

Mir-138 plays an important role in diabetic nephropathy through SIRT1-p38-TTP regulatory axis

Fengxun Liu

Zhengzhou University

Jia Guo

Core unit of national clinical medical research center of kidney disease

Yingjin Qiao

Zhengzhou University First Affiliated Hospital

Shaokang Pan

key laboratory of precision diagnosis and treatment for chronic kidney disease in henan province

Jiayu Duan

key laboratory of precision diagnosis and treatment for chronic kidney disease in henan province

Dongwei Liu

Zhengzhou University First Affiliated Hospital

Zhangsuo Liu (✉ zhangsuoliu@zzu.edu.cn)

first affiliated hospital of zhengzhou university <https://orcid.org/0000-0002-2916-8371>

Research

Keywords: MiRNA-138, SIRT1, TTP, Diabetic nephropathy, Podocytes

Posted Date: January 23rd, 2020

DOI: <https://doi.org/10.21203/rs.2.21672/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Journal of Cellular Physiology on April 12th, 2021. See the published version at <https://doi.org/10.1002/jcp.30238>.

Mir-138 plays an important role in diabetic nephropathy through SIRT1-p38-TTP regulatory axis

Fengxun Liu², Jia Guo⁴, Yingjin Qiao¹, Shaokang Pan³, Jiayu Duan³, Dongwei Liu¹ (✉), Zhangsuo Liu¹ (✉)

¹ Department of Nephrology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, P. R. China

² Research Institute of Nephrology, Zhengzhou University, Zhengzhou 450052, P. R. China

³ Key Laboratory of Precision Diagnosis and Treatment for Chronic Kidney Disease in Henan Province, Zhengzhou 450052, P. R. China

⁴ Core Unit of National Clinical Medical Research Center of Kidney Disease, Zhengzhou 450052, P. R. China

Authors' information

Fengxun Liu: **E-mail**, nephrology003@outlook.com

Jia Guo: **E-mail**, zzuzhangyu@foxmail.com

Yingjin Qiao: **E-mail**, nagato@csu.edu.cn

Shaokang Pan: **E-mail**, glomerulus001@outlook.com

Jiayu Duan: **E-mail**, Lu1994March@163.com

Dongwei Liu: **E-mail**, liu-dongwei@zzu.edu.cn

Zhangsuo Liu: **E-mail**, zhangsuoliu@zzu.edu.cn

Corresponding author

✉ **Dongwei Liu**, Department of Nephrology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, P. R. China. **E-mail**: liu-dongwei@zzu.edu.cn

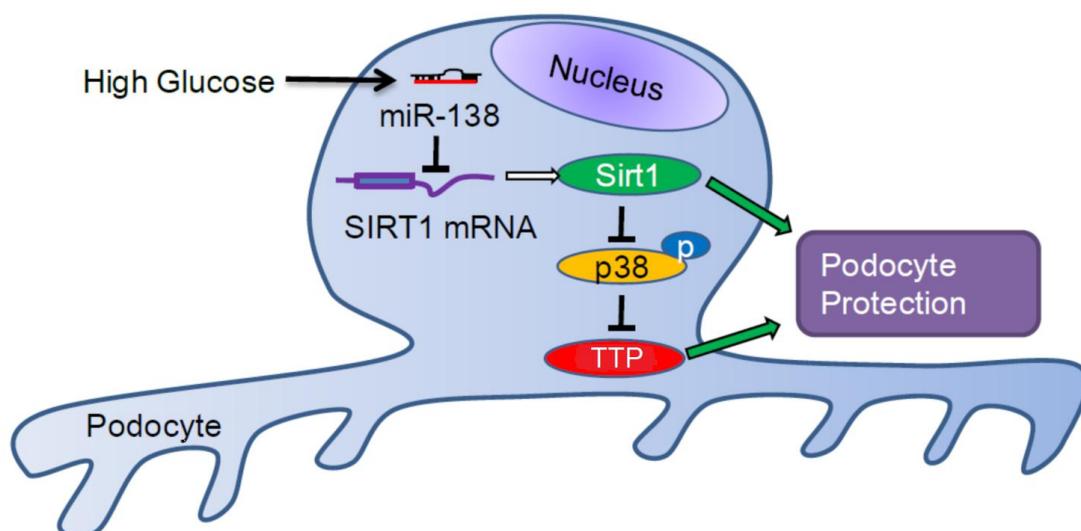
✉ **Zhangsuo Liu**, Department of Nephrology, the First Affiliated Hospital of Zhengzhou University; Research Institute of Nephrology, Zhengzhou University; Key Laboratory of Precision Diagnosis and Treatment for Chronic Kidney Disease in Henan Province; Core Unit of National Clinical Medical Research Center of Kidney Disease, No. 1, Jianshe East Road, Zhengzhou 450052, Henan Province, P. R. China. **E-mail**: zhangsuoliu@zzu.edu.cn

Abstract

Background: Diabetic nephropathy (DN) is the main cause of chronic kidney disease (CKD) and is one of the most common and serious complications of diabetes mellitus (DM). SIRT1 and TTP are two important protective factors in DN, however, the regulatory relationship between SIRT1 and TTP and the underneath mechanism are interesting but still unclear. Identifying the key factors that regulate SIRT1 or TTP may be of great value to the understanding and treatment of the DN. **Methods:** in this study, through systematic experimental methods, we found that the expression of miR-138 was significantly up-regulated in DN clinical patients samples, and our experimental results suggested that miR-138 could bind the 3'UTR of SIRT1 and inhibit its expression in both cultured podocytes and db/db mice kidney tissues. **Results:** furthermore, our *in vitro* and *in vivo* data also indicated miR-138 could target SIRT1 and affect TTP through p38 pathway. And down-regulation of miR-138 attenuated podocyte injury and showed some extend of therapeutic effects in DN mice models. **Conclusion:** our findings reveal that the regulatory axis of miR-138-SIRT1-p38-TTP might play a key role in DN. We believe these findings may be of some value for deepening the understanding of DN and may serve as a reference for future treatment of this disease.

Key words: MiRNA-138; SIRT1; TTP; Diabetic nephropathy; Podocytes

Graphic Abstract



Introduction

Diabetic nephropathy (DN) is one of the most common and serious complications of diabetes mellitus (DM) [1]. The incidence of DN is increasing year by year in both developed and developing countries. It has become the main cause of chronic kidney disease (CKD) [2]. DN has the characteristics of rapid progress and low replacement treatment rate, so it is particularly urgent to strengthen the prevention and clinical treatment of DN [1-3].

The occurrence and progression of DN are related to podocytes injury with participation of many factors, such as stimulation of high glucose environment, changes in hemodynamics, and involvement of inflammatory factors [1,3]. Recently, more and more evidence shows that SIRT1, which is a key molecule in glucose, lipid, and energy metabolism, is an important protective factor of DN [4-7]. Identifying the key factors that regulate SIRT1 may be of great value to the understanding and treatment of the disease. It has been reported that microRNA-138(mir-138)can regulate the expression of SIRT1 and participate in nerve regeneration in mammals [8]. However, in DN, whether miR-138 plays a role in regulating SIRT1 and participates in the pathogenesis of this disease is still not clear.

As an important anti-inflammatory protein, Tristetraprolin (TTP) recently has been identified as another protective factor of DN [9,10]. TTP is an AU-enriched region binding protein that mediates the degradation of m RNA and regulates the expression of post-transcriptional proteins [11]. Decreased expression of TTP leads to an increase in the expression of inflammatory factors, which causes the podocytes to remain in an inflammatory state for a long time, losing normal morphology and function. Whether there is a regulatory relationship between SIRT1 and TTP and the underneath mechanism are also interesting but still unclear.

In this study, we attempt to clarify the issues mentioned above through in vivo and in vitro experiments, and provide experimental reference for further understanding the pathological mechanism of DN and finding clue to improve its diagnosis and treatment in the future.

Materiaks And Methods

Human studies

Renal tissue specimens of patients were collected. All patients provided informed consent, and the study was approved by an ethics committee. In total, 20 renal tissue specimens were collected: 18 from DN patients (15 with macroalbuminuria and 3 with microalbuminuria), 3 from diabetes mellitus (DM) patients, and 9 control samples from healthy renal tissue around the cancerous tissue of radical nephrectomy patients. Blood and urine samples were collected from 40, 18, 13, and 17 patients in the control, DM, DN with microalbuminuria, and DN with macroalbuminuria groups, respectively. All experiments were carried out in accordance with the guidelines of Ethics Committee of Scientific Research and Clinical Trial of the First Affiliated Hospital of Zhengzhou University.

Animal studies

All procedures involving animals were conducted in accordance with the guidelines prescribed by Animal Care Committee of Zhengzhou University. Mice were purchased from the Model Animal Research Institute of Nanjing University. All mice were raised in a laboratory animal room at the Medical Science Research Institute, Henan Province. Five mice were raised per cage in IVC-II independent supply air isolation cages. All bedding, feed, and drinking water were disinfected with ultraviolet light at appropriate dose intensity with a traffic limit device.

We used mice with a mutation of the leptin receptor gene in BKS.Cg-Dock7^m +/+ Lepr^{db}/J mice (db/db mice). We purchased 5-week-old mice and allowed 1-week adaptation period. In diabetic mice, blood glucose increases spontaneously at week 5 or 6, with proteinuria and renal impairment appearing at 8–10 weeks. The 60 db/db mice were randomly divided into three groups (n = 20 each): db/db group, no treatment/intervention; db/db+veh (vehicle) group, nonsense sequence of miR138 encapsulated by lentivirus were injected into the blood through the mouse tail vein; and db/db+LV (lentivirus) group, miR-138 shRNA encapsulated by lentivirus were injected into the blood through the mouse tail vein.

The first injections were performed when the mice were 10 weeks old, and the injection dose was 40 μL and the titer was 2×10^7 . At 10 weeks, before miR-138 shRNA injection, three mice were sacrificed in each group and marked as B-10w. After injection, they were marked as A-10w. The second injections were performed when the mice were 14 weeks old, at the same dose and titer to achieve the desired concentration in vivo. At 8 weeks post injection, that is, at 22 weeks old, model mice and control mice were euthanized and renal tissues were obtained (**Figure 4A**). Control group included db/m mice of the same age and weight ($n = 12$).

Cell culture

Cells in this experiment were derived from permanent mouse kidney podocyte cell lines, which was authorized by Peter Mundel of Mount Sinai Medical School in the United States.

Culture, proliferation, and differentiation of podocytes

A podocyte cell culture medium (containing 10% fetal bovine serum, 5.6 mmol/L glucose, 4 ng/mL mouse γ -interferon, and RPMI 1640 medium) was prepared at 33°C. Microscopic observation showed that podocyte proliferation significantly slowed 1 week later, podocytes were irregular in shape, and numerous foot processes extended into the surroundings. Podocytes matured approximately 14 days after differentiation. For the glucose treatment experiments, mice podocytes were cultured at a concentration of 5.6 mmol/L glucose (NG group), 30 mmol/L glucose (HG group) and 5.6 mmol/L glucose + 44.4 mmol/L mannitol (NG+ Mannitol group).

Transfection

The differentiated mature podocytes were routinely digested before transfection and evenly inoculated into sterile 6-well plates. Transfection was performed when the cells grew to 40%–50% confluence, i.e., after 24 h. To mediate transfection, 4 μL Lipofectamine 2000 was gently mixed with 200 μL Opti-MEM transfection medium and incubated for 5 min at room temperature. Then, 5 μL each of miR-138 shRNA and Scramble were mixed with 200 μL Opti-MEM transfection medium and incubated at room temperature for 5 min. The shRNA transfection reagent mixture was produced by mixing

the Lipofectamine 2000 and the shRNA solutions together at room temperature for 20 min. Then, the medium in the 6-well plate was discarded, the plate was washed thrice with sterile PBS, 1.6 mL Opti-MEM transfection medium and siRNA transfection reagent mixture were added, the plate was gently shaken, and it was placed in an incubator at 37°C and 5% CO₂.

Protein extraction and Western-blot

The culture plate was placed on ice, and the cells were lysed with RIPA lysate. Then, 1 mL RIPA was supplemented with 10 µL PMSF (100 nM) (PMSF:RIPA, 1:100), 10 µL protease inhibitor cocktail, and 10 µL phosphatase inhibitor cocktail. After complete mixing, 100 µL solution was added to each well of a 6-well plate and placed on ice for 30 min. To ensure complete lysis, a cell scraper was used to scrape the cell debris from the 6-well plate. Then, the lysate containing the cell debris was transferred into a 1.5-ml centrifuge tube using a pipette. All procedures were performed on ice as far as possible. Then, 5 µL of the supernatant after centrifugation (4°C, 12,000 rpm, 15 min) was used to detect protein concentration using a BCA protein concentration assay kit. Rabbit anti-mouse IL-18 polyclonal, rabbit anti-mouse desmin polyclonal, rabbit anti-mouse nephrin polyclonal, rabbit anti-mouse podocin polyclonal, and rabbit anti-mouse GAPDH polyclonal antibodies were purchased from Santa Cruz Biotechnology or Abcam, USA. All the grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

RNA extraction and real-time RT-PCR

One mL Trizol lysate was added to the 6-well plate, the mixture was gently mixed with a pipette, and placed horizontally on ice for 10 min, which facilitated complete contact between the Trizol and cells. Chloroform was pipetted into a non-enzymatic EP tube with an enzyme-free pipette tip (1 mL Trizol:200 µL chloroform); then, this mixture was rapidly shaken for 15 s and set on ice for 5 min before being centrifuged in a cryogenic centrifuge at 4°C and 12,000 rpm for 10 min. This divided the sample into three layers; the upper layer was an RNA layer (colorless), which was transferred into a new non-enzymatic EP tube and centrifuged repeatedly under the same conditions. Finally, 30 µL DEPC water was added to the precipitate and gently mixed by pipetting with a sterile pipette tip. Then, the c-DNA was reverse transcribed and the corresponding fragments

were amplified in an RT-PCR machine. To reduce human error, three replicate wells were set for each target gene.

Immunohistochemistry and PAS staining

Tissue samples were baked at 60°C for 30 min, dewaxed, hydrated, and placed in a plastic container containing Tris-EDTA Antigen Recover. The tissue was covered with 0.3% Triton X-100 and incubated for 15 min at room temperature. Then, it was blocked with 5% PSA, incubated with the primary antibody overnight at 4°C and the secondary antibody for 1 h at room temperature, and finally observed and photographed under a fluorescent microscope.

Statistical analysis

Experiments were performed at least thrice, and values reported are mean \pm SD. Data were analyzed using IBM SPSS Statistics 19.0. Statistical significance was assessed using the Student's *t*-test, one-way ANOVA, the LSD *t*-test, and two-way ANOVA. *P*-values < 0.05 were considered statistically significant.

Results

MiR-138 is highly expressed in DN patients and associated with podocyte injury

In this study, we first focused on microRNA-138 and tried to determine whether microRNA-138 has changed in DN clinical case samples. This is an important question that needs to be answered at the most basic level, which was not clear before. To achieve this, subjects of the First Affiliated Hospital of Zhengzhou University from March 2016 to September 2017 were selected and separated into 4 groups: Control, DM, DN1 (DN with microalbuminuria), and DN2 (DN with macroalbuminuria groups). We first analyzed the blood and urine indexes of each group (Control, n=40; DM, n=18; DN1, n=13; DN2, n=17), and found that the exchanges of urinary albumin excretion rate, serum creatinine levels and blood glucose levels were consistent with the characteristics of each group (**Figure S1 A-C**). Then, the PAS staining results and electron microscopic examination results also showed typical morphological changes of the 4 groups (**Figure S1D**). These data indicated

that the grouping was appropriate for the present study. After further RT-PCR analysis of blood specimens, we found the expression of miR-138 in the DM and DN groups (i.e., high-glucose environments) was significantly higher than in the control group, moreover, we found that the expression of microRNA-138 increased with the severity of the disease, which was the highest in DN2 group (**Figure 1A**). Moreover, we found that the exchange trend of inflammatory-related factors IL18 and podocyte-specific factor desmin in each group also has a trend of miR-138 (**Figure 1B-C**). Additionally, the Masson's stain of paraffin-embedded kidney specimens were also shown the degree of fibrosis in the DN group was higher than that in the DM and control groups (**Figure 1D**). These results suggest that miR-138 is highly expressed in DN patients and may be associated with inflammation, podocyte injury, and renal fibrosis in this disease.

MiR-138 can down-regulate SIRT1 and promote podocytes injury in DN models.

Next, we tested whether microRNA-138 could target and down-regulate SIRT1 *in vitro* and *in vivo*, which is an important protective factor of DN [4-7]. In cultured mice podocytes, we found that SIRT1 was down-regulated under high glucose treatment (HG group, 30 mmol/L glucose). Additionally, over-expression of microRNA-138 mimics could down-regulate the expression of SIRT1 whether or not under high glucose stimulation, while knock-down of miR-138 using inhibitor or shRNA could up-regulate the expression of SIRT1 (**Figure 2A-C**). Then, to further confirm, we constructed a SIRT1-3'UTR-reporter which the SIRT1-3'UTR was cloned at the downstream of luciferase element, and SIRT1-3'UTR-muta-reporter which lost the predicted miR-138 binding site (**Figure 2D**). The dual-luciferase reporter assay showed that miR-138 can down-regulate the expression of luciferase of SIRT1-3'UTR-reporter but not SIRT1-3'UTR-muta-reporter (**Figure 2E**). For *in vivo* experiments, we used 5-week-old male diabetic db/db mice as DN models, which develop spontaneous renal impairment at 8–10 weeks of age, and male non diabetic db/m mice as control (**Figure S2**). And we found the expression levels of SIRT1 were decreased at 22 weeks in db/db mice when compared to db/m mice, and knock down of miR-138 by injecting shRNA-expression lentivirus caused obvious SIRT1 expression recovery (**Figure 2F**). All these results suggested that miR-138 can target and down-regulate SIRT1 in mice podocytes.

Additionally, in *in vitro* experiment, we also found miR-138 over-expression up-regulated the expression of Desmin, which is the marker for podocytes injury (**Figure 2B**). In *in vivo* experiment, we found miR-138 knock-down up-regulated the expression of synaptopodin, which is the marker for differentiated podocytes and its down-regulation indicates podocyte injury (**Figure 2F**). Therefore, these results suggested that miR-138 might play a role in promoting podocytes injury in DN.

MiR-138 could inhibit TTP through targeting SIRT1

We then wanted to study the in-depth mechanism by which miR-138 target SIRT1 to affect podocytes. Although SIRT1 has been reported to have protective effects in podocyte injury during DN, it is worthwhile to explore new downstream pathways. As an important anti-inflammatory protein, Tristetraprolin (TTP) recently has been identified as another another protective factor of DN [9, 10]. Whether there is a regulatory relationship between SIRT1 and TTP and the underneath mechanism are also interesting but still unclear. To investigate the direct relationship between SIRT1 and TTP, we utilized the SIRT1 agonist SRT1720 which dose-dependently promote the expression of SIRT1 protein (**Figure 3 A**). And we found TTP is indeed upregulated after the addition of SRT1720 (**Figure 3 B-C**). Additionally, EX527, a specific inhibitor of SIRT1, was found to down-regulate TTP expression (**Figure 3 B-C**). Since we have found that miR-138 can inhibit SIRT1, it should be seen that knocking down miR-138 can up-regulate TTP, which was confirmed by the experimental results, whether *in vitro* (**Figure 3D**) or *in vivo* (**Figure 5F-G**). These results suggest that SIRT1 could regulate TTP, and miR-138 could inhibit TTP through targeting SIRT1.

MiR-138 could target SIRT1 and affect TTP through p38 pathway

Then, who is the intermediate key regulator of mir-138 that affects SIRT1 and then TTP? Previous studies have shown that SIRT1 reduce phosphorylated p38 (p-p38) expression in astrocyte [12, 13] and neural progenitors [14]. Additionally, it is known that TTP could be regulated by p38 MAPK pathway [15, 16]. Based on these researches, we hypothesized that: in DN, high glucose stimulation could up regulate miR-138, which inhibits SIRT1, leading to the p38 phosphorylation, causing the inhibition of TTP, in the end, up regulates the expression of inflammatory molecules; and this mir-138- SIRT1-p38-TTP regulatory axis plays a key role in podocyte injury (**Figure 4F**). To verify this hypothesis, we first

found that high glucose stimulation led to an up-regulation of miR-138 (**Figure 3C**) *in vitro*, and knockdown of miR-138 resulted in an up-regulation of SIRT1, a decrease in phosphorylation of p38, and an up-regulation of TTP and Nephlin expression. In addition, the expression of TNF- α and IL-18 was down-regulated, and the nephlin marker of podocyte was up-regulated after miR-138 knockdown, which alleviated the damage of podocyte (**Figure 4A-E**). Moreover, *in vivo* experiments, we found SIRT1 was decreased (**Figure 5C-D**), but p38 phosphorylation (**Figure 5C, E**) and Desmin (**Figure 5C, F**) were increased in db/db mice kidney tissues when compared to db/m mice at age 10 week which was just before the lentiviral injection. 12 weeks after lentiviral intervention, we found that knockdown of miR-138 could decrease p38 phosphorylation (**Figure 5F, I**), and increase TTP expression (**Figure 5G, H**) in db/db mice. In addition, the expression of TNF- α was down regulated (**Figure 5G, J**), and the expression of nephlin in podocytes was up regulated (**Figure 5B, G**), suggesting that the damage of podocytes was reduced. All these data indicated that miR-138-SIRT1-p38-TTP regulatory axis exist in podocyte and plays a key role in podocyte injury in DN.

Down-regulation of miR-138 showed therapeutic effects in DN mice models

According to the above experimental results, miR-138 can target and down regulate SIRT1, and miR-138 can also down regulate TTP through SIRT1-p38 pathway. Since SIRT1 and TTP are key protective factors in DN, negative intervention of miR-138 may have a certain therapeutic effect on DN. So we further tested the related indexes of DN model mice treated with miR-138 shRNA virus. It was found that down regulation of miR-138 had no significant effect on blood glucose and body weight in DN model mice (**Figure 6A-B**), but significantly reduced proteinuria and serum creatinine (**Figure 6C-D**), and PAS staining and electron microscopy results of renal tissue also showed therapeutic effect after miR-138 shRNA virus treatment (**Figure 6E**). Furthermore, immunohistochemistry showed that miR-138 knock-down suppressed podocyte injury marker Desmin but increased the expression of Podocin, which is the marker for differentiated podocytes (**Figure S3**).

Discussion

In this study, we first found that the expression of miR-138 was significantly up-regulated in DN clinical patients samples, then we verified miR-138 could bind the 3'UTR of SIRT1 and inhibit its expression in both cultured podocytes and db/db mice kidney tissues. We also revealed the important role of miR-138 -SIRT1-p38-TTP regulatory axis in podocytes injury through in vivo and in vitro experiments, and the down-regulation of miR-138 showed therapeutic effects in DN. We believe these findings may help for better understanding of DN pathology and may have some values on the treatment of this disease in the future.

DN is one of the important complications of diabetes. Its clinical manifestations include microalbuminuria and progressive renal function loss. Studies have shown that there are numerous proteins, including nephrin and podocin, that are important molecular barriers that prevent protein leakage in the kidney. The development of DN is closely related to podocyte injury [17,18]. However, podocytes are terminal differentiation cells, meaning that they cannot be regenerated after injury, which is an irreversible step in the development of DN. Our findings reveal that the regulatory axis of miR-138 -SIRT1-p38-TTP might play a key role in podocytes injury of DN. These results might have potential value to deepen the understanding of the molecular mechanism of podocyte injury in DN and to propose targeted interventions. Of course, our research is still relatively simple, more in-depth and systematic large sample research is still needed in the future.

SIRT1 regulates metabolism and modulates metabolic diseases such as diabetes, and is an important protective factor in DN [4-7] [19]. Cellular studies have shown that SIRT1 modulates fat accumulation, regulates mitochondrial biogenesis, and activates fatty acid oxidation. Mouse studies have revealed the importance of the physiological effects of SIRT1, including its effect on the metabolism of mice during caloric restriction. Transgenic mice overexpressing SIRT1 are protected from some pathological conditions including insulin resistance and glucose tolerance [20]. Identifying the key factors that regulate SIRT1 may be of great value to the understanding and treatment of the disease. MicroRNAs play important roles in controlling renal function [21], more than 16 miRNAs modulate SIRT1 expression [22], however, the important miRNAs that play an important role in DN through regulating SIRT1 is still not fully understood. Here, we show that miR-138 is up-regulated under high glucose stimulation, and it could negatively regulate

SIRT1 in podocytes and DN model mice. And further experiments indicated miR-138 could be a pretend target for DN treatment, however, new SIRT1 regulating factors still need to be identified in the future. Considering the key role of SIRT1 in many fields, such as stem cells [23], metabolism [24], cancer [25] , miR-138 may play a role in a broader area, which, of course, requires more research support.

Increased DN inflammation was associated with the downregulation of the RNA-binding protein tristetraprolin (TTP), also known as zinc finger protein 36 homolog (ZFP36). TTP has been reported to regulate the stability of multiple target mRNAs, including proinflammatory cytokines and chemokines such as TNF α , IL-6, and IL-8 [26,27]. TTP-deficient mice exhibit a severe inflammation syndrome characterized by cachexia, polyarthritis, and autoimmunity. Thus, as an important anti- inflammation, TTP not only play important roles in DN, but also play key roles in many other diseases. Identification of the factors and mechanisms regulating TTP may be valuable in a wide range of fields. Here, our *in vitro* and *in vivo* data also indicated miR-138 could target SIRT1 and affect TTP through p38 pathway. Our findings reveal that the regulatory axis of miR-138 - SIRT1-p38-TTP might play a key role in DN. These findings may be of some value for DN understanding and future treatment. Whether this regulatory axis also play roles in other diseases and bio-progress still need to be systematically investigated in the future.

Key of Definitions for Abbreviations

Abbreviation	full name
DM	Diabetes mellitus
DN	Diabetic nephropathy
SIRT1	sirtuin 1
TTP	Tristetraprolin
RIPA	Radio Immunoprecipitation Assay
PMSF	Phenylmethanesulfonyl fluoride
PAS	Periodic acid-Schiff
OCT	Optimal Cutting Temperature

ZFP36	zinc finger protein 36
MAPK	mitogenactivated protein kinase

Ethical Approval and Consent to participate

The human and animal blood, urine, kidney tissue specimen collection in this study was approved by the Ethics Committee of the The First Affiliated Hospital of Zhengzhou University(Research-2018-LW-035). Written informed consent was obtained from all patients.

Consent for publication Declarations

All authors read the final manuscript and consent for publication.

Availability of supporting data

Availability of data and supporting materials section: Please contact author for data requests. Please contact the email: nephrology003@outlook.com.

Competing interests

The authors declare no competing interests.

Funding

This study was supported by a grant from National Key Research and Development Program (No.2016YFC1305404), Henan Joint Funds of the National Natural Science

Foundation of China (No. U1604284), and the National Natural Science Foundation of China (No.81670663).

Authors' contributions

Designed by Zhangsuo Liu, Dongwei Liu, and Jia Guo; written by Dongwei Liu and Fengxun Liu, and Yingjin Qiao; directed by Zhangsuo Liu, Dongwei Liu, and Jia Guo; result collation by Yingjin Qiao, and Jiayu Duan; Medical statistics by Jiayu Duan; figures were processed by Fengxun Liu. The experiments were performed by Fengxun Liu, Shaokang Pan.

All authors read and approved the final manuscript.

Acknowledgements

All authors read and approved the final manuscript.

Authors' information

Fengxun Liu is Chronic Diabetes Major. Working in Research Institute of Nephrology, Zhengzhou University. **E-mail:**nephrology003@outlook.com

Jia Guo is good at treating Prevention of Chronic Nephropathy research. Working in Core Unit of National Clinical Medical Research Center of Kidney Disease. **E-mail:**zzuzhangyu@foxmail.com

Yingjin Qiao is good at Medical statistics research. Being employed in Department of Nephrology, the First Affiliated Hospital of Zhengzhou University. **E-mail:**nagato@csu.edu.cn

Shaokang Pan is Chronic Diabetes Major. Working in Key Laboratory of Precision Diagnosis and Treatment for Chronic Kidney Disease in Henan Province. **E-mail:**glomerulus001@outlook.com

Jiayu Duan is good at Medical statistics research. Working in Key Laboratory of Precision Diagnosis and Treatment for Chronic Kidney Disease in Henan Province. **E-mail:**Lu1994March@163.com

Dongwei Liu is good at treating prevention and treatment of chronic kidney disease and early diagnosis research. Working in Department of Nephrology, the First Affiliated Hospital of Zhengzhou University. **E-mail:**liu-dongwei@zzu.edu.cn

Zhangsuo Liu is good at treating prevention and treatment of chronic kidney disease and early diagnosis research. Being employed in Department of Nephrology, the First Affiliated Hospital of Zhengzhou University. **E-mail:**zhangsuoliu@zzu.edu.cn

FIGURES AND FIGURE LEGENDS

Figure 1. MiR-138 is highly expressed in DN patients and associated with podocyte injury. (A-C) Expression levels of miR-138(A), Desmin (B) and IL-18(C) in the four groups of clinical blood samples tested by RT-PCR. Control, n=40; DM, n=18; DN1, n=13; DN2, n=17. U6 or GAPDH were used as endogenic control. *: P< 0.05. **(D)** Paraffin-embedded kidney specimens stained by Masson's stain to observe glomerular fibrosis. Tissue section thickness 3 μ m. The degree of fibrosis in the DN group was higher than in the DM and control groups (scale bar, 30 μ m). Con: control; DM: diabetes mellitus; DN1: diabetic nephropathy with microalbuminuria; DN2: diabetic nephropathy with macroalbuminuria. *: P< 0.05.

Figure 2. miR-138 can down-regulate SIRT1 and might promote podocytes injury in DN. (A) The expression levels of SIRT1 in cultured mice podocytes after miR-138 mimics or shRNA introduction tested by Western-blot. **(B)** The expression levels of SIRT1, Desmin in cultured mice podocytes in HG or NG condition after miR-138 mimics transfection, tested by Western-blot. HG: high glucose; NG: normal glucose. **(C)** The expression levels of SIRT1 in cultured mice podocytes after miR-138 mimics or inhibitor introduction tested by Western-blot. **(D)** Schematic diagram of the plasmid vectors of SIRT1-3'UTR-reporter and SIRT1-3'UTR-muta-reporter. The SIRT1-3'UTR was cloned at the downstream of luciferase element in SIRT1-3'UTR-reporter. SIRT1-3'UTR-muta-reporter lost the predicted miR-138 binding site. **(E)** Results of the dual-luciferase reporter

assay using the plasmid vectors of SIRT1-3'UTR –reportor SIRT1-3'UTR-muta-reportor and miR-138 mimics. *: P< 0.05. **(F)** Immunofluorescence results of the expression and position of SIRT1 and synaptopodin(SYNPO) in glomeruli of mice models. (scale bar, 20µm). LV: lentivirus expressing miR-138 shRNA, which knock down miR-138; w: week; db/db : diabetic db/db mice (BKS.Cg-Dock7^m +/+ Lepr^{db}/J mice), which develop spontaneous hyperglycemia at 5–6 weeks of age followed by proteinuria and renal impairment at 8–10 weeks of age; db/m: nondiabetic mice, n=20 in each group. The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

Figure 3. MiR-138 could inhibit TTP by targeting SIRT1.(A) Western-blot result of the protein levels of SIRT1 in cultured mice podocytes after treatment of SRT1720 in different concentration. SRT1720 is a specific SIRT1 agonist. **(B)** Western-blot result of the protein levels of TTP and Podocin in cultured mice podocytes after treatment of SRT1720 and EX527. EX527 is a specific inhibitor of SIRT1.**(C)**RT-PCR results of RNA expression levels in cultured mice podocytes after different treatment.U6 or GAPDH were used as endogenic control.**(D)** Immunofluorescence results SIRT1and TTP in cultured mice podocytes after different treatment.LV: lentivirus expressing miR-138 shRNA, which knock down miR-138. The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

Figure 4. MiR-138 could inhibit TTP by targeting SIRT1 *in vitro*. (A) Western-blot result of the protein levels in cultured mice podocytes after treatment of miR-138 shRNA. **(B-E)** Grayscale analysis results of the Western-blot results in (A). *: P< 0.05. **(F)** Schematic display of mir-138- SIRT1-p38-TTP regulatory axis. in DN, high glucose stimulation could up regulate miR-138, which inhibits SIRT1, SIRT1 inhibits the p38 phosphorylation, phosphorylated p38 inhibits TTP. Both SIRT1 and TTP are protective factor that reduce podocytes injury in DN. The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

Figure 5. MiR-138 could inhibit TTP by targeting SIRT1 *in vivo*. (A) Schematic diagram of mir-138 treatment in diabetic db/db mice. Db/db mice were divided into three groups: db/db mice, vehicle shRNA db/db mice, and db/db+LV mice (n = 20 in each group). MiR-138 downregulated lentivirus (LV) was injected into the tail vein of db/db mice at 10w and 14w. **(B)** Western-blot results of the protein levels in db/m mice (n=20,

non diabetic) or db/db mice treated with MiR-138 downregulated lentivirus (LV) as shown in (A). (C) Western-blot results of the protein levels in db/m mice or db/db mice before treated with MiR-138 downregulated lentivirus (at 10w). (D-F) Grayscale analysis results of the Western-blot results in (C). (G) Western-blot result of the protein levels in db/m mice or db/db mice after treatment of miR-138 downregulated lentivirus (at 22w). (H-J) Grayscale analysis results of the Western-blot results in (F). The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

Figure 6. Downregulation of miR-138 showed some therapeutic effects in DN mice models. (A-D) Blood glucose levels(A) , Changes in body weight(B), urinary albumin excretion rate (C) and serum creatinine (D) of mice in 4 groups. The mice were grouped and treated as in Figure 5. *: P< 0.05. (E) PAS staining showed that the mesangial matrix and glomerular basement membrane of the db/db group and vehicle group had thickened and that tubular and interstitial fibrosis was present, unlike in the db/m and db/db+LV groups at the same age. After 12 weeks of treatment with the miR-138 shRNA, the globules and tubules had significantly improved (scale bar, 50 μ m). Electron microscopy showed that the fusion of the foot process was significantly improved in the lentiviral group (scale bar, 5 μ m). *: P< 0.05.

Reference

1. Forbes, J.M. and M.E. Cooper, *Mechanisms of diabetic complications*. *Physiol Rev*, 2013. **93**(1): p. 137-88.
2. Heerspink, H.J.L., et al., *Atrasentan and renal events in patients with type 2 diabetes and chronic kidney disease (SONAR): a double-blind, randomised, placebo-controlled trial*. *Lancet*, 2019. **393**(10184): p. 1937-1947.
3. Rask-Madsen, C. and G.L. King, *Kidney complications: factors that protect the diabetic vasculature*. *Nat Med*, 2010. **16**(1): p. 40-1.
4. Canto, C., et al., *AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity*. *Nature*, 2009. **458**(7241): p. 1056-60.
5. Wang, W., et al., *Management of diabetic nephropathy: the role of sirtuin-1*. *Future Med Chem*, 2019. **11**(17): p. 2241-2245.

6. Liu, H.W., H.H. Kao, and C.H. Wu, *Exercise training upregulates SIRT1 to attenuate inflammation and metabolic dysfunction in kidney and liver of diabetic db/db mice*. Nutr Metab (Lond), 2019. **16**: p. 22.
7. Wang, W., et al., *Role of sirtuin-1 in diabetic nephropathy*. J Mol Med (Berl), 2019. **97**(3): p. 291-309.
8. Liu, C.M., et al., *MicroRNA-138 and SIRT1 form a mutual negative feedback loop to regulate mammalian axon regeneration*. Genes Dev, 2013. **27**(13): p. 1473-83.
9. Guo, J., et al., *MiRNA-29c regulates the expression of inflammatory cytokines in diabetic nephropathy by targeting tristetraprolin*. Sci Rep, 2017. **7**(1): p. 2314.
10. Liu, F., et al., *The Expression of Tristetraprolin and Its Relationship with Urinary Proteins in Patients with Diabetic Nephropathy*. Plos One, 2015. **10**(10): p. e0141471.
11. Patial, S. and P.J. Blackshear, *Tristetraprolin as a Therapeutic Target in Inflammatory Disease*. Trends Pharmacol Sci, 2016. **37**(10): p. 811-821.
12. Greenhill, C., *Non-coding RNA: Exosomal microRNAs as novel adipokines*. Nat Rev Genet, 2017. **18**(4): p. 212.
13. Li, D., et al., *Interactions between Sirt1 and MAPKs regulate astrocyte activation induced by brain injury in vitro and in vivo*. J Neuroinflammation, 2017. **14**(1): p. 67.
14. Rafalski, V.A., et al., *Expansion of oligodendrocyte progenitor cells following SIRT1 inactivation in the adult brain*. Nat Cell Biol, 2013. **15**(6): p. 614-24.
15. Tiedje, C., et al., *The RNA-binding protein TTP is a global post-transcriptional regulator of feedback control in inflammation*. Nucleic Acids Res, 2016. **44**(15): p. 7418-40.
16. Thapar, R. and A.P. Denmon, *Signaling pathways that control mRNA turnover*. Cell Signal, 2013. **25**(8): p. 1699-710.
17. Koshikawa, M., et al., *Role of p38 mitogen-activated protein kinase activation in podocyte injury and proteinuria in experimental nephrotic syndrome*. Journal of the American Society of Nephrology Jasn, 2005. **16**(9): p. 2690.
18. Zhou, S., et al., *Genetic and Pharmacologic Targeting of Glycogen Synthase Kinase 3 β Reinforces the Nrf2 Antioxidant Defense against Podocytopathy*. Journal of the American Society of Nephrology Jasn, 2015. **27**(8): p. 2289.

19. Lagouge, M., et al., *Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha*. *Cell*, 2006. **127**(6): p. 1109-1122.
20. Cantó, C., et al., *AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity*. *Nature*, 2009. **458**(7241): p. 1056-1060.
21. Putta, S., et al., *Inhibiting MicroRNA-192 Ameliorates Renal Fibrosis in Diabetic Nephropathy*. *Journal of the American Society of Nephrology Jasn*, 2012. **23**(3): p. 458-69.
22. Mitomo, S., et al., *Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines*. *Cancer Science*, 2008. **99**(2): p. 280–286.
23. Igarashi, M. and L. Guarente, *mTORC1 and SIRT1 Cooperate to Foster Expansion of Gut Adult Stem Cells during Calorie Restriction*. *Cell*, 2016. **166**(2): p. 436-450.
24. Arumugam, T.V. and B.K. Kennedy, *H2S to Mitigate Vascular Aging: A SIRT1 Connection*. *Cell*, 2018. **173**(1): p. 8-10.
25. Brooks, C.L. and W. Gu, *How does SIRT1 affect metabolism, senescence and cancer?* *Nat Rev Cancer*, 2009. **9**(2): p. 123-8.
26. Ray, D., et al., *Tristetraprolin Mediates Radiation-Induced TNF- α Production in Lung Macrophages*. *Plos One*, 2013. **8**(2): p. e57290.
27. Shi, J.X., et al., *MK2 posttranscriptionally regulates TNF- α -induced expression of ICAM-1 and IL-8 via tristetraprolin in human pulmonary microvascular endothelial cells*. *American Journal of Physiology Lung Cellular & Molecular Physiology*, 2012. **302**(8): p. L793.

Figures

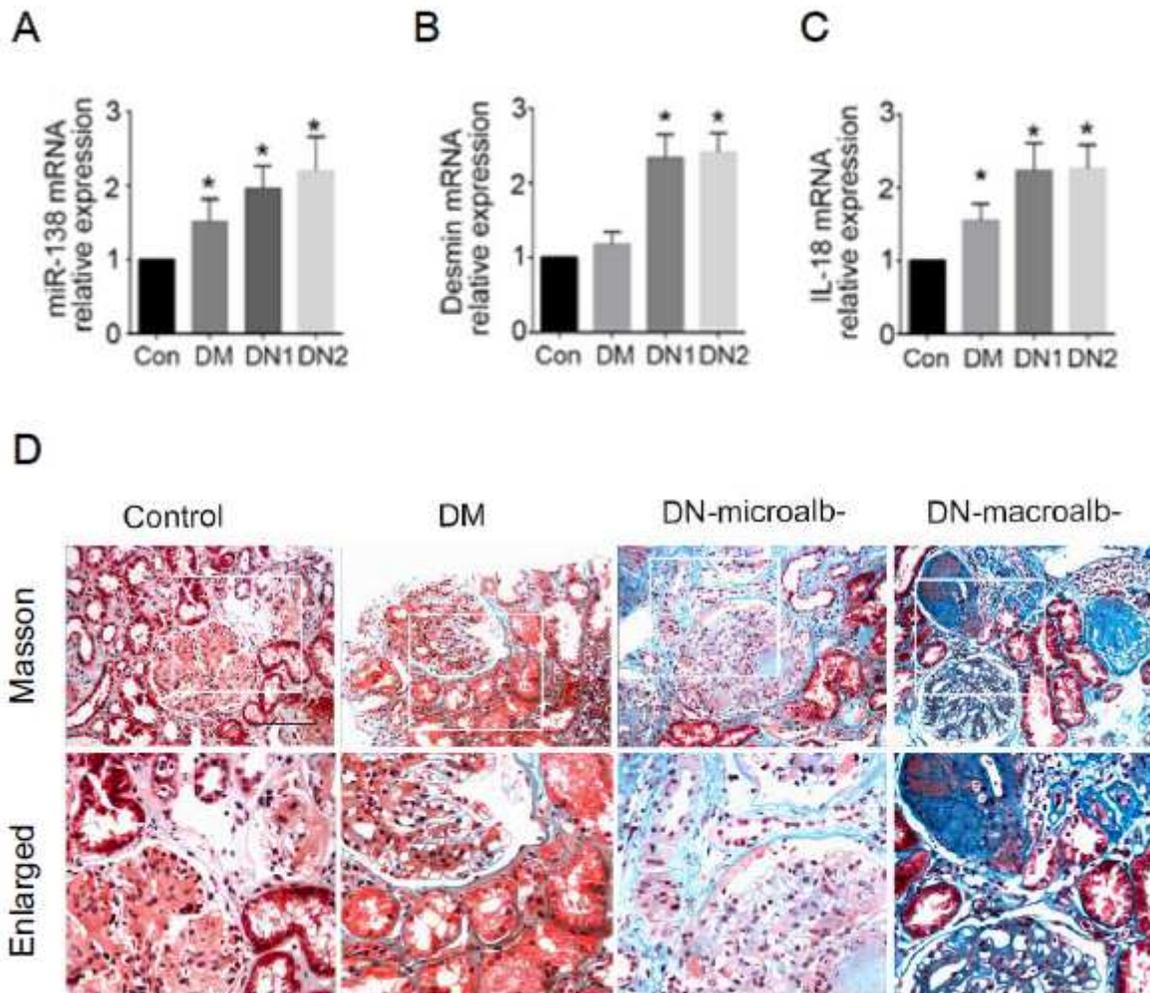


Figure 2

MiR-138 is highly expressed in DN patients and associated with podocyte injury. (A-C) Expression levels of miR-138(A), Desmin (B) and IL-18(C) in the four groups of clinical blood samples tested by RT-PCR. Control, n=40; DM, n=18; DN1, n=13; DN2, n=17. U6 or GAPDH were used as endogenic control. *: $P < 0.05$. (D) Paraffin- embedded kidney specimens stained by Masson's stain to observe glomerular fibrosis. Tissue section thickness 3 μm . The degree of fibrosis in the DN group was higher than in the DM and control groups (scale bar, 30 μm). Con: control; DM: diabetes mellitus; DN1: diabetic nephropathy with microalbuminuria; DN2: diabetic nephropathy with macroalbuminuria. *: $P < 0.05$.

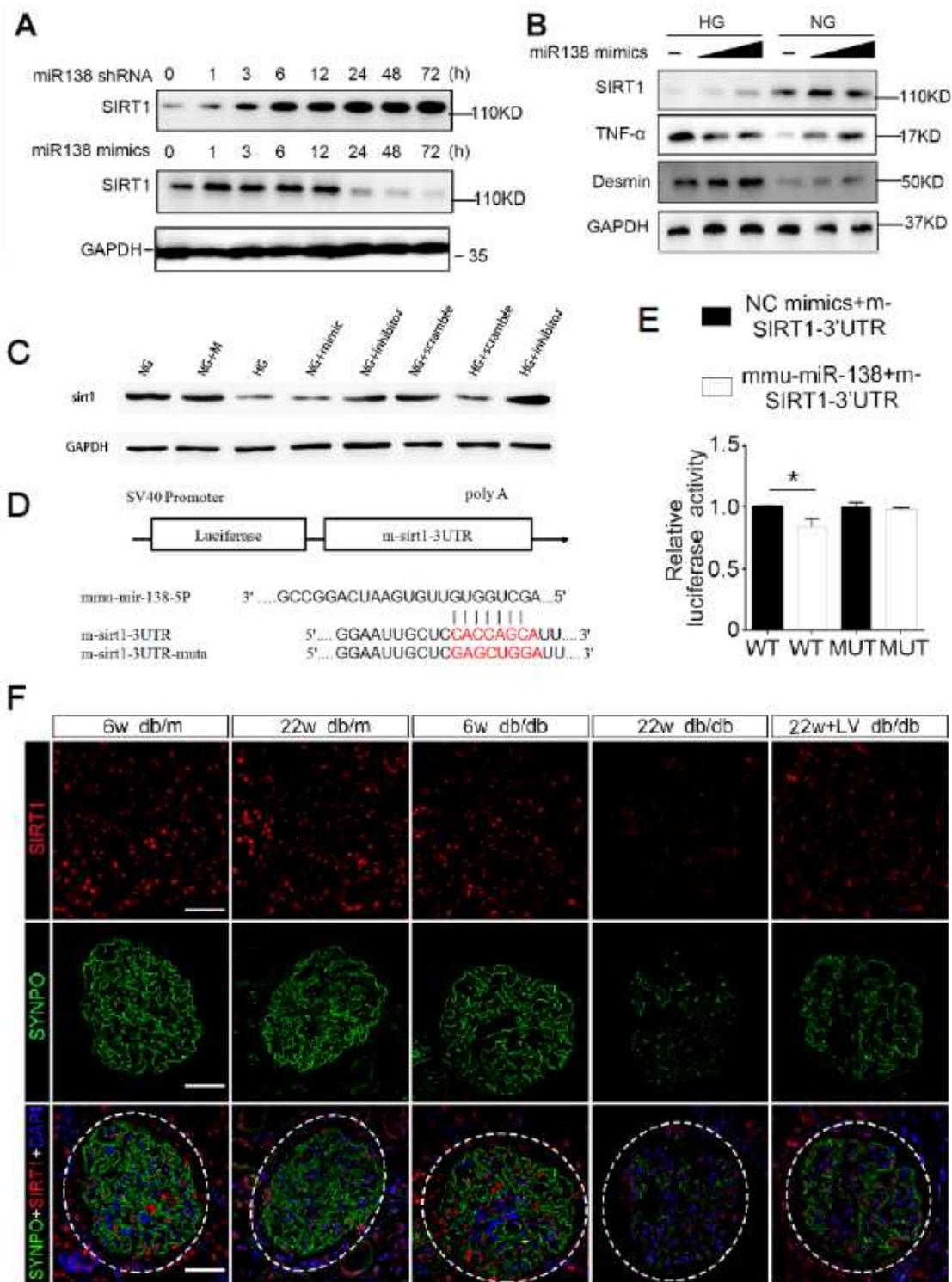


Figure 4

miR-138 can down-regulate SIRT1 and might promote podocytes injury in DN. (A) The expression levels of SIRT1 in cultured mice podocytes after miR-138 mimics or shRNA introduction tested by Western-blot. (B) The expression levels of SIRT1, Desmin in cultured mice podocytes in HG or NG condition after miR-138 mimics transfection, tested by Western-blot. HG: high glucose; NG: normal glucose. (C) The expression levels of SIRT1 in cultured mice podocytes after miR-138 mimics or inhibitor introduction tested by

Western-blot. (D) Schematic diagram of the plasmid vectors of SIRT1-3'UTR -reporter and SIRT1-3'UTR-muta-reporter. The SIRT1-3'UTR was cloned at the downstream of luciferase element in SIRT1-3'UTR -reporter. SIRT1-3'UTR-muta-reporter lost the predicted miR-138 binding site.(E) Results of the dual-luciferase reporter assay using the plasmid vectors of SIRT1-3'UTR -reporter SIRT1-3'UTR-muta-reporter and miR-138 mimics. *: $P < 0.05$. (F) Immunofluorescence results of the expression and position of SIRT1 and synaptopodin(SYNPO) in glomeruli of mice models. (scale bar, $20\mu\text{m}$). LV: lentivirus expressing miR-138 shRNA, which knock down miR-138; w: week; db/db : diabetic db/db mice (BKS.Cg-Dock7m +/+ Leprdb/J mice), which develop spontaneous hyperglycemia at 5–6 weeks of age followed by proteinuria and renal impairment at 8–10 weeks of age; db/m: nondiabetic mice, n=20 in each group. The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

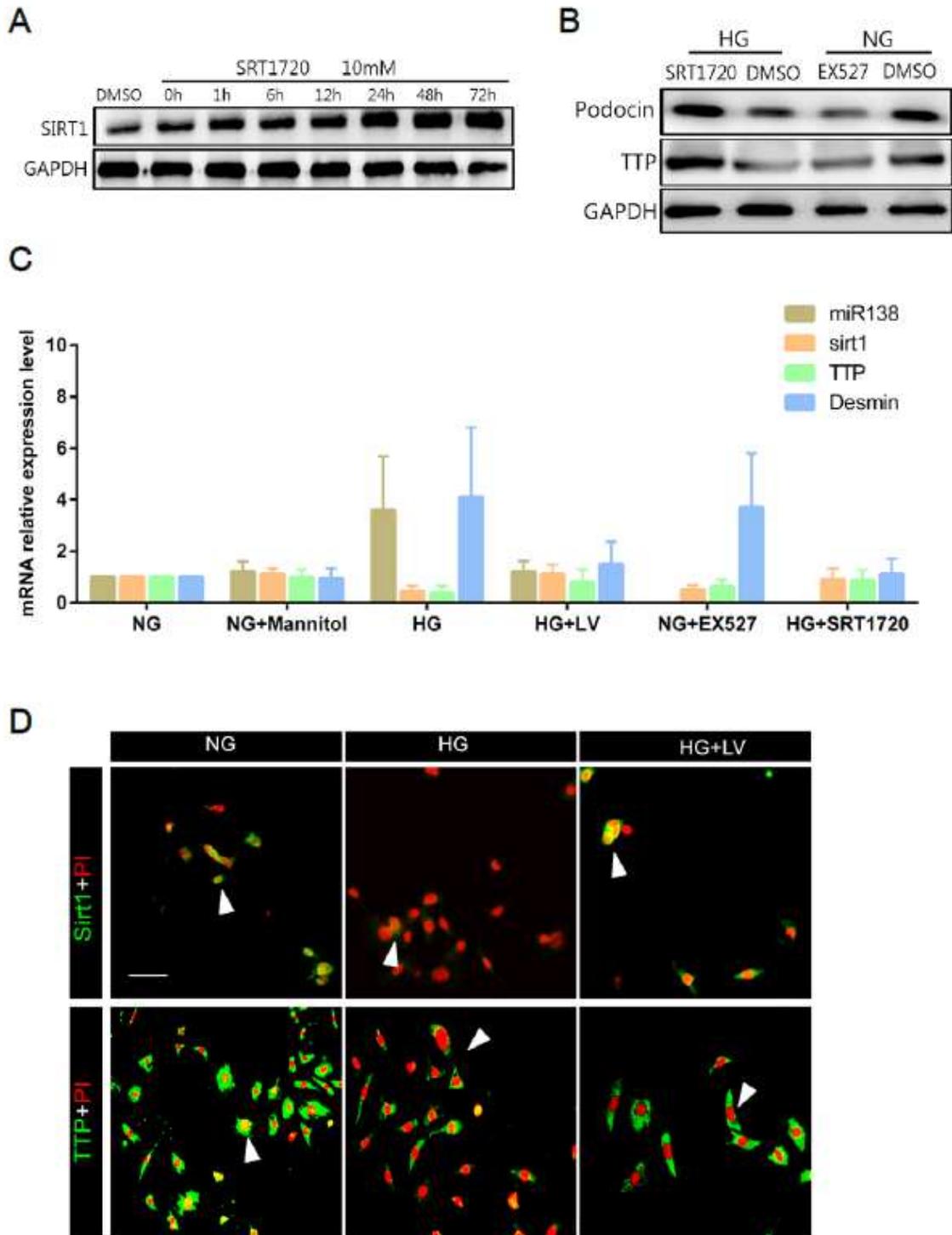


Figure 6

MiR-138 could inhibit TTP by targeting SIRT1. (A) Western-blot result of the protein levels of SIRT1 in cultured mice podocytes after treatment of SRT1720 in different concentration. SRT1720 is a specific SIRT1 agonist. (B) Western-blot result of the protein levels of TTP and Podocin in cultured mice podocytes after treatment of SRT1720 and EX527. EX527 is a specific inhibitor of SIRT1. (C) RT-PCR results of RNA expression levels in cultured mice podocytes after different treatment. U6 or GAPDH were

used as endogenic control.(D) Immunofluorescence results SIRT1 and TTP in cultured mice podocytes after different treatment.LV: lentivirus expressing miR-138 shRNA, which knock down miR-138. The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

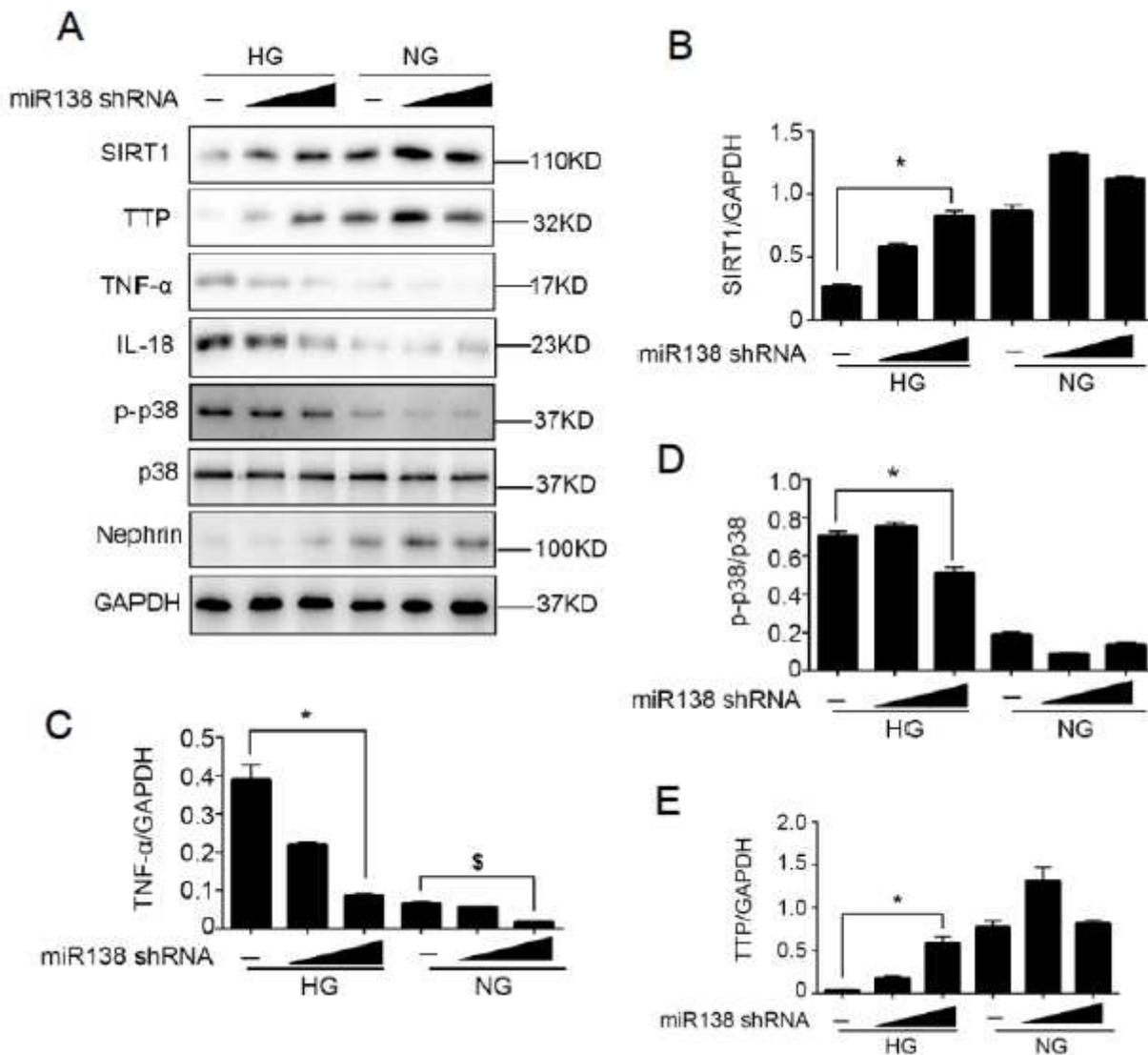


Figure 8

MiR-138 could inhibit TTP by targeting SIRT1 in vitro. (A) Western-blot result of the protein levels in cultured mice podocytes after treatment of miR-138 shRNA. (B-E) Grayscale analysis results of the Western-blot results in (A). *: $P < 0.05$. (F) Schematic display of mir-138- SIRT1-p38-TTP regulatory axis. in DN, high glucose stimulation could up regulate miR-138, which inhibits SIRT1, SIRT1 inhibits the p38 phosphorylation, phosphorylated p38 inhibits TTP. Both SIRT1 and TTP are protective factor that reduce podocytes injury in DN. The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

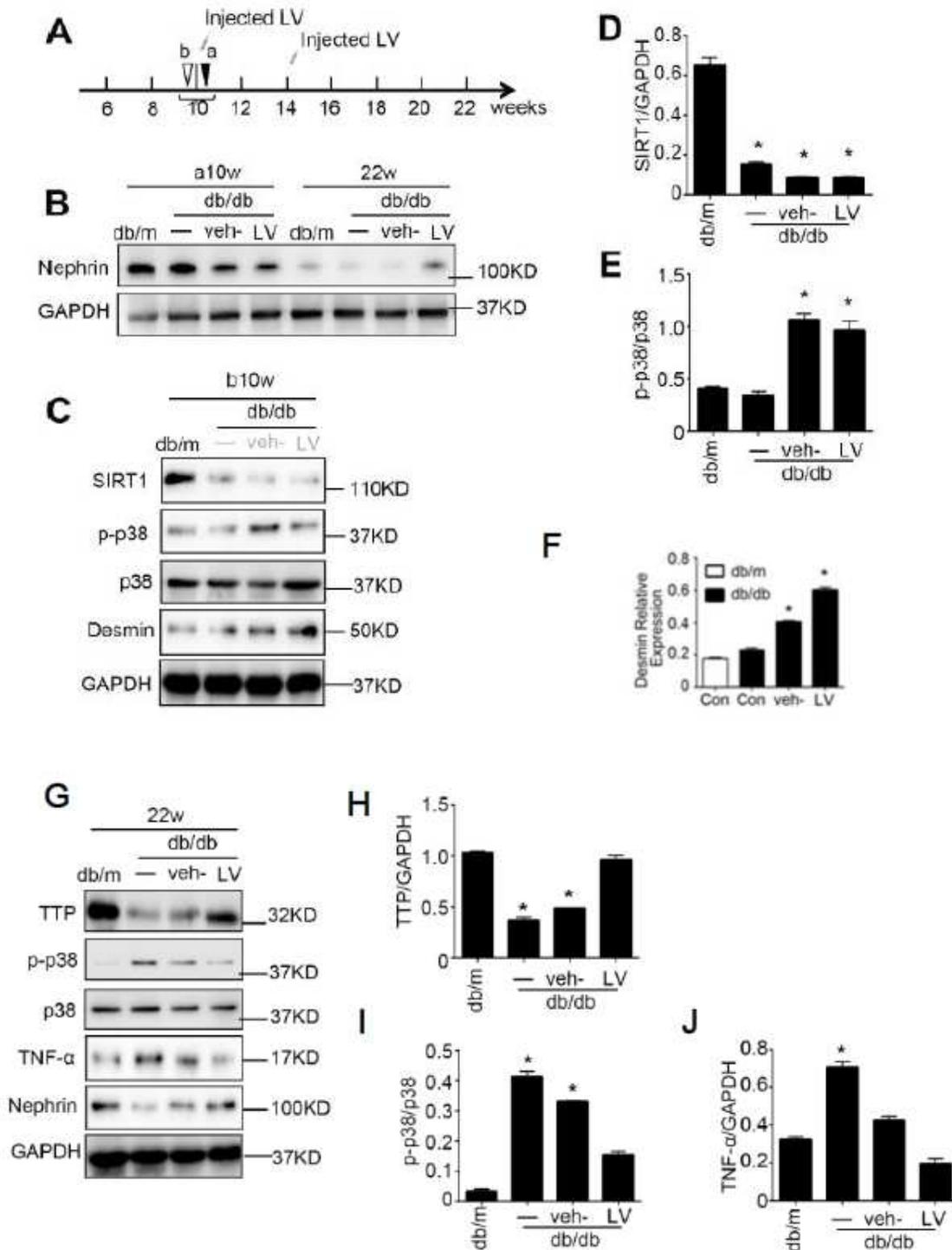


Figure 10

MiR-138 could inhibit TTP by targeting SIRT1 in vivo. (A) Schematic diagram of miR-138 treatment in diabetic db/db mice. Db/db mice were divided into three groups: db/db mice, vehicle shRNA db/db mice, and db/db+LV mice (n = 20 in each group). MiR-138 downregulated lentivirus (LV) was injected into the tail vein of db/db mice at 10w and 14w. (B) Western-blot results of the protein levels in db/m mice (n=20, non diabetic) or db/db mice treated with MiR-138 downregulated lentivirus (LV) as shown in (A). (C)

Western-blot results of the protein levels in db/m mice or db/db mice before treated with MiR-138 downregulated lentivirus (at 10w). (D-F) Grayscale analysis results of the Western-blot results in (C). (G) Western-blot result of the protein levels in db/m mice or db/db mice after treatment of miR-138 downregulated lentivirus (at 22w). (H-J) Grayscale analysis results of the Western-blot results in (F). The grouping blots in Western-blot results were cropped from different parts of the same gel or from different gels.

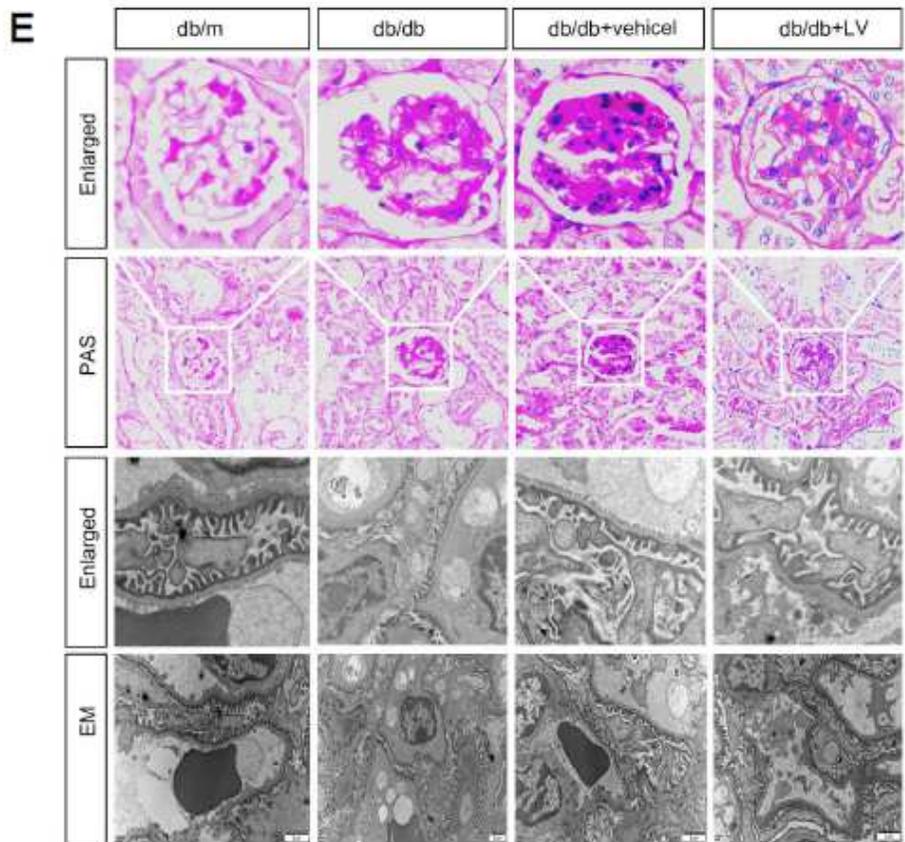
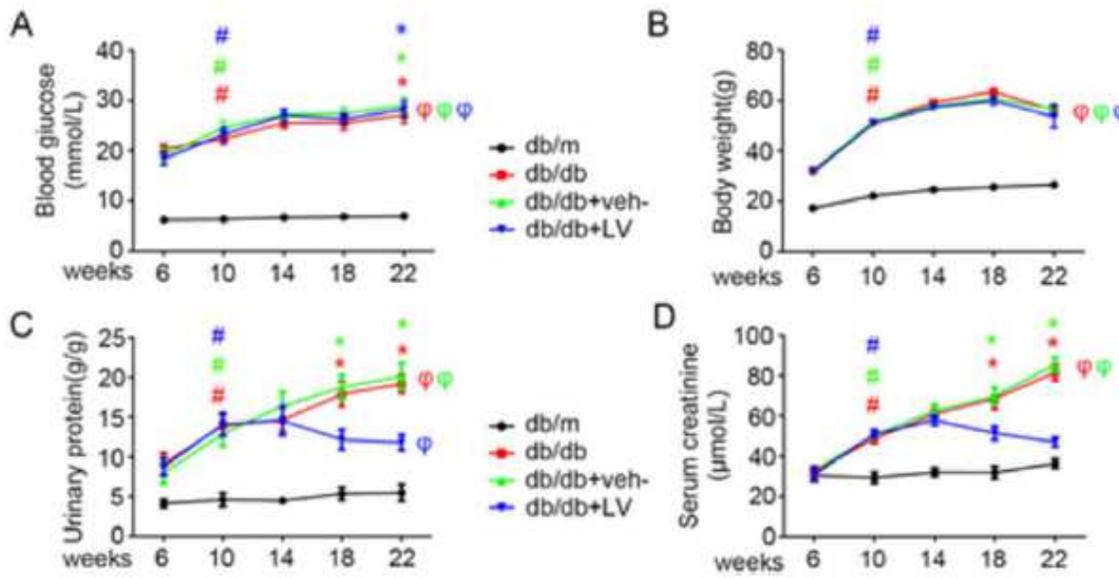


Figure 12

Downregulation of miR-138 showed some therapeutic effects in DN mice models. (A-D) Blood glucose levels(A) , Changes in body weight(B), urinary albumin excretion rate (C) and serum creatinine (D) of mice in 4 groups. The mice were grouped and treated as in Figure 5. *: P< 0.05. (E) PAS staining showed that the mesangial matrix and glomerular basement membrane of the db/db group and vehicle group had thickened and that tubular and interstitial fibrosis was present, unlike in the db/m and db/db+LV groups at the same age. After 12 weeks of treatment with the miR-138 shRNA, the globules and tubules had significantly improved (scale bar, 50 μ m). Electron microscopy showed that the fusion of the foot process was significantly improved in the lentiviral group (scale bar, 5 μ m). *: P< 0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ethicsapprovalform.pdf](#)
- [ethicsapprovalform.pdf](#)
- [supplementarymaterials.pdf](#)
- [supplementarymaterials.pdf](#)