Dear editors and reviewers,

First of all, I am glad to receive valuable comments from reviewers. The following are the answers to the questions raised by reviewers.

Reviewer 1:

Question: the most western blot results presented in the manuscript (Figures 1b, 1c, 2a, and 3a) exhibit over heavy background signals, which could potentially compromise the accuracy of the quantitative analysis performed using ImageJ. This issue may be attributable to the quality of the antibodies used, and it is recommended that the authors consider optimizing antibody selection or adjusting experimental conditions to reduce background noise and enhance the reliability of the data. Additionally, the methods section, particularly the description of the serum biochemistry analysis, is overly simplistic. For example, The authors state, "Mouse serum was sent to Wuhan Service Biotech Co., Ltd. for measurement of creatinine and blood urea nitrogen." This description lacks detail and does not provide sufficient information on the methodologies used for these critical measurements. It would be beneficial for the authors to elaborate on the specific techniques employed by the external laboratory, including any relevant details such as the type of assays performed.

Answer:

1.The background signal of the manuscript has been readjusted, and figures 1B, 1C, 2A and 3A have been modified. The adjusted results are as follows:

Figure 1

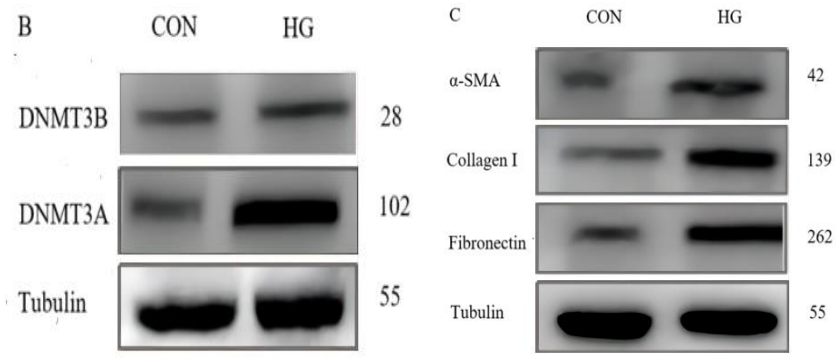


Figure 2

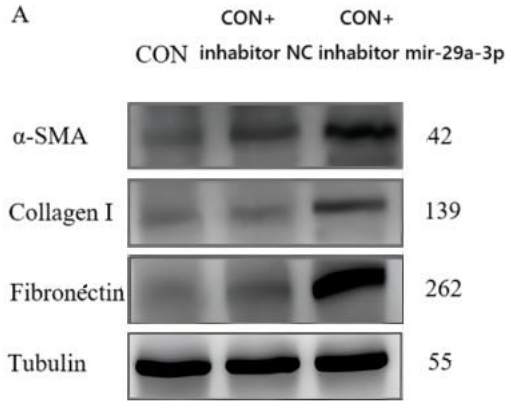
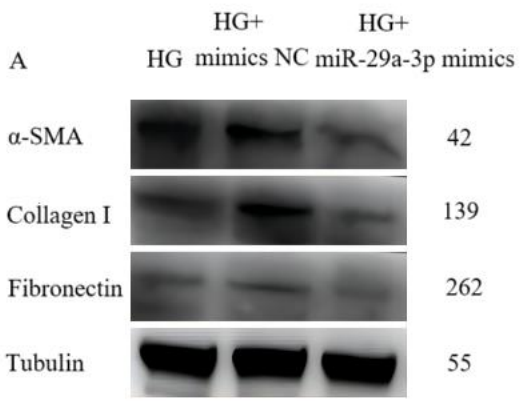


Figure 3

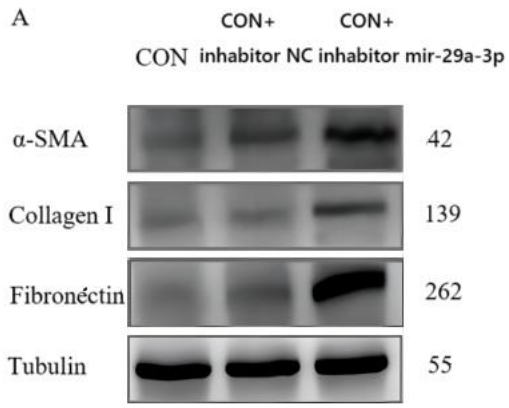


2.The simplistic description of serum biochemical analysis has been modified as follows:
Serum biochemistry analysis: Mouse Blood was placed in an evacuated heparinized tube on ice for 30 min. The serum in the supernatant was collected through centrifugation at 4000 r/min for 10 min and stored in a refrigerator at -80°C for further detection. The final samples were sent to Wuhan Service Biotech Co., Ltd. and the corresponding operations were carried out in strict accordance with the operating rules. The serum indexes creatinine and blood urea nitrogen were detected by using the detection data of automatic biochemical analyzer (Johnson VITROS5600).

Reviewer 2:
Question: There were still some typographic, grammar, and format errors observed in this manuscript. For example, the format in Indentation of subheadings, western bolt, RT-qPCR and collagen type I were not uniform. Authors have to check and revise these errors carefully. Why did the authors use two internal parameters in their experiments? Some studies have shown that in some cells, due to tissue hypoxia, diabetes and other factors will lead to increased expression of GAPDH, not suitable for internal reference. and the molecular weight is incorrectly labeled in the Figure2. In terms of the statistics of the data we were only told that unpaired t-tests were used but there was no indication of how the chi-square was verified. Sample size of mice not indicated in the methodology

Answer:
1. It has been revised to solve the problem of inconsistent indentation in the article

2.The experiment has been re-used with Tubulin as internal reference, and the molecular weight in the figure has been modified, as follows:



3.The statistical methods used have been elaborated in detail in the paper,as follows:
Statistical analysis: Data analysis was performed using GraphPad Prism8 software. All data were expressed as mean ± standard deviation. Inter-group comparisons were performed using the unpaired t-test, correlation analysis using chi-square test. Statistical significance was indicated as ap < 0.05, bp < 0.01, and cp < 0.001.

4.Twenty 6-week-old male mice were purchased from SPF Biotechnology Co., Ltd, Beijing.

JOURNAL EDITORIAL BOARD'S REVIEW REPORT

1. Please send the revised manuscript to the previous reviewers: (1) either to both reviewers (Reviewer's codes: 08188182; 02536281) who reviewed the original manuscript or only to the first reviewer (Reviewer's codes: 08188182) since this reviewer indicated willing to "Re-review" the revised manuscript. After the previous reviewers provided the comments, please send the revised manuscript and comments from reviewers to me. Thanks! 2. The revised manuscript was not well prepared since there was not enough information for (1) the materials and methods, and (2) Result description even the author provided certain amount of experimental data. 3. The reviewers have asked the author to provide animal information, the authors only mentioned in the authors' response, which was not provided in the revised manuscript's section of materials and methods.

Because we didn't carefully confirm the contents of the manuscript in the early stage, the materials, methods and results were too simplified. Now we will describe the two parts in detail:

MATERIALS AND METHODS

Materials

Mouse glomerular mesangial cells (SV40 MES 13) were purchased from Wuhan Procell Life Science & Technology Co., Ltd, while RNAiso Plus and reverse transcription reagents were obtained from Chengdu Weike Biotechnology Co., Ltd. Fluorescence quantitative PCR reagents, DNA extraction kits, fast bisulfite conversion kits, and methylation analysis kits were purchased from QIAGEN Taiwan Co., Ltd. MiR-29a-3p mimics, mimics NC, inhibitor-miR-29a-3p, inhibitor NC, agomiR-29a-3p, and agomir NC were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Refer to Table 1 for detailed information on the antibodies used in the study.

Methods

Cell culture and transfection: SV40 MES 13 cells were cultured in HyClone medium containing 5% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamine. The cells were seeded in culture plates and incubated in either 5.5 mmol/L (normal glucose group) or 30 mmol/L (high glucose group) glucose medium. In the normal glucose group, the cells were transfected with inhibitor-miR-29a or inhibitor NC (100 pmol/L) and then divided into CON, CON+inhibitor NC, and CON+inhibitor-miR-29a-3p groups.

In the high glucose group, the cells were transfected with miR-29a-3p mimics or mimics NC (50 pmol/L) and divided into HG, HG+mimics NC, and HG+miR-29a-3p mimics groups.

Experimental animals and specific grouping: Twenty 6-week-old male mice were purchased from SPF Biotechnology Co., Ltd, Beijing. The animals were housed under SPF conditions with free access to food and water. The experimental groups included the db/m group, db/db (CON) group, db/db (antagomir NC) group, and db/db (antagomiR-29a-3p) (CON, antagomir NC, and antagomiR-29a-3p) group. These mice received a dosage of 80 mg/kg and one injection per week for three consecutive weeks. The mice were weighed weekly. After four weeks, the mice were euthanized by cervical dislocation and blood collected by removing the eyeballs. Kidney tissues were then isolated and divided into two halves, with one half stored in liquid nitrogen in a freezing tube. Animal experiments were conducted in accordance with the eighth edition of the "Guidelines for the Care and Use of Experimental Animals" (2011). All procedures are carried out in accordance with the relevant laws and institutional guidelines and have been approved by the appropriate institutional committee: LL-202125.

Quantitative polymerase chain reaction (RT-qPCR): Total RNA was extracted from the transfected cells 24h post-transfection, using the stem-loop method for reverse transcription. U6 was used as an internal reference, with RT-qPCR performed using a fluorescent quantitative PCR kit. Refer to Table 2 for the primer sequences used in the study.

Western blotting: Total protein was extracted from mouse cells and kidney tissues using the T-PER method. Protein concentration was measured using the NanoDrop method. The proteins were then separated by SDS-PAGE electrophoresis and transferred onto a PVDF membrane by electro transfer. After blocking with 5% BSA, the membrane was incubated overnight at 4°C with primary antibodies (dilution ratio of 1:1000). After washing with TBST, the corresponding HRP-labeled secondary antibodies were added

and incubated for 30 min, followed by exposure, development, and fixation. The grayscale values of the bands were analyzed using ImageJ-win64 software.

Serum biochemistry analysis: Mouse Blood was placed in an evacuated heparinized tube on ice for 30 min. The serum in the supernatant was collected through centrifugation at 4000 r/min for 10 min and stored in a refrigerator at -80°C for further detection. The final samples were sent to Wuhan Service Biotech Co., Ltd. and the corresponding operations were carried out in strict accordance with the operating rules. The serum indexes creatinine and blood urea nitrogen were detected by using the detection data of automatic biochemical analyzer (Johnson VITROS5600).

Immunofluorescence analysis: The cells were treated with 100 μl of permeabilization buffer, incubated for 20 min, followed by the addition of 5% bovine serum albumin and further incubation for 60 min. The cells were then incubated overnight with primary antibody (dilution 1:200) in a humidification chamber at 4℃. After washing with PBS, the appropriate fluorescent secondary antibody was added and incubated for 60 min. The nuclei were stained with Hoechst 33258 dye. After washing with PBS, the liquid was aspirated and the samples mounted using an anti-fading mounting medium. The samples were then examined under a microscope and the images captured using a fluorescence microscope (Olympus BX51).

Hematoxylin and eosin (HE) staining: The fixed kidney tissue was embedded in paraffin and sliced using a microtome. The sections were then deparaffinized and rehydrated, followed by staining with hematoxylin for 3-5 min and eosin for 5 min. The samples were dehydrated and mounted, and then observed and imaged under a microscope.

Periodic acid-schiff（PAS）staining: The tissue sections were deparaffinized and rehydrated, then stained with PAS staining solution B for 10-15 min, followed by staining with PAS staining solution A for 25-30 min, and then with PAS staining solution C for 30

s. After bluing with ammonia water, the samples were dehydrated, mounted, and then observed and imaged under a microscope.

Immunohistochemistry: After deparaffinization and rehydration, the tissue sections were subjected to antigen retrieval by placing them in citrate buffer (pH 6.0) and heating in a microwave oven (medium heat for 8 min until boiling, then turned off for 8 min, followed by 7 min at low-medium heat, and naturally cooled to room temperature). After washing with PBS, endogenous peroxidase was blocked by adding 3% hydrogen peroxide solution, followed by the addition of 5% BSA and incubation at room temperature for 30 min. The primary antibody was then added at an appropriate dilution and incubated overnight at 4°C in a humidification chamber. After washing with PBS, the corresponding HRP-conjugated secondary antibody solution was added and incubated for 30 min. After a further wash with PBS, DAB staining was performed and the nuclei stained with hematoxylin. After bluing with ammonia water, the samples were dehydrated, mounted, and then observed and imaged under a microscope. ImageJ-win64 software was used for image quantification analysis.

Statistical analysis: Data analysis was performed using GraphPad Prism8 software. All data were expressed as mean ± standard deviation. Inter-group comparisons were performed using the unpaired t-test, correlation analysis using chi-square test. Statistical significance was indicated as $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$.

RESULTS

The expression of miR-29a-3p was down-regulated, while the expression of DNMT3A/3B and fibrosis-related molecules were up-regulated after high glucose induction: The expression level of miRNA has recently become a potential biomarker of various pathological conditions, while DNMT3A/3B and fibrosis-related molecules have become the key molecules in the occurrence and development of DKD fibrosis. In order to evaluate the role of miR-29a-3p in the pathogenesis of DKD, SV40 MES 13 cells were cultured with high glucose to establish an *in vitro* DKD cell model. Quantitative real-time

PCR analysis was used to detect the expression of miR-29a-3p, DNMT3A/3B and fibrosis-related molecules, Refer to Figure 1.

MiR-29a-3p negatively regulates the expression of α-SMA, collagen I and fibronectin under high glucose condition: DKD is characterized by glomerulosclerosis or fibrous changes. It is known that miRNA is involved in the process of fibrosis. In order to study the function of miR-29a-3p in DKD, miR-29a-3p inhibitors and miR-29a-3p mimics were added to SV40 MES 13 cells cultured under different glucose conditions, and the expression of fibrosis molecules was measured by Western blotting and immunofluorescence. First of all, we determined the relationship between miR-29a-3p and fibrosis-related molecules, and determined that miR-29a was involved in the fibrosis process. In normal glucose group, the expressions of α-SMA, collagen I and fibronectin ($cp < 0.001$) were significantly increased after transfection with inhibitor-miR-29a-3p (Figure 2). The results showed that the degree of cell fibrosis was enhanced after inhibition of miR-29a-3p. In HG group, the expression of α-SMA, type I collagen and fibronectin decreased after adding miR-29a-3p mimic ($cp < 0.001$, Figure 3). With regard to the progress of cellular fibrosis, miR-29a-3p mimics improve the progression of fibrosis. The overexpression of miR-29a in HG group can improve the expression of fibrosis-related markers.

Negative regulation of DNMT3A/3B expression by miR-29a-3p under high glucose condition: After transfection with inhibitor-miR-29a-3p, the expression of miR-29a-3p in normal glucose group was significantly decreased by qRT-PCR ($cp < 0.001$), while the expression of DNMT3A and DNMT3B ($cp < 0.001$) was significantly up-regulated (Figure 4A-B). Similarly, we found that after HG was transfected with miR-29a-3p mimics, the expression of miR-29a-3p was significantly increased ($cp < 0.001$, Figure 4C), and the expression of DNMT3A/3B was significantly down-regulated ($bp < 0.01$, Figure 4D). It is suggested that miR-29a-3p negatively regulates the expression of DNMT3A/3B protein in SV40 MES 13 cells.

Inhibition of DNMT3A and DNMT3B can reverse the effect of down-regulation of miR-29a-3p on DN-related fibrosis in SV40 MES 13 cells cultured with high glucose: In order to further study whether miR-29a-3p regulates DKD by targeting DNMT3A and DNMT3B, SV40 MES 13 cells were treated with SGI-1027 after transfection with miR-29a-3p inhibitor to inhibit the expression of DNMT3A and DNMT3B. The results of real-time quantitative PCR (Figure 5) showed that SGI-1027 treatment could significantly reduce the promoting effect of miR-29a-3p inhibitors on the expression of fibrosis-related molecules and enhance the effect of miR-29a-3p inhibitors. These results further suggest that miR-29a-3p can directly down-regulate DNMT3A and DNMT3B to inhibit DKD.

The effect of antagomiR-29a-3p on the kidney index, serum creatinine (CR) and blood urea nitrogen (BUN) levels, and renal histopathological changes in db/db mice: The above in vitro experimental results demonstrated that miR-29a-3p negatively regulated the expression of DNMT3A/3B in SV40 MES 13 cells, with high expression effectively alleviating cell fibrosis. To further elucidate the role of miR-29a-3p in animals, db/db mice were used as a model of DKD, with db/m mice as normal controls. At the 10th week, we found that the diabetic mice exhibited renal pathological changes such as glomerular hypertrophy, mesangial matrix proliferation, and mesangial area widening. With increasing age, these renal pathological changes worsened, with pathological changes such as glomerulosclerosis appearing by the 16th week(Figure 6A). We also measured Scr (normal range: 10.81-34.74 mg/dL) and BUN (normal range: 10.91-85.09μ/L) which were shown to be outside the normal range(Figure 6B-C). Therefore, we were able to determine that the db/db mice had already developed kidney disease at the 10th week and were in the early stage of DKD. These results provided a time basis for subsequent experiments. In our previous study, we intervened in the 10th week by injecting agomiR-29a-3p into db/db mice. Four weeks later, we showed that the kidney index of the agomiR-29a-3p group mice was increased significantly, indicating that kidney atrophy had reduced after treatment(Figure 6D). Serum biochemical tests showed that the levels of Scr and BUN in all groups of the db/db mice were within the normal range, indicating that renal function in all groups of mice was in the

compensatory period(Figure 6E-F). The blood glucose levels of db/m mice were normal, while db/db mice showed a significant increase, although the difference between the groups was not statistically significant(Figure 6G). HE and PAS staining showed that after treatment with agomiR-29a-3p, pathological changes such as glomerular hypertrophy and mesangial area widening in db/db mice had improved significantly, with no obvious glomerulosclerosis being observed. In contrast, the mice who had not received agomiR-29a-3p treatment developed glomerulosclerosis(Figure 6H,6J). Taken together, these results indicate that overexpression of miR-29a-3p delays the kidney structural changes associated with DKD, although whether the signaling pathway was inactivated by DKD methyltransferase requires validation in future experiments.

MiR-29a-3p negativity regulates the expression of DNMT3A/3B and fibrosis-related molecules in db/db mice: In the in vitro experiments, overexpression of miR-29a reduced the expression of DNMT3A/3B and fibrosis-related molecules. Therefore, we speculated whether this was also the case in an in vivo environment. We showed that treatment with agomiR-29a-3p significantly decreased the expression of DNMT3A/3B and fibrosis-related molecules (α-SMA, collagen type I, and fibronectin)(Figure 7). These results indicated that high expression of miR-29a-3p reduced the expression of renal DNA methyltransferases in DKD mice, delaying the progression of renal fibrosis. This finding was consistent with the results of the in vitro experiments.

The expression of miR-29a-3p negative regulation DNMT3A/3B in db/db mice is involved in the pathogenesis of renal fibrosis through Wnt/β-catenin and JAK/STAT signal pathways: In order to determine potential signaling pathways that may be regulated, we examined the expression of Wnt3a, β-catenin, JAK2, and STAT3 in the Wnt/β-catenin and JAK/STAT signaling pathways. The results showed that the expression of these signaling molecules in db/db mice was significantly higher than that measured in the normal control group, and that after treatment with agomiR-29a-3p, their expression was significantly inhibited(Figure 8). Therefore, overexpression of miR-29a-3p may participate in the development of kidney fibrosis by inhibiting the

Wnt/β-catenin and JAK/STAT signaling pathways.

Please explain in details: How the background signal of the figures 1B, 1C, 2A and 3A were readjusted?

We optimized the western blotting results by optimizing the sealing conditions, adjusting the concentration of primary antibody, increasing the time and times of membrane washing, keeping the membrane moist and ensuring the consistency of experimental conditions.