MicroRNA 1228 Mediates the Viability of High Glucose-Cultured Renal Tubule Cells through Targeting Thrombospondin 2 and PI3K/AKT Signaling Pathway

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Keywords
MicroRNA 1228 · Thrombospondin 2 · Proliferation · Apoptosis

Abstract
Aim: The present study aimed to elucidate the potential function of microRNA 1228 (miR-1228) on the high glucose (HG)-damaged human renal proximal tubule cells (HK-2) and the underlying mechanism.

Methods: The datasets GSE47185 and GSE51674 were downloaded from the Gene Expression Omnibus database for mining differently expressed mRNAs and miRNAs, respectively. Bioinformatics online tools were applied to predict the binding sites between miR-1228 and thrombospondin 2 (THBS2), which was confirmed by dual-luciferase assay. Real-time quantitative polymerase chain reaction was used to detect the miRNA level of miR-1228/THBS2. Western blot was used to detect the protein level of THBS2 and the PI3K/AKT signaling pathway-associated markers. HK-2 cells were cultured in HG (30 mM) to mimic hyperglycemia. Cell counting kit 8 and flow cytometry assays were utilized to determine the cell proliferation and apoptosis.

Results: The expression of THBS2 was significantly upregulated in diabetic nephropathy (DN) based on bioinformatics tools and identified as a direct target of miR-1228. miR-1228 was downregulated in DN and HG-damaged HK-2 cells. HG notably reduced HK-2 cell proliferation. This negative effect was attenuated by transfecting with an miR-1228 mimic and aggravated by transfecting with an miR-1228 inhibitor. However, under basal condition, there was no significant effect on the HK-2 cell proliferation among blank control, mimic, and inhibitor groups. Overexpression of THBS2 abolished the elevating effect of the miR-1228 mimic on the HG-damaged HK-2 cell proliferation, while restored the inhibitory effects of the miR-1228 mimic on the cell apoptosis. On the contrary, the suppressive effects on the proliferation and the enhancive effects on the apoptosis by silencing miR-1228 in HK-2 cells stimulated with HG can be weakened by recommendation of THBS2 small interference RNAs. Furthermore, we also found that HG significantly enhanced the phosphorylation levels of PI3K and AKT. In terms of overexpression and knockdown experiments, Western blot analysis further revealed that miR-1228 inhibited the activation of the PI3K/AKT signaling pathway in HG-damaged HK-2 cells by regulating THBS2. Conclusion: The findings illustrated that miR-1228 improved survivability and inhibited apoptosis in HK-2 cells stimulated with HG partly by restraining the activation of the PI3K/AKT signaling pathway.

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Introduction

DN is one of the most common and severe chronic complications of diabetes [1]. Clinically, even strict monitoring of blood pressure and blood sugar cannot effectively prevent the progression of DN, and about one-third of diabetic patients will eventually enter the stage of end-stage kidney disease [2]. DN is mainly characterized by renal tubulointerstitial fibrosis, glomerulosclerosis, and renal vascular disease [3]. Among them, the tubulointerstitium accounts for >90% of the renal parenchyma and is responsible for many important functions [4]. In the diabetic environment, under the influence of metabolic disturbance, inflammatory state, changes in urine composition, hemodynamics, etc., renal tubular epithelial cells exhibit oxidative stress response, secreting various cytokines, which lead to interstitial inflammation and fibrosis and mediate the occurrence and development of DN [5]. Therefore, it is of great significance to probe the molecular mechanism of renal tubular injury for determining drug intervention targets and delaying the progression of DN.

Thrombospondins (THBSs) are a family of glycoproteins expressed in the extracellular matrix (ECM) which play a regulatory role in cell-to-cell and cell-to-matrix relationships and interact with other ECM molecules to influence their function [6]. Among the 5 types of THBSs, THBS2, also known as tumor suppressor region 2, has been mined to be involved in multiple biological functions including cell adhesion, angiogenesis, proliferation, and ECM modeling [7, 8]. Over the past few years, several pieces of evidence from multiple studies have been paid to the role of THBS2 in various fields, including cardiovascular diseases [9, 10], cancers [11], renal diseases [12], and metabolic diseases [13, 14]. A study revealed that overexpression of THBS2 caused increased oxidative stress in diabetic mice [14]. Moreover, THBS2 levels were notably higher in proliferative diabetic retinopathy vitreous samples than in control patients without diabetes [13]. Besides, the levels of THBS2 were notably higher in type 2 diabetes mellitus patients with nephropathy [15], suggesting that THBS2 might participate in the progression of DN. However, its functional role and the molecular mechanism in DN have not yet been fully illuminated.

MicroRNAs (miRNAs) are a class of small and noncoding RNAs with about 22 nucleotides in length [16]. They are well known as master regulators of the human genome and regulate gene expression by inducing degradation or translational repression of the target mRNA in a sequence-specific manner [17]. In mammals, miRNAs have been proven to be involved in the regulation of almost every cellular process studied to date [18]. Massive miRNAs have been disclosed to participate in the development of DN, such as miR-31 [19], miR-27a [20], miR-124a [21], and miR-455-3p [22]. In regard to miR-1228, little is known about its role in diseases. Only a few studies have focused on its promoting effects on cancers, including hepatocellular carcinoma [23], breast cancer [24], and lung cancer [25]. Noticeably, miR-1228 was downregulated in biopsies and urines of patients with DN [26]. However, whether miR-1228 involves in DN dysfunction remains unknown, and if so, the underlying mechanism was investigated.

Thus, in the present study, we aimed to determine the expression of miR-1228 and THBS2 in DN and to evaluate their regulatory relationship. Furthermore, we assessed the effects of miR-1228/THBS2 on proliferation and apoptosis using the high glucose (HG)-induced human renal proximal tubule cells (HK-2). Since differentially expressed genes (DEGs) were mainly enriched in the phosphatidylinositol 3-kinase/AKT serine/threonine kinase 1 (PI3K/AKT) signaling pathway according to the public GSE47185 dataset through bioinformatics analysis, we explored the effect of miR-1228/THBS2 on the PI3K/AKT signaling pathway-associated markers.

Materials and Methods

Data Collection and Processing

The mRNA expression data (series accession number: GSE47185) associated with DN were downloaded from the Gene Expression Omnibus database for mining DEGs which were analyzed using the Linear Models for Microarray Data package of Bioconductor as previously described [27]. Genes meeting \( p < 0.05 \) and \(|\log (\text{FC})| \geq 2\) were screened as DEGs. To explore the signaling pathways of DEGs, the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was implemented using the Database for Annotation, Visualization, and Integrated Discovery online tools [28]. The cutoff value for a significant signaling pathway was set with \( p < 0.05 \) and gene count \( \geq 2 \). The miRNA expression data (series accession number: GSE51674) were obtained to identify differently expressed miRNAs with the screening conditions \( p < 0.05 \) and \(|\log (\text{FC})| \geq 2\). Besides, the dataset GSE47185 contained a total of 24 samples, including 6 normal samples of the renal tubule and 18 renal tubule samples in patients with DN, and the dataset GSE51674 included 6 renal tubule samples of DN patients and 4 normal controls.

Cell Culture

Human renal proximal tubule cells (HK-2) (American Type Culture Collection, Manassas, VA, USA) were incubated in Dulbecco’s modified eagle medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum supplemented with 5.5 mM glucose (NG) at 37°C in an incubator with 5% CO₂. Since 30 mM glucose concentration has the greatest effect on the downstream related genes compared to other concentrations (5, 10, and 20 mM),...
numerous works in the literature have used 30 mM glucose concentration to expose cells to high concentrations of glucose [29–31]. In the present study, when cells had reached ~60% confluence, they were subjected to serum starvation for 12 h and then exposed to 30 mM glucose (HG).

Transfection

For gene disruption experiments, cells were transfected with the miR-1228 mimic, miR-1228 inhibitor, irrelevant-targeting negative control (NC), pcDNA3.1-THBS2, pcDNA3.1 empty vector, THBS2 siRNA, or scrambled control siRNA (si-con) using Lipofectamine 3000 reagent (Invitrogen). After transfection for 48 h, the relative luciferase activities were determined by using the dual-luciferase reporter assay system (Promega).

Real-Time Quantitative Polymerase Chain Reaction

Total RNA from cultured cells was prepared using the TRIzol (Invitrogen) reagent. For cDNA preparation, reverse transcription polymerase chain reaction was carried out by using the MiScript Reverse Transcription kit (Qiagen, Hilden, Germany) for miR-1228 and the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan) for THBS2. Real-time qPCR was carried out using the MiScript SYBR Green PCR kit (Qiagen) reagent. For cDNA preparation, reverse transcription polymerase chain reaction was carried out by using the MiScript Reverse Transcription kit (Qiagen, Tokyo, Japan) for miR-1228 and the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan) for THBS2. Real-time qPCR was carried out using the MiScript SYBR Green PCR kit (Qiagen) for miR-1228 and SYBR Premix Ex Taq II (Takara) for THBS2. The expression levels of miR-1228 and THBS2 were normalized to U6 and GAPDH, respectively, and calculated by the cycle threshold method (2−ΔΔCq) [24, 32]. Primer sequences used in the present study are listed in Table 1.

Table 1. Primer sequences in the present study

| Name   | Forward                       | Reverse                       |
|--------|-------------------------------|-------------------------------|
| miR-1228 | 5′-GGGCCCTCACACCTGCCTCG-3′     | 5′-GGAGACATGTCTGGTATCTC-3′    |
| U6     | 5′-CTCGTCCTGCACGCAATACATT-3′   | 5′-AGGATTTGCTGGTGTC-3′        |
| THBS2  | 5′-CAGTCTGAGCAAGTGCTCAACC-3′  | 5′-TTCGACGAGCTTCGTGTA-3′      |
| GAPDH  | 5′-TGTTCCGGTCTGGATCTGA-3′     | 5′-CCGTCTACCACCTTCTGTA-3′     |

Western Blot

Total protein was extracted, separated, and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA), as previously described [33]. These membranes were then incubated with blocking buffer for an hour and subjected to the appropriate primary antibodies for 24 h at 4°C. The primary antibodies included anti-THBS2 (1:1,000, ab112543; Abcam, Cambridge, UK), anti-GAPDH (1:2,500, ab9485; Abcam), anti-p-PI3K (1:1,000, AF5905; Beyotime), anti-GAPDH (1:2,500, ab9485; Abcam), anti-p-PI3K (1:1,000, AF5905; Beyotime), anti-p-AKT (1:1,000, AF1546; Beyotime), and anti-PI3K (1:1,000, AF7742; Beyotime). After centrifuging again, the supernatant was discarded. Next, the cells were resuspended in 1× binding buffer adjusted to an appropriate concentration (1–5 × 10^6/mL). Annexin V-fluorescein isothiocyanate (5 μL) and propidium iodide (10 μL) were added in 100 μL cell suspension, which was then incubated for 10 min at room temperature. Next, after adding 400 μL PBS, the cells were analyzed using the flow cytometry method (BD Biosciences, San Jose, CA, USA).

Statistical Analyses

The unpaired Student’s t test was adopted for comparison of the 2 groups. One-way analysis of variance was used to compare the difference of 3 or more groups, with the Bonferroni post hoc test. All the analyses were performed using the GraphPad Prism 6.0 software (San Diego, CA, USA) and SPSS 22.0 software (IBM SPSS, Armonk, NY, USA). Data were expressed as mean ± standard deviation of 3 independent experimentations. A p value < 0.05 was considered as statistically significant.

Results

THBS2 Is Upregulated in DN according to the Bioinformatics Analysis

Overall, according to GSE47185, a set of 91 genes were identified to be differentially expressed based on the cutoff criteria between DN tissue samples and normal tissue.

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Fig. 1. THBS2 was upregulated in DN and enriched in the PI3K/AKT signaling pathway. a KEGG analysis of differentially expressed genes according to the GSE47185 dataset. b Higher expression of THBS2 was identified in the DN tissues than normal control tissues according to the GSE47185 dataset. Data were analyzed using the unpaired Student’s t test. KEGG, Kyoto Encyclopedia of Genes and Genomes; THBS2, thrombospondin 2; DN, diabetic nephropathy; PI3K/AKT, phosphatidylinositol 3-kinase/AKT serine/threonine kinase 1.

Fig. 2. miR-1228 directly targeted THBS2. a The hub miRNA was identified between the downregulated differentially expressed miRNAs in GSE51647 and the predicted miRNAs binding to THBS2. b The putative binding sites between miR-1228 and 3′-UTR THBS2. c Downregulation of miR-1228 was observed using the dataset GSE51647. d Luciferase reporter assay was used to determine the luciferase activity of HK-2 cells co-transfected with the miR-1228 mimic, inhibitor, or NC and THBS2-Wt or THBS2-Mut vectors. **p < 0.01 versus the NC group. e–j miR-1228 negatively regulated the expression of THBS2 determined by real-time qPCR and Western blot. *p < 0.05 and **p < 0.01 versus control, #p < 0.05 and ##p < 0.01 versus NC, *p < 0.05 versus vector, ^^p < 0.01 versus si-con, *p < 0.05 and **p < 0.01 versus mimic or inhibitor. Difference between the 2 groups was analyzed using Student’s t test, and differences among 3 or more groups were analyzed using one-way analysis of variance followed by the Bonferroni post hoc test. DN, diabetic nephropathy; THBS2, thrombospondin 2; NC, negative control; miRNA, microRNA; UTR, untranslated region; HK-2, human renal proximal tubular epithelial cell; Wt, wild type; Mut, mutant.

(For figure see next page.)
miR-1228/THBS2 Contributes to HK-2 Cell Progression

![Diagram showing DEG-Down and Prediction with percentages and numbers]

![Graph showing expression of miR-1228 with p < 0.0001 and data from GSE51674]

![Bar graph showing relative luciferase activity with NC, mimic, and inhibitor groups]

![Graph showing relative THBS2 mRNA level with NC, mimic, vector, THBS2, and mimic-THBS2]

![Western blot images of THBS2 and GAPDH with corresponding protein expression graphs]

![Graph showing relative THBS2 mRNA level with inhibitor, inhibitor-THBS2, and inhibitor-st-THBS2]

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samples, of which 51 were upregulated and 40 were downregulated. Next, the Kyoto Encyclopedia of Genes and Genomes pathway analysis of these 91 DEGs revealed that the DEGs were remarkably enriched in the PI3K-AKT signaling pathway (Fig. 1a). A total of 8 DEGs including TNC, COL1A2, COL6A3, THBS2, PDGFRA, ITGB6, DDIT4, and G6PC were annotated to the PI3K-AKT pathway. Through retrieving the literature, THBS2 was also found to be upregulated in type 2 diabetes mellitus with nephropathy based on bioinformatics analysis [15]. However, its specific mechanism has not been studied. Additionally, considering the relatively high logFC ranking of THBS2, THBS2 was chosen as the research target, and its high expression is shown in Figure 1b (**p < 0.01).

miR-1228 Directly Targets THBS2 and Negatively Regulates Its Expression

A total of 152 differently expressed miRNAs were identified using the dataset GSE51674 according to the cutoff criteria, of which 121 were upregulated and 31 were downregulated. To elucidate the molecular mechanism of THBS2 in the progression of DN, 5 online prediction tools (miRWALK, Targetscan, PITA, miRBD, and miRanda) were used to identify the upstream regulated miRNAs of THBS2. Then, 26 miRNAs were predicted to have a binding site with the 3’UTR of THBS2. Using the Venn diagram, miR-1228 was identified by intercrossing the 26 targeted miRNAs and 31 downregulated miRNAs (Fig. 2a). The binding sites between miR-1228 and THBS2 are shown in Figure 2b, and the downregulation of miR-1228 is displayed in Figure 2c (**p < 0.01). To evaluate whether miR-1228 directly targets THBS2, we conducted dual-luciferase reporter assay. The data indicated that the relative luciferase activity was notably reduced when HK-2 cells were co-transfected with THBS2-Wt and miR-1228 mimic, whereas co-transfection of THBS2-Wt and miR-1228 inhibitor significantly elevated the luciferase activity (**p < 0.01, Fig. 2d). However, the luciferase activity showed no discernible change when cells were co-transfected with THBS2-Mut and miR-1228 mimic or inhibitor.

Next, we performed real-time qPCR and Western blot to measure miR-1228-induced dysregulation of THBS2 expression. The detections showed that the miR-1228 mimic notably decreased the mRNA expression levels of THBS2 in HK-2 cells compared with the blank control or NC group (**p < 0.05, Fig. 2e), whereas introduction of the THBS2 pcDNA-3.1 vector rescued the expression of THBS2 (**p < 0.05, Fig. 2e). On the contrary,
miR-1228/THBS2 Contributes to HK-2 Cell Progression

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the miR-1228 inhibitor remarkably increased the mRNA expression of THBS2 compared with the blank control or NC group (**p < 0.01, &p < 0.01), while introduction of THBS2 siRNA significantly weakened this increase (**p < 0.01, Fig. 2h). In addition, mRNA levels of THBS2 were significantly elevated after THBS2 overexpression compared with the blank control or pcDNA3.1 empty vector group (**p < 0.01, ^p < 0.05, Fig. 2e), whereas reduced after THBS2 knockdown compared with the blank control or si-con group (**p < 0.01, ^^p < 0.01, Fig. 2h). We noted that among blank control, NC, and vector/si-con groups, no significant changes were observed; thus, we selected a control group, blank control, for the following Western blot assay. The change trend of THBS2 protein level was consistent with that of mRNA level in the same group (**p < 0.01, **p < 0.01, Fig. 2f, g, i, j). Overall, all these detections indicated that miR-1228 directly targeted THBS2 and negatively mediated its expression.

miR-1228 Is Downregulated in HG-Cultured HK-2 Cells and Increases Their Proliferation

To further characterize the functional role of miR-1228 in DN, we constructed an HG model in vitro using the HK-2 cells. Real-time qPCR revealed that HG significantly reduced the expression of miR-1228 compared with relative controls (**p < 0.01, **p < 0.01, Fig. 3a), which was in agreement with the results by previous bioinformatics analysis. Moreover, the cell viability was significantly decreased under HG treatment, indicating that we have successfully established the model (**p < 0.01, Fig. 3b). Meanwhile, compared with NC, cell viability was notably enhanced after transfected with the miR-1228 mimic under HG condition (**p < 0.05), while it was obviously reduced upon transfection with the miR-1228 inhibitor (**p < 0.05, Fig. 3b). Additionally, we also evaluated the effect of miR-1228 on the HK-2 cell proliferation under basal condition and found no significant differences among blank control, miR-1228 mimic, and miR-1228 inhibitor groups (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000516791). Taken together, these findings illustrated that miR-1228 was downregulated in HG-cultured HK-2 cells and promoted cell viability.

THBS2 Rescues the Effects by miR-1228 on HG-Damaged HK-2 Cell Proliferation and Apoptosis

Since miR-1228 targeted THBS2 and displayed a role in protecting HK-2 cells from HG damage, we wondered whether this is involved in THBS2, and a rescue CCK8 assay was implemented. We noted that in HG-cultured HK-2 cells, transfection with THBS2 pcDNA3.1 vectors significantly reduced cell viability (**p < 0.05, Fig. 4a) while silencing THBS2 significantly elevated cell viability (**p < 0.05, Fig. 4b), suggesting that knockdown of THBS2 may protect the HK-2 cells avoiding HG damage. Moreover, the experimental results showed that in HG-induced HK-2 cells, overexpression of THBS2 attenuated the pro-proliferation effects caused by the miR-1228 mimic (**p < 0.01, Fig. 4a), whereas knockdown of THBS2 restored the anti-proliferation effects caused by the miR-1228 inhibitor (**p < 0.05, Fig. 4b).

Next, we performed flow cytometry assay to examine the effect of miR-1228/THBS2 on the HK-2 cell apoptosis. As expected, HG-cultured HK-2 cells displayed a higher apoptotic rate compared with the normal control group (**p < 0.01, Fig. 4c–e), while transfection of miR-1228 mimic or THBS2 siRNA reduced this effect (**p < 0.01, Fig. 4c–e). However, the anti-apoptosis effect by the miR-1228 mimic can be rescued by introducing THBS2 pcDNA3.1 vectors in HG-induced cells (**p < 0.01, Fig. 4c, d). On the other hand, transfection of the miR-1228 inhibitor or THBS2 pcDNA3.1 vectors significantly strengthened the apoptosis of HG-cultured cells (**p < 0.01, Fig. 4c–e). Taken together, these results indicated that restoration of THBS2 attenuated miR-1228-mediated apoptosis in HG-cultured HK-2 cells.

miR-1228/THBS2 Inhibited the PI3K/AKT Signaling Pathway

Previous literature studies have reported that HG induces ECM deposition in the glomerulus by activating the PI3K/AKT pathway [34]. Our abovementioned results illustrated that THBS2 was enriched in the glomerulus and owned a PI3K/AKT signaling pathway-related markers using the HG-cultured HK-2 cells. The data showed that HG significantly enhanced the phosphorylation (p) levels of PI3K and AKT compared with the blank control group (**p < 0.01, online suppl. Fig. 2). However, the expression levels of PI3K and AKT in HK-2 cells were not significantly changed under basal and HG conditions (online suppl. Fig. 2). Moreover, the results indicated that, under HG condition, THBS2 was responsible for activation of the PI3K/AKT pathway, as displayed that upregulation of THBS2 enhanced the pro-
miR-1228/THBS2 Contributes to HK-2 Cell Progression

Figure 5. miR-1228/THBS2 affected the PI3K/AKT signaling pathway in HG-induced HK-2 cells. a–j Western blot determined the effects of miR-1228/THBS2 on the levels of p-PI3K, PI3K, p-AKT, and AKT in HK-2 cells treated with HG, HG + miR-1228 mimic, and HG + miR-1228 mimic + THBS2 (a–e) or control, HG, HG + miR-1228 inhibitor, and HG + miR-1228 inhibitor + si-THBS2 (f–j). a, f Representative protein bands. b–e Quantification of a. g–j Quantification of e. Multiple group analysis was performed using one-way analysis of variance followed by the Bonferroni post hoc test. **p < 0.01 and *p < 0.05 versus control. ***p < 0.01 and #p < 0.05 versus HG + mimic or HG + inhibitor. HG, high glucose; THBS2, thrombospondin 2; PI3K/AKT, phosphatidylinositol 3-kinase serine/threonine kinase 1.

Protein levels of p-PI3K and p-AKT (**p < 0.01, Fig. 5a, b, d), while downregulation of THBS2 reduced the protein levels of these 2 markers (*p < 0.05, Fig. 5f, g, i). Meanwhile, we also found that miR-1228 significantly inactivated the PI3K/AKT signaling pathway, as confirmed by the reduced protein levels of p-PI3K and p-AKT after transfection with the miR-1228 mimic (*p < 0.05, Fig. 5a, b, d) and the elevated protein levels of both p-PI3K and p-AKT af-
stronger mechanisms of DN progression [20, 22, 36]. Urinary the dysregulated expression of miRNAs is related to vari-
ted with HG in vitro. Our investigations provide a the-
tation of the potential mechanisms may contribute to the
diagnosis and effective treatment for DN. Here, we mon-
tored the functional role of the miR-1228/THBS2 axis
and its underlying mechanism using the HK-2 cells stim-
ulated with HG in vitro. Our investigations provide a the-
oretical basis for the application of miR-1228/THBS2 in
procession of kidney fibrosis in DN [26]. However, the
miR-1228 has been reported to be correlated with the
progression of HG-damaged HK-2 cells [48, 49]. Consistently, our results also showed
that HG elevated the expression of p-PI3K and p-AKT,
suggesting that HG causes kidney tubular epithelial in-
jury. Moreover, our initial gene set enrichment analysis
revealed that THBS2 was annotated to the PI3K-AKT
signaling pathway caused by the miR-1228 inhibitor.
THBS2 knockdown enhanced cell proliferation but reduced cell
apoptosis after HG treatment, suggesting that silencing
THBS2 might protect HK-2 cells from HG injury. This is
consistent with a report that showed THBS2-knockdown
hydrogels composed of solubilized decellularized ECM
notably improved diabetic wound healing [40]. Further-
more, through rescue experiments, we also proved that
knockdown of THBS2 attenuated the HG-induced tubule
knockdown of THBS2 attenuated the HG-induced tubule
cells injury caused by the miR-1228 inhibitor.
The PI3K/AKT signaling axis plays an important role
in promoting cellular proliferation and growth and inhib-
iting apoptosis [41]. It has been shown that the PI3K/
AKT pathway is involved in the initiation and develop-
ment of human renal diseases, including renal tubule in-
jury [42, 43]. The activated PI3K converts the plasma
membrane lipid phosphatidylinositol-4,5-bisphosphate
(PI(4,5)P2) into phosphatidylinositol-3,4,5-trisphos-
phate (PI(3,4,5)P3), which acts as a second messenger to
activate the downstream signaling molecules [44]. AKT,
generally considered to be one of the main effectors of
PI3K, is best known for its anti-apoptotic/pro-survival
action [44]. Downstream products of PI3K/AKT have a
regulatory role in the utilization of glucose [45]. The
PI3K/AKT signaling pathway is the main pathway of in-
sulin effect signal transduction [46]. Abnormalities in any
of its links will interfere with the physiological function
of insulin, thus leading to diabetes and its complications.
In diabetic environment, the inhibition of p-PI3K/p-AKT
was triggered [47]. Previous studies revealed that HG en-
hanced the phosphorylation levels of PI3K and AKT in
HK-2 cells [48, 49]. Consistently, our results also showed
that HG elevated the expression of p-PI3K and p-AKT,
suggesting that HG causes kidney tubular epithelial in-
jury. Moreover, our initial gene set enrichment analysis
revealed that THBS2 was annotated to the PI3K-AKT
pathway, plus that THBS2 was a target of PI3K/AKT,
acting as a master regulator in the utilization of glucose in
DKD. The activated PI3K converts the plasma
membrane lipid phosphatidylinositol-4,5-bisphosphate
(PI(4,5)P2) into phosphatidylinositol-3,4,5-trisphos-
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suggesting that HG causes kidney tubular epithelial in-
jury. Moreover, our initial gene set enrichment analysis
revealed that THBS2 was annotated to the PI3K-AKT
pathway, plus that THBS2 was a target of miR-1228, re-
minding us that miR-1228/THBS2 may be associated
with the PI3K/AKT signaling pathway in DKD. Thus, we
measured the effects of miR-1228/THBS2 on the expres-
sion of p-PI3K, PI3K, p-AKT, and AKT using the HG-
induced HK-2 cells in vitro. Our measurements indicated
that downregulation of miR-1228 or overexpression of
THBS2 can elevate the phosphorylation of PI3K and AKT
in HG-damaged HK-2 cells. Importantly, downregu-
lation of THBS2 inhibited the activation of the PI3K/AKT
signaling pathway caused by the miR-1228 inhibitor. Be-
sides, THBS2 knockdown has been proven to restrain the
proliferation of gastric cells through the PI3K/AKT path-
way [50] and is involved in ECM function that activates
PI3K in colorectal cancer, illustrating that THBS2 has a

Discussion

So far, multifactor interventions to manage blood glu-
cose, lipid, and blood pressure are still the main treatment
options for DN [35]. Hopefully, with the development of
medical technology, the value of molecular targets in the
treatment of DN is gradually becoming evident. Explora-
tion of the potential mechanisms may contribute to the
diagnosis and effective treatment for DN. Here, we mon-
tored the functional role of the miR-1228/THBS2 axis
and its underlying mechanism using the HK-2 cells stim-
ulated with HG in vitro. Our investigations provide a the-
oretical basis for the application of miR-1228/THBS2 in
DN treatment in the future.

Accumulating pieces of evidence have revealed that
the dysregulated expression of miRNAs is related to vari-
ous mechanisms of DN progression [20, 22, 36]. Urinary
miR-1228 has been reported to be correlated with the
progression of kidney fibrosis in DN [26]. However, the
role of miR-1228 and the potential molecular mechanism
in DN have not been fully evaluated. Consistently, down-
regulation of miR-1228 in this study was also observed in
DN tissues and in HG-subjected HK-2 cells. Using the
functional experiments, miR-1228 displayed a protective
role in HK-2 cells against HG injury, such as increasing
proliferation in mimic-transfected cells and decreasing
proliferation in inhibitor-transfected cells.

Strong pieces of evidence have shown that THBS2 regu-
lates a variety of biological functions, including prolifera-
tion, apoptosis, angiogenesis, and ECM modeling [37, 38].
A study showed that THBS2, as a matricellular protein, af-
facts the apoptosis-proliferation balance in endothelial
cells [39]. However, the role of THBS2 in DN or DN-relat-
ed cells is still unclear. In our study, THBS2 was upregu-
lated in DN tissues and as a target of miR-1228 involved in
the progression of HG-damaged HK-2 cells. THBS2
knockdown of THBS2 attenuated the HG-induced tubule
cells injury caused by the miR-1228 inhibitor.

The PI3K/AKT signaling axis plays an important role
in promoting cellular proliferation and growth and inhib-
iting apoptosis [41]. It has been shown that the PI3K/
AKT pathway is involved in the initiation and develop-
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action [44]. Downstream products of PI3K/AKT have a
regulatory role in the utilization of glucose [45]. The
PI3K/AKT signaling pathway is the main pathway of in-
sulin effect signal transduction [46]. Abnormalities in any
of its links will interfere with the physiological function
of insulin, thus leading to diabetes and its complications.
In diabetic environment, the inhibition of p-PI3K/p-AKT
was triggered [47]. Previous studies revealed that HG en-
hanced the phosphorylation levels of PI3K and AKT in
HK-2 cells [48, 49]. Consistently, our results also showed
that HG elevated the expression of p-PI3K and p-AKT,
suggesting that HG causes kidney tubular epithelial in-
jury. Moreover, our initial gene set enrichment analysis
revealed that THBS2 was annotated to the PI3K-AKT
pathway, plus that THBS2 was a target of miR-1228, re-
minding us that miR-1228/THBS2 may be associated
with the PI3K/AKT signaling pathway in DKD. Thus, we
measured the effects of miR-1228/THBS2 on the expres-
sion of p-PI3K, PI3K, p-AKT, and AKT using the HG-
induced HK-2 cells in vitro. Our measurements indicated
that downregulation of miR-1228 or overexpression of
THBS2 can elevate the phosphorylation of PI3K and AKT
in HG-damaged HK-2 cells. Importantly, downregu-
lation of THBS2 inhibited the activation of the PI3K/AKT
signaling pathway caused by the miR-1228 inhibitor. Be-
sides, THBS2 knockdown has been proven to restrain the
proliferation of gastric cells through the PI3K/AKT path-
way [50] and is involved in ECM function that activates
PI3K in colorectal cancer, illustrating that THBS2 has a
miR-1228/THBS2 Contributes to HK-2 Cell Progression

connection with the PI3K/AKT signaling pathway [51]. Taken together, we concluded that overexpression of miR-1228 could ameliorate HG-induced tubule cell injury partly through inhibiting the PI3K/AKT signaling pathway via targeting THBS2.

One limitation of the present study is that only cells were used in the experimental part to investigate and evaluate the influence of miR-1228/THBS2 on the PI3K/AKT pathway. Therefore, further in vivo investigation is needed.

In conclusion, we found that miR-1228 is downregulated and THBS2 is upregulated in DN tissues. Upregulation of miR-1228 elevated the proliferation and reduced the apoptosis of HG-cultured renal tubule cells partly through suppressing the PI3K/AKT signaling pathway-related proteins via downregulation of THBS2, which was confirmed as a target gene of miR-1228. Our study may provide a novel insight into the therapy of DN in the future.

**Statement of Ethics**

The study did not involve any in vivo experiments or human studies. The study is exempt from ethics committee approval.

**References**

1. Vinik AI, Nevoret ML, Casellini C, Parson H. Diabetic nephropathy. *Endocrinol Metab Clin North Am*. 2013;42:747–87.
2. Alicic RZ, Rooney MT, Tuttle KR. Diabetic kidney disease: challenges, progress, and possibilities. *Clin J Am Soc Nephrol*. 2017;12:2032–45.
3. Gregg EW, Williams DE, Geiss L. Changes in diabetes-related complications in the United States. *New Engl J Med*. 2014;371(3):286–7.
4. Tzimolas K, Athyros VG, Diabetic nephropathy: new risk factors and improvements in diagnosis. The review of diabetic studies. *Rev Diabet Stud*. 2015;12:110–8.
5. Morosanova MA, Plotnikov EY, Zorova LD, Osmozenko VA, Grechko AV, Orekhov AN. Thrombospondin proteins in renal injury. Biochemistry. 2016;81:1240–50.
6. Mustonen E, Ruuskoaho H, Rysä J. Thrombospondins, potential drug targets for cardiovascular diseases. *Basic Clin Pharmacol Toxicol*. 2013;112:4–12.
7. Armstrong LC, Bornstein P. Thrombospondins 1 and 2 function as inhibitors of angiogenesis. *Matrix Biol*. 2003;22:63–71.
8. Liu JF, Lee CW, Tsai MH, Tang CH, Chen PC, Lin LW, et al. Thrombospondin 2 promotes tumor metastasis by inducing matrix metalloproteinase-13 production in lung cancer cells. *Biochem Pharmacol*. 2018;155:537–46.
9. Chatila K, Ren G, Xia Y, Huebener P, Bujak M, Frangogiannis NG. The role of the thrombospondins in healing myocardial infarcts. *Cardiovasc Hematol Agents Med Chem*. 2007;5:21–7.
10. Chistjakov DA, Melnichenko AA, Myasoedova VA, Grechko AV, Orekov AN. Thrombospondins: a role in cardiovascular disease. *Int J Mol Sci*. 2017;18(7):1540.
11. Kazeroonian S, Yee KO, Lawler J. Thrombospondins in cancer. *Cell Mol Life Sci*. 2008;65:700–12.
12. Hugo C, Daniel C. Thrombospondin in renal disease. *Nephron Exp Nephrol*. 2009;111:661–6.
13. Abu El-Asrar AM, Nawaz MI, Ola MS, De Hertogh G, Opdenakker G, Geboes K. Expression of thrombospondin-2 as a marker in proliferative diabetic retinopathy. *Acta Ophthalmol*. 2013;91:e169–77.
14. Bae ON, Wang JM, Baek SH, Wang Q, Yuan H, Chen AF. Oxidative stress-mediated thrombospondin-2 upregulation impairs bone marrow-derived angiogenic cell function in diabetes mellitus. *Arterioscler Thromb Vasc Biol*. 2013;33:1920–7.
15. Yeh SH, Chang WC, Chang H, Huang HC, Liu RT, Yang KD. Differentiation of type 2 diabetes mellitus with different complications by proteomic analysis of plasma low abundance proteins. *J Diabetes Metab Disord*. 2015;15:24.
16. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–97.
17. Rana TM. Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol*. 2007;8:23–36.
18. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*. 2008;9:102–14.
19. Rovira-Llopis S, Escribano-Lopez I, Diaz-Morales N, Iannantuoni F, Lopez-Domenech S, Andujar I, et al. Downregulation of miR-31 in diabetic nephropathy and its relationship with inflammation. *Cell Physiol Biochem*. 2018;50:1005–14.
20. Zhou Z, Wan J, Hou X, Geng J, Li X, Bai X. MicroRNA-27a promotes podocyte injury via PPARy-mediated β-catenin activation in diabetic nephropathy. *Cell Death Dis*. 2017;8:e2658.
21. Sun J, Zhao F, Zhang W, Lv J, Lv J, Yin A. BMSCs and miR-124a ameliorated diabetic nephropathy via inhibiting notch signalling pathway. *J Cell Mol Med*. 2018;22:4840–55.
22. Wu J, Liu J, Ding Y, Zhu M, Lu K, Zhou J, et al. MiR-455-3p suppresses renal fibrosis through repression of ROCK2 expression in diabetic nephropathy. *Biochem Biophys Res Commun*. 2018;503:977–83.

**Conflict of Interest Statement**

The authors declare no conflicts of interest.

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**Author Contributions**

T.R.M. and J.L. designed the study. T.R.M., Q.F., X.Y.H., and Y.F. performed the experiments and collected the data. T.R.M., Q.F., and J.L. analyzed the data. T.R.M. wrote the manuscript. All authors read, commented, and accepted the final version.

**Data Availability Statement**

The data of this paper is available from the corresponding author on reasonable request.
23 Zhang Y, Dai J, Deng H, Wan H, Liu M, Wang J, et al. miR-1228 promotes the proliferation and metastasis of hepatoma cells through a p53 forward feedback loop. Br J Cancer. 2015; 112:365–74.
24 Lin L, Liu D, Liang H, Xue L, Su C, Liu M. MiR-1228 promotes breast cancer cell growth and metastasis through targeting SCAI protein. Int J Clin Exp Pathol. 2015; 8:6646–55.
25 Chen D, Ma W, Ke Z, Xie F. CircRNA hsa_circ_100395 regulates miR-1228-3p to inhibit lung cancer progression. Cell Cycle. 2018; 17:2080–90.
26 Conserva F, Barozzino M, Pesce F, Divella C, Oranger A, Papale M, et al. Urinary miRNA-27b-3p and miRNA-1228-3p correlate with the progression of kidney fibrosis in diabetic nephropathy. Sci Rep. 2019; 9:11357.
27 Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004; 3:Article3.
28 Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000; 28:27–30.
29 Bai X, Hou X, Tian J, Geng J, Li X. CDK5 promotes renal tubulointerstitial fibrosis in diabetic nephropathy via ERK1/2/PPARγ pathway. Oncotarget. 2016; 7:36510–28.
30 Su J, Ren J, Chen H, Liu B. MicroRNA-140-5p ameliorates the high glucose-induced apoptosis and inflammation through suppressing TLR4/NF-κB signaling pathway in human renal tubular epithelial cells. Biosci Rep. 2020; 40:40.
31 Huang C, Zhang Y, Kelly DJ, Tan CY, Gill A, Cheng D, et al. Thioredoxin interacting protein (TXNIP) regulates tubular autophagy and mitophagy in diabetic nephropathy through the mTOR signaling pathway. Sci Rep. 2016; 6:29196.
32 Jiao H, Zeng L, Zhang J, Yang S, Lou W. THBS2, a microRNA-744-5p target, modulates MMP9 expression through CUX1 in pancreatic neuroendocrine tumors. Oncol Lett. 2020; 19:1683–92.
33 Liu X, Li J, Li X. miR-142-5p regulates the progression of diabetic retinopathy by targeting IGF1. Int J Immunopathol Pharmacol. 2020; 34:2058738420900041.
34 Zang XJ, Li L, Du X, Yang B, Mei CL. LncRNA TUG1 inhibits the proliferation and fibrosis of mesangial cells in diabetic nephropathy via inhibiting the PI3K/AKT pathway. Eur Rev Med Pharmacol Sci. 2019; 23:7519–25.
35 Rossing P, Persson F, Frimodt-Møller M. Prognosis and treatment of diabetic nephropathy: recent advances and perspectives. Nephrol Ther. 2018; 14 Suppl 1:S31–7.
36 Zhang LY, Wang Y, Yang YR, Shao JJ, Liang B. MiR-135a regulates renal fibrosis in rats with diabetic kidney disease through the notch pathway. Eur Rev Med Pharmacol Sci. 2020; 24:1979–87.
37 Lawler PR, Lawler J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. Cold Spring Harb Perspect Med. 2012; 2:a00627.
38 Nakao T, Morita H. Thrombospondin-2. Int Heart J. 2019; 60:235–7.
39 Lopes N, Gregg D, Vasudevan S, Hassanain H, Goldschmidt-Clermont P, Kovacic H. Thrombospondin 2 regulates cell proliferation induced by Rac1 redox-dependent signaling. Mol Cell Biol. 2003; 23:5401–8.
40 Morris AH, Lee H, Xing H, Stamer DK, Tan M, Kyriakides TR. Tunable hydrogels derived from genetically engineered extracellular matrix accelerate diabetic wound healing. ACS Appl Mater Interfaces. 2018; 10:41892–901.
41 Patel RK, Mohan C. PI3K/AKT signaling and systemic autoimmunity. Immunol Res. 2005; 31:47–55.
42 Sugimoto H, LeBleu VS, Bosukonda D, Keck P, Tan H, Bechtel W, et al. Activin-like kinase 3 is important for kidney regeneration and reversal of fibrosis. Nat Med. 2012; 18:396–404.
43 Zhao S, Zhu L, Duan H, Liu S, Liu Q, Liu W, et al. PI3K/Akt pathway mediates high glucose-induced lipid accumulation in human renal proximal tubular cells via spliced XBP-1. J Cell Biochem. 2012; 113:3288–98.