, miR-29a inhibitor, and miR-29a-NC into MIN6 cells when the cell confluency reached 75%, and extracted the proteins for each group after 40 h. The western blot results showed that, compared with the Cdc42 protein expression in the miR-29a NC group, the expression in the miR-29a mimic group significantly decreased ($P < 0.01$), whereas the expression in the miR-29a inhibitor group significantly increased ($P < 0.01$) (Figure 3(a)). These results indicated that miR-29a negatively effects Cdc42 protein expression.

3.3.2. miR-29a Binding Site in the Cdc42 mRNA 3′-UTR. Based on the negative effect of miR-29a on Cdc42 protein expression and in order to confirm that Cdc42 mRNA is a target of miR-29a, the miR-29a binding site in the Cdc42 mRNA 3′-UTR was identified in a bioinformatics analysis and a luciferase reporter assay was performed (Figures 3(b) and 3(c)). The bioinformatics analysis showed that the Cdc42 mRNA 3′-UTR was targeted by the complementary sequence of miR-29a (Figure 3(b)). The luciferase reporter assays showed that the miR-29a mimic significantly decreased the luciferase activity of MIN6 cells expressing the nonmutated Cdc42 mRNA 3′-UTR, but it had no effect on the luciferase activity of MIN6 cells expressing the mutated Cdc42 mRNA 3′-UTR (Figure 3(c)). These results showed that Cdc42 mRNA is

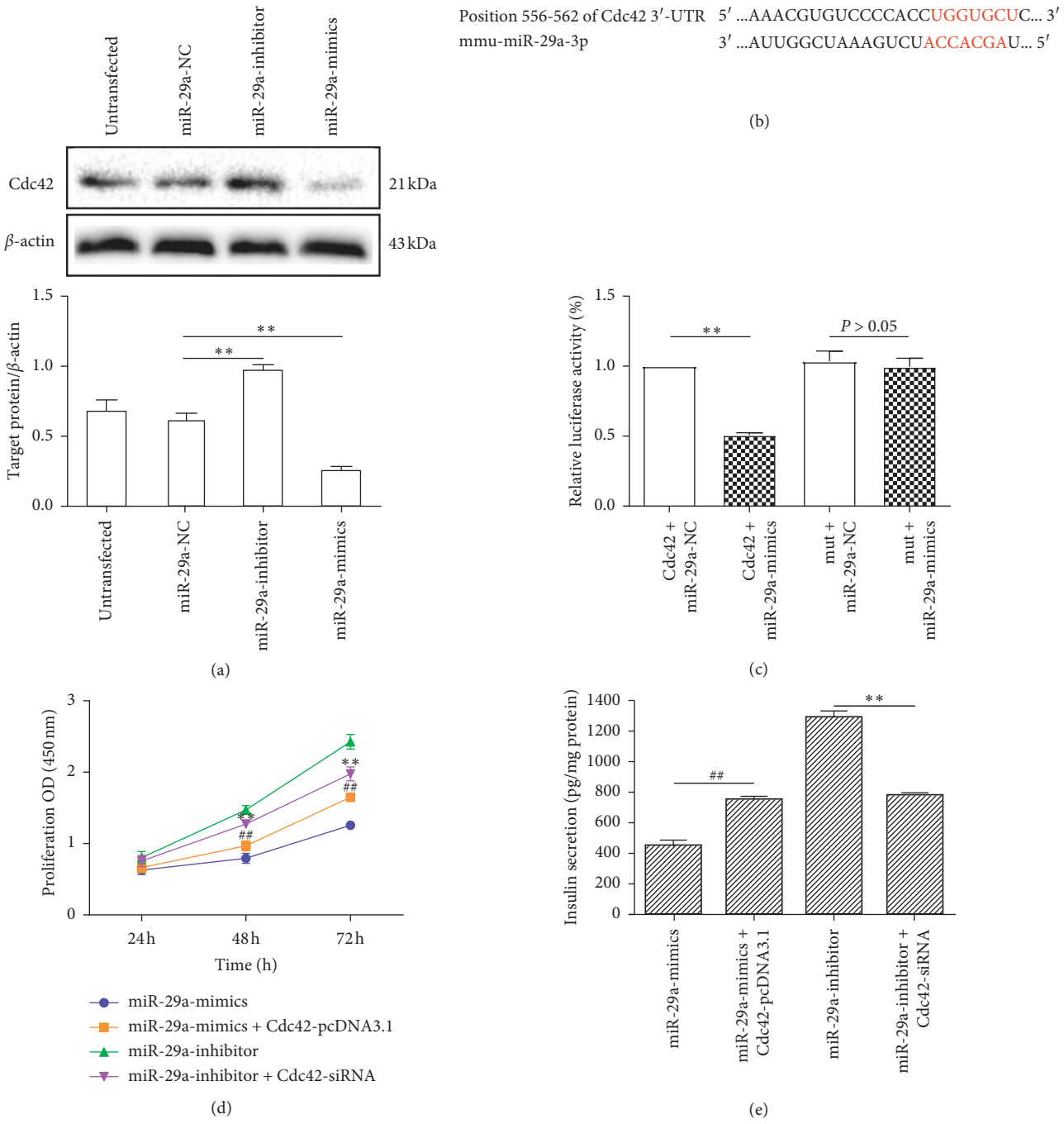

Figure 3: Effects of miR-29a/Cdc42 on MIN6 cells. (a) Effects of miR-29a on Cdc42 protein expression. Western blot to detect Cdc42 protein expression after transfection with miR-29a mimic and inhibitor ($n = 3$). $^{**}P < 0.01$, compared with miR-29a NC group, as assessed by paired Student's t-test. (b) Bioinformatics analysis of the miR-29a binding site in the Cdc42 mRNA 3′-UTR. (c) Luciferase reporter assays indicate that miR-29a binds to Cdc42 mRNA in MIN6 cells. $^{**}P < 0.05$, compared with nonmutated Cdc42 3′UTR + miR-29a-NC group, as assessed by paired Student's t-test. (d) Effects of miR-29a/Cdc42 on MIN6 cell proliferation. CCK assay to detect MIN6 cell proliferation after simultaneous overexpression of miR-29a and Cdc42, and after simultaneous interference with miR-29a and Cdc42 expression ($n = 3$). $^{**}P < 0.01$, compared with miR-29a mimic group, and $^{\#\#}P < 0.01$, compared with miR-29a inhibitor group, as assessed by one-way ANOVA, followed by Fisher's least significant difference test. (e) Effects of miR-29a/Cdc42 on insulin secretion by MIN6 cells. ELISA to detect insulin secretion by MIN6 cells after transient transfection with miR-29a mimic + Cdc42-pcDNA3.1 and miR-29a inhibitor + Cdc42-siRNA under high-glucose (20.0 mM) stimulation ($n = 3$). $^{**}P < 0.01$, compared with miR-29a inhibitor group, and $^{\#\#}P < 0.01$, compared with miR-29a mimic group, as assessed by paired Student's t-test. Data are shown as mean ± SD. NC: negative control; mmu: mouse lemur; 3p: mmu-miR-29a is produced from the 3′ end arm of the double strand of miR-29a; mut: mutated.

a potential downstream molecule of miR-29a, and the miR-29a/Cdc42 axis may be involved in diabetes.

3.3.3. Effects of miR-29a/Cdc42 on MIN6 Cell Proliferation. To identify the effects of miR-29a/Cdc42 on MIN6 cell proliferation, we transfected miR-29a inhibitor + Cdc42-siRNA-643 and miR-29a mimic + Cdc42-pcDNA3.1 into MIN6 cells. The absorbance at 450 nm was measured at 24, 48, and 72 h after transient transfection, and corresponding cell growth curves were plotted. The CCK results showed that after 48 and 72 h, simultaneous overexpression of miR-29a and Cdc42 reversed the effect of the miR-29a mimic regarding MIN6 cell proliferation inhibition ($P < 0.01$). Simultaneous interference with miR-29a and Cdc42 expression reversed the effect of the miR-29a inhibitor regarding MIN6 cell proliferation promotion ($P < 0.01$) (Figure 3(d)). These results further illustrated that Cdc42 mRNA is a downstream molecule of miR-29a and indicated that miR-29a can inhibit MIN6 cell proliferation by downregulating Cdc42 expression.

3.3.4. Effects of miR-29a/Cdc42 on Insulin Secretion by MIN6 Cells. To identify the effects of miR-29a/Cdc42 on insulin secretion by MIN6 cells, we transiently transfected miR-29a inhibitor + Cdc42-siRNA-643 and miR-29a mimic + Cdc42-pcDNA3.1 into MIN6 cells, and then stimulated them with KRBH containing 20.0 mM glucose for 1 h. The amount of secreted insulin in the supernatant was measured by ELISA. The results showed that simultaneous overexpression of miR-29a and Cdc42 reversed the effect of the miR-29a mimic regarding insulin secretion inhibition ($P < 0.01$), and simultaneous interference with miR-29a and Cdc42 expression reversed the effect of the miR-29a inhibitor regarding the promotion of insulin secretion under high-glucose stimulation ($P < 0.01$) (Figure 3(e)). These results further indicated that miR-29a can inhibit insulin secretion by MIN6 cells under high-glucose stimulation by downregulating Cdc42 expression.

3.4. miR-29a/Cdc42/β-Catenin is a Potential Signaling Cascade in MIN6 Cells. Many studies have demonstrated that β-catenin expression can be regulated by Cdc42 [47–49]. Therefore, we hypothesized that β-catenin is a downstream molecule of miR-29a/Cdc42, and miR-29a/Cdc42/β-catenin is a potential signaling cascade in MIN6 cells. We transfected miR-29a mimic, miR-29a inhibitor, Cdc42-pcDNA3.1, and Cdc42-siRNA-643 into MIN6 cells. The western blot results showed that compared with β-catenin expression in the miR-29a mimic and inhibitor NC, miR-29a downregulation significantly increased β-catenin expression ($P < 0.01$), whereas miR-29a overexpression significantly decreased β-catenin expression ($P < 0.01$) (Figure 4(a)). Conversely, Cdc42 overexpression significantly increased β-catenin expression ($P < 0.01$), whereas Cdc42 downregulation significantly decreased β-catenin expression ($P < 0.01$) (Figure 4(b)). These results indicated that miR-29a can inhibit but Cdc42 can promote β-catenin protein expression in MIN6 cells.

4. Discussion

Apart from being one of the leading causes of death worldwide, hyperglycemia in diabetic patients endangers microvessel in various target organs; for example, the brain, heart, kidneys, and eyes [50]. Proliferation impairment of β cells is the main cause of T1DM and GSIS impairment in β cells is the main cause of T2DM [1, 31, 51]. Therefore, it is important to understand the underlying mechanisms that affect proliferation and GSIS of β cells in order to improve the development of pharmacological agents for diabetes treatment. In most cases, miRNAs act as negative regulators and affect protein-coding genes; therefore, abnormal miRNA expression interferes a variety of physiological and pathophysiological processes, including insulin secretion. Many studies have shown that Cdc42 is an important regulatory gene in GSIS [25], via activating its downstream effector p21-activated kinase (Pak1; a Ser/Thr protein kinase) during the second phase of GSIS [52–54]. Cdc42 locates on insulin secretory granules, and it participates in exocytosis of insulin vesicles by regulating F-actin and its associated pathways [55].

In this study, the MIN6 cell line was selected to investigate cell proliferation and insulin secretion *in vitro* for the following four reasons: (a) the MIN6 cell line was established from pancreatic tumors of transgenic non-obese diabetic mice, and the insulin secretory function of MIN6 cells is highly similar to that of the pancreas; (b) islet β cells are the most abundant cells among islet cells, accounting for about 70% of the total; (c) the insulin secretory function of MIN6 cells is highly similar to that of the pancreas; and (d) most of the related studies in the literature used this cell line to study insulin secretion and related signaling pathways, so the body of literature on MIN6 cells is relatively rich. Both T1DM and T2DM involve insulin deficiency, and T1DM also involves loss of β cells, so the study of the role of miR-29a/Cdc42/β-catenin in MIN6 cell proliferation and GSIS will be helpful to explore the molecular mechanisms of T1DM and T2DM. Our experiment first explored effects of miR-29a and Cdc42, and the mechanism of miR-29a/Cdc42/β-catenin pathway on MIN6 cells proliferation and GSIS under high-glucose condition.

The CCK and ELISA results showed that miR-29a inhibits MIN6 cell proliferation and insulin secretion under high-glucose stimulation, but there were no significant differences under basal-glucose stimulation. This result is consistent with the results of an in vitro study by Bagge et al. [24] on the INS-1E cell line, but it conflicts with the conclusions of an in vivo study by Dooley et al. [20],in which miR-29a/b-1 was knocked out because it was not possible to knockout only miR-29a. Thus, these conflicting results may be caused by knocking out miR-29b-1 or by the differences between the *in vitro* and *in vivo* approaches.

The inhibition of Cdc42 protein expression using siRNA-569 appeared to be as effective as that using siRNA-643. Compared with the siRNA-NC group, the inhibition of Cdc42 mRNA expression using siRNA-569 was also effective, but there was no statistical difference. The possible reason was that our experiment was only performed three

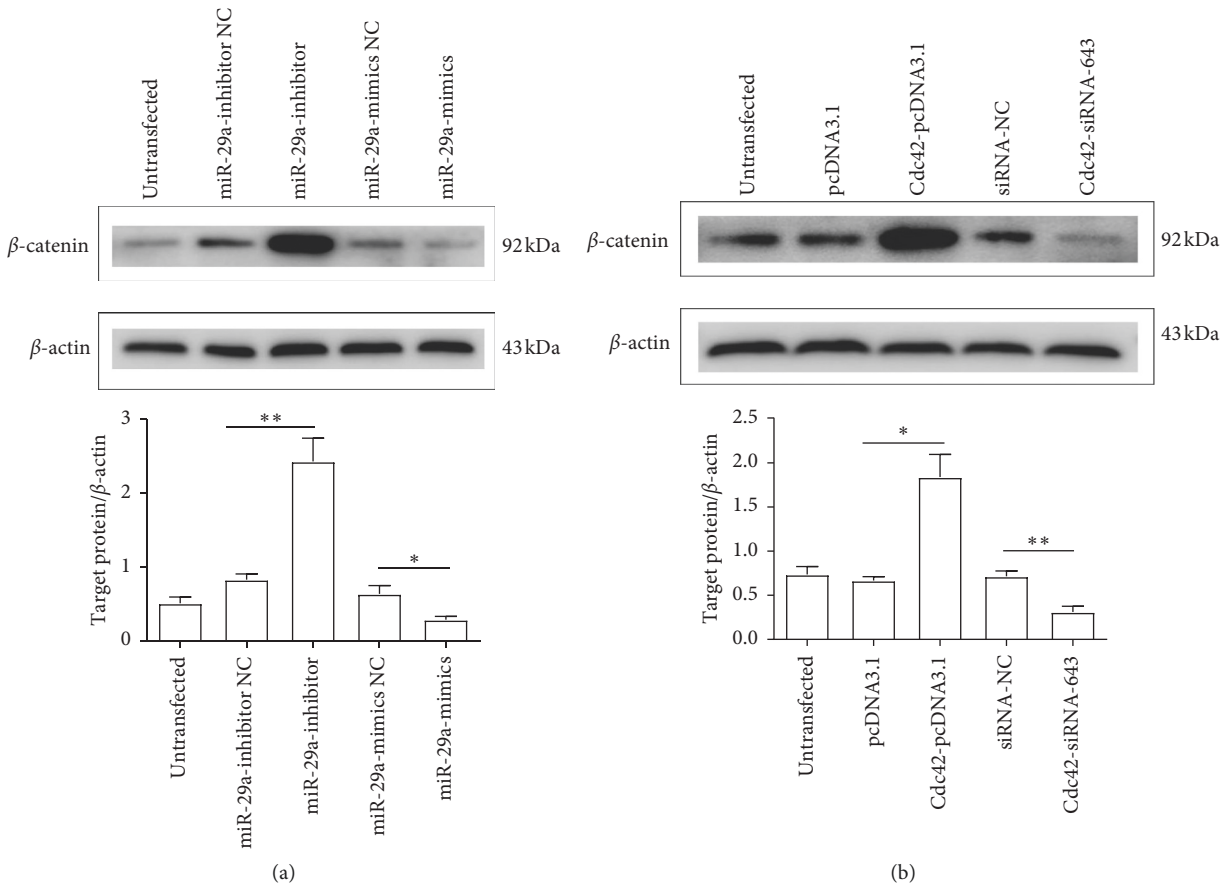

Figure 4: Effects of miR-29a and Cdc42 on β-catenin expression. (a) Effects of miR-29a on β-catenin expression. Western blot to detect β-catenin expression after transfection with miR-29a mimic and inhibitor ($n = 3$). $^{*}P < 0.01$, compared with miR-29a mimic NC, and $^{**}P < 0.01$, compared with miR-29a inhibitor NC, as assessed by paired Student's t-test. (b) Effects of Cdc42 on β-catenin expression. Western blot to detect β-catenin expression after transfection with Cdc42-pcDNA3.1 and siRNA-643 ($n = 3$). $^{*}P < 0.01$, compared with pcDNA3.1, and $^{**}P < 0.01$, compared with siRNA-NC, as assessed by paired Student's t-test. Data are shown as mean ± SD. NC: negative control.

times. And siRNA-643 showed the best inhibitory effect on Cdc42 mRNA and protein expression among the screened siRNA fragments; therefore, siRNA-643 was used for subsequent experiments. The CCK and ELISA results showed that Cdc42 promotes MIN6 cell proliferation and insulin secretion under high-glucose stimulation, but there was no significant difference under basal-glucose stimulation. The results of this experiment are consistent with current mainstream thinking in this field. Current researchers generally believe that Cdc42 plays a positive regulatory role in GSIS and interfering with its expression decreases insulin secretion [25]. Cdc42 can affect insulin secretion by regulation of insulin vesicle fusion, exocytosis, and cytoskeletal rearrangement [56], but the specific pathways involved in this process require further research.

In addition, we investigated whether miR-29a affects MIN6 cell proliferation and insulin secretion by interfering with Cdc42 expression. The western blot results showed that miR-29a has a negative regulatory role regarding Cdc42 expression in MIN6 cells, which is similar to the findings for cancers such as nonsmall-cell carcinoma, stomach cancer, and breast cancer [42, 43, 57]. Furthermore, the bioinformatics analysis and luciferase reporter assay showed that there is a miR-29a binding site in the Cdc42 mRNA 3′-UTR, and miR-29a can therefore affect the expression of Cdc42 and downstream molecules, ultimately exerting biological effects. And Cdc42 can reverse the effects of miR-29a on MIN6 cell proliferation and GSIS under high-glucose condition. It is worth mentioning that regardless of whether miR-29a or Cdc42 was up- or downregulated, there were no effects on GSIS under basal-glucose stimulation; therefore, effects of miR-29a/Cdc42 on MIN6 cells GSIS under basal-glucose stimulation study seems less necessary. These results suggest that Cdc42 is a direct effector of miR-29a *in vitro*, and miR-29a can suppress MIN6 cells proliferation and GSIS via negatively regulating Cdc42 expression.

Many studies have demonstrated that β-catenin can be regulated effectively by Cdc42 [47–49], and we hypothesized that miR-29a/Cdc42/β-catenin is a potential signaling cascade involved in diabetes progression. Collectively, miR-29a can negatively affect the expression of Cdc42 and downstream molecule β-catenin, and, therefore, suppress F-actin remodeling, insulin granules mobilization, and cell-to-cell interaction, and ultimately inhibit GSIS by MIN6 cells

[39, 58]. Besides, a low β-catenin protein expression may inhibit β-catenin nuclear translocation and cyclins D1, D2, and c-Myc gene expression, so miR-29a can negatively affect the proliferation rate by MIN6 cells [59]. Therefore, upregulation of miR-29a associated with inhibition of Cdc42/β-catenin signaling may be potential factors in MIN6 cells proliferation and GSIS suppression.

5. Conclusions

In conclusion, the current study reports the role of miR-29a in MIN6 cell proliferation and GSIS, which involves regulating Cdc42 and β-catenin expression. The results indicate that miR-29a inhibits MIN6 cells proliferation and GSIS and negatively regulates Cdc42 expression. In contrast, Cdc42/β-catenin is a miR-29a downstream signaling that promotes MIN6 cells proliferation and GSIS. To summarize, miR-29a can negatively affect GSIS and MIN6 cell proliferation via Cdc42/β-catenin signaling. miR-29a/Cdc42/β-catenin may be involved in diabetes progression. However, further animal experiments and studies of clinical samples from patients are needed to validate the function of miR-29a/Cdc42/β-catenin, and whether other miRNAs and downstream molecules play crucial roles in diabetes progression also requires further studies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Jing Duan, Xian-Ling Qian, and Jun Li contributed equally to this work.

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Review Article

The Role of Occupational Therapy in Secondary Prevention of Diabetes

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Diabetes mellitus is becoming a global health concern due to its prevalence and projected growth. Despite a growing number of interventions for secondary prevention of diabetes, there is a persistent poor glycemic control and poor adherence to the prescribed diabetes management regimen. In light of the tremendous costs of diabetes to both individuals and the society, it is pressing to find effective ways to improve diabetes self-management (DSM) and treatment adherence. Occupational therapists can bring values to the diabetes care team by evaluating multiple levels of influence on DSM, addressing personal and environmental barriers to well-being and DSM, and supporting patients to develop of a highly complex competences and skills to satisfactorily self-manage diabetes. This article summarizes two evidence-based, well-structured occupational therapy (OT) programs that use activity-based treatments and psychosocial strategies, respectively, to improve DSM abilities and to enhance quality of life. As the needs of adolescents with diabetes are quite different from other diabetic populations, this article also provides a summary of pediatric OT interventions that aim to facilitate autonomy and development of DSM ability among adolescents with diabetes. Evidence indicates that OT interventions can improve the quality of life and treatment adherence in patients with diabetes and hence should be continued and built on to address the increasing needs of diabetic populations.

1. Background

Diabetes mellitus is a growing health concern around the world. In 2016, 415 million people were afflicted with diabetes worldwide, and this number is expected to increase to 642 million, or one in every ten adults by the year 2040[1]. Diabetes, as a complex chronic disease, requires continuous medical care with multifactorial, risk-reducing approaches beyond glycemic control [2]. Therefore, ongoing patient self-management education and support are critical to preventing acute complications and reducing the risk of long-term complications [2]. The costs of diabetes are tremendous to both individuals and the society. Poor management of diabetes may give rise to an array of secondary complications with devastating consequences, including microvascular (such as retinopathy, neuropathy, and nephropathy) and macrovascular complications (such as myocardial infarction, angina pectoris, and stroke) [3], which adversely affect the well-being of individuals living with diabetes [4]. Globally, the costs of diabetes are expected to increase substantially from 1.3 trillion to 2.2 trillion U.S dollars in the baseline by 2030, which is equivalent to an increase in costs as a share of global GDP from 1.8% to 2.2% [5]. It is therefore pressing that effective ways of managing diabetes are found to reduce secondary complications and reduce the economic burden.

2. Diabetes Self-Management (DSM) Is Challenging

Landmark studies have clearly demonstrated that intensive glycemic control can significantly reduce the risk of developing microvascular and macrovascular complications in diabetes [6–8]. Since the risk of developing diabetes-related complications increases when values of glycated hemoglobin (HbA1c) are in excess of 6.5%, an HbA1c of less than 7% is generally a target goal for diabetes management [9, 10]. Given the nature of diabetes being a chronic disease, the potential

to achieve the optimum glycemic control (HbA1c<7%) roots in patients' ability to consistently carry out self-management activities according to the prescribed diabetes management regimen [11].

Diabetes self-management (DSM) refers to that patients take on responsibility for nutrition, physical activities, insulin therapy, and glucose monitoring, to maintain a good metabolic control and reduce diabetes complications [12, 13]. DSM is not a single behavior but rather a complex, dynamic constellation of behaviors influenced by changes in social, environmental, and individual circumstances across the lifespan [14]. Patients are required to take an active role in restructuring daily habits and routines to take medications, monitor blood glucose, follow a diet, and exercise regularly [6, 7]. Such massive behavioral changes for anyone could be difficult. Firstly, patients' psychosocial well-being is particularly challenged when they are confronted with the burden of changing their current self-care regimen, especially among older adults who have to modify their longstanding habits acquired during their lifetime [15]. Secondly, taking diabetes medications, following a diet, and constantly monitoring glucose levels are difficult activities that necessitate ongoing motivation and gradual readiness [16]. Without these abilities, many patients, children and young adults in particular, have found DSM activities burdensome and challenging to persist [17, 18]. Thirdly, there are limited resources to support health professionals in addressing patients' psychosocial needs [17, 19]. Many health professionals reported feeling uncomfortable and unconfident in discussing diabetes-related psychosocial concerns [19]. It is, therefore, not surprising that many patients are experiencing what Rubin called "Diabetes overwhelms" which refers to patients' reacting to prescribed self-care changes with feelings of distress or burnout [20].

As a result of the aforementioned reasons, poor DSM is common among patients despite our growing understanding of diabetes. Researchers reported that in 2002 less than 50% of individuals receiving diabetes education had carried out self-management activities successfully in accordance with their diabetes management regimen, and only around 50% of patients had achieved the optimum glycemic control (HbA1c<7%) [21]. This unsatisfactory glycemic control had changed a little over the past decade. Taking the U.S., for example, only 51% of patients who had been treated with diabetes medications achieved the optimal glycemic control for the period of 2011-2014, which is about the same as the percentage for the period of 2007-2010 (52%) [22–24].

Unsatisfactory glycemic control as a result of poor DSM may cause devastating consequences including elevated rates of comorbidity, greater risks of secondary complications, more visits to urgent care settings, increased hospitalizations, and higher medical costs [25–28]. Considering the persistent poor glycemic control and the considerable biopsychosocial demands to carry out DSM activities, there has been a call for effective approaches that could help patients deal with the daily challenges relating to diabetes, improve their adherence to the recommended diabetes management regimen, and sustain the improvements in glycemic control achieved in DSM interventions [18].

3. Who May Help?

As described previously, patients living with diabetes must develop a broad range of competencies that could enable them to carry out DSM activities, to solve diabetes-related social issues, and to maintain psychosocial and physical well-being [29]. Occupational therapists are known to provide activity-focused treatments and psychosocial interventions by targeting multiple levels of influence, including individual capacity, family, organization, and community factors, to facilitate DSM and psychosocial adjustments to chronic disease [29, 30]. Occupational therapists' unique role in diabetes care has been increasingly recognized for their contribution to improvement in treatment adherence, DSM abilities, and health-related quality of life [31].

4. Occupational Therapy (OT)

Occupational therapy (OT) is a person-centered healthcare profession that aims to enhance patients' well-being by maximizing their ability to choose, organize, and satisfactorily perform the activities, or 'occupations' in OT term, that patients need or want to do on a daily basis [11]. The core philosophic underpinning of OT is that humans are occupational beings whose ability to involve in desired and meaningful activities is essential to their well-being and health [11]. OT treatments are informed by activity analysis where demands of desired activities are broken down at the level of individual (e.g., spiritual, affective, cognitive, and physical components), environment (e.g., cultural, institutional, physical, and social environments), and tasks (e.g., steps required to perform the desire activity) [11]. Barriers at each of these levels are then identified and addressed to develop person-centered interventions to promote activity performance [11]. Central to the focus of OT interventions is developing health-promoting habits and routines, which is a key mechanism by which health-promoting behaviors can be sustained over time [32, 33]. Despite the fact that some of the strategies used in OT are shared across disciplines, occupational therapists remain irreplaceable in diabetes health care teams because they use DSM activities as the unit of analysis and as the intervention to achieve the overarching goal of facilitating diabetes self-management[11].

5. Occupation-Based Treatments

5.1. Resilient, Empowered, Active Living with Diabetes (REAL Diabetes) [11]. REAL Diabetes is a structured, manualized one-on-one OT program for patients who struggle in carrying out DSM activities. A randomized control trial was conducted recently to investigate the effectiveness of REAL Diabetes in diabetes management. In this study, eighty-one ethnically diverse young adults (aged 22.6 ± 3.5 years; English and/or Spanish speakers) were recruited [11]. They all live in Los Angeles county, have low socioeconomic status (household income ≤ 250% of the federal poverty level) and have type 1 or type 2 diabetes ([HbA1c] = 10.8%/95mmol/mol ± 1.9%/20.8mmol/mol) [11]. Participants were then randomly allocated to the intervention group (IG) to receive biweekly

OT sessions guided by the REAL Diabetes manual and to the control group (CG) to receive standardized educational materials published by the National Diabetes Education Program and biweekly follow-up phone calls for 6 months [11]. Findings of this study showed a significant improvement in IG group, as compared to CG, in glycemic control ($[HbA_{1c}]$ = -0.57%/6.2mmol/mol vs. +0.36%/3.9mmol/mol, $p = 0.01$), diabetes-related quality of life (+0.7 vs. +1.7, $p = 0.04$), and habits strength for monitoring blood glucose level (+3.9 vs. +1.7, $p = 0.05$) [11]. These results suggest that structured OT DSM interventions, such as REAL Diabetes, could contribute to improving the glycemic control as well as psychosocial outcomes among patients with diabetes.

REAL Diabetes is an individually tailored OT intervention program that centers on promoting patient autonomy and establishment of health-promoting habits and routines to manage diabetes. REAL Diabetes is grounded in an adapted, diabetes-focus Lifestyle Redesign OT framework that applies activity analysis to the DSM tasks [11]. In this program, occupational therapists delivered seven constituent content modules in accordance with patients' treatment goals in patients' homes and community settings over 6 months in a minimum of 10 hours of treatment session depending on the complexion of individuals' care needs and the progress toward their goals [11]. For patients who had identified social support as a challenge, their family members were encouraged to participate in diabetes-related education workshops [11]. Moreover, a multidisciplinary care team composed of an endocrinologist and a social worker was formed for consultations regarding medical and social issues outside the scope of OT practice [11].

The intervention consists of seven content modules [11]: (1) assessment and goal setting; (2) basic self-management knowledge and skills; (3) self-advocacy in health care and community settings; (4) establishment and maintenance of health-promoting habits and routines; (5) seeking and receiving social support; (6) enhancing emotional well-being; (7) self-reflection and strategies to maintain long-term health. After completing the initial evaluation and establishment of goals in module 1, occupational therapists personalized the interventions by using content from the remaining modules that are relevant to patients' individual goals and are in line with their personal factors, including their readiness to change, personal preferences, and their prescribed diabetes management regimen [11]. REAL Diabetes is therefore a menu of possible treatment activities that can be selected to meet the needs of individual patient, rather than a fixed curriculum that every participant need to complete. In this way a person-centered intervention program was provided to address unique needs of each patient.

6. OT Psychosocial Interventions

Several studies have presented a viewpoint that psychosocial support is essential to dealing with the daily challenges of diabetes [17, 34]. For many patients, living with diabetes is a complex, lifelong process where the psychological consequences of continuous DSM activities and pressures from family, friends, and health care professionals can generate stress and burnout in daily life [35, 36]. In addition, patients' experiences of their body's reaction to episodes of low or high blood glucose levels could result in a fear of hypoglycemia and a loss of confidence in their bodily functions [37–41]. Individuals with diabetes hence must develop a broad range of competencies that could support them to deal with diabetes-related social issues, to redesign lifestyle and daily routines, and to maintain psychosocial and physical well-being [42–45]. Scientific evidence has shown that occupational therapists are competent to provide psychosocial interventions that target multiple levels of influence to facilitate psychosocial adjustments to diabetes and thus improve patients' ability in DSM [30, 42–45].

A pilot study has proved the efficacy of a structured peer-led group program supervised indirectly by an occupational therapist in improving adherence to the diabetes management regimen among 16 Mexican-American older adults aged 60-85 [46]. All participants have type 2 diabetes and lack motivation to establish health-promoting habits [46]. After two months, these participants saw improved glycemic control and improved self-perception of being prepared to manage diabetes. HbA1c test results were significant at the $p < 0.05$ level between pretest and 2-month posttest with a stabilizing effect found at the 6-month posttest [46]. Furthermore, changes in responses on the Diabetes Self-Efficacy Scale, the Diabetes Attitude Scale, and the Diabetes Empowerment Scale were highly significant at the 2-month, 4-month, and 6-month posttests at the $p < 0.0005$ level [46].

This peer-led OT psychosocial intervention program adopted "Bridges Diabetes Support Group Manual (BDSGM)" to structure the intervention. Each BDSGM chapter used stories to teach concepts and asked introspective questions at the end of each chapter to encourage readers to reflect on their motivations for changes and self-preservation behaviors, and to refine their problem-solving and social skills, which are considered essential competences in adhering to the prescribed diabetes management regimen [46]. The intention of this manual is to counteract negative thinking and correct faulty information by exposing participants to different viewpoints, and to provide topics for shared discussion in group sessions led by peer mentors [46]. During phase one, an occupational therapist served as a peer role model for the interventions using BDSGM [46]. A train-the-trainers strategy was used to enable selected peer mentors to run subsequent mentee groups where the occupational therapist only stood by on the premises [46]. To facilitate mentees' acceptance of and adjustment to diabetes, key issues such as health care beliefs, values clarification, changing habits, developing goals, stages of adaptation, and social assertiveness were explored introspectively and were discussed in group sessions [46].

Occupational therapists have a long history of using peer support groups to address individuals' psychosocial needs and encourage behavioral changes [42–45]. The above diabetes peer support program borrows a concept from Social Learning Theory that social support can be a powerful tool for motivation for changes because vicarious learning

occurs when one recognizes and imitates others' success [47–50]. Bringing patients facing similar diabetes-related challenges together and encouraging them to share lived experiences regarding emotional and practical challenges of diabetes, this peer support program could elicit hope and motivate patients to improve their DSM abilities [51–53]. In this program, patients could give and receive emotional support from peers. The interconnections of people alike could reduce the feeling of being alone in their struggle with diabetes [54]. In addition, patients could learn how others have coped with the conditions they are struggling with [53]. Participants who have successfully managed diabetes, in particular, can deliver powerful and inspiring messages to their peers [53]. Feedback from the participants is positive. They acknowledged that social support is needed at any stages of their illness and agreed that given the topical guidelines and rules of interaction to follow, peers with diabetes can provide meaningful support to each other [46].

7. Pediatric OT DSM Interventions

Occupational therapists are able to support patients with childhood diabetes to develop and improve DSM skills [55]. Similar to OT DSM programs targeting adult populations, adolescent patients in pediatric DSM programs participate in individual OT sessions to learn basic DSM knowledge and skills [56–63]. What makes the pediatric programs unique is that parents are often included as indispensable part of the intervention. The goal is to adjust parental involvement in DSM to promote patients' independence in DSM and to improve patients' adherence to their diabetes management regimen [56–63]. For instance, the parent-child group interventions aim to improve parents' perceptions of their children's ability to effectively manage diabetes by providing education about the developmental aspects of DSM in adolescence [60, 64]. Occupational therapists also initiate parent-child negotiation regarding proper parental involvement in DSM and encourage renegotiation of DSM responsibilities between parents and their children during adolescence [64]. To further promote adolescent patients' independence and autonomy in DSM, occupational therapists use a variety of creative ways, such as using formal behavioral contracts for parents to reduce nagging [57] and fortnightly reminders to parents to provide positive affirmations to their children [59].

Additionally, technology is used to deliver important parts of the intervention to support the development of DSM skills of teenagers with diabetes [56–65]. For instance, in some OT programs, a cell phone meter, monitored by an occupational therapist, for blood glucose values was provided to adolescent patients [56, 57]. Parents were also given a link to access and monitor children's blood glucose data and were encouraged to cease their cues for self-management, which could also promote children's independence in DSM [56, 57]. Post-treatment outcome surveys suggested that most of the adolescent patients felt more independent in DSM and agreed that the cell-phone-based blood sugar systems had helped them establish DSM habits [57]. Occupational therapists also use online platform to promote the development of DSM skills among adolescents with type 1 diabetes [65]. Patients are encouraged to involve in the online discussion forums, trouble-shoot diabetes-related challenges, and review multimedia stories focusing on common barriers to DSM [65]. Treatment outcomes suggested a significant improvement in DSM (d = 0.30; p = 0.02), and an important improvement in HbA1c (d = -0.28; p = 0.27) in intervention group, as compared to control group [65].

8. OT Practice Challenges

Despite the fact that occupational therapists have a long history of working with a variety of populations with chronic diseases, including diabetes, most of the OT interventions are directed to the disabling consequence of diabetes, such as upper limb pain, poor vision, amputation, and depression, rather than to the secondary prevention of diabetes to address challenges of DSM [46]. This is because interventions for disabilities following uncontrolled diabetes are reimbursed while DSM treatments to prevent diabetes complications may be not [46]. Although the reimbursement systems are not yet in place in many countries, occupational therapists still have the responsibility and play a significant role in facilitating patients to manage diabetes and to continually live the life they want to live [32, 46]. Furthermore, the current secondary OT prevention services may require further refinements in delivery modality in order to reach wider diabetic populations, especially those with poor access to health care [11].

9. Conclusion

Against the backdrop of a global rise in the incidence of diabetes and poor adherence to the prescribed diabetes management regimen, it is urgent to refine current DSM interventions. Occupational therapists can bring values to the diabetes care team by evaluating multiple levels of influence that interact to influence patients' DSM abilities, and addressing barriers at each of these levels to improve glycemic control and enhance well-being. Occupational therapists can also support patients to develop highly complex competences and skills to self-manage diabetes and can help with planning activities of daily living in a systematic way for optimum health outcomes. Scientific evidence supports that OT interventions can improve the quality of life and adherence in people with diabetes. Therefore, it is suggested that OT interventions should be continued and built on to help address the increasing needs of populations with diabetes.

Abbreviations

DSM: Diabetes self-management
OT: Occupational therapy
HbAlc: Hemoglobin A1c.

Data Availability

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

Ethical Approval

The study complied with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Zhongshan Hospital Xiamen University (Xiamen, China).

Consent

Written informed consent was obtained from all the study participants.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xizi Shen is study leader and contributes to writing of paper; Xingping Shen is study leader and corresponding author. All authors read and approved the final manuscript.

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Review Article

Effect of Social Factors and the Natural Environment on the Etiology and Pathogenesis of Diabetes Mellitus

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Type 2 diabetes mellitus (T2DM) is currently a public health problem worldwide and a threat to human health and social development. The incidence rate of the disease is steadily increasing. Various genetic and environmental factors have been established as influencing the pathogenesis of this disease. However, the influence of social factors and the natural environment on DM incidence should also be considered. Low-grade inflammation could represent a central point of connection integrating all these potential triggers, being partly responsible for the development of insulin resistance. This paper aims to elaborate on the impact of the natural environment and social factors on DM development, with a special focus on six aspects of the pathogenesis of DM: pollution, radiation, psychology, drink, sleep, and exercise. We identified a two-way relationship between T2DM and social and natural environments. Changes in these environments may lead to low-grade inflammation, which in turn induces or aggravates T2DM and vice versa. Poor lifestyle may lead to increased insulin resistance and promote DM development. Improvements in blood glucose control can be achieved through nonenvironmental and behavioral interventions.

1. Introduction

Diabetes mellitus (DM) is currently the third most dangerous chronic noncommunicable disease in terms of its effect on human health [1]. Worldwide, the prevalence of the disease is continuously increasing, owing in part to rapid social economy developments, improved living standards, acceleration of urbanization, industrialization, and increase in the proportion of aging individuals. According to the World Health Organization, about 422 million people are currently living with diabetes; this number is expected to increase to 600 million by 2040 [2]. The prevalence rate of DM among adults in China is 11.6% [3]. The dramatic increase in the number of people with DM is among the greatest challenges facing the healthcare sector today. The etiology and pathogenesis of DM are extremely complicated. At present, genetic factors, environmental factors (obesity), and interaction of these two factors have been established as increasing the risk of type 2 diabetes mellitus (T2DM) [4]. However, various social factors and the natural environment may also affect the synthesis and secretion of insulin. Some scholars have discovered that, in a similar setting, low-grade inflammation could represent a central point of connection integrating all these potential triggers, being partly responsible for the development of insulin resistance in recent studies, and may represent a sensor of the consequence of metabolic imbalance [5, 6]. T2DM is associated with an increased inflammatory score. The term 'metaflammation' was coined to indicate metabolically triggered inflammation [7]. These six factors are mainly related to T2DM. This paper mainly aims to demonstrate the impact of external and internal factors on the development of T2DM from the perspective of the influence of the natural environment and social factors on individual behavior.

2. Air Pollution

Air pollution is becoming an increasingly serious problem worldwide. Air pollutants can be classified as gaseous pollutants (sulfur compounds, nitrogen compounds, carbon oxides, etc.) and atmospheric particulate matter (PM_{10} and $PM_{2.5}$). Several studies have found that environmental factors play an important role in the pathogenesis of T2DM; specifically, air pollution has been shown to be an important catalyst [8–10]. Andersen et al. [11], in their 9.7-month follow-up study of 51,818 volunteers, demonstrated that people with healthy lifestyles and who regularly exercised had a lower incidence of T2DM than those without these habits in a population of individuals with long-term exposure to traffic-related pollutants. Traffic-related pollutants are among the potential risk factors for T2DM development. Dijkema et al. [12] conducted a cross-sectional study in which 8,018 elderly people were screened for DM. They concluded that the odds of developing type 2 DM increased to 1.39 when NO_2 level in the atmosphere increased to 10 $mg \cdot m^{-3}$. A Chinese study [13] with a large sample showed that the type 2 DM risk index was 1.11 (95% confidence interval [CI]: 1.02–1.21) when the $PM_{2.5}$ level increased to an average concentration of 10 $\mu g \cdot m^{-3}$. Evidence-based medicine supports the idea that air pollution is a risk factor for T2DM, while epidemiological evidence indicates that greater increases in the level of air pollution can cause or increase the prevalence of T2DM. The pathogenesis associated with this increase in the prevalence of T2DM may be attributed to stress-mediated insulin resistance and/or decreased insulin sensitivity, which results in T2DM development through inflammatory response, oxidative stress, and endoplasmic reticulum. The presence of air pollutants in the lungs can stimulate alveolar epithelial cells and macrophages to produce inflammatory factors, such as interleukin and macrophage inflammatory protein 2, and lead to the disorderly interaction of other mediators of systemic inflammatory response [14]. During the process, islet inflammation leads to the destruction or apoptosis of islet cells and insulin sensitivity is reduced, which affects the utilization of glucose by peripheral tissues, thereby increasing the risk of DM. At the same time, when the lungs are stimulated by external pollutants (such as $PM_{2.5}$), they release reactive oxygen species (ROS) rapidly; the overaccumulation of ROS to levels that exceed those that can be normally removed by the body disrupts the balance between the oxidation and antioxidant systems, resulting in oxidative damage to the tissues and organs [15]. Islet β-cells, which are sensitive to ROS, have low levels of antioxidant enzymes and poor antioxidant capacity. As a result, ROS can directly damage β-cells and lead to DM. Studies have shown that PM_{10} can increase the proportion of immune cells, malondialdehyde, and neutrophil chemotactic factors in the bronchoalveolar lavage fluid in rats, proving that it can enhance inflammation and the role of oxidative stress in rodents [16]. Islet β-cells are rich in endoplasmic reticulum, and air pollution can mediate endoplasmic reticulum stress. If the stress persists for long periods, the cells initiate an unfolded protein response, activate the apoptotic pathway, induce islet β-cell apoptosis, and affect insulin secretion, leading to T2DM.

3. Radiation Environment

Mobile phones, computers, electronic watches, and other communication equipment have become indispensable in daily life. Radiotherapy is an important treatment modality for cancer. The development of the military industry and demand for alternative energy sources have also led to the establishment of a large number of nuclear power plants. However, long-term exposure to cell phone towers, smart meters, and other radiation-emitting devices can adversely affect human health. Most people know little about the possible relationship between radiation exposure and diabetes [17]. For example, among 8 articles including a systematic review and meta-analysis of 1863 T2DM patients reported by Pettit et al. which identified and analyzed the current evidence on glycemic control (HbA1c) during and after cancer treatment, the effect of radiation on glycemic control is not mentioned [18]. However, it has been considered by the International Commission for Radiation Protection as one factor involved in multifactorial diseases [19].

Existing studies have shown that cancer patients receiving radiotherapy have an increased risk of developing insulin resistance and T2DM [20]. Patients undergoing radiotherapy in whom a dose to the pancreatic tail is 10 Gy or higher have a 11.5 times higher risk of developing DM than those without radiation therapy, suggesting that ionizing radiation exposure may lead to T2DM [20]. In China, a survey has shown that the incidence rates of obesity and metabolic syndrome among those working in nuclear power plants are significantly higher than the national average, indicating that such workers, even after retirement, may have a higher risk of DM than the general population in the corresponding age group [21]. Radiation is a major inducer of inflammatory responses [22]. While radical therapy is over within a short time of radiation exposure, the ensuing inflammatory response perpetuates the response by generating recurring waves of ROS, cytokines, chemokines, and growth factors with associated inflammatory infiltrates [23]. Systemic low-grade inflammatory response and a large number of inflammatory mediators are known pathological features of diabetes [7]. Overexpression of inflammatory factors such as serum C-reactive protein, interleukin (IL)-6, and tumor necrosis factor-α can inhibit insulin secretion and islet function and leads to insulin secretion disorder and insulin resistance, resulting in insufficient insulin secretion and inability to lower blood glucose levels, leading to T2DM and metabolic syndrome [24]. Therefore, we consider that inflammatory responses may be the putative mechanism linking radiation and T2DM.

While radiation exposure increases the risk of T2DM development, T2DM, in turn, increases the degree of radiation damage among cancer patients undergoing radiation therapy. Due to the presence of autonomic neuropathy in diabetic patients, the ability of the parasympathetic nerves to regulate bronchial activity is reduced, and the permeability of patients' blood vessel walls and sensitivity to ionizing radiation are enhanced, which is not conducive to the absorption and dissipation of inflammatory reactions. Ma et al. [25] showed that DM can aggravate, to an extent, the symptoms

of radiation pneumonitis in lung cancer patients. Few studies have focused on the incidence of radiation-induced DM. However, through our review of the aforementioned studies, it can be concluded that radiation can cause diabetes. Diabetes onset may further aggravate the damage caused by radiation, forming a vicious circle. Radiation damage can stimulate the pathogenesis of diabetes; the mutual influence of the two requires further exploration.

4. Psychological Factors

The influence of psychological factors such as depression and anxiety on DM is increasingly being investigated, owing to gradual changes in the social medical model and increasing pressure of life. The "biological-psychological-social medical model" has led to the realization that social and psychological factors play a vital role in the process of DM development and the associated outcomes. According to a report published by the World Health Organization Diabetes Expert Committee, work-related and other psychological burdens may aggravate psychological and social pressures. Furthermore, this situation may induce and produce glucose tolerance-related abnormalities through hormonal action on insulin secretion and glucose metabolism [26]. Engum et al. showed that an increase in the incidence of type 2 DM in people with depressive symptoms regardless of sex in a follow-up survey of 37,291 people conducted over 10 years. Another conclusion is that depression is a significant risk factor for DM development [27]. A meta-analysis of 20 articles, including 45,514 people, showed that the risk of DM in people with stress was 1.80 times higher than that in the normal population [28], showing that blood glucose control alone is not sufficient among people with DM. One of the focus areas of current medical research is understanding the various forms of communication among individuals with diabetes and their family members, partners, friends, and health providers, including the reception of love, care, supervision, motivation, and education [29], and conducting in-depth analyses of the possible associated effects and intervention effects on T2DM; the results may have extensive social benefits as well as research and application prospects. The currently known psychological stress-related mechanisms in terms of T2DM development mainly pertain to the autonomic nervous pathways, neuroendocrine mechanisms, and direct effects on the pancreas. Long-term psychological depression promotes the hypothalamic-pituitary-adrenal axis activity to increase cortisol secretion. The process not only reduces glucose utilization and promotes gluconeogenesis, but also raises blood glucose levels by antagonizing insulin production and inhibiting blood glucose utilization [26]. Tomita et al. [30] showed that depression is a chronic inflammatory response through animal experiments, and changes in inflammatory factor levels can affect the normal metabolism of brain tissue cells. Long-term depression and loss may also lead to pessimism, which could, in turn, lead to lower adherence rates to doctors' instructions, and the destruction of the original nursing pattern is likely to have a negative impact on blood glucose control. Similarly, some patients who require insulin monitoring and metering adjustments may experience insulin performance task interruptions or a lack of implementation due to emotional factors. Recent studies have pointed out [31] that the occurrence of depressive symptoms is also closely related to the increase of serum inflammatory factors. It can be speculated that DM has a common mechanism of action with depression. By inhibiting the level of serum inflammatory factors, it can not only effectively improve pathological changes caused by T2DM, but also effectively improve depressive symptoms. Some scholars have suggested that there may be a third factor in the mechanism of the interaction between T2DM and psychological stress. Further research should focus on whether the third factor can produce the same results as psychological depression [32].

5. Alcohol Intake

Alcohol intake has become a way of life, with people consuming alcohol to alleviate social pressure. Previous studies have shown a positive correlation between alcohol intake and T2DM development [33], with high intake levels having the potential to damage the liver. The liver is a crucial organ that regulates glucose homeostasis through glycogen synthesis and catabolism. Liver injury can lead to glucose metabolism disorders, resulting in impaired glucose tolerance and DM development. Pathological and ultrastructural abnormalities in chronic alcoholic steatohepatitis are associated with sustained hepatic insulin resistance and proinflammatory cytokine activation [34]. Ethanol intake reduces the sensitivity of islet cells through interference with muscarinic signaling and insulin signaling, resulting in a decrease in the rate of basal insulin secretion. It can be concluded that high alcohol intake levels are closely related to T2DM occurrence. However, another study showed that ethanol intake promotes the expression of insulin signaling and selectively upregulates the insulin transduction pathway by upregulating the expression of intracellular phosphorylated protein kinase B, phosphatidylinositol kinase, and transcription factor pFOX01 and by downregulating the phosphorylation of insulin receptor protein 1 and insulin receptor substrate protein 2 [35]. Moderate drinking can reduce the risk of T2DM. The mechanism is as follows: while moderate drinking can increase the level of high-density lipoprotein, it can lead to significantly reduced levels of serum C-reactive protein, neutrophils, and neutrophil CD64, which may have an anti-inflammatory effect (it is widely accepted that DM is a chronic low-grade inflammatory disease). In addition, moderate drinking can increase the sensitivity of insulin, and ethanol intake can selectively upregulate the insulin signaling pathway in the case of normal blood glucose levels. Therefore, there is a certain dose-effect relationship between alcohol intake and DM development. A cohort study in Korea showed that mild (intake < 5 g/day) and moderate ($5 \leq$ intake < 30 g/day) alcohol intake reduced the risk of T2DM in men, while high (intake ≥ 30 g/day) alcohol consumption led to increases in the risk of T2DM among men. Therefore, it can be concluded that there is a "J"-type relationship between alcohol consumption and T2DM risk among men [36]. A follow-up survey conducted by Cullmann et al. [37] also showed that heavy drinking increased the risk

of pre-diabetes, while the risk of pre-diabetes was lower in those with low or moderate alcohol consumption levels. In women, the risk of DM and its complications is not alleviated by moderate alcohol consumption [38, 39].

6. Lack of Exercise

Rapid economic and technological developments have greatly changed the way people commute, resulting in a significant decline in the levels of daily physical activity. Simultaneously, people also do not have the time for regular exercise owing to the accelerated pace of life or are not interested due to various social pressures. It has been concluded that a lack of exercise is among the risk factors for T2DM, and exercise-based intervention is indispensable for diabetic patients. A meta-analysis performed in the United Kingdom of 1,261,991 people enrolled in 28 cohort studies showed that the risk of T2DM associated with moderate-intensity exercise was reduced by 26% with participation in exercise for 150 min per week, by 36% with participation in exercise for 300 min per week, and by 53% with participation in exercise for 800 min per week compared with that in individuals who did not exercise [40]. The American Diabetes Association and the American Movement recommend that diabetic patients participate in at least 150 min of moderate-to-high-intensity exercise per week [41]. Exercise can accelerate the metabolism of glucose and energy, lead to the consumption of a large amount of glycogen, increase the proportion of capillary and muscle fibers, and promote the intake of glucose in the blood. On the completion of an exercise session, blood glucose is stored in the form of glycogen, leading to further blood glucose reduction. Consumption of glycogen reduces the secretion of insulin, promotes the corresponding receptor binding of insulin in the blood circulation to improve insulin resistance, and enhances glucose metabolism. Exercise can also alleviate tension, improve social adaptability, and alter bad lifestyle-related behavior, which can be crucial for the recovery of people with diabetes. The intensity, duration, and volume of exercise should be gradually increased according to a patient's personal situation, and the load should not exceed the patient's ability to withstand it. For patients with DM, daily training should be adhered to. Exercise can be performed across two or three sessions daily (planned such that they do not coincide with the one or two hours following meal intake) and should combine aerobic and anti-resistance movements. Tai Chi, Baduanjin, Five Animal Qigong, and other traditional Chinese exercises have the characteristics of low-intensity and long-term aerobic exercise. These exercise forms are safe and associated with improved health. By coordinating and adjusting limb movements, one can harmonize body parts to echo his/her physiological status, therefore relaxing muscle tissue and bringing further correction of uncoordinated body posture and spirits. Ultimately, one can react to a status in which mind and body are relaxed and coordinated [42, 43]. In recent years, research studies have reported that Qigong training has certain curative effects in cases of chronic diseases such as T2DM [31]. For instance, the sixth section of Baduanjin, a traditional Chinese Qigong, involves the movement "reaching bilateral hands down to feet to nourish kidneys." By performing waist flexion and extension, as well as massaging the posterior waist and lower limbs, one's governor meridian and bladder meridian will be exercised and exerted. This facilitates the flow of Qi-blood circulation and can cause sympathetic nerve excitation and stimulation of the hypothalamic-pituitary-adrenal pathway, promote increases in insulin secretion by β-cells, and lower blood sugar levels; this exercise form also has the effect of regulating water, electrolyte, and acid-base balance [44]. Tai Chi is a traditional Chinese martial art. A large number of studies have confirmed that participation in Tai Chi improves the levels of blood glucose, glycosylated hemoglobin, cholesterol, and other indicators of T2DM and enhances the mechanism of oxidative damage, regulates the balance of the sympathetic and parasympathetic nervous systems, improves immune function, and reduces psychological pressure [45, 46].

7. Sleep-Related Factors

In recent years, people's sleep time has shown a downward trend. The average daily sleep time of Chinese residents is 7.20 h, and the proportion of people with sleep insufficiency is 23.60% [47]. Similarly, the proportion of young Americans who sleep for less than 7 hours/day has risen from 15.6% to 37.1% in the last 40 years [48]. The relationship between sleep and T2DM is a topic of international concern, as a lack of sleep can easily lead to a gradual acceleration in the body's insulin resistance. Katano et al. showed that sleep disorders showed a clear association with DM [49]. A cross-sectional study found that people with sleep deprivation had fasting blood glucose levels that were increased by 23%, fasting insulin levels that were 48% higher, and an insulin resistance index (homeostatic model assessment of insulin resistance) that was 82% higher compared to those with sufficient sleep [50]. Lack of sleep is also associated with various metabolic disorders, as it enhances sympathetic activity, boosts catecholamine levels, and inhibits pancreatic function, thereby reducing insulin secretion. Current studies have shown that increased levels of inflammatory factors and mediators in patients with sleep disorders, including tumor necrosis factor-α, IL-6, IL-8, high-sensitivity C-reactive protein, transcription factors, and adhesion factors, may affect human health through low-grade inflammation pathways [51, 52]. Increased levels of inflammatory factors are involved in the promotion of insulin resistance by sleep disorders [53]. Difficulties in sleeping reduce the brain's glucose ingestion and insulin sensitivity, impair glucose tolerance, and induce insulin resistance to some extent. The irritability caused by long-term difficulty in falling asleep affects hypothalamic activity, especially in the hypothalamic-pituitary-adrenal axis that is associated with stress [54]. Glucocorticoids are not only responsible for the destructive action of transmembrane glucose transporter 4 (due to which glucose cannot be transported to the cell surface and utilized by the body) but also directly inhibit insulin secretion by islet β-cells. Therefore, elevated cortisol levels caused by sleep problems can also trigger T2DM. Other studies have shown that sleep

disorders can lead to metabolic disorders; resistin, leptin, adiponectin, and other cytokines have been shown to mediate insulin resistance, also affecting blood glucose levels [55].

8. Summary

In summary, there is a two-way relationship between T2DM and the social and natural environments; changes in these environments may induce or aggravate T2DM and vice versa. Poor lifestyle can lead to increased insulin resistance and promote T2DM development in some risk groups or environments. However, improvements in blood glucose control can be achieved through interventions that do not rely on environmental and social aspects. Although some epidemiological and mechanistic studies have shown that the natural environment, social factors, and personal behavior are related to T2DM, existing data are insufficient, and results of studies are currently inconsistent.

Some limitations of this study must be considered when interpreting the results. First, the current epidemiological investigation is mainly based on T2DM, and there are many patients with type 1 diabetes mellitus (T1DM) and pre-diabetes. Therefore, it is necessary to broaden the scope of the study to T1DM and gestational diabetes mellitus and pre-diabetes. Most recent studies are cross-sectional in design. Thus, the causal implications of environment and behavior factors for T2DM should be carefully considered. Prospective studies are needed in the future to determine the causal relationship between these risk factors and diabetes. A third limitation is that the results of one measurement do not necessarily reflect the long-term exposure of the human body, so multiple follow-up and determination are required. Fourth, some studies are unable to distinguish individuals with T1DM from those with T2DM, nor can they prove that the investigators evaluated all participants during the relevant survey. Given that the proportion of T1DM patients among all diabetic patients in Korea is less than 1%, we suspect that their inclusion would have had little effect in some studies [56]. Finally, age, gender, and lifestyle habits mentioned in the experimental and epidemiological studies vary widely and may affect the promotion and comparison of the above risk factors with recent study results. For instance, hormonal effects and low statistical power may have contributed to the differences in findings according to sex; thus, the role of endogenous sex hormones in the development of T2DM may have influenced the gender differences noted in this study [57]. Additionally, the insufficient number of females likely resulted in the negative relationship between high-risk female drinkers and the prevalence of T2DM.

Therefore, it is important to perform a more in-depth exploration of the relationship between the aforementioned factors and T2DM. Moreover, there is an urgent need to conduct systematic and deeper research focusing on the pathological mechanism that connects the environment and individual behavior to T2DM. Future clinically relevant and epidemiological studies aimed at achieving comprehensive, optimized interventions can provide a reliable basis for diagnosis and treatment among clinicians.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Junping Wei proposed the paper topic, contributed to this work, and is the corresponding author; Guangtong Dong wrote the paper and is first author; Lianlian Qu searched and reviewed the literature; Xuefeng Gong and Weitian Yan revised the paper. Bing Pang was responsible for subsequent revision of the manuscript.

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